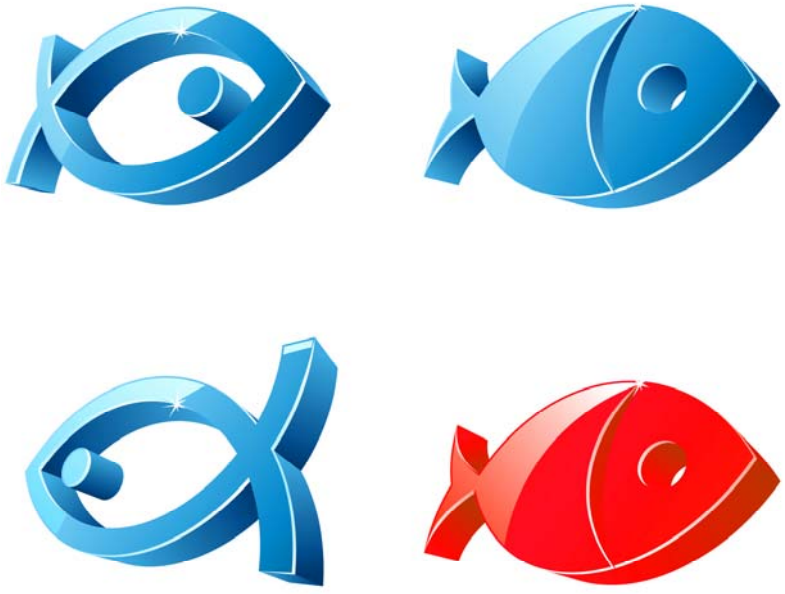
larvi 2013

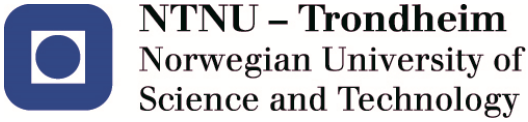
6th fish & shellfish larviculture symposium

gent, belgium, september 2-5, 2013



editor

c.i. hendry



technical editing: chris hendry
technical support: marc verschraeghen

printed in belgium, september 2013

isbn: xxx-xx-xxxxxxx-xx

EDITORIAL

This digital version contains the mini-papers of the poster contributions and the extended abstracts of the oral papers, presented at the occasion of larvi 2013, the sixth symposium on fish & shellfish larviculture, organized on September 2-5, 2013 at Ghent University, Belgium.

As in the previous larvi conferences, this digital version primarily provides the participants with detailed information on the scientific contents of the meeting, especially of the poster displays. Additionally, and in a broader sense, it can be used, both by participants and others, as a publication reflecting – in a condensed form – the present state of fish and shellfish larviculture.

The papers, included on this digital version, have been retained by the scientific committee in function of their relevance within the scope of the conference. Though not peer-reviewed, they have passed through a limited editing process in order to improve, where needed, compliance with the editors' scientific and technical guidelines and uniformity of formatting.

Finally we would like to acknowledge the secretarial staff of the Laboratory of Aquaculture & Artemia Reference Center, Ghent University for their continuous help: without Marc Verschraeghen the realization of this book would not have been possible.

Gent, August 9, 2013

The Editor

TABLE OF CONTENTS

(in alphabetical order)

Editorial I

D. Adjei-Boateng and J.G. Wilson
Gametogenic development and spawning of the freshwater clam, *Galatea paradoxa* (Born 1778) from the Volta river estuary, Ghana 2

E. Ahmadian, N. Agh, A. Tokmechi, and R. Jalili
Effect of dietary bovine lactoferrin on rainbow trout (*Oncorhynchus mykiss*) fecundity and larval quality 6

J.A. Alfredsen, M.O. Alver, and T.I. Eriksen
Architecture for automation and telepresence in a marine hatchery laboratory 10

M. Alix, B. Schaerlinger, Y. Ledoré, D. Chardard, and P. Fontaine
Developmental staging and deformities characterization of the Eurasian perch, *Perca fluviatilis* 12

C. Arndt, M. Moison, and U. Sommer
Providing harpacticoid copepods via floating sieve improves fish larval feeding success 15

K. Attramadal, G. Øie, E. Kjørsvik, G. Minniti, J. Skjermo, I. Bakke, M.-A. Østensen, Y. Olsen, and O. Vadstein
Control of the selective pressure on microbes of the incoming water increases survival of marine fish larvae 17

E.G. Ayala-Borboa, D. Tovar-Ramírez, R. Civera-Cerecedo, M. Rojas-Contreras, M.A. Cadena-Roa, H. Nolasco- Soria, and E. Goytortúa-Bores
Effect of red crab meal (*Pleuroncodes planipes*) on growth and digestive enzyme expression in the intestine of white shrimp (*Litopenaeus vannamei*) 19

I. Bakke, J. Skjermo, K. Attramadal, E. Coward, E. Kjørsvik, Tu Anh Vo, Y. Olsen, and O. Vadstein
Bacterial community assembly in developing cod larvae (*Gadus morhua*) 23

K. Baruah, P. Norouzitallab, P. Sorgeloos, and P. Bossier
Inducer of heat shock protein 70: a new disease preventive option in aquaculture production systems 25

S.C. Battaglene, C.J. Simon, Q.P. Fitzgibbon, G.G. Smith, and C.G. Carter
Could the eastern rock lobster, *Sagmariasus verreauxi,* be the best spiny lobster for aquaculture? 27

T. Benítez-Santana, R. Saleh, M.B. Betancor, A. Mesa, C.M. Hernández-Cruz, and M. Izquierdo
Effects of combined phospholipids and selenium dietary contents on patterns of bone formation in the axial skeleton of *Sparus aurata* 29

E. Bequé, R. Phuthongphan, R. Wouters, and G. Rombaut
Effect of phytochemicals on stress tolerance of *Penaeus vannamei* postlarvae 33

Ø. Bergh
Experimental challenge: the quest for virulence – and protection 37

N. Berkovich, I. Meiri-Ashkenazi, V. Zlatnikov, A. Corriero, C. Bridges, C. Mylonas, R. Vassallo Aguis, F. De La Gándara, A. Belmonte, A. Elizur, H. Gordin, and H. Rosenfeld
The key neuroendocrine regulators of the onset of puberty in the Atlantic bluefin tuna (*Thunnus thynnus*) 39

M.B. Betancor, M.J. Caballero, and M.S. Izquierdo
Oxidative stress in sea bass *Dicentrarchus labrax* larvae: interaction of high dietary DHA contents and several antioxidant nutrients 41

A. Boglino, A. Wishkerman, M.J. Darias, A. Estévez, K.B. Andree, and E. Gisbert
High dietary levels of arachidonic acid not only affects the normal pigmentation patterns in post-metamorphic Senegalese sole larvae, but also disrupts the process of eye migration in pseudo-albino fish 43

S. Bolla, O. Nicolaisen, and O.-L. Brekke
Effect of broodstock diet on hepatic status and reproductive performance in Atlantic halibut (*Hippoglossus hippoglossus* L.) females 45

K. Bonacic, R. Valles, X. Gómez-Arbonés, A. Estevez, and S. Morais
Fluorescent microspheres – a new approach to quantifying live feed intake in larval fish 48

M. Bouilliart, J. Tomkiewicz, P. Lauesen, and D. Adriaens
Exploring the feeding mystery of leptocephalus larvae: a mouth full of teeth 52

I.A.E. Butts, S.R. Sørensen, S.N. Politis, P. Lauesen, and J. Tomkiewicz
Development of techniques and technology for embryonic and larval rearing of the European eel 54

E. Cabrita, S. Martínez-Páramo, P. Gavaia, T. Pacchiarini, D.G. Valcarce, C. Sarasquete, and V. Robles
Factors affecting sperm quality and emerging tools for sperm analysis 58

C. Campos, L.M.P. Valente, L.E.C. Conceição, S. Engrola, and J.M.O. Fernandes
Epigenetic regulation of muscle development and growth in Senegalese sole larvae 60

P. Canada, S. Engrola, A.F. Lopes, N. Richard, L.M.P. Valente, and L.E.C. Conceição
Does dietary amino acid profile modulate Senegalese sole larvae protein metabolism? 62

J. Carmona-Contreras, D. Tovar-Ramírez, R. Civera-Cerecedo, M. Rojas-Contreras, M. A. Cadena-Roa, H. Nolasco-Soria, and E. Goytortúa-Bores
Gene expression profile of *Litopenaeus vannamei* juveniles fed different protein sources and detection of digestive-related genes by functional genomics 66

R. Chakrabarti and I.M. Lukram
Influence of feeding regimes on the digestive enzyme profile and ultrastructure of digestive tract of *Catla catla* 70

Y.H. Chang, J.C. Kuo, and Y.M. Chen
Vibrio static activity in the culture of the *Picochlorum* sp. s1b can be attributed to the bacteria associated with the microalga 73

M. Christou, M. Iliopoulou, and G. Koumoundouros
Towards the development of new quality indices in juvenile fish – relationship of caudal-fin morphology with the thermal history of juveniles 77

J.M. Cobcroft, S.C. Battaglene, J.C.G. Biggs, and D.S. Fielder
Investigating causes of skeletal malformation in yellowtail kingfish *Seriola lalandi* 81

M.L. Cobo, R. Wouters, M. Wille, S. Sonnenholzner, J. Calderón, and P. Sorgeloos
Intensification of *Litopenaeus vannamei* larviculture 85

M. Corteel, G. Franchi, L. Chiappi, A. Rekecki, T. De Wolf, and G. Rombaut
Effect of Sanolife MIC-F as larval fish probiotic on host stress resistance, histology, and microbiota in sea bream..... 87

X. Cousin, M.G. Claros, D. Mazurais, R.Bautista, H. Benzekri, M.-L. Bégout, M. Ponce, P. Armesto, J. Zambonino, J.V. Planas, and M. Manchado
Genome-wide gene expression analysis during *Solea* sp. embryo-larval development..... 91

C.A. Cuenca-Soria, D.T. Ramírez, J.L. Ortiz-Galindo, E. Gisbert, and C.A. Álvarez-González
Studies of digestive physiology during early ontogeny of the Mayan cichlid *Cichlasoma urophthalmus* 93

M.E. Cunha, M. Gamboa, S. Castanho, H. Quental-Ferreira, L. Ribeiro, F. Soares, P. Gavaia, M. Mateus, P. Moura, N. Cordeiro, R. Gonçalves, and P. Pousão-Ferreira
Fish larval performance fed with copepods (*Acartia grani*) and the dinoflagellate (*Oxyrrhis marina*) as supplement: the case of dusky grouper (*Epinephelus marginatus*) 97

P. D'Alvise, S. Lillebø, H. Wergeland, K.F. Nielsen, Ø. Bergh, and L. Gram
Control of bacterial disease in cultures of marine larvae and live feed organisms by a probiotic bacterium..... 101

K. Dabrowski, M. Korzeniowska, T. Farmer, S. Ludsin, and E. Marschall
The function of wax esters in larval fish transition from endogenous to exogenous nutrition – are freshwater fish the exception or the rule? 102

A. Davie, E. Leclerq, M.V. Medeiros, C. Talbot, and H. Migaud
Digital image analysis to aid broodstock management and egg quality assessment 104

P. De Schryver
The application of ecological theory for microbial control in aquaculture..... 106

T. De Wolf, P. Cecconi, S. Lenzi, I. Labaere, and G. Rombaut
The development of new enrichment products and strategies for live feed in fish hatcheries 108

N. El-Bermawi
Effects of dietary *Dunaliella salina* extract and highly unsaturated fatty acids on the fecundity and lipid content of pond-reared *Penaeus japonicus* broodstock 111

S. Engrola, N. Richard, A.F. Lopes, W. Pinto, and L.E.C. Conceição
Linking weaning success to larval digestive capacity using radiolabelled peptide fractions 115

J. Estefanell, J. Socorro, B. Ramírez, M. Izquierdo, and J. Roo
Fatty acid profile in eggs and newly hatched paralarvae of *Octopus vulgaris* collected from the wild, and after 1-5 days starvation 119

I. Fernández, E. Gisbert, and P. Gavaia
Vitamin A and K, two fat-soluble vitamins required for harmonic fish larval development 123

S. Fontagné-Dicharry, S. Godin, H. Liu, P. Antony Jesu Prabhu, B. Bouyssiere, M. Bueno, P. Tacon, F. Médale, and S. Kaushik
Influence of the forms and levels of dietary selenium on oxidative stress in rainbow trout fry 125

T. Forberg and O. Vadstein
Lessons learned from gnotobiotic systems on the effect of bacteria on growth, survival, and gene expression in marine larvae 129

S. Fragkoulis, P. Kokkinias, and G. Koumoundouros
Correlation of saddleback syndrome with deformities of the pelvic fins and lateral line in European sea bass, *Dicentrarchus labrax* 131

M.A. Galaviz, L.M. López, A. García Gasca, E. Gisbert, A.C. Álvarez, G. Sandoval, and C.D. True
Expression and activity of three digestive proteases in larvae of the totoaba (*Totoaba macdonaldi*) 135

J. García-Manchón, J. Socorro-Cruz, Á. Segade Botella, F. Otero-Ferrer, A. Mesa, and L. Molina Domínguez
First feeding regimes for long-snout seahorse *Hippocampus reidi* larvae 139

E. Georgakopoulou, M. Loizides, M. Christou, M. Iliopoulou, I. Papadakis, P. Katharios, P. Divanach, and G. Koumoundouros
Thermally-induced phenotypic plasticity in gilthead sea bream 143

A.N. Georgiou, M. Christou, M. Sxoinaraki, P. Kokkinias, and G. Koumoundouros
Larval diet determines juvenile and adult phenotype in zebrafish (*Danio rerio*)..... 147

S. Georgiou, E. Sarropoulou, D.M. Power, H. Alami-Durante, Z. Mamuris, and K.A. Moutou
Expression of skeletal myosin light chain 2 in gilthead sea bream (*Sparus aurata*, L.): regulation and correlation to growth markers 151

M. Gerdol, G. De Moro, V. Torboli, C. Manfrin, U. Rosani, P. Venier, and A. Pallavicini
Genomics in bivalve aquaculture..... 153

I. Geurden, P. Borchert, M. Balasubramanian, J. Schrama, M. Dupont-Nivet, E. Quillet, S. Kaushik, P. Panserat, and F. Médale
Early-feeding exposure to a plant-based diet improves its future acceptance and utilization in rainbow trout 157

R.A.Y.S.A. Gunasekara, C. Casteleyn, P. Bossier, and W. Van den Broeck
Surface area estimation of the gut segments of *Artemia franciscana* nauplii fed with mnn9 yeast vs. wild type yeast 161

R. Gustiano, E.K Kontara, H. Wahyuningsih, J. Subagja, S. Asih, and A. Saputra
Domestication of mahseer (*Tor soro*) in Indonesia 165

A. Hagemann, G. Øie, Y. Attramadal, R. Veiseth, and J.O. Evjemo
Content of essential fatty acids in cultivated *Acartia tonsa* nauplii fed a DHA-deficient *Tetraselmis* sp. Concentrate..... 169

A. Hagiwara, S. Wullur, N. Hirai, and Y. Sakakura
Euryhaline rotifer *Proales similis* as initial live food for rearing fish larvae with small mouths..... 173

K. Hamre, S. Penglase, J. Rasinger, and P.A. Olsvik
Ontogeny of the redox balance in relation to organogenesis in Atlantic cod (*Gadus morhua*) larvae 175

B.W. Hansen, G. Drillet, and P.M. Jepsen
Combating some of the crucial bottlenecks for calanoid copepod cultivation for live feed 176

M.R. Hauville, J.L. Zambonino-Infante, H. Migaud, J.G. Bell, and K.L. Main.
Effects of probiotics on pompano (*Trachinotus carolinus*), common snook (*Centropomus undecimalis*), and red drum (*Sciaenops ocellatus*) larvae 180

M. Hawkyard, B. Laurel, Y. Barr, and C. Langdon
Microparticulate enrichment of rotifer live diets with taurine and nutritional effects on northern rock sole (*Lepidopsetta polyxystra*) larvae 184

P.I. Hilder, J.M. Cobcroft, N.S. Hart, S.P. Colin, and S.C. Battaglene
Is sinking mortality in southern bluefin tuna larvae caused by high light intensity? 186

B. Hu, P. Bossier, and P. Sorgeloos
Effect of heat shock protein 70 (hsp70) homologue dnak on gene expression of prophenoloxidase and transglutaminase in haemocytes of *Litopenaeus vannamei* 190

N.T. Hua and H. Ako
Maturation and spawning induction in Hawaiian opihi *Cellana* spp. by hormone GnRH 194

Huynh Thanh Toi, Nguyen Van Hoa, P. Bossier, P. Sorgeloos, and G. Van Stappen
Promoting of bacteria growth by manipulating carbon/nitrogen ratio and use as microalgae substitution for filter feeders: a demonstration on *Artemia* culture 198

E. Jiménez-Fernández, E. Zuasti, A. Ruyra, N. Roher, C. Infante, and C. Fernández-Díaz
Nanoparticles as a novel delivery system for vitamin c administration in aquaculture 202

I. Karunasagar., S.K. Girisha, M.N. Venugopal, and M. Biswajit
Bacteriophage application as a management strategy in shrimp hatcheries 204

E.E. Khurshut
Reproductive capacity of *Hemiculter leucisculus* (Basilewsky, 1855) in Uzbekistan 206

S. Kolkovski and J. Kolkovski
Development of maturation diet for penaeid shrimp using herbal extracts..... 208

S. Kolkovski, J. King, N. Watts, M. Natale, J. Bannister, and F. Stephens
Associated effects of bacteria on *Octopus tetricus* larvae rearing..... 211

M. Komrakova, U. Kuembet, and W. Holtz
The effect of ph on the fertilizability of rainbow trout (*Oncorhynchus mykiss*) eggs stored in a chilled state 215

V. Kostopoulou, A. Tsopelakos, E. Zogopoulou, H. Miliou, P. Divanach, and P. Katharios
The effect of different phytoplankton species and commercial enrichment products on the fatty acid profile, enzyme activity, and overall condition of the rotifer *Brachionus plicatilis*..... 219

N.P. Kovatcheva, R.R. Borisov, A.V. Parshin-Chudin, R.O. Lebedev, and D. S. Pechonkin
Culture systems in the coastal complexes which are used in the process of red king crab artificial reproduction in Russia 223

S. Krejszeff, D. Żarski, P. Gomułka, K. Kupren, Palińska-Żarska, J. Nowosad, K. Stańczak, and D. Kucharczyk
Dynamic of efficiency of ms-222 as an anaesthetic for tench *Tinca tinca* (L.) larvae 227

J. Król, Z. Zakęś, and P. Kestemont
Emergence of cannibalism in European percid fish – size heterogeneity or natural born killers consequence? 231

K. Kupren, M. Prusińska, D. Żarski, S. Krejszeff, and D. Kucharczyk
Allometric growth in the *Nannacara anomala* regan, 1905 (Cichlidae, Pisces) 233

N.J. Leitão, J.B. Rodrigues, C.R.F. Grosso, M. Dal Pai-Silva, and M.C. Portella
Microencapsulated diets for altricial freshwater fish larvae: production and evaluation..... 237

K. Li, M.-A. Østensen, K. Attramadal, P. Winge, T. Sparstad, I. Bakke, A.M. Bones, O. Vadstein, E. Kjørsvik, and Y. Olsen
Gene regulation of Atlantic cod (*Gadus morhua*) larvae with focus on lipid digestion and phospholipid metabolism 241

X. Li, T. Defoirdt, Q. Yang, P. Bossier, and K. Dierckens
Host-induced increase of sea bass mortality in a gnotobiotic challenge test with *Vibrio anguillarum*..... 245

D.J. López-Peraza, M. Hernández-Rodríguez, and B. Barón-Sevilla
Ontogeny of the digestive system of *Octopus bimaculatus* paralarvae 249

G. Ludevese-Pascual, M. de la Peña, O. Reyes, and J. Tornalejo
Low-cost production of the marine thraustochytrid isolate, *Schizochytrium* sp. ley7 as larval live feed enrichement for the mangrove snapper, *Lutjanus* sp. 253

I. Lund, E. Höglund, and P.V. Skov
Importance of DHA for first feeding pikeperch larvae – influence on behavioural responses 257

P. Makridis, N. Papandroulakis, and P. Divanach
Use of *Phaeobacter* sp. probiotic bacteria for the rearing of sea bass larvae (*Dicentrarchus labrax*) 259

S.N.M. Mandiki, C. Mathieu, F. Chaffa, T.M. Rakotomalla, J. Douxfils, and P. Kestemont
Digestive and immune responses to probiotics or prebiotics in percid larvae 262

S. Martínez-Páramo, F. Arfuso, S. Engrola, J. Dias, C. Faggio, and C.Aragão
Do immunostimulants affect sperm quality in Senegalese sole?..... 265

J.A. Mata, G. Martínez-Rodríguez, F.J. Moyano, and M. Yúfera
Evaluation of daily rhythms in feeding activity and digestive functions in gilthead sea bream (*Sparus aurata*) larvae 269

A.C. Mendes, S. Castanho, M. Gamboa, J. Coutinho, N. Bandarra, L. Conceição, S. Morais,and P. Pousão-Ferreira
Advances in larval rearing protocols of sole, *Solea senegalensis* 271

A. Mesa-Rodriguez, C. Hernández-Cruz, M. Izquierdo, H. Fernández-Palacios, and J. Roo
Effect of increasing DHA content in new weaning diets for longfin yellowtail (*Seriola rivoliana)* 275

G. Mitra, J.K Jena, P. Jayasankar, P.C. Das, and S.K. Swain
Evaluation of colour development of rosy barb, *Puntius conchonius* (Hamilton) during ontogeny 279

A. Miyashima, T. Kamiya, H. Fushimi, N. Sato, T. Kotani, and T. Kusano
DHA requirement of larval Japanese flounder *Paralichthys olivaceus* in the rotifer feeding period 283

Ó. Monroig, F. Hontoria, I. Varó, D.R. Tocher, and J.C. Navarro
Investigating the essential fatty acid requirements of the cephalopod *Sepia officinalis*: a molecular approach 286

S. Morais, A. Candeias Mendes, F. Castanheira, J. Coutinho, N. Bandarra, J. Dias, L.E.C. Conceição, and P. Pousão-Ferreira
Effect of Senegalese sole broodstock nutrition on early larval performance and metabolism of long-chain polyunsaturated fatty acids (DHA and EPA)..... 290

M. Moren, K. Kvalheim, A. Nordgreen, T. Harboe, and K.K. Lie
Gene expression is strongly regulated by nutrients in first feeding Atlantic cod larvae (*Gadus morhua*) 294

K. Nagasawa, C. Presslauer, G. Yoshizaki, M. Miwa, J.M.O. Fernandes, and I. Babiak
Identification and migration of primordial germ cells in Atlantic salmon (*Salmo salar*) and Atlantic cod (*Gadus morhua*) 296

C. Navarro-Guillén, S. Engrola, F. Castanheira, N. Bandarra, I. Hachero-Cruzado, L.E.C. Conceição, and S. Morais
Interaction between dietary levels of LC-PUFA and vegetable oil sources in Senegalese sole (*Solea senegalensis)* post-larvae: puzzling results suggesting complete biosynthesis pathway from c18 PUFA to DHA 300

Ngo Thi Thu Thao and Le Quang Nha
Effects of glucose and probiotic supplementation in nursing juvenile clam, *Meretrix lyrata*..... 304

Nguyen Thi Hong Van, Nguyen Van Hoa, P. Bossier, P. Sorgeloos, and G. Van Stappen
Effect of environmental factors on heritability and its biometrics of *Artemia franciscana* Vinh Chau by mass selection of small sized cysts 308

Nguyen Thi Ngoc Anh, Tran Thi Thanh Hien, and Tran Ngoc Hai
Potential uses of gut weed *Enteromorpha* spp. as a feed for herbivorous fish 312

Nguyen Thi Xuan Hong, D. Vanrompay, and P. Bossier
Influence of virulent and avirulent bacterial strains on hsp70 content of *Artemia* instar ii larvae..... 316

Nguyen Van Hoa, Tran Huu Le, Nguyen Thi Hong Van, P.Sorgeloos, and G. Van Stappen
New approaches for *Artemia* pond culture 320

Nguyen Van Hung, P. De Schryver, P. Bossier, and N. Nevejan
Use of poly–β-hydroxybutyrate in bivalve larviculture..... 324

O. Nicolaisen, M. Cuny, and S. Bolla
Improving visual environment in cod larval rearing by factorial designs 328

Y. Niu, T. Van de Wiele, and P. Bossier
The protective and intestinal microbiota steering effect of a novel heat shock inducer on brine shrimp larvae 330

C.C.V. Oliveira, A. Davie, E. Cabrita, M.F. Castanheira, M.T. Dinis, and H. Migaud
Ontogeny of *kiss2* and *kiss1r* gene expression in gilthead sea bream (*Sparus aurata*) larvae 334

K. Palińska-Żarska, D. Żarski, S. Krejszeff, J. Nowosad, M. Biłas, and D. Kucharczyk
Tank wall color affects swimbladder inflation in Eurasian perch, *Perca fluviatilis* L., under controlled conditions..... 338

K. Palińska-Żarska, D. Żarski, I. Trąbska, S. Krejszeff, B. Łączyńska, R. Deptuła, and D. Kucharczyk
Burbot, *Lota lota*, L., larvae requirements for *Artemia* sp. nauplii during experimental rearing in laboratory conditions..... 342

G.S.J. Pande, P. Bossier, and T. Defoirdt
The impact of quorum sensing-disrupting compounds on survival and growth of giant freshwater prawn (*Macrobrachium rosenbergii*) larvae 346

B. Paz-Raymundo, D. Tovar-Ramírez, E. Palacios-Mechetnov, and J.C. Pérez-Urbiola
Effect of DHA on the expression of the δ6-desaturase during larval development of yellow snapper, *Lutjanus argentiventris*..... 350

S. Peixoto, J. Vogeley, R. Nery, C. Barros, J.V. Lima, and R. Soares
Selection and identification of probiotic bacteria for use in the marine shrimp culture 354

R. Peña, S. Dumas, I. Moguel-Hernández, and N. García-Aguilar
Amino acid profile and consumption during the embryonic development and yolk-sac larvae of Pacific red snapper *Lutjanus peru* 358

D. Pham, D. Ansquer, A. Chevalier, A. Peyramale, C. Dauga, N. Wabete, and Y. Labreuche
Selection study of potential probiotic bacteria for shrimp hatcheries in New Caledonia 362

Pham Thi Tuyet Ngan, Trương Quoc Phu, Tran Suong Ngoc, and Nguyen Huu Hiep
Identification of nitrifying bacteria in intensive shrimp ponds in Soc Trang province, Vietnam by biochemical test and molecular technique..... 364

W. Pinto, P. Diogo, G. Martins, S. Martínez-Páramo, P. Gavaia, L. Conceição, and J. Dias
Can dietary phospholipid and trace mineral supplementation influence zebrafish reproductive performance?..... 368

A. Plovie, F. Gonzaga, N. Nevejan, and P. Bossier
The development of an axenic blue mussel (*Mytilus edulis*) larvae test system 372

M.C. Portella, N.J. Leitão, O.C.C. Menossi, T.M. Freitas, J.T. Kojima, T.S. Lopes, R.K. Jomori, J.A. Clavijo-Ayala, and D.J. Carneiro
Review on the ontogeny of larvae from neotropical freshwater fishes: the pacu model..... 376

P. Pousão-Ferreira, S. Castanho, L. Ribeiro, J. Coutinho, N.M. Bandarra, and A.C. Mendes
Larval rearing protocols for meagre *Argyrosomus regius*..... 378

L. Prestinicola, C. Boglione, and S. Cataudella
Effects on the skeleton development in reared gilthead sea bream (*Sparus aurata*) 382

Z-Z. Qi, S-Q. Chen, A-G. Zou, L. Tang, M-M. Sun, H-Y. Zhang, C-L. Liu, J-P. Wang, and Y. Han
Bacterial community compositions of ejected intestine of juvenile sea cucumber and the effect of sea sediment bacillus on in vivo antagonism against pathogenic *Vibrio splendidus* 384

S. Rehberg-Haas, S. Meyer, S. Lippemeier, and C. Schulz
Pavlova – a "new" microalgae candidate species for live feed cultivation and fish larvae nutrition 388

D.B. Reis, N.G. Acosta, E. Almansa, J.C. Navarro, D.R. Tocher, O. Monroig, J.P. Andrade, A.V. Sykes, and C. Rodríguez
Endogenous metabolism of unsaturated fatty acids in *Artemia* nauplii as determined through incubation with ^{14}C-labelled fatty acid substrates 392

N. Richard, S. Engrola, P. Palma, W. Pinto, D. Simes, and L. Conceição
Assessment of protein digestive capacity and utilisation during ontogeny of Senegalese sole larvae: a tracer study using in vivo produced radiolabelled peptide fractions 396

J. Roo, A. Mesa-Rodriguez, C. Hernández-Cruz, M. Izquierdo, and H. Fernández-Palacios
Recent advances in *Seriola dumerilli* culture 398

M. Rozihan, E. Ismail, and B. Akbar John.
Genetic variability of wild horseshoe crabs (*Tachypleus gigas*) from west coast of peninsular Malaysia 399

H.A.D. Ruwandeepika, T. Defoirdt, T. S. P. Jayaweera, I. Karunasagar, and P. Bossier
Virulence genes and quorum sensing of *Vibrio harveyi* 403

Ø. Sæle, K.E.L. Rød, and S. Farber
Lipid digestion in first feeding larvae – visualization in vivo 407

Y. Sakakura, K. Suga, A. Kanamori, and A. Hagiwara
The self-fertilizing mangrove killifish *Kryptolebias marmoratus* as a model fish for aquacultural study 409

R. Saleh and M. Izquierdo
Optimum phospholipids and antioxidant levels to develop novel microdiets for gilthead sea bream larvae 411

Ahmed. Md. Salem, A.M. Nour, S.S. Assem, T.M. Srour, H.S. El-Sayed, and H.A. Ibrahim
European sea bass larval early weaning development using greenwater and synbiotic in Alexandria, Egypt 413

S.M. Sharifuzzaman and S. Adhikari
An assessment of biosecurity and hazard management practices in the larviculture of shrimp (*Penaeus monodon*), Bangladesh 417

M.L. Situmorang, K. Dierckens, and P. Bossier
The effects of dietary poly-β-hydroxybutyrate on growth, feed utilization, and survival of nile tilapia (*Oreochromis niloticus*) fry 421

T.H. Skaalsvik, S.L. Bolla, and I. Babiak
Quantitative characteristics of Atlantic halibut (*Hippoglossus hippoglossus* l) egg production throughout the reproductive season and their relationship to embryo and larval quality 425

F. Soares, S. Castanho, M. Moreira, A.C. Mendes, and P. Pousão-Ferreira
Microbial characterization of enriched *Artemia* sp. at two different temperatures and enrichments 429

G. Suantika, P. Aditiawati, D.I. Astuti, A. Sjarmidi, N. Lim, and Z.F. Khotimah
Evaluation of probiotic bacteria against aeromonads syndrome in common carp (*Cyprinus carpio* L.) in simple axenic larviculture 433

L. Sui, G. Chang, Y. Liu, and Y. Deng
Effect of poly-hydroxybutyrate on growth and enzymatic activity of Chinese mitten crab, *Eriocheir sinensis*, juvenile 437

B. Sumares and N. Nogueira
Egg production, egg hatching success, and population growth of the calanoid copepod *Acartia grani* (Calanoida, Acartiidae) fed with eight different diets 441

M. Tamtin, M. Wille, J. Saeton, K. Kademuan, V. Tanasomwang, P. Sobhon, and P. Sorgeloos
Current status of crab larviculture in Thailand and development of a diet for domesticated broodstock 445

A. Teles, W.M. Costa, D. Ammar, E.M. Nazari, Y.M.R. Muller, and V.R. Cerqueira
Ontogenetic development of the digestive system in reared fat snook (*Centropomus parallelus*) larvae 447

R. Tillner, I. Rønnestad, P. Dhert, and B. Ueberschär
Cholecystokinin and tryptic enzyme activity in sea bass (*Dicentrarchus labrax*) larvae: a regulatory loop and the impact of feeding regimes 451

J. Tomkiewicz, J.G. Støttrup, G. Corraze, S. Kausik, L.K. Holst, F. McEvoy, S. Dufour, A.G. Lafont, J. Asturiano, S. R. Sørensen, H. Tveiten, P. De Schryver, I.A.E. Butts, P. Munk, J. Zambonino-Infante, S.N. Politis, M. Krüger-Johnsen, and P. Lauesen
Reproduction of European eel and larval culture – state of the art..... 455

Tran Suong Ngoc, Nguyen Van Hoa, and Vu Ngoc Ut
Applying of freshwater rotifer (*Brachionus angularis*) in rearing newly hatching fries of marble goby (*Oxyeleotris marmoratus*) 457

T. van der Meeren, Ø. Karlsen, A. Mangor-Jensen, I. Rønnestad, and K. Hamre
Copepods enhance growth and development in Atlantic cod (*Gadus morhua* L.) larvae 461

S. Vanmaele, T. Defoirdt, and P. Bossier
A gnotobiotic model system: the case of *Artemia franciscana* 465

C. Vargas Cecilia, Ø. Hagen, S. Peruzzi, and C. Solberg
Muscle growth of triploid Atlantic cod larvae (*Gadus morhua*) 469

I. Varó, J.C. Navarro, J. Iglesias, J.J. Otero, J. Sánchez, E. Almansa, O. Monroig, F. Hontoria, A.E. Morales, and G. Cardenete
Studies on the characterisation of biomarkers of nutritionally-derived stress in paralarval cultures of the common octopus (*Octopus vulgaris*) 473

Vu Ngoc Ut, Nguyen Phi Long, and Tran Suong Ngoc
Effects of feeding time, rates, and frequencies on survival rate of stripped catfish fry (*Pangasianodon hypophthalmus*) fed by freshwater rotifers (*Brachionus angularis*) 477

C.N. Walpita, A.D. Crawford, and V.M. Darras
Thyroid regulation in teleost embryonic and larval development – can it be a promise for aquaculture?..... 481

B. Weber, G.A. Santos, M. Mohnl, and G. Schatzmayr
Water management and biocontrol – selection of probiotic strains 485

R. Wouters and E. Naessens
Low *Artemia* consumption strategies in larval shrimp rearing 489

D. Xu, L.L. Wang, X.Z. Zhang, J. Wang, and X.N. Zang
Orthogonal analysis of elements affecting the formation of cystocarps on female gametophyte of *Gracilariopsis lemaneiformis* 493

E.N.Yaacob, B. De Geest, D. Vanrompay, K. Dierckens, and P. Bossier
Microparticles delivery system in gnotobiotic European sea bass larvae (*Dicentrarchus labrax* L., 1758) 496

H.L. Yang, H.Y. Lin, and C.C. Lin
Development of an NNV-free larvae rearing system and production of SPR grouper fingerlings 500

Q. Yang, P. Bossier, and T. Defoirdt
The impact of quorum sensing on motility in *Vibrio harveyi* 501

O. Yilmaz, F. Prat, A.J. Ibáñez, H. Amano, S. Koksoy, and C.V. Sullivan
Multiple vitellogenin yolk precursors in European sea bass (*Dicentrarchus labrax*) 505

T. Yoshimatsu
Effect of light conditions on the population growth of rotifers 509

M. Yúfera, V. de las Heras, J.A. Martos-Sitcha, C. Sarasquete, G. Martínez-Rodríguez, and J.B. Ortiz-Delgado
Ontogeny of the digestive tract of the omnivorous fish *Chelon labrosus* 513

J. Zwielehner, T. Suyawanish, G.A. Santos, and P. Encarnação
Application of a multi-strain probiotic improves utilization of microalgae in *Litopenaeus vannamei* post-larvae 517

Index of Authors 521

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

GAMETOGENIC DEVELOPMENT AND SPAWNING OF THE FRESHWATER CLAM, *GALATEA PARADOXA* (BORN 1778) FROM THE VOLTA RIVER ESTUARY, GHANA

D. Adjei-Boateng[1] and J.G. Wilson[2]

[1] Department of Fisheries and Watershed Management, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana
[2] Zoology Department, TCD, Dublin 2, Ireland

Abstract

The study focused on the reproductive cycle of *Galatea paradoxa* (Born 1778), a major species for artisanal fishery in the Volta River estuary, Ghana. Condition indices and histological observation of the gonads revealed that *G. paradoxa* has a single spawning event between July and October. Gametogenesis started in December progressing steadily to a peak in June-July when spawning began until November when individuals were spent. Condition and gonadal indices showed a clear relationship with the gametogenic stages.

Introduction

The freshwater clam *G. paradoxa* (Born 1778) is a bivalve mollusc belonging to the Order Veneroidea and Family Donacidae (Purchon, 1963). It is endemic to the West African sub-region and restricted to the lower reaches of a few rivers including the Volta (Ghana), Cross and Nun (Nigeria), and Sanaga (Cameroon) (Etim and Brey, 1994). Despite its wide distribution, little information exists on its ecology and biology (Purchon, 1963). For decades, *G. paradoxa* has supported a thriving artisanal clam fishery and the livelihood of 1000-2000 young men and women (Lawson, 1963) and is an important protein source to these communities (King, 2000). Commercial extinction of *G. paradoxa* is imminent in Ghana as a result of habitat alteration and overfishing. Loss of the fishery would have socio-economic consequences for villages and especially the women whose livelihoods depend on it. *G. paradoxa* has been found to be a suitable species for culture (Attipoe and Amoah, 1989). As a first step to developing sustainable management and ultimate culture of *G. paradoxa* in the Volta River, it is imperative to determine its reproductive cycle. Thus, the focus of this study was to determine the gametogenic cycle and spawning season of *G. paradoxa* in the Volta River.

Materials and methods

Study area
The study was conducted at Ada (10km) (05°49' 10" N, 0°38' 38" E) and Aveglo (15km) (5˚ 52′ 54″ N, 0˚ 38´55″ E) from the mouth of Volta River, Ghana, from March 2008 to February 2010.

Condition Indices
Sixty specimens per site were sampled monthly. Shell length was measured (±0.01mm) with digital calipers and total wet weight (shell+flesh; ±0.1g) was recorded. The shell-free dry weights (oven-dried at 60°C for 48h) were recorded (±0.0001g). Ash content of the dried body tissue was determined after burning the sample in a muffle furnace at 500°C for 12h. The ash-free body weight was computed for each specimen as:

$$\text{Ash-free dry body weight (AFDW)/ shell weight (SW)} \times 100$$

Histological examination of gonads
The gametogenic cycle of *G. paradoxa* was determined by standard histological procedures.

The following environmental variables were measured monthly: temperature, salinity, conductivity, dissolved oxygen, and chlorophyll a.

Data presentation and statistical analysis
Condition indices and water quality are presented using the mean and standard error (SE). A chi-square goodness of fit test ($\alpha = 0.05$) was used to test the hypothesis that there was an equal representation of males and females in the population (samples were pooled across all sampling dates).

Results

G. paradoxa collected from the Volta estuary over the study period ranged from 20-82mm in length. A total of 2820 and 278 specimens were sampled for condition indices and histological analyses, respectively. Out of 278 clams examined, 223 (80.2%) were females, 29 (10.4%) were males, and 26 (9.4%) were hermaphrodites. The overall ratio of males to females to hermaphrodites was 1:7.7:1 ($X^2 = 275$; $P < 0.001$, d.f. = 2, n = 278).

Condition indices
Variation in AFDW/SW, rose from 2.83±0.12 in March 2008 to 5.72±0.50 in August 2008, then declined until a minimum of 2.10±0.12 in January 2009. The same pattern was repeated during subsequent months of 2009, increasing to a peak of 5.47±0.25 in September 2009 and declining to a low value of 2.25±0.10 in January 2010.

Temporal distribution of gametogenetic stages

Gametogenic development in *G. paradoxa* was synchronous between the sexes. Five gonad development stages were determined from histological sections for both sexes: (I) start of gametogenesis, (II) advanced gametogenesis, (IIIA) ripe, (IIIB) start of spawning, and (IV) spent as illustrated in Figure 1. Clams with gonads at sexual rest (stage 0) were absent from the samples. Gametogenesis started during December (stage I) and progressed steadily through March to stage II in May (80% of clams) to a peak in June-July when a majority (60-90%) of individuals were ripe (stage IIIA). Spawning began in June (10% of clams) with the peak of the spawning event occurring in September (IIIB). A small percentage (<20%) of partially spent clams were recorded in September and by November (stage IV) 80% of individuals were spent

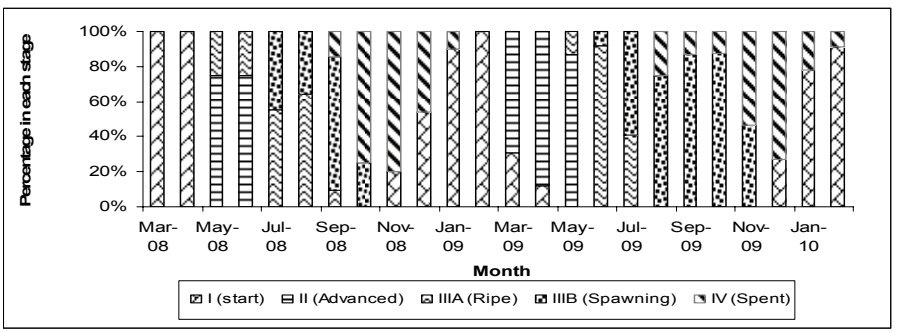


Fig. 1. Percentage of *G. paradoxa* in various reproductive stages at the Volta estuary from March 2008 to February 2010.

Variations in environmental variables

Monitored environmental variables were similar at Ada and Aveglo over the study period except for conductivity and to lesser extent salinity which was elevated at high tide. Water temperature was between 27.3 and 29.6°C. Salinity was at 0.03 practical salinity units (psu) during low tides at both sites, however during HT values as high as 1.5psu were recorded. Mean conductivity was 57.9µS.cm^{-1} during low tides at Ada and Aveglo. However, during high tide the mean was 583.4µS.cm^{-1} at Ada. The maximum conductivity recorded at Ada was 2879µS.cm^{-1}. Thus, the clam beds at Ada were tidally exposed to a variable salinity/conductivity regime.

Discussion

Gametogenesis in *G. paradoxa* commences at the beginning of the dry season (December) and progresses with the onset of rains (March-April) until the clam is ripe (June-July). Spawning occurred from July to October. Rainfall, which is directly related to the flooding and nutrient dynamics of the river, appears to be an overriding factor influencing spawning in *G. paradoxa*. During the rainy sea-

son, increase in run-off water carries nutrients and debris from land into the river (Moses, 1987; Etim and Sankare, 1998). Etim and Taege (1993) observed that during the rainy season, there was an increase in water depth of the Cross River, Nigeria from 4 to 12m, a lowering of conductivity and a decrease in water temperature from 32°C in March to 20°C in July. These observations were, to a small extent, seen at the Volta estuary during the rainy/flooding season. The synergistic effect of several factors – a faster current, a slight drop in water temperature, lower food availability due to dilution of water, and lower DO levels experienced at the peak of the rainy season – may have acted as trigger for spawning in *G. paradoxa.* The findings of this study are in contrast to earlier work (Pople, 1966; Whyte, 1981) who indicated that the species spawns during the dry season (November-March). The pattern of gametogenesis and spawning observed in this study is in agreement with the findings of Etim (1996) who studied a population of *G. paradoxa* in the Cross River, Nigeria, using similar methods. He found that *G. paradoxa* spawned at the peak of the rainy season from June to October. Furthermore, the results of this study are corroborated by Lawson (1963) who observed that, during a mark-recapture experiment in the Volta River, recaptured specimens showing reduced growth during the flood months of July to December. It was concluded that the species was reproducing during that period. The condition indices employed in this study showed a clear relationship with the gametogenic stages and could be a useful and simple technique in the selection of broodstock for future hatchery operations as it is far less expensive and time consuming compared to histological methods. The data presented in this study provide valuable information on the timing and initiation of spawning in *G. paradoxa* in the Volta River which is necessary for developing sustainable management strategies and selection of broodstock for aquaculture.

Acknowledgement

The authors are grateful to Kwame Nkrumah University of Science and Technology, Kumasi, Ghana and the International Foundation of Science (IFS) for providing material and financial support (A/4421-1) to conduct the research.

References

Etim L. and T. Brey. 1994. Growth, productivity, and significance for fishery of the bivalve Egeria radiata (Donacidae) in the Cross River, Nigeria. ARCH. FISH. MAR. RES. 42(1): 63-75, 1994.

Etim L. and M. Taege. 1993. Comparison of condition indices and their seasonal variation in the freshwater clam, *Egeria radiata,* from the Cross River, Nigeria. Aquaculture and Fisheries Management 24, 603 -612.

King R.P. 2000. Poulation structure, growth performance and mortality rates of the freshwater clam *Galatea paradoxa* Born 1778) in Nun River, Nigeria. Archive of Fishery and Marine Research 48(1): 21-30.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EFFECT OF DIETARY BOVINE LACTOFERRIN ON RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) FECUNDITY AND LARVAL QUALITY

E. Ahmadian[1], N. Agh[2], A. Tokmechi[2], and R. Jalili[1]

[1] Department of Fisheries, Faculty of Natural Resources, Urmia University, Urmia, Iran
[2] Artemia and Aquatic Animals Research Institute, Urmia University, Urmia, Iran

Introduction

Fish egg quality can be affected by maternal age, condition factor, the timing of the spawning cycle, genetic factors, over-ripening processes, and stress (Swain et al., 2009, Çoban et al., 2011). However, research findings have proven that genetic background, physiological and environmental condition (stress and disease), general health status, and nutrition can influence the gamete production in fish (Swain et al., 2007; Quintero et al., 2009; Marimuthu et al., 2009).

Several immunostimulants are used in fish and shrimp including synthetic chemicals and natural biological substances (Galindo-Villegas and Hosokawa, 2004; Bricknell and Dalmo, 2005). Natural immunostimulants are easily degradable and safe for human health and the environment (Esteban et al., 2001; Kumari et al., 2003).

Antimicrobial activity and immunoregulatory and anti-stress properties of Lactoferrin (Lf) have been recognized during the last decade (Yokoyama et al., 2006; Lonnerdal 2009). This study was designed to check the probable positive effects of dietary Lf on improving the egg quality of rainbow trout.

Materials and methods

Forty-eight randomly selected four-year-old broodstock (24 males and 24 females) were transferred into rectangular polyethylene tanks of 1000-l capacity (6 pairs in each tank) containing 800 l water. The experiment was performed in a flow-through system with water coming from a deep well at a rate of 5 $l.min^{-1}$. The tanks were aerated well and physico-chemical parameters of water temperature, dissolved oxygen, and pH were maintained at 14.3±0.5°C, 8.8±0.5$mg.l^{-1}$, and 7.8±0.1, respectively. The photoperiod was maintained on at 6h:18h light:dark period.

Commercial trout feed (38% crude protein, 12% crude lipid, 10% ash, 3.5% fiber) was crushed and supplemented with bovine Lf (Biopole SA, Belgium) at levels of 0 (control), 100, 200, and 300mg.kg^{-1} diet. The diets were repelleted using a lab-scale extruder to obtain 8-mm-sized pellets. Diets were oven-dried (40°C) and stored at 4°C until used. The fish were fed the experimental diets to apparent satiation once daily for 90 day.

By the end of the feeding experiment (four weeks before the full maturation of females), males and females were transferred to separate tanks and checked for ripeness weekly. Ovulated females were separated and non-ovulated fish were returned to the tanks. Eggs were collected from the ripe females and total fecundity (TF), absolute fecundity (AF), relative fecundity (RF), and individual weight of eggs were measured. The fertilization rate, survival of larvae after eye formation until hatching, and hatching percentage were also measured.

Results and discussion

Results of this study showed that total fecundity was significantly higher in fish fed 200mg Lf.kg^{-1} compared to the control group ($P < 0.05$), but no significant differences among fish fed three different levels of Lf (Table I). There was no significant difference in individual weights of the eggs produced by experimental fish compared to the control group. Absolute fecundity was significantly higher in fish fed Lf compared to the control fish ($P < 0.05$), but no significant differences among fish fed three different levels of Lf. Relative fecundity was significantly higher in fish fed 200 and 300mg Lf.kg^{-1} compared to the control ($P < 0.05$). Results of this study also showed that bovine Lf supplementation decreased spawning time in rainbow trout especially in those fed 300mg.kg^{1} Lf (Table II).

Table I. Mean±std deviation, measured parameters of rainbow trout broodstock (Duncan test $P < 0.05$).

Treatment	Fish early weight (g)	Total fecundity (g)	Egg weight (g)	Absolute fecundity (N)	Relative fecundity (N.kg^{-1} bw)	Larval Survival (%)	Hatching %
Control	2661±233	304.8±57^{a}	0.067±0.007	4562±850^{a}	1650±341^{a}	91.8±3.3^{a}	67.49±5.17^{a}
100mg Lf.kg^{-1}	2816±366	397.5±53^{b}	0.066±0.008	6731±176^{b}	2197±660^{ab}	98±0.7^{b}	85.95±5.11^{b}
200mg Lf.kg^{-1}	2615±390	400.2±84^{b}	0.068±0.01	6509±1001^{b}	2381±308^{b}	98.1±1.1^{b}	88.93±3.66^{b}
300mg Lf.kg^{-1}	2474±474	381.08±92^{b}	0.069±0.008	6626±1658^{b}	2533±613^{b}	98.7±0.1^{b}	89.18±2.49^{b}

Different superscripts in each column indicates significant differences

Table II. Percent of broodstock spawning each week.

Treatment	Week 1	Week 2	Week 3	Week 4	Week 5	Total
Control	25	25	16.66	25	8.33	100
100mg Lf.kg^{-1}	33.33	33.33	33.33	0	0	100
200mg Lf.kg^{-1}	33.33	50	0	16.66	0	100
300mg Lf.kg^{-1}	66.66	0	33.33	0	0	100

The results showed that Lf has no significant effect on egg size, fertilization rate, and egg water absorption level, but fecundity increased significantly in fish fed 200mg Lf.kg^{-1} diet compared to control group. The results also showed that total fecundity increased significantly in all experimental groups fed Lf compared to control, however, relative fecundity were significantly higher only in groups fed 200 and 300mg Lf.kg^{-1} diet compared to control fish. Crude protein, total lipid, and total n-3 fatty acids increases significantly in fish fed 200mg Lf.kg^{-1} diet compared to control fish. Significantly higher survival of embryos during incubation period and hatchability of eggs were observed in all groups fed Lf compared to fish received commercial diet. There is no published literature on effect of Lactoferrin on fish fecundity to compare the results obtained. However, the broodstock used in this study were brought up right from the larval stage of the same batch of larvae collected from a local rainbow trout hatchery; therefore the fish were of the same age and almost same size.

The proven role of Lf on stimulating immunity and reducing stress in fish (Esteban et al., 2005; Yokoyama et al., 2006; Lonnerdal, 2009; Rahimnejad et al., 2012) allows us to assume that in the current experiment improving fecundity factors and acceleration of the sexual maturity could have resulted due to stress reducing and immunity improving role of Lf. Results of this study may be comparable with effects of probiotic (as an immune stimulator and stress reducer) on improving reproduction and fecundity (Lombardo et al., 2011). Based on the results obtained, supplementation of 200mg Lf.kg^{-1} diet of rainbow trout broodstock may be recommended for improving the fecundity rate, egg quality, and survival of embryos. These results can assist in laying the groundwork for future research on supplementation of immunostimulants in broodstock diet for improving reproductive output.

References

Bricknell I and RA Dalmo. 2005. The use of immunostimulants in fish larval aquaculture. Fish & Shellfish Immunology 19(5):457-72.

Çoban D., H.O. Kamaci, C. Suzer, Ş. Yıldırım, G. Arda, A.Y. Korkut, Ş Saka, and K. Fırat. 2011. Effect of Some Morphometric Characteristics on Egg Quality in Common Dentex, *Dentex dentex* (Linnaeus, 1758). Turkish Journal of Fisheries and Aquatic Sciences 11: 425-431.

Esteban M.A., A. Rodríguez, A. Cuesta, and J. Meseguer. 2005. Effects of lactoferrin on non-specific immune responses of gilthead seabream (*Sparus aurata*). Fish & Shellfish Immunology 18:109-124

Esteban M.A., A. Cuesta, J. Ortuño, and J. Meseguer. 2001. Immunomodulatory effects of dietary intake of chitin on gilthead seabream (*Sparus aurata* L.) innate immune system. Fish & Shellfish Immunology 11:303-315.

Galindo-Villegas J. and H. Hosokawa. 2004. Immunostimulants: Towards temporary prevention of diseases in marine fish. In: Proceeding of VII International Symposium on Aquaculture Nutrition, 16-19 Nov, 2004, Hermosillo, Sonora, Mexico, pp 279-319.

Kumari J., T. Swain, and P.K. Sahoo. 2003. Dietary bovine lactoferrin induces changes in immunity level and disease resistance in Asian catfish *Clarias batrachus*. Veterinary Immunology and Immunopathology 94:1-9.

Lonnerdal B. 2009. Nutritional roles of lactoferrin. Current Opinion in Clinical Nutrition& Metabolic Care 12:293–297.

Marimuthu K., A. Jesu Arokiaraj, and M.A. Haniffa. 2009. Effect of Diet Quality on Seed Production of the Spotted Snakehead (*Channa Punctatus*) (Bloch) American-Eurasian Journal of Sustainable Agriculture 3(3):344-347.

Quintero H.E., E. Durland, D. Allendavis, and R. Dunham. 2009. Effect of lipid supplementation on reproductive performance of female Channal catfish, *Ictalurus punctatus*, induced and strip-spawned for hybridization. Aquaculture Nutrition doi: 10.1111/j.1365-2095.2009.00709.

Rahimnejad S., N. Agh, M.R. Kalbassi, and S. Khosravi. 2012. Effect of dietary bovine lactoferrin on growth,haematology and non-specific immune response in rainbow trout (*Oncorhynchus mykiss*). Aquaculture Research 43:1451-1459.

Swain P and Nayak S.K., 2009. Role of maternally derived immunity in fish. Fish & Shellfish Immunology 27: 89–99.

Swain P., S. Dash, P.K. Sahoo, P. Routray, S.K. Sahoo, S.D. Gupta, P.K. Meher, and N. Sarangi. 2007. Non-specific immune parameters of brood Indian major carp Labeo rohita and their seasonal variations. Fish & Shellfish Immunology 22:38-43.

Yokoyama S., S. Koshio, N. Takakura, K. Oshida, K. Ishikawa, M. Gallardo-Cigarroa, J. Francisco, M.R. Catacutan, and S. Teshima. 2006. Effect of dietary bovine lactoferrin on growth response, tolerance to air exposure and low salinity stress conditions in orange spotted grouper *Epinephelus coioides*. Aquaculture 255:507–513.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

ARCHITECTURE FOR AUTOMATION AND TELEPRESENCE IN A MARINE HATCHERY LABORATORY

J.A. Alfredsen[1], M.O. Alver[1,2], and T.I. Eriksen[1]

[1] Norwegian University of Science and Technology (NTNU), Department of Engineering Cybernetics, NO-7491 Trondheim, Norway
[2] SINTEF Fisheries and Aquaculture, PO Box 4762 Sluppen, 7465 Trondheim, Norway

The CodTech hatchery automation laboratory was established by NTNU and SINTEF to explore ICT and process automation principles in the context of marine larval and juvenile fish production. This was motivated by the prospects of achieving higher survival rates and juvenile quality through better tools for continuous monitoring and control of hatchery processes, as well as relieving hatchery personnel from laborious and repetitive tasks. These are benefits that are well-known from other industries where automation has been more extensively employed. Process automation has also enabled new modes of hatchery operation and exploring rearing strategies that were previously unfeasible using manual labour, e.g., closed-loop control of the live feed density in larval tanks.

With networked computers monitoring and controlling an increasing number of laboratory tasks, the question of providing the lab operator with access to real-time information about the state of the hatchery processes from remote locations, as well as the ability to manipulate them, is getting more relevant. The use of telepresence in the laboratory adds flexibility to the operation of the facility and may allow closer and more continuous follow-up of experiments. However, making real-world equipment remotely accessible through the internet raises a number of serious issues with respect to safety and security that must be treated with utmost care. The question of adding telepresence to the CodTech laboratory increased in importance when the facility became part of the EU FP7 Capacities project Aquaculture infrastructures for excellence in European fish research (www.aquaexcel.eu), where transnational access for researchers to the facility must be handled efficiently. Here we present the architecture for automation and telepresence which has been implemented in the CodTech laboratory.

The laboratory includes an automated rotifer production unit, a robotic larval feeding system, automatic rotifer density sensing in rotifer cultures and larval tanks, as well as instruments for inspecting, measuring, and controlling the tank environment. Digital cameras covering the lab hall, the feeding robot, and all larval tanks provide the operator with remote visual input from the laboratory

environment. The total system comprises a diverse set of instrumentation and equipment from different providers, and each subsystem typically has its own user interface and way of operating, and connecting them together is usually not straightforward. Providing remote access to the laboratory without a common point of entry would appear awkward and confusing for the operator, and safe operation and security could potentially be at risk. The different subsystems of the CodTech laboratory are therefore tied together and integrated using a supervisory control and data acquisition (SCADA) system and user interface based on the Proview open-source SCADA and HMI platform. Proview is able to communicate with most types of process equipment and instrumentation, and provides the tools needed to build a consistent and coherent interface to the system for different groups of laboratory operators (regular users, lab managers, maintenance personnel, etc.).

As regards the remote access to the CodTech laboratory, both the physical layout of the network as well as the software design has been done in a way that seeks to minimize the risk of security breaches. The first step is to limit the parts of the system which are exposed to the internet to a single point of entry. This means that all communication between the control system and the outside world is tunnelled through this point. The entry point is secured by a firewall in terms of a dedicated security appliance, with a VPN login solution that requires valid credentials to gain access. When a user logs in through the VPN portal, the system is set up to only grant the user access to specific web pages which are used for controlling the system. Any login to the system is being logged to a separate file, and there is an alarm system which can be used to notify a network administrator of any potential breaches.

Beyond the entry point, there is a completely separate security layer within Proview. Access to the system can be controlled on a per-user basis, with different levels of access ranging from monitoring simple variables, to full control of the system. This mechanism will for instance be employed to allow a remote user to monitor variables, but not change them. The existence of users within Proview, also allows logs to be kept of individual user's actions within the system, a feature that can be valuable in case of any breaches or irregularities. For the CodTech laboratory, it was decided that the best approach is to allow users logging in through the remote portal to monitor the system directly. This means that only a single set of credentials is necessary to monitor the status of an experiment. Users that need access to change variables is required to use two separate credentials, which further heightens the security without causing unnecessary work for lower risk operations.

Based on user experiences gathered during the AQUAEXCEL project the potential benefit of the CodTech e-infrastructure will be evaluated and made available to laboratories and hatcheries planning to build similar capacity.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

DEVELOPMENTAL STAGING AND DEFORMITIES CHARACTERIZATION OF THE EURASIAN PERCH, *PERCA FLUVIATILIS*

M. Alix, B. Schaerlinger, Y. Ledoré, D. Chardard, and P. Fontaine

University of Lorraine, UR AFPA, Vandoeuvre-les-Nancy, F-54506, France

Introduction

Understanding the mechanisms regulating reproduction is one of the most important steps in controlling fish breeding in aquaculture, and can affect both broodstock and offspring. Several fish species exhibit high mortality and deformity rates following embryogenesis or at the larval stages. However, few studies have focused on early embryogenesis to better understand the reasons for this. This information is, however, important when linking broodstock physiology, gamete quality, and developmental defects observed later at hatching. Several genetic and environmental parameters are known to affect fish embryogenesis, but the knowledge of the effects of factors linked to broodstock management and status (e.g., domestication level) is still sparse. For example, a recent study showed that domesticated coho salmon lay smaller eggs with faster early development compared to their wild counterparts (Neely et al., 2012). This type of study requires preliminary knowledge of normal embryonic development. Generally, fish embryogenesis can be divided into five steps: cleavage (cell division), gastrulation (cell movements to surround the yolk), organogenesis, hatching, and free larvae (before first feeding), but the timing of each step is different between species. A first description of *Perca fluviatilis* development focused on late embryonic and larval developments (Konstantinov, 1957). In this study, we aimed at defining key steps of the embryonic development of *P. fluviatilis* and then describing the occurrences of early stages of lethality and/or deformities.

Material and methods

Two experiments have been conducted to determine mortality and deformity impacts. The first was performed in 2010 on domesticated fish (Geneva Lake origin) provided by Lucas Perches (Hampont, France) and wild animals originated from the Geneva lake (fertilized eggs supplied by INRA, Thonon-les-bains, France). Reproduction was induced indoors using a photo-thermal program (Abdulfatah et al., 2013). Artificial fertilizations were performed to determine the beginning of development. The second experiment was performed in 2013 on two other groups: domesticated animals from the same origin, reared in

the same conditions, and a wild stock from Lorraine ponds (provided by GAEC Piscicole du Saulnois, France). Both populations were transferred to outdoor facilities in March one month before the spawning period. Artificial spawning was performed according to Zarski et al. (2012). Embryos were then transferred and maintained in the hatchery (13°C) until hatching. The early embryogenesis was first analyzed by time-lapse-video using a light upright microscope and the NIS BR software (Nikon). Randomly chosen samples of each spawn were monitored daily for 3 days (around 150-300 eggs per spawn) to determine mortalities during this period. The hatching rates were established with 3 samples of 150 eggs (separated from spawning after fertilization to avoid any manipulation during the development). The experiments were stopped after hatching and each larva was collected at hatch and fixed in 4% paraformaldehyde.

Results and discussion

We first determined the main duration of each step of *P. fluviatilis* development from fertilization to hatching. We found that embryos hatched from 6 to 12 days post-fertilization (dpf) independent of breeder origin. This is short compared to the closely related yellow perch *P. flavescens* which hatches after 25-27dpf at temperatures ranging from 8-12°C (Mansueti, 1964). The cell cleavage begins a few hours after fertilization and lasts for one day before the start of gastrulation (around 24h pf). By the end of the second day, organogenesis begins with the definition of the antero-posterior axis and lasts until hatching. Interestingly, the duration of the hatching period is constant regardless of when hatching starts (6-9dpf depending on the spawn, mean hatching duration = 3.7±0.65d, n=11 spawn) and embryos seem to hatch at different developmental stages based on their eye pigmentation. This agrees with a previous study proposing 4 stages of development at hatching related to larval size for *P. fluviatilis* (Konstantinov, 1957). In addition, Mansueti (1964) observed that *P. flavescens* hatches around 3 days with diverse yolk volumes between the first and last hatched embryos. The accurate knowledge of these developmental steps allows better definition of the timing of mortality or occurrence of abnormalities. Indeed, numerous studies define the fertilization rate after 3 days of development. Our data show that within this period a large number of cellular processes have already occurred. It suggests that calculated fertilization rates at 3 days may better provide information about fertilization and early development impairment than solely fertilization success. In order to check this hypothesis, we carefully analyzed this period by checking daily the mortality rate. Our data were variable depending on the spawner (whatever the breeder origin) and showed several main mortality stages. First, 35% of the spawn didn't undergo any embryogenesis and thus completely lacked fertilization, 45% died before hatching, and 20% hatched (n=20). Among the embryos that died before hatching, most of them died within the 24 first hours, with a less important mortality rate between 24 and 48h. When we looked more closely to the associated phenotypes, we observed a large diversity of

cleavage defects. This could be due to defects in several important pathways involved in the cell division (e.g., orientation, cell cycle control, etc.). Moreover, several embryos stopped during gastrulation, leading to an extra mortality occurrence between 48 and 72h. At this stage, developmental defects may involve other pathways controlling cell movements. Later on, embryos still died due to defects in the organogenesis process but with a less important occurrence.

Conclusion

As a conclusion, our work first demonstrates that *P. fluviatilis* embryogenesis last for 6 to 12 days but with short cleavage and gastrulation periods compared to organogenesis. The hatching duration is very constant regardless of the date of first hatching and embryos can hatch at diverse developmental stages. Moreover, we showed that the calculation of fertilization rate after 3 days of development is not accurate enough to properly characterize fertilization defects as severe mortality occurrences appear during this period. During the first cell divisions, embryos are still under the control of maternal genes, thereafter the midblastula transition occurs in order to switch under the control of zygotic embryonic genes. This transition is a very important stage as many cellular pathways can be impaired during this process. Up to now, we have few data about the timing of this transition in different fish species but it seems to occur between the middle of the cell cleavage and the onset of the gastrulation. Further work is needed to better understand the reasons of early mortality and associated deformities.

References

Abdulfatah A., P. Fontaine, P. Kestemont, S. Milla, and M. Marie. 2013. Effects of the thermal threshold and the timing of temperature reduction on the initiation of reproduction cycle in female of Eurasian perch *Perca fluviatilis*. Aquaculture 376-379:90-96.

Konstantinov K.G. 1957. A comparative analysis of the morphology and biology of the yellow perch (*Perca fluviatilis*), the European pike-perch (*Lucioperca lucioperca*) and the Volga pike-perch (*Lucioperca volgensis*) at different stages of development. Academy of Sciences of USSR, Trudy Institute of Animal morphology 16:181-202.

Mansueti A.J. 1964. Early development of the yellow perch, *Perca flavescens*. Chesapeake Science 5:46-66.

Neely K.G., J.M. Myers, and J.J. Hard. 2012. A comparison of early development between a domesticated stock of coho salmon and its parental stock. Transactions of the American Fisheries Society 141:1504-1509.

Zarski D., A. Horvath, L. Kotrik, K. Targonska, K. Palinska, S. Krejszeff, Z. Bokor, B. Urbanyi, and D. Kucharczyk. 2012. Effect of different activating solutions on the fertilization ability of Eurasian perch, *Perca fluviatilis* eggs. Journal of Applied Ichthyology 28:967-972.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

PROVIDING HARPACTICOID COPEPODS VIA FLOATING SIEVE IMPROVES FISH LARVAL FEEDING SUCCESS

C. Arndt[1*], M. Moison[1,2], and U. Sommer[1]

[1] Helmholtz Centre for Ocean Research (GEOMAR), Düsternbrooker Weg 20, 24105 Kiel, Germany (*corresponding author: Tel.: +49 431 6004410; fax: +49 431 6004402. E-mail address: carndt@geomar.de)
[2] Current address: Institute of Oceanography and Institute of Ecology and Evolutionary Biology, National Taiwan University, No. 1, Sec. 4, Roosevelt Rd. Taipei, 10617, Taiwan

Introduction

Rotifers (*Brachionus* sp.) are the classically used and easy-to-rear live food species but are often of suboptimal food quality for fish. Copepods are known to improve survival, growth, and development of fish larvae by increasing the food quality. Harpacticoid copepods may have an advantage over calanoid copepods (diverse food sources, higher culture density, lower escape abilities). However, their orientation towards the surface of the larval fish tank wall is potentially posing a problem for fish larvae searching pelagic food. It is therefore of interest for the aquaculture industry whether harpacticoid copepods are sufficiently available for fish larvae. The obstacle of low prey availability might be overcome by providing the copepods via a floating sieve.

Consequently, the swimming behaviour of herring larvae (*Clupea harengus*) together with their feeding success was analysed in relation to two different food sources (harpacticoid copepods *Tachidius discipes* as well as the rotifer *Brachionus plicatilis*) and food supply methods (via sieve) using a 2D video analysis by addressing following questions: (1) are pelagic fish larvae able to perceive and feed on benthic prey, and (2) is the sieve supply method improving the larval feeding success?

Materials and methods

The behaviour of herring larvae (*C. harengus*) in relation to different prey was tested in a video experiment. *T. discipes*, a harpacticoid copepod, and *B. plicatilis* were offered as prey. The video analyses were conducted at a larval age of 10dph at 11°C. 30 fish larvae were transferred into a 3-l glass aquarium containing filtered seawater. The 2D video set-up consisted of a single camera oriented

orthogonally to the aquarium. Three different food conditions were tested: (1) *T. discipes*, (2) *T. discipes* via a floating sieve to analyse the influence of an indirect supply method; and (3) *B. plicatilis* to compare a commonly used live feed species with harpacticoid copepods. The tracks of fish larvae were analysed for swimming speed and swimming direction and five swimming states were defined. Subsequently, the percentage of larvae which had at least one prey item in the gut (feeding success) was determined for each feeding condition.

Results and discussion

Directly *Tachidius*-fed larvae never entered the slow swimming state during experimental observation, but spent more time in the normal swimming state than *Brachionus* and *Tachidius* via sieve-fed larvae (Fig. 1). Both swimming states are considered to signify searching in herring larvae. Consequently, directly copepod-fed larvae spent probably more energy for searching than rotifer-fed larvae, because normal swimming consumes more energy than slow swimming.

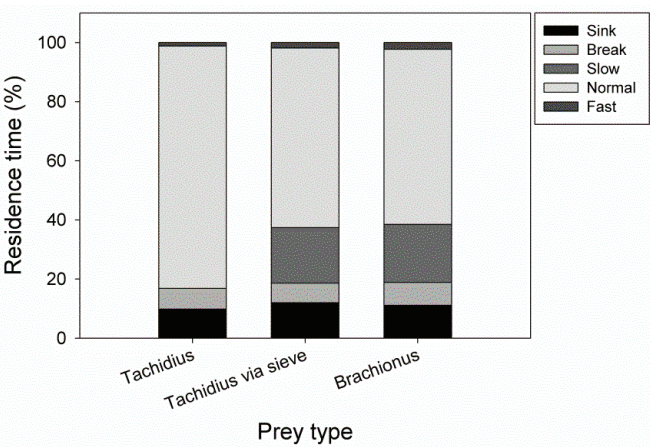


Fig. 1. Larval swimming behaviour of 10 days old herring larvae (*Clupea harengus*) in dependence of prey type (*Tachidius discipes*, *Brachionus plicatilis*) and supply method (*T. discipes* offered via a floating sieve). Mean of residence time (%) spent in each of the five states.

Furthermore, the feeding success of larvae fed *Tachidius* directly was lowest (22%). The provision of *Tachidius* via a sieve led to an increased feeding success of 35%.

The provision of harpacticoid copepods via a sieve combines the advantageous characteristic of rotifer-fed larvae, i.e., high capture success and presumably low energy expenditure but also the high nutritional value of copepods. Consequently, when harpacticoid copepods are used as food organisms, their provision via a floating sieve is recommended to improve the rearing of marine fish larvae.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

CONTROL OF THE SELECTIVE PRESSURE ON MICROBES OF THE INCOMING WATER INCREASES SURVIVAL OF MARINE FISH LARVAE

K. Attramadal[1*], G. Øie[2], E. Kjørsvik[1], G. Minniti[1], J. Skjermo[2], I. Bakke[3], M.-A. Østensen[2], Y. Olsen[1], and O. Vadstein[3]

[1] Department of Biology, NTNU, 7491 Trondheim, Norway
[2] SINTEF Fisheries and Aquaculture, Department of Marine Resources Technology, 7465 Trondheim, Norway
[3] Department of Biotechnology, NTNU, 7491 Trondheim, Norway

For most intensive aquaculture systems, selection pressure on microbes of the incoming water to the fish tanks differs from that of the rearing water. Efforts made in microbial management are most commonly directed towards control of the incoming water (e.g. disinfection, removal of organic matter). However, larvae are affected by the microbial composition in the tank, which is often very different from that of the incoming water. Through experiments comparing different methods to apply selective pressure on microbes of the incoming water, increased control of the microbial community composition of the rearing tanks may be achieved by minimizing the difference between the selective environment in the incoming water and the tank water. Two important factors influencing this difference are the microbial carrying capacity (supply of organic matter) and the number of competing bacteria present. Water treatment of the incoming water studied included recirculating aquaculture systems (RAS) with different types of or no disinfection, and microbial maturation of water with and without feeding of organic matter to the maturation unit. The microbial control of the tank water was improved by these treatments, with beneficial effects on the fish. The microbial community in the RAS treatment loop has the possibility to stabilize at a similar carrying capacity as in rearing tanks, which could limit the chances of proliferation of opportunistic microbes in the rearing water. In Experiment 1, the development of the microbial community in a RAS with moderate ozonation was compared to that of a conventional flow-through system (FTS) for Atlantic cod (*Gadus morhua*). The RAS developed and maintained a more diverse and stable microbial community composition compared to the FTS. Water treatment regime explained most of the variation in microbial composition during the live feed period, and accounted for a tenfold higher variation in the composition of bacteria in the FTS than in the RAS. The RAS had a more even microbial community structure with higher species diversity. The average

survival was 11.5% in the RAS and 2.3% in the FTS 30 days post-hatching (dph). In Experiment 2 with Atlantic cod larvae, two different marine RAS, one with moderate ozonation (RAS_{O3}), and one with high intensity UV-irradiation (RAS_{UV}) were compared with a FTS. The two RAS developed a different and more stable microbial community than the FTS. The density and the growth of bacteria were higher in the rearing tanks than in the incoming water in the RAS_{UV}, whereas for the RAS_{O3}, both densities and growth of bacteria were similar, indicating low disinfection efficiency. As a consequence, the RAS_{O3} showed a more mature and stable microbial community than the RAS_{UV}. Survival of larvae was 21±2%, 36±12%, and 16±2% on day 30dph for the RAS_{UV}, RAS_{O3}, and FTS, respectively. Experiment 2 suggested that strong disinfection within the RAS loop may reduce the maturing effect of the microbial community. In Experiment 3 with Atlantic cod larvae, a RAS without disinfection was compared to a FTS and a microbially matured flow-through system (MMS) where incoming water passed a maturing unit (biofilter) before entering the larval tanks. Abundance, diversity, and growth of bacteria in the rearing water were higher in the RAS than in the other systems. The gap in microbial carrying capacity between the incoming water and the rearing water was reduced in the RAS compared to the FTS or MMS, as the incoming water of the RAS showed higher abundance and growth of bacteria. The microbial composition of the incoming water differed significantly between treatments, and the microbial composition of the incoming water in the RAS and MMS was more stable over time than that in the FTS. The survival of larvae was significantly higher in the RAS (29±3%) and MMS (28±5%) than in the FTS (17±4%). In Experiment 4, two MMS with Ballan wrasse (*Labrus bergylta*) were compared: one was fed with fish feed to increase the carrying capacity of the maturing unit (FMS), whereas the other was matured without enhancing the carrying capacity of the incoming water (MMS). The number of bacteria was equal or higher in the incoming water than in the fish tanks for the FMS. In comparison, the number of bacteria was always significantly higher in the fish tanks of the MMS than in the incoming water. The microbial growth potential in the water of the fish tanks (growth in sample incubated 3 days compared to sample fixated immediately) was stable and low in the FMS, indicating a crowded environment that is resistant to invasion. In the MMS, the microbial growth potential of the tank water varied considerably, it was never lower than that in the FMS, and was sometimes very high. This indicates a less controlled system. There was a high similarity between the microbial composition of the incoming water and the tank water in the FMS compared to the MMS. The larvae in the FMS showed lower survival (5±1% at day 27dph) compared to the MMS (20±3%) due to high initial mortality related to H_2S in the FMS. The surviving FMS larvae showed significantly higher growth and stress tolerance than those in the MMS. These four experiments support the hypothesis that the similarity of the selective conditions for the microbes in the incoming water and rearing water is key to microbial control in the fish tanks, and justifies optimization of water treatment of the incoming water to benefit larviculture.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EFFECT OF RED CRAB MEAL (*PLEURONCODES PLANIPES*) ON GROWTH AND DIGESTIVE ENZYME EXPRESSION IN THE INTESTINE OF WHITE SHRIMP (*LITOPENAEUS VANNAMEI*)

E.G. Ayala-Borboa[1*], D. Tovar-Ramírez[1], R. Civera-Cerecedo[1], M. Rojas-Contreras[2], M.A. Cadena-Roa[2], H. Nolasco-Soria[1], and E. Goytortúa-Bores[1]

[1] Centro de investigaciones Biológicas del Noroeste, La Paz, B. C. S., México. *eayala@cibnor.mx
[2] Universidad Autónoma de Baja California Sur, La Paz, B. C. S., México

Introduction

The white shrimp (*Litopenaeus vannamei*) is one of the most important commercially cultured species. As the industry grows, the need for artificial diets and animal protein is more relevant, however at a significant cost. Aquaculture consumes about 10% of the world production of fishmeal, calling for the exploration of new sources of quality and affordable protein. In Mexico, *Pleuroncodes planipes* is a natural resource with great potential to be used as source of protein (Ehrahdt and Ramírez, 1982).

The enzymes activity in the digestive tract of any organism plays a key role in the assimilation and adaptation of nutrients from food, and for this reason it is necessary to understand the mechanisms of gene expression, regulation, and activity of these enzymes. Catabolic digestive functions of the shrimp are based mostly in the digestive gland; however, more studies are needed to know the role of intestinal digestive enzymes and those responsible for the absorption and assimilation of nutrients. The objective of this study was to assess the activity and expression of genes coding for the main digestive enzymes from the intestine of the white shrimp (*L. vannamei*) fed with red crab meal, using microarrays and real-time PCR.

Materials and methods

Shrimp (*L. vannamei)* post-larvae were donated by Acuacultura Mahr (La Paz, B.C.S., Mexico). The organisms were acclimated in plastic with 2000-l^{-1} tanks, at 27°C and salinity of 35UPS, at the UABCS, Pichilingue unit. Shrimp were fed twice a day with a commercial diet with 35% protein food, until they reached the size required for the experiment of 0.2-0.5g (juveniles). Juvenile shrimps were fed with red crab meal or fish meal as control for 31 days in a recirculatory sys-

tem. At the beginning of the experiment, food was delivered at a rate of 10% of the total biomass and then adjusted according to daily consumption ad libitum. In this work we used 360 shrimp with average weight of 0.25±0.01g, randomly distributed among twelve 200-l tanks to a density of 30 organisms per tank. Six tanks were fed with diet control (fish meal) and six with the experimental diet (Crab meal). The food was distributed manually in two daily rations (50% at 8am and 50% at 3pm) for 31 days. Growth, survival, and feed conversion rate (FCR) were measured at the final of experiment. At 0 and 31 days of feeding, shrimp in intermolt stage were euthanized and were sampled the intestines to RNA extraction (Trizol®).

TRIzol® Reagent (Invitrogen,) was used for total RNA extraction. Then, 1µg total RNA was reverse-transcribed to cDNA with Improm II kit and conjugated with Alexa555 and Alexa 647 to be hybridized on a microchip of the total genome (15 139 genes) of the fruit fly *Drosophila melanogaster*. The reading was conducted in Genepix 4100 A and reader-generated files were processed with Genepix software. The overexpressed and/or underregulated genes were obtained by means of a threshold set at 2 (Z score) in the Genarise software. cDNA pooled from shrimp fed red crab or control diet were used to construct standard curves for each analyzed gene using specific primers for amylase, lipase, trypsin, aminopeptidase, and chymotrypsin according to Tovar et al. (2010). The Ef1á was chosen as a reference gene. The relative gene expressions were determined according to the ÄÄCq method using a CFX96 touch real time PCR system and SsoAdvanced SYBR green supermix (Bio-Rad).

A statistical design was used with six replicates for both treatments. STATISTICA software was used for data analysis, consisting in a Kolgomorov-Smirnov, the Levene test for homoscedasticity, as well as the ANOVA analysis to determine significant differences between the two treatments and the Tukey test, with a confidence level of 95%.

Results and discussion

The growth and survival were affected by the inclusion of red crab meal in the food. The weight gain was significantly higher ($P \leq 0.05$) in shrimp fed with red crab meal (Fig. 1) during the experiment; and survival was greater than 90% and 79% for the shrimps fed with fish meal and red crab meal, respectively. The feed conversion ratio was not different based on feed type ($P > 0.05$). A value of 1.61±0.10 was obtained for crab meal and 1.80±0.16 for fishmeal. It is known that the red crab meal inclusion into the diet gives not only a greater protein digestibility but also an attractability and also allows better growth, an increase in proteolytic activity in the digestive gland, and improved survival.

Not only was an improvement in the growth rate observed in this study, but there was also an increase in food consumption. This could indicate that the increase in growth rate is a consequence of increase in food consumption according to Cruz-Suarez et al. (1993).

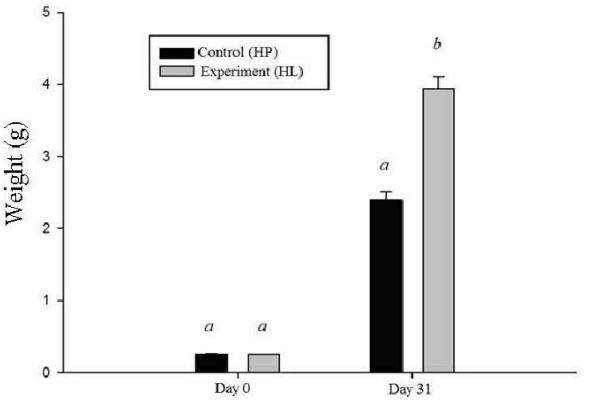


Fig. 1. Effect of two diets on weight gain. The average ± S.D. show different letters indicate significant differences. (P≤0.05).

The microarray analysis of gene expression using DAVID (Functional Annotation Bioinformatics Microarray Analysis) showed 416 overexpressed genes, where only 238 were annotated and 68 genes were involved in metabolic functions related with digestive enzymes such as phospholipase, triacylglycerol lipase, peptidase, and lipase. Alternatively, 302 genes were underexpressed and 173 were only analyzed. Relative expression patterns were obtained by using the elongation factor 1 (EF1α) as reference gene and Sybr green by RTqPCR. (Fig. 2).

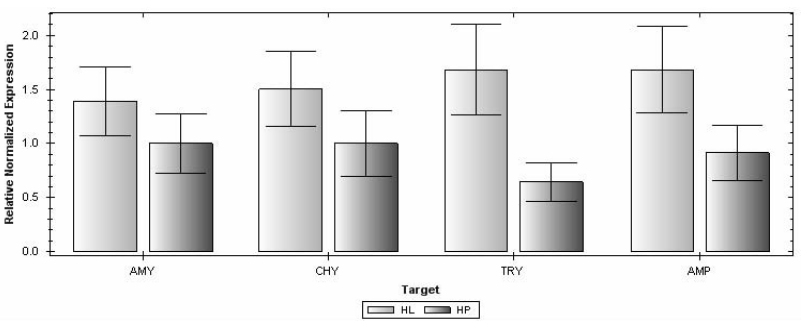


Fig. 2. Expression level of amylase (AMY), chymotrypsin (CHY), trypsin (TRY), and aminopeptidase (AMP) relative to elongation factor 1 (EF1α) in shrimp fed red crab meal (HL) and fishmeal (HP).

Our results show that expression is higher, although not statistically different (P<0.05), with shrimp fed crab meal. Digestion and assimilation of nutrients re-

quires the action of a large number of digestive enzymes that are located in the interior of cells that are found in all organs involved in digestion as the stomach, the digestive gland, intestine, and the pyloric caeca. Since 1967, studies on the digestive capacity of the shrimp have only focused on the digestive gland, so there are no studies of enzyme activity or gene expression in the intestine related to digestive functions of shrimp. At present, the interest in microbiota in the nutrient processing is increasing using genomic approaches (Rungrassamee et al., 2013). This work is the first study demonstrating the existence of gene expression coding for the main digestive enzymes in the shrimp *P. vannamei* intestine.

Conclusion

The results show that red crab meal can be considered as a source of high quality protein and a good substitute for fishmeal since it promotes the growth of shrimp through better use of food nutrients and better digestive enzymatic capacity.

Acknowledgments

The UABCS for the facilities and support provided to perform the bioassay and CONACYT by scholarship 416918. Grant 2009-C02-126427 SAGARPA-CONACYT. Thanks for Technical assistance to Biol. Patricia Hinojosa Baltasar, Biol. Mar. Hever Lastisnere Barragan, M. C Gutiérrez Rivera, Jesús Neftalí, and Dr. Jorge Ramírez.

References

Cruz-Suárez L.E., D. Ricque-Marie, J.A. Martinez-Vega, and P. Wesche-Ebeling. 1993. Evaluation of two shrimp by-product meals as protein sources in diets for Penaeus vannamei. Aquaculture 115:53-62.

Ehrarhdt N.E and P. Ramírez. 1982. Evaluación de los recursos demersales accesibles y redes de arrastre de fondo de la península de B.C, México. INP. Serie científica 23:10-46.

Heid C.A., J. Stevens, K.J. Livak, and P.M. Williams. 1996. Real time quantitative PCR. Genome Research 6: 986-994.

Rungrassamee W., A. Klanchui, S. Chaiyapechara, S., Maibunkaew, S. Tangphatsornruang, P. Jiravanichpaisal, and N. Karoonuthaisiri. 2013. Bacterial Population in Intestines of the Black Tiger Shrimp (Penaeus monodon) under Different Growth Stages. PLoS ONE 8(4): e60802. doi:10.1371/journal.pone.0060802.

Tovar-Ramírez D., D. Mazurais, J.F. Gatesoupe, P. Quazuguel, C.L. Cahu, and J.L. Zambonino-Infante. 2010. Dietary probiotic live yeast modulates antioxidant enzyme activities and gene expression of sea bass (Dicentrarchus labrax) larvae. Aquaculture 300:142-147.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

BACTERIAL COMMUNITY ASSEMBLY IN DEVELOPING COD LARVAE (*GADUS MORHUA*)

I. Bakke[1], J. Skjermo[2], K. Attramadal[3], E. Coward[4], E. Kjørsvik[3], Tu Anh Vo[3], Y. Olsen[3], and O. Vadstein[1]

[1] Department of Biotechnology, NTNU, 7491 Trondheim, Norway
[2] SINTEF Fisheries and Aquaculture, Department of Marine Resources Technology, 7465 Trondheim, Norway
[3] Department of Biology, NTNU, 7491 Trondheim, Norway
[4] Department of Cancer Research and Molecular Medicine, NTNU, 7491 Trondheim, Norway

In marine fish larviculture, negative bacteria-larva interactions are known to cause problems with low and unpredictable growth and survival. Healthy microflora could increase the resistance of larvae against detrimental bacteria. However, how environmental factors and ecological processes govern the assembly of the gastrointestinal (GI) microflora in developing fish larvae is poorly understood. We addressed this subject with results from several studies of the microflora associated with cod larvae.

In Experiment 1, the effect of diet was studied by rearing cod larvae on a diet consisting of copepods (*Acartia tonsa*) cultivated on the algae *Rhodomonas baltica* and diets comprised of rotifers (*Brachionus* 'Nevada') cultivated with either *R. baltica* (CR) or standard yeast/lipid (RR) from 3-22 days post-hatch (dph). Each diet study was done in triplicate rearing tanks. At 18dph, larvae in all tanks were fed *Artemia franciscana*. The microbial communities for individual larvae were assessed using PCR/DGGE. Statistical tests were applied to investigate differences in the larval microflora between groups of individuals. Although the microflora associated with the diets were very different, our results indicated that live feed was not a major influence on the composition of larval microflora. Several findings support this conclusion. First, no significant differences were found between microflora of larvae fed different diets, except at 8dph. Second, despite a change of live feed organism for all larvae at 18dph, the larval microflora was strikingly similar at 17 and 32dph. Third, the larval microflora was generally more similar to the water microflora than to the live feed microflora.

In Experiment 2, cod larvae were cultivated with rearing water from three different water treatment systems from hatching until 30dph: a flow-through system (FTS), a microbially matured flow-through system (MMS), and a recirculation

system (RAS). For all systems, larvae were reared in three replicate tanks. After 30dph, all tanks received MMS water. The microbial composition of the rearing water differed significantly between the FTS, MMS and RAS. The microflora of individual larvae were investigated by PCR/DGGE. Statistical analyses demonstrated that the larval microflora differed significantly between systems at 8, 17, and 30dph. However, at 46 and 60dph, no significant differences in larval microflora were found between the three systems. These results indicate that the larval microflora was influenced by the rearing water since (1) distinct rearing water resulted in different larval microflora, and (2) after change to MMS water to all tanks at 30dph, differences in larval microflora could no longer be observed.

Finally, we performed deep sequencing of barcoded 16S rDNA amplicons to characterize the composition of the microflora associated with individual cod larvae at different ages (8, 17, 32, and 61dph) at a high taxonomic resolution. The v4 region of the 16S rRNA gene was amplified and sequenced for individual cod larvae from two of the rearing tanks in Experiment 1. Also the microflora of the rearing water and live feed cultures were characterized. A large number of sequence reads were obtained (an average 6250 reads per larva after quality trimming), allowing for a detailed characterization of the microflora associated with larvae. Among 15 observed phyla, Proteobacteria was the most abundant, followed by Firmicutes, Bacteroidetes, and Actinobacteria. The richness and diversity of the larval microflora was highest at 61dph and lowest at 17 and 32dph. The composition of the microflora clearly changed with larval age. Characteristics of the 8dph larval microflora were a high abundance of *Pseudomonas* (mainly represented by one OTU), the presence of diverse β-proteobacteria, and a relatively high abundance of bacilli. At 17dph, the composition of the larval microflora was dramatically changed. High abundance (≤78% of total reads) of *Arcobacter* (ε-proteobacteria) was characteristic to the microflora of 17 and 32dph larva. Typically *Arcobacter* and γ-proteobacteria constituted >90% (≤98%) of the microfloras at these sampling times. At 61dph, the larval microflora was more diverse, and the γ-proteobacteria was represented by a variety of genera like *Colwellia*, *Photobacterium*, *Leucothrix*, *Vibiro*, and *Pseudomonas*. There was a relatively high abundance of Rhodobacteraceae (α-proteobacteria). Comparisons of larval, water, and live feed microfloras revealed that the most abundant OTUs in the 8dph larvae constituted only minor fractions of the live feed and water microfloras (0-0.1%). At 61dph, however, the larval microflora exhibited the highest similarity to the water microflora. Statistical analyses will be performed to investigate the effect of factors such as age, developmental stage of the gut, water microflora, and live feed microflora. Preliminary results suggest that the developmental stage of the gut system exerts strong selection on the larval microflora, particularly in the early stages. These results are consistent with the hypothesis that rearing water is more important than live feed as a source of bacteria for the cod larval microflora, and selection exerted by the larval gut system influences on microflora composition.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

INDUCER OF HEAT SHOCK PROTEIN 70: A NEW DISEASE PREVENTIVE OPTION IN AQUACULTURE PRODUCTION SYSTEMS

K. Baruah*, P. Norouzitallab, P. Sorgeloos, and P. Bossier

Laboratory of Aquaculture & *Artemia* Reference Center, Ghent University, Rozier 44, 9000 Ghent, Belgium. *baruahkartik4@rediffmail.com

Aquaculture has made very effective use of antibiotics either as a growth promoter or as a therapeutic in maintaining fish/shellfish health for half a century. However, now their usage is under severe scientific and public scrutiny due to their links to the development of resistant strains, which pose a great threat to human health. As a result of such concerns, the European Union imposed a complete ban on antibiotic growth promoters in animal production sector on January 1, 2006. Although a complete ban on the use of antibiotics has not been implemented in many countries, international pressure and public health concerns are likely to lead to such a scenario. However, there are also good indications that this (complete) ban could result in a higher frequency of pathogenic bacteria (such as *Salmonella* spp. and *Vibrio* spp.), which in turn could lead to a higher frequency of infections in animals as well as in consumers. Consequently, alternative anti-infective strategies must be developed to address public health concerns without compromising the efficiency of animal production. During the last two decades, data in the literature points to the role of heat shock proteins (Hsps) – a suite of evolutionary conserved proteins of varying molecular weight (16-100kDa) produced in all cellular organisms when they are exposed to cellular stress – as an attractive strategy for disease prevention in both humans and agriculture (aquaculture) animal species. Despite the ample success of Hsps in experimental therapies of various aquaculture animal diseases to date, there are still many impediments remaining toward developing a strategy for enhancing the availability of Hsps within the host or for delivering Hsps into the animals. So far, the classic stress inducer (a mild thermal shock) has generally been used because of the ease of application of such shocks to the cells, tissues or individual animals. However, such shock in an aquaculture system is not the best way to enhance Hsp production because acute temperature shifts are often detrimental, adversely affecting physiological balance and causing significant mortality of the cultured organisms. It has therefore become useful and beneficial to find less traumatic approaches for up-regulation of Hsp expression in aquatic organisms. Here, using examples from our series of in vivo studies in fish and crusta-

cean, we discuss the possible applications of an Hsp70 inducer as new protective modality for bacterial diseases in aquaculture production systems.

An Hsp inducer is a compound that can activate Hsp transcription factors (mainly HSF1, primary stress-inducible transcription factor) and induce Hsps by itself. Recently, the product Pro-Tex®, which contains the active compound Tex-OE®, a patented extract from the skin of the prickly pear fruit, *Opuntia ficus indica*, and which can act as a non-stressful effector that induces high levels of endogenous Hsps in animal tissues, has become available for use in fish and shellfish. To date, the principal commercial use of this product in aquaculture has been for reduction of transport stresses in salmon and sea bass culture. In a series of experimental studies using *Artemia* as a model organism, where we aimed to elucidate whether this product could also confer protection to abiotic and pathogenic biotic stressors, we showed that pretreatment of the crustacean with Tex-OE®, raised the Hsp70 levels and also provided protection against thermal and hypersalinity stresses. Results also demonstrated that Tex-OE® pretreatment remarkably protects *Artemia* against *V. campbellii* and *V. harveyi* challenges by eliciting the important components of the crustacean's innate immune response – the prophenolxidase (proPO) system – at both mRNA and protein activity levels, and nitric oxide level. Interestingly, this increase in the immune responses in the *Vibrio*-challenged *Artemia* pretreated with Tex-OE® was accompanied by a significant decrease of *Vibrio* count. In another study, Tex-OE® was shown to induce Hsp40, Hsp70, and Hsp90 in common carp and cross-protect the fish against various abiotic stressors, indicating that Tex-OE® is a potential inducer of stress proteins and thus could be an ideal candidate for use as an anti-stressor during various aquaculture practices.

Overall results suggested that Tex-OE® is undoubtedly beneficial for the induction of protective immunity in both fish and crustacean against various causative factors (abiotic and pathogenic biotic stressors) of diseases and thus it can potentially be a useful preventative treatment modality for (bacterial) diseases in aquaculture.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

COULD THE EASTERN ROCK LOBSTER, *SAGMARIASUS VERREAUXI,* BE THE BEST SPINY LOBSTER FOR AQUACULTURE?

S.C. Battaglene, C.J. Simon, Q.P. Fitzgibbon, G.G. Smith, and C.G. Carter

Institute for Marine and Antarctic Studies, University of Tasmania, Nubeena Crescent, Taroona, Tasmania, Australia, 7053

The commercial aquaculture of spiny lobsters (from egg) remains one of the great challenges in larviculture. Spiny lobsters are one of the most desired and expensive seafood worldwide, and consumer demand, especially from China, is growing rapidly. The complex and protracted life larval cycle of lobsters makes them especially difficult to culture. Aquaculture has been pioneered by the Japanese at a laboratory scale for >100 years. More recent research had been carried out in Australia centered on overcoming poor health, efficient feeding and upscaling systems. Most commercial attention has been paid to tropical species but one of the better biological candidates for commercial aquaculture is the temperate packhorse or eastern rock lobster *Sagmariasus verreauxi.* A native of eastern Australia and New Zealand it is the largest of the world's spiny lobsters, has a relatively short larval cycle, is gregarious (less cannibalistic than tropical species) and is one of the easiest species to culture. A review of seven years of temperate spiny lobster culture suggests *S. verreauxi* have all the attributes for commercial seed production.

From 2006 to 2010 newly-hatched phyllosoma larvae were mass-cultured in 200 to 500-l tanks. Stocked initially at 10 phyllosoma.l^{-1} cultures were maintained under a 12h light: 12h dark photoperiod and then reduced to 18:6 L:D during the late stages. Phyllosoma diet was supplemented with 2-mm pieces of blue mussel gonad at 0.025ml l^{-1} daily. From 2010 some larvae were also reared on a formulated feed. Larvae were cultured at 23°C. Seawater was highly filtered and ozonated to an oxidation-reduction potential (ORP) of 650mV. Seawater in larval tanks was reduced to enter at 350 to 450mV ORP. Dissolved oxygen was maintained between 105-110% saturation, salinity between 33-35 and total ammonia below 0.5mg.l^{-1}. Tanks were cleaned once a fortnight.

Overall survival from hatch to final stage phyllosoma rose to >20% and subsequent survival through metamorphosis >80% with an average larval duration of ~230 days in 2010. Improved survival during the early and mid-stages was due to better health management, feeding, and husbandry. In 2010, survival at meta-

morphosis was increased from <20% to >80% resolving a major bottleneck and resulting in a juvenile production of >250 juveniles. Growth rates were similar in 2006, 2008, and 2009 taking on average between 220 and 240 days to reach the final phyllosoma stage.

The challenges in *S. verreauxi* larviculture have primarily centered on maintaining good health through control of the microbial environment, improving larval nutrition, growth, and survival at molting. A principal cause of lobster mortality was the outbreak and spread of infectious diseases. The development of better systems to prevent bacterial infection of phyllosoma within intensive culture is important for achieving commercial spiny lobster production. We used a multi-pronged approach to combat bacterial disease in phyllosoma culture including the examination of ozonation water treatment, chemical disinfectants and probiotics. While disease outbreaks still occurred, mainly due to *Vibrio* contamination from *Artemia,* effective and cost efficient treatments are now available.

Mortality at metamorphosis has been a consistent problem encountered by all lobster research institutions across a range of species and would be debilitating for commercial production due to the loss of the large resource investment into the final stages after >200 days culture. Improved metamorphosis survival in *S. verreauxi* was achieved through the multiple application of better feeding strategies, health management, and improved provision of abiotic requirements through detailed experimentation. Photoperiod was particularly important in triggering metamorphosis and adjusting photoperiod reduced metamorphosis mortality.

We have now demonstrated that temperate lobsters can be reliably cultured through to juveniles at commercially sustainable survival rates. Considerable further research is required to improve economic viability of hatchery production. In particular, the development of an economical feed source to replace the use of live *Artemia* and mussel gonad is required to improve the commercial sustainability of culture. Furthermore, the development of up-scaled culture systems is required for large-scale production. The consistent and steady success achieved demonstrates sufficient "proof of concept" that *J. verreauxi* can be intensively cultured and thus potentially can be an exciting new option for aquaculture and possible stock-enhancement.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EFFECTS OF COMBINED PHOSPHOLIPIDS AND SELENIUM DIETARY CONTENTS ON PATTERNS OF BONE FORMATION IN THE AXIAL SKELETON OF *SPARUS AURATA*

T. Benítez-Santana*, R. Saleh, M.B. Betancor, A. Mesa, C.M. Hernández-Cruz, and M. Izquierdo

Grupo de Investigación en Acuicultura. ULPGC & ICCM. P.O. Box 56, 35200, Telde, Las Palmas, Canary Islands, Spain. (*Email: tibi.benitez.santana@gmail.com)

Introduction

Many genetic and epigenetic factors have been linked to skeletal abnormalities in cultured teleost fish (Lewis-McCrea and Lall, 2010). Among nutritional factors, several vitamins and minerals were recognized early to influence the occurrence of bone malformations (Betancor et al., 2012). Other nutrients such as dietary lipids have been also related to skeletal malformations (Cahu et al., 2003; Roo et al., 2009; Lewis-McCrea and Lall, 2010). Dietary phospholipids reduce the incidence of skeletal malformations in ayu larvae (*Plecoglossus altivelis;* Kanazawa et al., 1981), carp (*Cyprinus carpio;* Geurden et al., 1995), European sea bass (*Dicentrarchus labrax;* Cahu et al., 2003; Villeneuve et al., 2005), and gilthead seabream (Saleh et al., in press). Moreover, DHA-deficient dietary levels delay early mineralization and increase the risk of cranial and axial skeletal deformities (Izquierdo et al., 2012). Contrarily, excessive DHA levels without an adequate balance of antioxidant nutrients increase the production of free radicals damaging cartilaginous structures prior to bone formation (Izquierdo et al., 2012). Thus, the inclusion of complementary antioxidative factors such as selenium (Se) could counteract the high oxidation risk in early weaning diets high in polyunsaturated fatty acids (PUFA). The objective of the present study was to investigate the effect of graded levels of Se-derived yeast with krill PL (KPL), on skeletal development and its influence on bone formation in the axial skeleton, survival, and biochemical composition of sea bream larvae.

Material and methods

Sea bream larvae were completely weaned at 16dph and fed five isoenergetic and isoproteic microdiets for 30 days with different levels of Se: 2SE, 4SE, 6SE, 8SE, and 12SE (1.73, 3.91, 6.41, 8.47, 11.65 $mg.kg^{-1}$ dietary dry weight, respectively) and the same level of dietary phospholipids (PL): 4% krill PL (8% total dietary PL). To determine incidences of malformations, 100 larvae from each tank at 46dph were fixed in 10% buffered formalin. Prior to staining, larvae were

measured under a Profile Projector (Mitutoyo, PJ 3000, Japan) and divided in four size classes (<7.0mm, 7.0-9.0mm, 9.0-11.0mm and >11.0mm total length, TL) and mineralized bone stained with Alizarin red (Izquierdo et al., 2012). The different regions of the axial column were divided according to Boglione et al. (2001). The presence of supernumerary vertebral bodies, the presence of urinary calculus, and anomalies different from the ones described by Boglione et al. (2001) were analyzed separately. Observations were performed on the right side of the stained samples under a stereomicroscope (Wild, LEITZ). The anatomical terminology is according to Harder (1975) and Matsuoka (1987), with the exception of terminology for caudal fin structures, which is according to Schultze and Arrantia (1989). The numerical data set obtained was processed to calculate incidences and perform a descriptive analysis for each descriptor (anomaly typology) and diet. The surface corresponding to bone in whole coloured larvae was visualized and quantified using a computerized image analysis package (Image-Pro Plus®, Media Cybernetics, Maryland, USA). Selecting ranges of pixel values in colour images allowed the pixels associated with red to be distinguished. The number of selected pixels was then quantified using particle analysis and by counting the area of all bright objects (in pixels). Larval size was estimated by calculating the surface areas (in pixels) covered by whole stained larvae.

Results and discussion

Increasing dietary Se levels significantly improved larval survival, but did not affect larval growth. For fish size classes higher than 9mm TL, dietary Se inclusion up to 6SE significantly increased larval Se content, denoting the progressive absorption of this nutrient and markedly enhanced mineralization (Fig. 1). Anomalies were only determined on mineralized bones, and thus, larvae fed diets increasing in Se levels did not significantly affect vertebral anomalies in the pre-haemal region, dorsal fin, and cranium. However, larvae fed 6SE levels significantly reduce the presence of scoliosis and tended to reduce the axial skeletal deformities. The presence of polar lipids rich in n-3 HUFAs improves the incorporation of radiolabelled EPA into total and polar lipids of gilthead sea bream larvae (Izquierdo and Koven, 2011). Essential fatty acid deficiency has been associated with reduction in bone mineralization and increased renal calcification (Izquierdo et al., 2012). Similarly, in the present study feeding krill-derived PLs rich in n-3 HUFAs together with 6SE increased the larval content of EPA and DHA, particularly in the polar fraction, increasing mineralization. In summary, these results denoted the high efficiency of Se as an antioxidant factor and the importance of the inclusion of adequate levels in early weaning diets.

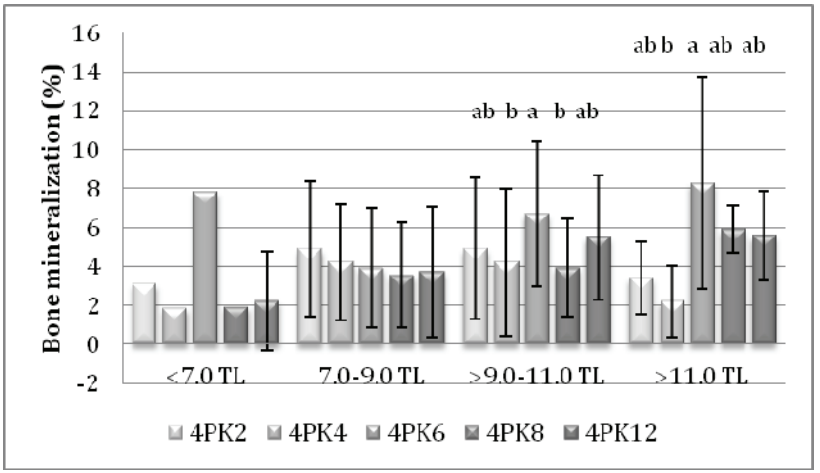


Fig. 1. Bone mineralization per larvae of each size class.

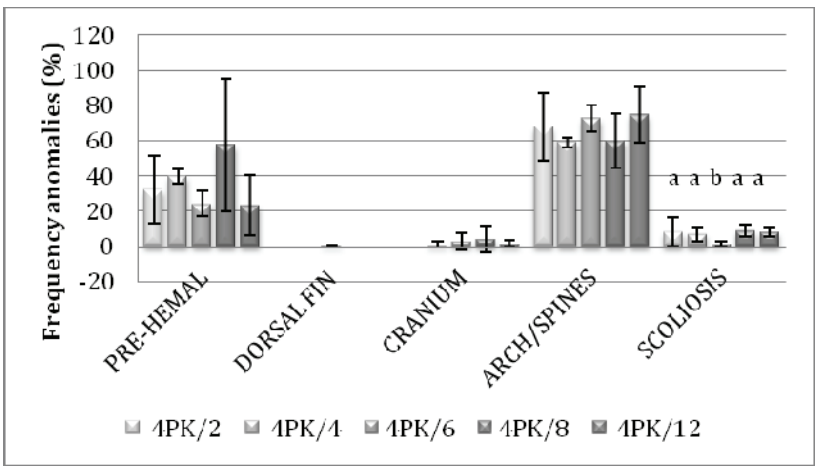


Fig. 2. Skeletal anomalies in gilthead sea bream larvae fed several levels of dietary Se (mean±standard deviation, different letters denote significant differences).

References

Betancor M.B., M.J. Caballero, G. Terova, S. Corà, R. Saleh, T. Benitez-Santana, G.J. Bell, C.M. Hernández-Cruz, and M.S. Izquierdo, M.S. 2012. Vitamin C enhances vitamin E status and reduces oxidative stress indicators in sea bass larvae fed high DHA microdiets. Lipids DOI 10.1007/s11745-012-3730-x

Boglione C., F. Gagliardi, M. Scardi, and S. Cataudella. 2001. Skeletal descriptors and quality assessment in larvae and post-larvae of wild caught and

hatchery reared gilthead sea bream (*Sparus aurata* L. 1758). Aquaculture 192:1-22.

Cahu C., J. Zambonino-Infante, and T. Takeuchi. 2003. Nutritional components affecting skeletal development in fish larvae. Aquaculture 227:245-258.

Geurden I., J. Raduenz-Neto, and P. Bergot. 1995. Essentiality of dietary phospholipids for carp (*Cyprinus carpio* L.) larvae. Aquaculture 131:303–314.

Harder W. 1975. Anatomy of Fishes. Part I. Text.E. Schweizerbart'sche Verlagsbuchhandlung (Nägele u. Obermiller), Stuttgart, 612 pp.

Izquierdo M.S. and W.M. Koven. 2011. Lipids. In: Larval Fish Nutrition (Holt, J. ed.), pp. 47–82. Wiley-Blackwell, John Wiley and Sons Publisher Editor, Oxford, United Kingdom.

Izquierdo M.S., M. Scolamacchia, M.B. Betancor, J. Roo, M.J. Caballero, G. Terova, and P.E. Witten. 2012. Effects of dietary DHA and a-tocopherol on bone development, early mineralisation and oxidative stress in Sparus aurata (Linnaeus, 1758) larvae. British Journal of Nutrition doi: 10.1017/S0007114512003935.

Kanazawa A., S. Teshima, S. Inamori, T. Iwashita, and A. Nagao. 1981. Effects of phospholipids on growth, survival rate and incidence of malformation in the larval ayu. Mem. Fac. Fish., Kagoshima Univ. 30:301–309.

Lewis-McCrea L.M. and S.P. Lall. 2010. Effects of phosphorus and vitamin C deficiency, vitamin A toxicity, and lipid peroxidation on skeletal abnormalities in Atlantic halibut (*Hippoglossus hippoglossus*). J. Appl. Ichthyol. 26:334-343.

Saleh R., M. Betancor, J. Roo, V. Benítez-Dorta, M.J. Zamorano, J.G. Bell, and M. Izquierdo. Effect of krill phospholipids vs. soybean lecithin in microdiets for gilthead seabream (*Sparus aurata*) larvae on molecular markers of antioxidative metabolism and bone development. Aquaculture Nutr. in press

Roo J., C.M. Hernández-Cruz, J. Socorro, H. Fernández-Palacios, D. Montero, and M.S. Izquierdo. 2009. Effect of DHA content in rotifers on the occurrence of skeletal deformitiesin red porgy Pagrus pagrus (Linnaeus, 1758). Aquaculture 287:84-93.

Schultze H.P. and G. Arrantia. 1989. The composition of the caudal skeleton of teleosts (*Actinopterygii. Osteichthyes*). Zoology Journal of Linnean Society 97:189-231.

Villeneuve L., E. Gisbert, H. Le Delliou, C. Cahu, and J. Zambonino-Infante. 2005. Dietary levels of all-trans retinol affect retinoid nuclear receptor expression and skeletal development in European sea bass larvae. British Journal of Nutrition 93:1-12.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EFFECT OF PHYTOCHEMICALS ON STRESS TOLERANCE OF *PENAEUS VANNAMEI* POSTLARVAE

E. Bequé[1], R. Phuthongphan[2], R. Wouters[1], and G. Rombaut[1]

[1] INVE Technologies, Hoogveld 93, 9200 Dendermonde, Belgium
[2] Shrimp Culture Research and Development (SCRD), 471 Bond Street, Tambon Bangpood, Amphur Pakkred, Nonthaburi 11120, Thailand

Introduction

Disease and microbial interference remain major causes of mortalities, unpredictable output, or reduced quality in shrimp aquaculture (Anderson, 2012). Until now, treatments were largely limited to the use of antibiotics (Serrano, 2005), disinfectants, or probiotics (Verschuere et al., 2000). It is also generally recognized that host-microbial interactions play an important role in aquaculture and even more during the hatchery cycle where high rearing densities result in suboptimal environmental conditions and stress (Vadstein et al., 2013). Therefore, it is imperative to promote innovative concepts that can improve the robustness of the host.

An interesting approach in this perspective is immunostimulation and increase of stress resistance in shrimp by phytochemicals, which include compounds with various biological properties (e.g., antimicrobial, antifungal, and antioxidant) that have presumably evolved, in part, to allow plants to cope with environmental challenges. At relatively small doses, these phytochemicals can induce cellular stress responses in a host organism (Calabrese et al., 2008). This cellular stress response generally translates in a gene expression leading to up-regulation of a group of universally conserved stress proteins (Kültz, 2005). In cases where disease outbreaks or stressful situations can be predicted, phytochemicals may be used in anticipation of events to elevate the non-specific defense mechanism and thus prevent losses from diseases.

Materials and methods

Screening of phytochemicals
10-day-old *P. vannamei* postlarvae (PL) were kept in 5-l beakers for 7 days at a density of 25PL.l^{-1}. Four different phytochemicals (P1, P2, P3, P4) were added to the culture water at different concentrations to evaluate toxicity and effect on stress resistance. Stress tolerance was evaluated by counting dead and live PL

following an exposure to three different stress situations for 30min, 1, 3, and 6h: osmotic stress - low salinity (0-1ppt); thermotolerance - high temperature (43°C), and ammonium stress - 150mg.l^{-1} ammonium chloride.

Transport trials
P. vannamei postlarvae (PL7) were transferred into 2-l airtight plastic bags at a density of 1000PL.l^{-1}. These transport bags were filled with 0.75 l of seawater and different doses of a phytochemical mixture; pure oxygen was added and 30 *Artemia* Instar I nauplii per PL were added to feed the PL during transport. The bags were kept in foam boxes at a temperature of 21±1°C during a 12-h transport period. Ten individual bags were used per treatment. After transport the PL were further evaluated in 5-l beakers for 7 days.

Results

Screening of phytochemicals
Optimal application ranges for phytochemicals (P1-4) were determined by exposing the postlarvae to different concentrations of each product. At a concentration of 10mg.l^{-1}, all tested products had a positive effect on the thermotolerance of the PL (Fig. 1) and no toxic effect was observed. A significant higher survival of the PL treated with 10mg.l^{-1} of each phytochemical was found (75.6%, 95.6%, 88.9%, and 95.6% for P1, P2, P3, and P4, respectively) compared to the survival of the control treatment (8.9%). All phytochemicals tested had a negative effect on PL survival at concentrations higher than 100mg.l^{-1} (data not shown). P2 had only a mildly negative effect on PL survival even at a concentration of 500mg l^{-1}.

Transport trial
Based on the initial screening tests, a phytochemical mixture was made and applied on shrimp to evaluate its effect during the stressful situation of transportation. Survival of treated postlarvae exposed to osmotic stress is presented in Fig. 2. These results show that osmotolerance was increased in those PL that were treated with different doses of the phytochemical mix during transport. The PL treated with 10mg.l^{-1} and 100mg.l^{-1} had a survival percentage of 63% and 76% respectively, which was significantly higher than the survival of untreated PL (45%) and similar or higher than survival in PL that did not undergo transportation (66%).

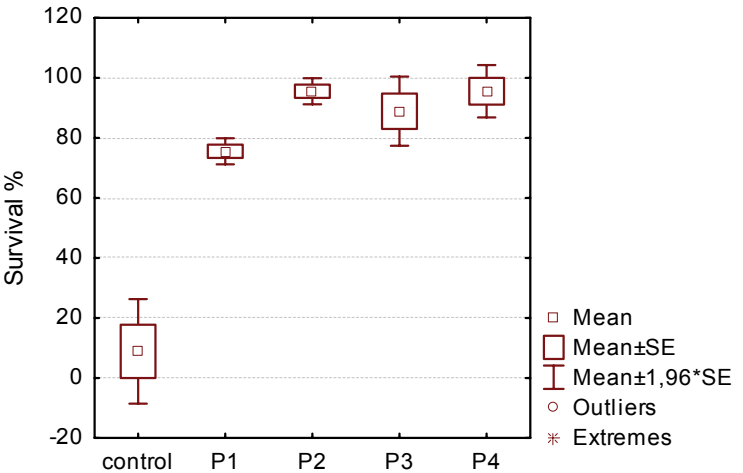


Fig. 1. Survival of PL17 in a 6-h temperature stress test following a 7-day exposure to four different phytochemicals at a dose of 10mg.l^{-1} versus a control treatment.

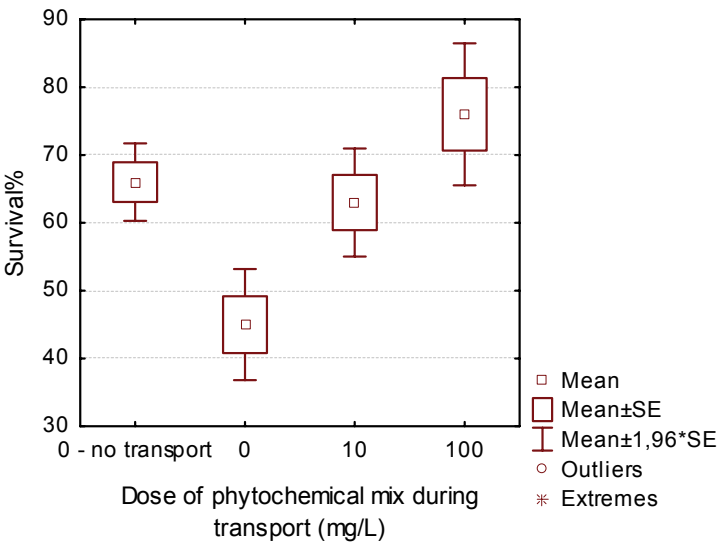


Fig. 2. Survival of PL14 in a 60-min salinity stress test. PL7 were transported for 12h and effects on stress evaluated after 7 days at PL14.

Discussion

The current study shows that various phytochemicals directly applied into the water increase the resistance of *Penaeus vannamei* postlarvae subjected to osmotic, temperature, and ammonium stress. A mixture of phytochemicals was

composed and showed to reduce stress during and after postlarvae transport. Our results show that osmotolerance after transport increased in those postlarvae that were treated with the mixture during transport and these effects remain for at least 7 days after transport. This demonstrates the potential of phytochemicals to improve the robustness of shrimp postlarvae and indicates that they could be used as prophylactic or metaphylactic agents to elevate the non-specific defense mechanism and thus prevent losses. More work is needed to elucidate the mode of action.

References

Anderson J.L. 2012. Fish to 2030, Shrimp Production Review. Presented at GOAL, Bangkok 2012

Calabrese V., C. Cornelius, C. Mancuso, G. Pennisi, S. Calafato, F. Bellia, T.E. Bates, A.M.G. Stella, T. Schapira, A.T.D. Kostova, and E. Rizzarelli. 2008. Cellular stress response: a novel target for chemoprevention and nutritional neuroprotection in aging, neurodegenerative disorders and longevity. Neurochemical research, 33(12):2444-2471.

Kültz D. 2005. Molecular and evolutionary basis of the cellular stress response. Annu. Rev. Physiol. 67:225-257.

Serrano P. H. 2005. Responsible Use of Antibiotics Aquaculture (Vol. 469). Food & Agriculture Org.

Vadstein O., Ø. Bergh, F.J. Gatesoupe, J. Galindo-Villegas, V. Mulero, S. Picchietti, G. Scapigliati, P. Makridis, Y. Olsen, K. Dierckens, T Defoirdt, N. Boon, P. De Schryver, and P. Bossier. 2013. Microbiology and immunology of fish larvae. Reviews in Aquaculture.

Verschuere L., G. Rombaut, P. Sorgeloos and W. Verstraete. 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiology and molecular biology reviews 64(4):655-671.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EXPERIMENTAL CHALLENGE: THE QUEST FOR VIRULENCE – AND PROTECTION

Ø. Bergh

Institute of Marine Research, PO Box 1870 Nordnes, 5817 Bergen, Norway

Despite major R&D efforts, culturing early life stages of fish and bivalves is often still troublesome. Bacterial infections of fish and shellfish larvae are often considered husbandry-related, rather than true diseases. Strong focus on rearing techniques and prophylactic countermeasures rather than treatments is a sound pragmatic implication. Improved rearing techniques are generally believed to cause improved survival and potential for survival throughout the production cycle. Not only the survival, but also the quality of larvae and fry are affected by pathogens. Or, if the problem is husbandry-related, are they really pathogens? The two views may be combined.

A too-strong focus on the husbandry aspect may be inconsistent with research trends in host-parasite ecology and molecular pathogenesis. Trends point out the importance of virulence factors, and on the interactions between the microorganisms and the ontogenetically primitive larvae. Furthermore, most work on the interactions between microorganisms and fish and shellfish larvae focus almost entirely on bacteria, thereby leaving some important pathogenic viruses in the dark. In contrast to most bacteria, viruses are obligate pathogens. A key question arises, on how to verify or falsify the hypothesis that the microorganism in question is virulent, i.e. capable of causing disease, and even death. Verification or falsification of such hypotheses requires challenge experiments. The difficulties on designing proper experimental models are, however, considerable. Therefore, few such experimental models exist, and surprisingly few bacteria (and even fewer viruses) have been verified as virulent to larvae. Are the other bacteria just secondary opportunists? In a controlled screening of a wide range of tentatively pathogenic *Vibrio* spp. isolated from cod larvae, only five out of 53 strains were found to cause mortality significantly higher than the unchallenged controls, four of them identified as *V. anguillarum*. The result might indicate that most strains isolated from dying larvae are not the causing agents of mortality. However, shortcomings of the presently available challenge models cannot be ruled out.

Furthermore, development of new prophylactic methods is to a large extent dependent on challenge experiments. Testing the relative protection of different

prophylactic protocols by their impact on larval survival/mortality in controlled challenge models makes it possible to optimize the prophylactic effect. In recent experiments we were able to identify the importance of tropodiethic acid (TDA) as an important contributor to the probiotic effect of *Phaeobacter gallaeciensis* to cod larvae, as well as microalgal and rotifer culture as TDA-negative mutants showed significantly less protective effect (in challenge experiments with *V. anguillarum*) than the wild type, producing TDA. However, the TDA-negative mutants did show significant protective effect compared to the control groups challenged with but not with probiont. Furthermore, significant differences among different *Phaeobacter* spp. could be found with respect to the protective effect on cod larvae, correlated with the antagonistic effect against *V. anguillarum* measured in vitro. Similarly, impact of immunoprophylactic methods needs evaluation by challenge protocols.

The development of protocols for gnotobiotic systems, allowing for complete control of the bacterial microflora is promising, however the strongly artificial nature of the models imply a continued need for supplementary protocols. Furthermore, there is a need to improve challenge protocols for unfed (yolk-sac) larvae as well as fed larvae.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

THE KEY NEUROENDOCRINE REGULATORS OF THE ONSET OF PUBERTY IN THE ATLANTIC BLUEFIN TUNA (*THUNNUS THYNNUS*)

N. Berkovich[1*], I. Meiri-Ashkenazi[1], V. Zlatnikov[1], A. Corriero[2], C. Bridges[3], C. Mylonas[4], R. Vassallo Aguis[5], F. De La Gándara[6], A. Belmonte[7], A. Elizur[8], H. Gordin[1], and H. Rosenfeld[1]

[1] Israel Oceanographic and Limnological Research, National Center for Mariculture, Laboratory of Reproduction, P.O. Box 1212, Eilat 8112, Israel. Fax +972-86361431 e-mail: hanna@ocean.org.il ;
[2] Department of Animal Health and Welfare, University of Bari, Italy
[3] Heinrich-Heine Universität, Institut für Zoophysiologie, Düsseldorf, Germany
[4] Institute of Aquaculture, Hellenic Center for Marine Research, Crete, Greece
[5] Malta Center for Fisheries Sciences, Marsaxlokk, Malta
[6] Instituto Español de Oceanografía, Centro Oceanográfico de Murcia, Puerto de Mazarrón (Murcia), Spain
[7] Tuna Graso S.A., Carretera de la Palma Km 7, Paraje la Estrella, Cartagena (Murcia), Spain
[8] Faculty of Science, Health and Education, University of the Sunshine Coast, Maroochydore DC, Maroochydore, Qld., Australia
[*] e-mail: nadiaberk@gmail.com, Fax +972-8-6375761. Department of Life Science, Ben-Gurion University of the Negev, P.O. Box 653, Beer-Sheva 84105, Israel

Recently, significant progress on spawning induction in captive bluefin tuna (BFT, *Thunnus thynnus*) has been achieved providing the basis for the species' domestication. To further promote the development of self-sustained BFT aquaculture, we investigated first sexual maturity in BFT reared from an immature stage in captivity. Accordingly, our major objectives were to evaluate (i) maturational status of the brain-pituitary-gonadal (BPG) axis, and (ii) responsiveness of the BPG to exogenous hormones. Special emphasis was given to characterize the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH) that act as central regulators of gonadal development and gamete maturation.

The growth parameters recorded for the captive BFT juveniles are consistent with the length-weight relationship established for wild Mediterranean BFT stocks. Histological analyses of the gonads indicate advanced sexual maturation in BFT males compared to females, yet it is not yet clear whether this phenomenon typifies wild stocks or is induced due to culture conditions. The hormone measurements show expression and accumulation of both gonadotropins in the pituitaries of immature and mature BFT. The pituitary LH content increased

concomitantly with the age of the fish, exhibiting sexually dimorphic patterns (i.e. 3-fold higher levels in females) in adult but not in juvenile BFT. The pituitary FSH levels, however, were elevated in 2Y immature males and in fully mature adults. Comparable to mammals, the intra-pituitary FSH:LH ratio was found to be higher (>1) in sexually immature than in maturing or pubertal BFT. Nevertheless, in the 3Y BFT females, which were all immature, the onset of puberty appears to require some other prerequisites, such as a rise in the LH storage above a minimal threshold. Our in vitro trials further demonstrated the capacity of rFSH and to a lesser extent that of rLH to stimulate cell proliferation in the immature ovarian and testicular fragments. Both rFSH and rLH have failed to stimulate steroidogenesis, yet pre-treatment with KiSS containing EVAc implants appeared to potentiate FSH-stimulated steroidogenesis in the immature testes. On the other hand, the expression levels of both the GtH-R and IGF I genes in the testicular fragments, derived from BFT juveniles and further exposed to the rLH treatment, showed dose-dependent pattern.

Future studies testing the effects of captivity and hormone-based treatments on precocious maturity at relatively small body size are expected to facilitate the handling in confined environments, and to greatly improve the cost-efficiency of BFT farming.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

OXIDATIVE STRESS IN SEA BASS *DICENTRARCHUS LABRAX* LARVAE: INTERACTION OF HIGH DIETARY DHA CONTENTS AND SEVERAL ANTIOXIDANT NUTRIENTS

M.B. Betancor*, M.J. Caballero, and M.S. Izquierdo

Grupo de Investigación en Acuicultura (GIA), ULPGC & ICCM, P.O. Box 35200 Telde, Canary Island, Spain. *Author present address: Institute of Aquaculture, University of Stirling. FK9 4LA, Stirling, UK

It is widely known that marine fish larvae have a huge requirement for n-3 long-chain polyunsaturated fatty acids (LC-PUFA) to reach a good culture performance. Among n-3 LC-PUFA, docosahexaenoic acid (DHA) seems to be more essential than other PUFAs and that is why it is included in high amounts in enrichment media. The objective this work was to study the effect of high dietary contents of DHA on sea bass (*Dicentrarchus labrax*) larvae in combination with different antioxidant nutrients, such as vitamin E (vitE), selenium (Se), or vitamin C (vitC). In a first study, three graded levels of DHA (1, 3 and 5%) were tested together with two levels of vitE (150 and 300 mg.100g^{-1}), paying attention to larval culture performance and to muscle morphology. The presence of muscular lesions, described as hyaline degeneration, was increased along with the DHA content. Nevertheless, vitE increase from 150 to 300mg.100g^{-1} helped to reduce the incidence of these lesions and enhanced larval survival and growth when a 3% of DHA was included in the diets. In the light of these results, a new experiment was planned, including a new diet, containing the highest content of DHA and vitE (5/300). However, histopathological evaluation proved that when a level of 5% of DHA was employed, the increase in vitE from 150 to 300mg did not prevent the appearance of muscular lesions. In addition, the oxidative status was altered, as indicated by TBARS content, in larvae fed with a high content of DHA, especially if compared to those fed a 1% of DHA, indicating that the oxidation of DHA and vitE was the cause of the muscular lesions. On the other hand, in the same work, more detailed features of the muscular lesions were shown through the thick and ultra-thin sections, proving that they show similarities with mammals muscle dystrophy. Semi-thin sections revealed focal lesions consisting of degenerated fibres with hypercontracted myofilaments and extensive sarcoplasm vacuolization affecting both red and white muscle. Ultra-thin sections of degenerating muscle fibres showed diffuse dilatation of sarcoplasmic reticulum, disorganized myofilaments, and autophagic vacuoles containing myelin figures and dense bodies.

Consequently, a molecular approach to these lesions was carried out by studying the expression of several genes related to cell proliferation (insulin-like growth factor I and II; IGFs) and muscle structure (myosin heavy chain, α-actin, and μ-calpain), as well as the possible regeneration after a washing out period. An overexpression of IGFs was found in the larval groups with the highest incidence of muscular lesions (those fed with a 5% of DHA), denoting an increased compensatory muscle mitogenesis. This hypothesis was reinforced by the enhanced expression of MyHC found in the same larvae. Switching larvae from a diet containing 5% to one with 1% of DHA, showed that only two weeks were necessary to take down DHA to the level of larvae fed a 5% of DHA.

At this point, it was evident that levels of DHA up to 5% lead to the appearance of muscular lesions regardless the content of vitE, but what would happen if high levels of other antioxidants are included in the diets? To answer this question, increased levels of Se and vitC were added to diets containing high contents of DHA and vitE (5/300). Apart from their antioxidant role, synergism and sparing mechanisms of these nutrients in relation to vitE have been described in juveniles and adults of several fish species. On another experiment, extra dosage of vitC reduced the incidence of muscular lesions and balanced the MDA levels to values similar to larvae fed the control diet. However, a clear effect of vitC on the expression of the antioxidant enzymes was not observed, indicating that vitC may act at a different level, probably by trapping ROS before they can attack the cellular membranes. On the other hand, the addition of vitC decreased the incidence of cranial deformities when high levels of DHA and vitE were included into the diet, by probably increasing the stability of the cartilage. Continuing with the addition of both Se and vitC, an overexpression of IGF-I and MyHC was related to larvae fed the diet with the highest contents of DHA and vitE, which also showed the highest incidence of muscular lesions, suggesting compensatory muscle growth. Conversely, addition of Se proved to reduce the expression of AOE, although Se did not seem to exert a direct effect on the expression on glutathione peroxidase (GPX). GPX uses this mineral at their active sites, but also Se can form other selenoproteins (SEP) with antioxidant potential, such as selenoprotein P (SEPP). Therefore, the positive antioxidant effect of Se on muscle may be related to the activity of some unidentified SEP.

These studies have shown that high dietary content of DHA has adverse effects on sea bass larvae, and that high doses of vitE are not able to completely counteract the adverse effects of ROS, indeed, they can contribute to alter the larvae oxidative status. Although Se and vitC have been proved to balance the removal of ROS, further studies are necessary to fully elucidate the interrelations between these nutrients, the molecular mechanisms by which they act and their adequate combination to completely avoid the adverse effects of oxidative stress in sea bass larvae culture.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

HIGH DIETARY LEVELS OF ARACHIDONIC ACID NOT ONLY AFFECTS THE NORMAL PIGMENTATION PATTERNS IN POST-METAMORPHIC SENEGALESE SOLE LARVAE, BUT ALSO DISRUPTS THE PROCESS OF EYE MIGRATION IN PSEUDO-ALBINO FISH

A. Boglino[1*], A. Wishkerman[1], M.J. Darias[2], A. Estévez[1], K.B. Andree[1], and E. Gisbert[1]

[1] IRTA, Centre of Aqüicultura, Ctra. Poble Nou km 5,5, 43540 Sant Carles de la Ràpita, Tarragona, Spain
[2] IRD, UMR 226 ISE-M, 361 rue Jean François Breton BP5095, 34196 Montpellier Cedex 5, France

In this presentation we review and present new unpublished data on the effects of arachidonic acid (ARA) on two distinct morphogenetic processes – pigmentation and eye migration – taking place during larval metamorphosis in Senegalese sole (*Solea senegalensis*, Kaup 1858). Pigmentary disorders and impaired eye migration in flatfishes are currently major problems in aquaculture, entailing economical, biological, and ethical issues; thus, they require extensive research on the causative factors responsible for disrupting the aforementioned morphogenetic processes. Thus, we decided to study the effects of high dietary ARA levels on Senegalese sole larvae by feeding from 2 to 50 days post-hatch (dph) with live prey enriched with an experimental emulsion containing high levels of ARA (ARA-H) versus a reference commercial enriching product (Algamac®). Tested diets affected the survival, but not larval growth performance at 50dph. The incidence of normally pigmented post-metamorphic larvae fed the control diet was 99.1±0.3%, whereas it was only 18.7±7.5% in fish fed ARA-H diet. The effects of ARA on normally pigmented and pseudo-albino fish were evaluated by means of the expression patterns of several gene markers involved in the pigmentation process and ARA metabolism, as well as the shape and density of melanophores in the dorsal skin of metamorphosed fish that was analyzed by means of texture and image segmentation analyses. Thus, new data on the regulation of molecular processes governing the transition from the larval to the adult skin pigmentation phenotype will be presented and discussed with regards to the frame of the study. In addition, the effects of high dietary ARA levels in the eye migration and cranial bone remodeling processes in postmetamorphic larvae was evaluated by means of geometric morphometric analyses and the staining of cranial skeletal elements. The frequency of fish presenting cranial deformities was

higher in fish fed ARA-H (95.1±1.5%) than those fed the control diet (1.9±1.9%) and significantly and negatively correlated with the incidence of normally-pigmented animals (R^2=-0.88, P<0.001). Pseudo-albino fish differed from the normally pigmented ones by the disposition of the eyes with regards to the vertebral column and mouth axes, and by the interocular distance and head height, as well as by the osteological development of some skeletal structures from the neuro- and splanchnocranium. Up to now, high dietary levels of ARA had only been correlated to pigmentary disorders, but this is the first study among flatfishes that describes the impaired migration of the ocular side eye to the ventral surface – the right eye in the case of Senegalese sole – whereas the left eye migrating into the ocular side occurred almost normally. In agreement with these morpho-anatomical and molecular results, significantly higher prostaglandin E2 (PGE2) production was observed in pseudo-albinos larvae than in normally pigmented larvae and higher PGE2 production in normally pigmented larvae fed ARA-H than in those fed the control diet. All the results are discussed considering the role of PGE2, an ARA-derivate hormone, on Senegalese sole larval metamorphosis and quality.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EFFECT OF BROODSTOCK DIET ON HEPATIC STATUS AND REPRODUCTIVE PERFORMANCE IN ATLANTIC HALIBUT (*HIPPOGLOSSUS HIPPOGLOSSUS* L.) FEMALES

S. Bolla[1], O. Nicolaisen[1], and O.-L. Brekke[2]

[1] Faculty of Biosciences and Aquaculture University of Nordland, N-8049 Bodø Norway
[2] Nordland Hospital, PO Box 1480, N-8092 Bodø, Norway

High quality egg supply is still recognized as a major bottleneck in juvenile production of marine fish. Gonadal development is a complex process under influence of environmental factors that trigger sexual maturation and nutritional status controlling fecundity and yolk composition. Several studies have lately shown differences in egg composition of farmed broodstocks compared with wild eggs and related to performances at early stages. So far, limited interest has been devoted to the liver, a major organ in ovoviviparous animals that synthesizes and exports eggshell components and vitellogenin under the influence of estrogens. In this regard the hepatic functionality is of major importance in the ability to synthesise the egg components and hepatic disorders might also affect their production. Several domesticated species including Atlantic halibut fed on compound diets develop a fatty liver. The origin of metabolic fatty liver and the ability of fatty liver to synthesize vitellogenin remain poorly understood. The functionality liver from domesticated females exposed to natural will be explored at late vitellogenesis and compared to wild specimens.

Two farmed groups of five fish each and six wild specimens (VF) were analysed. The farmed fish been fed on dry diet for three years followed by a moist diet (Fish Breed M , INVE; Belgium) for another three-year period (TM), while the other group had been only fed on the moist diet (M). The weight, length, HIS, and GSI were recorded. Blood was collected on EDTA at final concentration of 1.5mg.ml^{-1}. The classical hepatic tests used in human medicine were applied: aminotransferases (ASAT, ALAT, ALP, GT) and lipase in plasma as indicator of liver injury and pre albumin, major lipoproteins (LDL and HDL and lipoprotein A) to disclose potential hepatic dysfunction. Plasma concentrations of carbohydrates, cholesterol, and TAG will also be determined at the Medical Laboratory, Nordland Hospital. The circulating vitellogenin was indirectly determined according to Hallgren et al. (2009) modified for female fish. Liver and oocyte samples were snap frozen in LN_2 and stored at -80°C until analyses. Total lipid content was gravimetrically determined and separated into neutral lipid and

polar fractions on silica column after elution with chloroform and methanol. The FAME composition of the both fractions was determined by GC, the amino acid composition by HPLC.

The Atlantic halibut females were in the range 24-56kg. There were no significant differences in HIS or GSI. Plasma lipid in wild fish was slightly higher than in captive females, yet not significant. No differences were observed for ALP, LDH, and lipase, while GT could not be determined. The plasma glucose and aminotransferases (ALAT and ASAT) in wild-caught specimens were strongly elevated and significantly different from the farmed groups. Plasma CK and LDH were especially elevated in one wild caught specimen. These differences are probably the result of stress under capture resulting to some damages to the liver and muscle.

Plasma protein, TAG, HDL-, and LDL-Cholesterol as indicators for liver functionality was similar in farmed and wild specimens. However, plasma alkalilabile phosphate indirectly measuring the circulating vitellogenin was highly different between wild and farmed specimens (~two-fold higher), while no differences were observed between the farmed groups.

The fish from TM group showed the higher hepatic lipid, while the group fed moist diet had the lower content. In consequence, no significant differences were detected between wild caught and fish from group TM or between wild caught and the fish fed the moist diet. A significant difference existed between the TM and M farmed groups. The lipid content in oocyte was similar in all eggs.

Principal component analysis of amino acid composition
With PC1 (accounting for 84% of total variation) and PC2 (9% of total variation), the score plot showed clearest separation for eggs. On the same plot, the liver of fish fed on diet TM and M showed clear separation while the wild specimens were distributed between the two clusters. In plasma, the diets separated roughly into three clusters with wild specimens on one side, fish fed M diet on the other side, and the fish fed on PM diet in between. Amino acids contributing to variation belong to EAA group in addition to aspartic and glutamic acids.

Principal component analysis of FAMES composition in non polar lipids
With PC2 (accounting for 17% of total variation), the score plot of plasma samples showed two clusters, one including wild specimens and the other including the farmed fish with both diets TM and M.

Principal component analysis of FAMES composition in polar lipids
With PC2 (accounting for 20% of total variation), the score plot of plasma samples showed two closely related clusters: one including wild specimens one including the farmed fish with both diets TM and M.

The females fed on TM diet had the highest hepatic lipid content in the liver while the females from diet M had the lower. The liver of fish that had been fed on a dry diet retained more lipids after transfer to the M diet.

The plasma of wild specimens had higher levels of alkali-labile phosphate reflecting higher levels of circulating vitellogenin compared to farmed fish. However, the egg composition presented limited variation between the three groups. The PCA score plots of FAMEs in non-polar and polar lipids in plasma showed that farmed specimens cluster together and separately from the wild specimens. Indirect determination of circulating vitellogenin showed a two-fold difference between wild and farmed fish. These differences are probably of dietary origin, but could also be related endocrine disruption under farmed conditions.

Reference

Hallgren P., L. Mårtesnsson, and L.Mathiasson. 2009. Improved spectrophotometric determination via alkali-labile phosphate in fish plasma- a cost effective approach for assessment of endocrine disruption. Int. J. Environm. Analyt. Chem. 89:1023-1042.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

FLUORESCENT MICROSPHERES – A NEW APPROACH TO QUANTIFYING LIVE FEED INTAKE IN LARVAL FISH

K. Bonacic[1], R. Valles[1], X. Gómez-Arbonés[2], A. Estévez[1], and S. Morais[1]

[1] IRTA, Ctra. Poble Nou Km 5.5, 43540 Sant Carles de la Rápita, Spain
[2] Institut de Recerca Biomédica de Lleida, Universitat de Lleida, Spain

Introduction

Determining feed intake of larval fish has proven difficult due to the small size of the animals, the fact that most species do not grow well on inert microdiets, and the high cost, imprecision, or impracticality of existing methodologies (using radioactive or stable isotopes, or fluorescent markers (GFP) as tracers, measuring clearance rate of zooplankton from the water column, etc.). This study aims to develop and test a safe and easy methodology for the labeling of live prey using fluorescent microspheres, with applicability in studies of food intake and selectivity (due to the availability of microspheres containing different dye colours). In order to analyse and compare the results we have used different methods: fluorometry, cytometry, and fluorescent microscopy.

Materials and methods

Artemia salina (GSL SEP-art, INVE) cysts were hatched, harvested, and enriched under standard conditions. Enrichment was done with 0.6g.l^{-1} of a cod-liver oil (Fluka) emulsion for 16h. After this period, the *Artemia* were washed and resuspended in clean seawater in 100-ml beakers at 200metanauplii.ml^{-1}. Fluorescent microspheres (FluoSpheres® Blood Flow Determination Fluorescent polystyrene microspheres, 10µm, Invitrogen) were then added to the media. Two different concentrations of microspheres were tested: 5×10^3 and 1×10^4 spheres.ml^{-1}, and samples of 20ml were taken at two time points: 15 and 60min. Sampled *Artemia* were placed under a light source to separate live (fully active) from dead (or low performing). They were then thoroughly washed using a 50µm sieve, fixated in 70% ethanol and stored at 4°C in complete darkness. Fluorescent microscopy was used to assess the percentage of labeled *Artemia* and to count the number of spheres per *Artemia*. Additionally, some samples of pooled *Artemia* (around 120, counted exactly, in triplicate) were processed for spectrophotometry (using a sedimentation technique developed by Van Oosterhout, 1995), to compare and validate the two methods.

In order to test the method as a way to study feeding selectivity in fish larvae, an experiment was performed with meagre (*Argyrosomus regius*) larvae to show preferential ingestion depending on prey composition (three different enrichments) of its prey. At 30 days post hatching (dph), three groups of 150 meagre larvae which had been grown in different *Artemia* treatments – enriched with either *Nannochloropsis* (PHY), Easy DHA Selco mixed with olive oil (SOO), or Easy DHA Selco (S) – were each transferred to three 1-l beakers (50 larvae per beaker, 3 beakers per treatment) and left to acclimatise for 1h. To each beaker, a mixture containing 6.66ml of *Artemia* from each treatment, which were previously labelled for 1h with either blue-green, blue, or yellow-green microspheres (5×10^3 spheres.ml^{-1}; 200 *Artemia*.ml^{-1}), was added. Larvae were allowed to feed for 1h, after which they were sampled and processed for analysis by fluorometry and flow cytometry (each sample was divided in half, for each analysis). Four 1-ml samples of the labelled *Artemia* were equally processed after counting the exact number of *Artemia* in each sample (around 100).

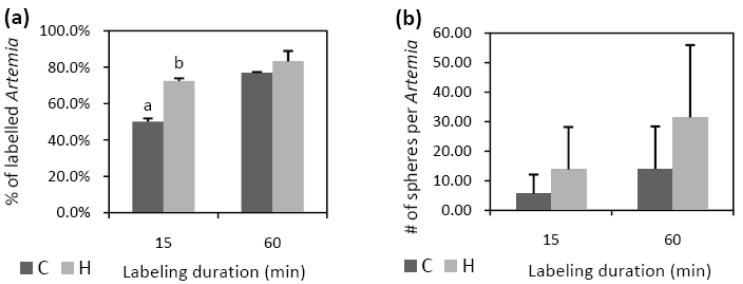


Fig. 1. (a) Labeling percentage (n=3) and (b) average number of microspheres in *Artemia* (n=140) from labeling solutions with different concentration of microspheres. Concentration of "C" was 5×10^3 and "H" 1×10^4 spheres.ml^{-1}.

Results and discussion

Increasing the concentration of microspheres in the labeling solution resulted in a statistically higher percentage of labeled *Artemia* (t-Student; P=0.003) at 15min of labeling, but at 60min there were no significant differences between the two microsphere concentrations (Fig. 1A). However, a very large variation was obtained in the number of microspheres per labeled *Artemia* (Fig. 1B and Fig. 2). There was a trend for the incorporation of a higher number of microspheres when *Artemia* were labeled at higher concentrations and after 60min. However, from a practical point of view, a higher concentration of microspheres was more difficult to wash out (possibly leading to inaccuracies in quantification) and would also increase the cost of the method. When later repeating the experiment using a concentration of 5×10^3 spheres.ml^{-1}, a labeling percentage of 84.7±3.0% was obtained after 15min and 87.8±0.5% after 60min. This represented an important improvement but also pointed out the variability of the

method. However, it is important to note that even though inter-experiment variability was important, replicates of treatments within a same experiment (i.e., using the same batch of *Artemia*) were very similar and perfectly comparable. With respect to the incorporation of microspheres per individual *Artemia*, once again the results denoted a high variability, independently of labeling time (Fig. 2).

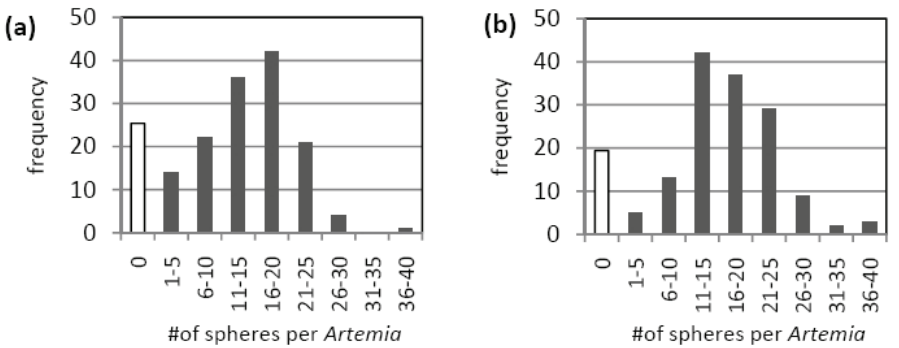


Fig. 2 Number of spheres per *Artemia* of the experiment repeated with 5×10^3 spheres.ml^{-1} after (a) 15min and (b) 60min of labeling.

Seeing that relatively high labeling percentages could also be achieved with shorter labeling time (15min) and lower concentrations (5×10^3 spheres.ml^{-1}), we opted for use of these more practical conditions.

Comparison of visual counts by fluorescent microscopy and the quantitative analysis using spectrophotometry showed a strong correlation between fluorescence intensity and number spheres per *Artemia* (Fig. 3).

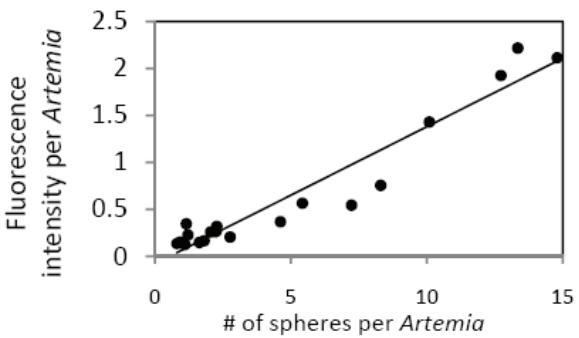


Fig. 3 Correlation between number of red spheres per *Artemia* and fluorescence intensity per *Artemia* (R=0.980).

The applicability of the method for selectivity studies, taking advantage of the possibility to label different treatments with diverse microsphere colors, was also

tested. Using flow cytometry we can rarely analyse a whole sample given that the analysis in based on a fixed number of counting "events" (i.e., particles). Therefore, even if not suitable to quantify ingestion, it can be used in selectivity studies, as it gives relative numbers of spheres with different absorbances/colors within a sample. Results obtained by cytometry were validated by comparing with fluorometric results quantified from the same samples (Fig. 4).

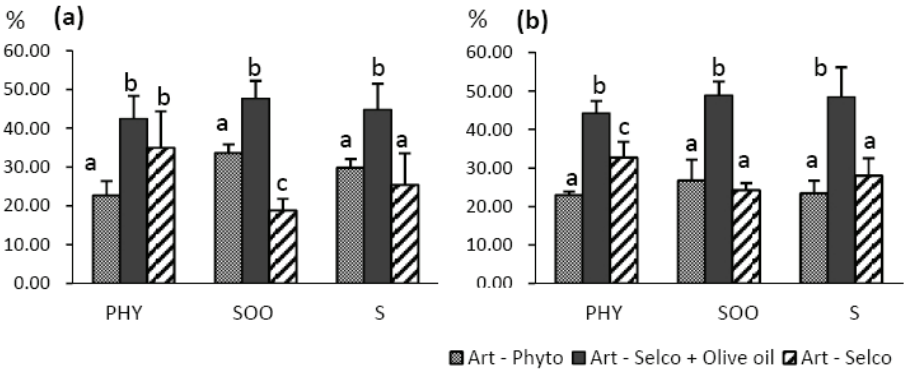


Fig. 4 Preference in % of meagre larvae towards 3 different preys quantified by (A) spectrophotometry and (B) cytometry. Three groups (PHY, SOO and S) were fed equal amounts of *Artemia* enriched with three different compounds (n=3).

Conclusion

As in previous methods (Morais et al., 2004), high variability of *Artemia* labeling, both in terms of labeling percentage and number of spheres per *Artemia*, is an issue. Regrettably this is a limitation with all larval nutrition studies that rely on such an active live organism. However, our experience based on multiple repetitions has shown that labeling percentage is very comparable between replicates within the same trial and the variability in the number of spheres per individual *Artemia* can be overcome by working with averages of pools containing a large number of individuals. Therefore, even if not adequate to exactly quantify absolute ingestion, this method shows promise in studies aiming to compare relative differences in ingestion between dietary treatments.

References

Morais S., E.C. Conceição, M.T. Dinis, and Rønnestad I. 2004. A method for radiolabeling *Artemia* with applications in studies of food intake, digestibility, protein and amino acid metabolism in larval fish. Aquaculture 231:1-4.

Van Oosterhout M.F., H.M. Willigers, R.S. Reneman, and F.W. Prinzen. 1995. Fluorescent microspheres to measure organ perfusion: validation of a simplified sample processing technique. Am J Physiol 269:H725-H733.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EXPLORING THE FEEDING MYSTERY OF LEPTOCEPHALUS LARVAE: A MOUTH FULL OF TEETH

M. Bouilliart[1], J. Tomkiewicz[2], P. Lauesen[3], and D. Adriaens[1]

[1] Research Group Evolutionary Morphology of Vertebrates, Ghent University, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium
[2] National Institute of Aquatic Resources – Section for Marine Ecology, Technical University of Denmark, Charlottenlund Slot Jaegersborg Allé 1, 2920 Charlottenlund, Denmark
[3] Billund Aquaculture, Klovermarken 27, 7190 Billund, Denmark

The leptocephalus larva is the unique larval stage of Elopomorph fishes. Since these larvae are completely transparent, small in size, and tied to the ocean's twilight zone, in-depth investigations of their anatomy, physiology, and behavior are rather hard to come by. On top of that, if researchers do succeed in examining one the aforementioned aspects, the obtained results are often inconclusive or even contradicting. The biggest contradiction, however, involves the larva's feeding strategy. Despite several decades of investigating plausible feeding methods, experimenting with all sorts of possible artificial food particles, and numerous attempts to uncover the identity of preferential prey items directly from nature itself, the how and what of their feeding strategy is elusive. This information is needed to complete the life cycle in captivity of the severely endangered European eel (*Anguilla anguilla* Linnaeus 1758; Actinopterygii, Anguillidae), an Elopomorph, which is necessary to protect the species from extinction. To gain additional information regarding the why and how of the failure to identify this feeding stategy, a functional morphological analysis of the larva's feeding apparatus at the onset of active feeding is performed in this study.

By combining morphology, kinematics, and biomechanics, it becomes possible to (1) model the theoretical bite force of the larva and (2) visualize its jaw and hyoid movability. This data subsequently allows assessing the larva's feeding capacity as well as alluding to preferential prey characteristics. In order to estimate the theoretical bite force, however, a static state equilibrium model based on 3D data of joints, levers, muscle insertions, and additional muscle data (volume and fiber length) is used. To obtain this required information, a graphical 3D reconstruction (based on a series of histological sections) of the musculoskeletal system of the larva is generated. The creation and use of this reconstruction is required as the overall size of the organism (<1cm) doesn't allow extracting the morphometric and 3D data directly from the larva itself. The re-

construction is also used in identifying the important contributors to the jaw opening and closing movements by comparing it with in vivo video recordings of leptocephalus larvae. These recordings also make the visualization of the jaw and hyoid movability possible, which grants us more general information about the maximum obtained gape angles and the sequence of operation.

As a result, an average gape angle of approximately 15° and an associated theoretical bite force of approximately 50μN are obtained. On top of that, two observations, which are derived directly from the reconstruction itself, deserve special attention. First, between the jaw and the hyoid, a ligament is present that couples the movements of both arches, thereby making the first-feeding leptocephalus larva owner of a hyoid four-bar coupling system that is involved in mouth opening. Second, although the larva possesses teeth, which fed the idea of calculating bite forces in the first place, their needle-like morphology, attachment to underlying elements, and forward-pointing orientation make most of them seem rather unfit for actually biting prey items. An exception is the first pair of teeth in both the upper and lower jaw. With their counterintuitive attachment locations and the absence of supporting bone, these pairs may be modified to perform a more biting or piercing related function.

To conclude, after combining the obtained gape angle and bite force, it becomes clear that if the first-feeding leptocephalus larva feeds by biting prey items, which will most probably be restricted to the first pair of upper and lower teeth, food particles have to be rather soft and/or small. These findings make small, gelatinous organisms like the Hydrozoa, Thaliacea, Ctenophora, and Polycystenia recently identified in the guts of these larvae their most likely prey candidates.

This study was part of the FP7 Project PRO-EEL, "Reproduction of European Eel: Towards a self-sustained Aquaculture" (Grant no. 245257)" funded by the European Commission.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

DEVELOPMENT OF TECHNIQUES AND TECHNOLOGY FOR EMBRYONIC AND LARVAL REARING OF THE EUROPEAN EEL

I.A.E. Butts[1*], S.R. Sørensen[1], S.N. Politis[1], P. Lauesen[2], and J. Tomkiewicz[1]

[1] Technical University of Denmark, National Institute of Aquatic Resources. Kavalergården 6, 2920 Charlottenlund, Denmark
[2] Billund Aquaculture, Kløvermarken 27, DK-7190 Billund, Denmark

Introduction

The decline of European eel (*Anguilla anguilla*) has brought attention to the need for sustainable exploitation of the stock (ICES, 2011). Eel is a valued species targeted for aquaculture production. Presently, aquaculture and restocking programs rely exclusively on the supply of glass eels, thus captive reproduction for a self-sustained production would be a major step forward. Recent experimental research in the international PRO-EEL project (www.pro-eel.eu) has led to the successful production of many large egg batches, with high fertilization rates, and viable embryos and larvae from both wild-caught and farmed eels. This promising progress urges the need for new and innovative techniques and technology for embryonic and larval rearing. Here, we evaluated the effects of oxygen (O_2) and water source on hatch success and light stimuli on larval activity. Our hope is that this work will shed light on new rearing protocols to produce sufficient larvae for future feeding trails.

Materials and methods

European eel broodstock were obtained from Vandet Lake, Denmark. Fish were transported to a research facility of the Technical University of Denmark where they were housed in 300l tanks. The salinity and temperature of the system ranged from 36.7 to 37.3% and 19.5 to 20.5°C, respectively. Photoperiod was 12h light and 12h dark at ~20lux. No feed was provided during the experiments as eels in silvering stage cease feeding. To produce viable gametes, fish received hormonal injections following Ohta et al. (1997) and Tomkiewicz (2012).

Effect of O_2 and water source on hatch success
Sperm in P1 media (Peñaranda et al., 2010) were immediately added to stripped eggs and quickly swirled together. Embryos were incubated in 24h darkness at 20°C in 36ppt (adjusted using Tropic Marin Sea Salt) filtered natural seawater. Prior to hatch (~45h post-fertilization), 50 to 90 embryos were collected using a

1ml syringe, and loaded into 200ml flasks, with seawater containing 40ppm penicillin and 65ppm streptomycin, from either Kattegat or the North Sea. For each water source, flasks were supplemented with O_2 (lightly bubbled for 8s) or without O_2.

Effect of light stimuli on larval activity
Larvae at 10 and 33h post-hatch were placed into a 300ml photographic glass tank. Prior to experimentation, larvae were kept in darkness for 30min. Digital video recordings and light treatments were started synchronously and turned off after 10s. Five light treatments were applied: blue, purple, red, white light at ~100lux, and white light 500lux. Videos were subsequent analyzed with respect to the number of larvae swimming as a reaction to light stimuli and expressed as a percentage.

Results

Effect of O_2 and water source on hatch success
Mean (±SEM) hatch success of the four different treatment combinations was 64.6±3.4% (ranged from 28.2 to 92.6%). For hatch success a significant O_2 × water source interaction was detected ($P<0.05$). Therefore, the model was revised to determine the effect of O_2 for each water source, and of water source for each O_2 treatment. When eggs were reared in Kattegat water the O_2 main effect was significant ($P<0.001$), such that eggs reared in oxygenated water had a lower hatch success (Fig. 1a). However, when the eggs were reared in North Sea water no significant difference in hatch success was detected between the two O_2 treatments ($P>0.05$; Fig. 1a). For both O_2 treatments, eggs reared in North Sea water had significantly higher hatch success than those reared in Kattegat water (Fig. 1b).

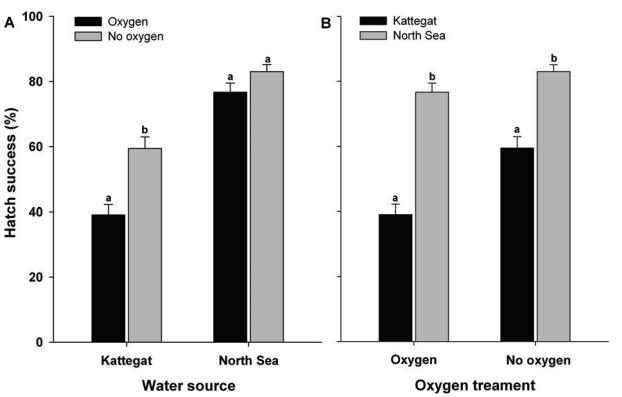


Fig. 1. Effects of O_2 and water source on hatch success. A significant interaction was detected. Therefore, the model was revised to determine the effect of O_2 for each water source (A), and of water source for each O_2 treatment (B).

Effect of light stimuli on larval activity

Swimming activity ranged from 8.4 to 13.07% at 10h post-hatch to 6.5 to 95.1% at 33h post-hatch. There was a significant post-hatch time × light interaction for larval activity ($P<0.001$). Therefore, the model was revised to determine the effect of light stimuli at 10 and 33h post-hatch. At 10h post-hatch, light stimuli had no effect on larval activity ($P>0.05$), while at 33h post-hatch it had an affect ($P<0.001$), such that blue and white light at 500lux increased the activity of larvae compared to purple, red, and white light at 100lux (Fig. 2).

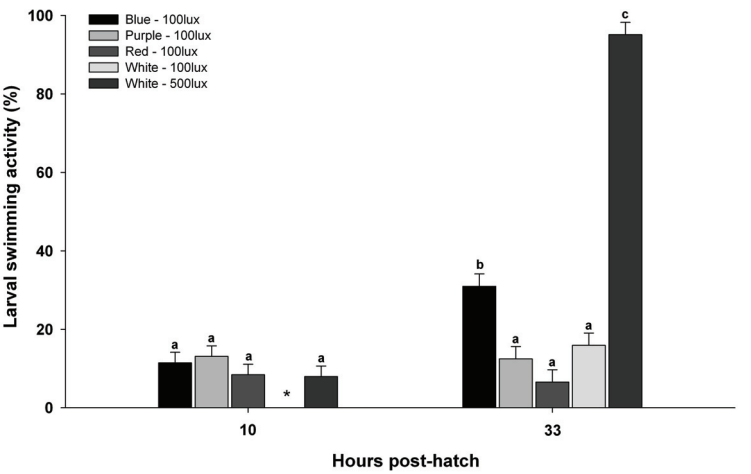


Fig. 2. Effect of light stimuli on larval swimming activity. * = no data available

Discussion

We found that low O_2 levels were more favorable for hatching embryos. In other fishes, reduced O_2 content has accelerated hatch by increasing secretion of the hatching enzyme (Korwin-Kossakowski, 2012), and a similar phenomenon may be occurring in eel. Water source also altered hatching, thus studies should investigate how seawater biochemistry influences embryonic development and ionic movement across the chorion.

For the majority of larvae, vision is the primary sense involved in the acquisition of prey (Blaxter, 1969); thus, suboptimal spectral conditions (among others) can negatively affect foraging efficiency and the physiological status of a larva. Here, we clearly show the first indication that eel larvae respond to light stimuli. Therefore, the effects of light intensity, spectral composition, and photoperiod need to be intensively investigated as these factors could have a large influence on growth and survival during early life history.

References

Blaxter J.H.S. 1969. Visual thresholds and spectral sensitively of flatfish larvae. Journal of Experimental Biology 51:221-230.

ICES. 2011. Report of the Joint EIFAAC/ICES Working Group on Eels (WGEEL), 5–9 September 2011, Lisbon, Portugal. ICES CM 2011/ACOM:18. International Council for the Exploration of the Sea, Copenhagen.

Korwin-Kossakowski M. 2012. Fish hatching strategies: a review. Reviews in Fish Biology and Fisheries 22:225-240.

Ohta H., H. Tanaka, H, K. Okuzawa, N. Linuma, and K. Hirose. 1997. Artificial induction of maturation and fertilisation in Japanese eel, *Anguilla japonica*. Fish Physiology and Biochemistry 17:163-169.

Peñaranda D.S, L. Pérez, V. Gallego, R. Barrera, M. Jover, and J.F Asturiano. 2010. European eel sperm diluent for short-term storage. Reproduction in Domestic Animals 45:407-15.

Tomkiewicz J. (Ed.). 2012. Reproduction of European Eel in Aquaculture (REEL): Consolidation and new production methods. DTU Aqua Report No 249-2012. National Institute of Aquatic Resources, Technical University of Denmark, Copenhagen Denmark. 47 p.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

FACTORS AFFECTING SPERM QUALITY AND EMERGING TOOLS FOR SPERM ANALYSIS

E. Cabrita[1,2], S. Martínez-Páramo[2], P. Gavaia[2], T. Pacchiarini[1], D.G. Valcarce[3], C. Sarasquete[1], and V. Robles[3]

[1] ICMAN.CSIC, Av Republica Saharaui 2, 11510 Puerto Real, Cádiz, Spain
[2] CCMAR, University of Algarve, Campus Gambelas, 8005-139 Faro, Portugal
[3] INDEGSAL and Dept of Molecular Biology, University of León, Campus Vegazana, 24071 León, Spain

The control of sperm quality is a major issue for the aquaculture industry both for the production of well-established and "new" species. The identification of predictive estimators or markers of sperm quality would also have major applications in research and biotechnological companies. It has been extremely difficult to accurately estimate the quality of sperm prior to fertilization, basically due to the need of a better understanding of the factors affecting gamete quality. Most of these factors are related with male breeder performance, life history, social context, or breeding conditions. This review gives an overview of methods to assess sperm quality and identifies important factors affecting sperm production and quality, including, epigenetics, environmental and spawning induction protocols, breeders provenance, broodstock nutrition, and sperm management (cryopreservation) and will be mostly focused in these two last aspects.

The quality of sperm can be assessed by analyzing different parameters from simple methods to very sophisticated approaches involving molecular tools. Parameters related with sperm composition or function (e.g. spermatozoa plasma membrane lipids, seminal plasma composition, motility activation) have successfully characterized a sperm sample but could not respond to the causes behind sperm defects. Reactive oxygen species (ROS) are one of the causes of the impairment of sperm traits. High contents of ROS are capable of producing cell apoptosis, DNA strand breakages, mitochondria function impairment, and changes in membrane composition due to sugars, lipids, and amino acid oxidation, affecting at later times sperm fertilization ability.

Recently, the importance of spermatozoa RNAs in the fertilization and early embryo development has been clearly demonstrated in different species, including fish. Spermatozoa delivers more than the paternal genome into the oocyte, carrying also remnant mRNA from spermatogenesis. These RNAs have been found in sperm from human, rodent, bovine, and recently in several fish species,

demonstrating the important predictive value of spermatozoa transcripts present only in those samples with high motility or from males with higher reproductive performance. The content of those transcripts can be changed during gametogenesis process influencing their content in spermatozoa. Breeder's nutrition can play an important role in this regard. The incorporation of antioxidant substances have proved to be favorable to sperm quality, especially when this quality needs to be reinforced to sustain manipulations such as cryopreservation. Sperm cryopreservation is a safe method to store and preserve the male genetic material. Its use should benefit the fish farm industry at different levels, from management of reproduction to genetic selection of males with high reproductive value. Protocols for fish sperm cryopreservation have been successfully developed in several teleost species, although some cell damage has been identified. Ongoing research in fish sperm cryopreservation highlights the potential of cryopreservation as a factor of transcript change which may have later implications during larval development. Although this issue has been a controversial aspect it cannot be neglected.

We will focus this review on sperm quality markers, in new trends on sperm analysis, and in the use of these tools for the identification of factors affecting gamete quality. The range of optimal indicators should be defined according to the different species, sperm fate or reproductive strategy such as artificial fertilization, cryopreservation, gene banking, or mass production. Basic research in this field is helping to develop appropriate quality evaluation methodologies and early biomarkers of reproductive success, with potential future industrial applications.

Project CRYOXI (AGL2011-28810) and LARVANET COST action. Fundación Ramón Areces and Ramón y Cajal Program, MICINN, Spain.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EPIGENETIC REGULATION OF MUSCLE DEVELOPMENT AND GROWTH IN SENEGALESE SOLE LARVAE

C. Campos[1,2,3], L.M.P. Valente[1], L.E.C. Conceição[2,4], S. Engrola[2], and J.M.O. Fernandes[3]

[1] CIIMAR/CIMAR, Centro Interdisciplinar de Investigação Marinha e Ambiental and ICBAS–Instituto de Ciências Biomédicas de Abel Salazar, Rua dos Bragas, 289, 4050-123 Porto, Portugal
[2] CCMAR/CIMAR, Centro de Ciências do Mar, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal
[3] Faculty of Biosciences and Aquaculture, University of Nordland, NO-8049 Bodø, Norway
[4] SPAROS Lda, CRIA - Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

Solea senegalensis embryos were incubated at different temperatures (15, 18, and 21ºC) and larvae were reared at 21ºC until 30 days post-hatch (dph). Higher embryonic temperatures promoted faster development and increased size of larvae. Muscle cellularity was affected by embryonic temperature during development, and by 30dph larvae from 18ºC had the highest number of fast fibres. However, at this age, 15ºC and 21ºC groups did not differ on fibre number or muscle area. Gene expression and miRNAs populations were also affected by embryonic temperature. Myf5, mrf4, and myHC were amongst the genes affected by temperature, and thermal plasticity of miRNAs such as miR-17, miR-26a, miR-181a, or miR-206, which are known to be related with development, might have potential implications on thermal gene regulation.

Senegalese sole larvae and juveniles originated from embryos incubated at 20ºC, reared at three different temperatures (15ºC, 18ºC, or 21ºC) during the pelagic phase, and then transferred to a common temperature (20ºC) from benthic stage until 121dph also had great differences on growth, gene expression and survival. In pelagic larvae, muscle growth increased with the increment of water temperature, with the concomitant changes in gene expression, particularly of myogenic genes. Lower rearing temperatures promoted DNA hypermethylation of the putative myogenin promoter in muscle of pelagic larvae, which coincided with lower myogenin expression levels at 15ºC compared to 18 or 21ºC. Nevertheless, a mechanism of compensatory growth was activated in the 15ºC group after transfer to 20ºC, since by 121dph no differences were found in size between the 15ºC and 21ºC treatments, and interestingly, both were larger than the 18ºC ju-

veniles. Muscle cellularity of early juveniles differed amongst temperature groups and the 18ºC group showed an intermediate growth capacity favouring fibre hypertrophy. Interestingly, protein absorption and retention were reduced in pelagic larvae from 15ºC when compared to fish reared at higher temperatures, but transfer to 20ºC enhanced their feed intake and protein digestibility, which is consistent with the increased growth of the 15ºC group. The putative poorer developed digestive system at 15ºC showed no long-term consequences and was probably quickly recovered once larvae were moved to 20ºC.

In conclusion, the present study shows that temperature during specific time-frames of ontogeny has both short-term and long-term effects on growth and muscle cellularity of Senegalese sole. However, Senegalese sole also seems to rapidly adapt to environmental temperature even in early developmental stages.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

DOES DIETARY AMINO ACID PROFILE MODULATE SENEGALESE SOLE LARVAE PROTEIN METABOLISM?

P. Canada[1,2], S. Engrola[2], A.F. Lopes[2], N. Richard[2], L.M.P. Valente[1], and L.E.C. Conceição[2]

[1] CIIMAR/CIMAR, Centro Interdisciplinar de Investigação Marinha e Ambiental and ICBAS–Instituto de Ciências Biomédicas de Abel Salazar, Rua dos Bragas, 289, 4050-123 Porto, Portugal
[2] CCMAR/CIMAR, Centro de Ciências do Mar, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

Introduction

Senegalese sole have a rapid and complex ontogeny accompanied with changing nutritional needs, namely amino acid (AA) requirements and the type of nitrogen source that can be digested by larvae throughout the development (Conceição et al., 2007). When formulating diets for sole larvae it is easy to fall into a mismatch of the ideal AA profile and nitrogen source, as these might also change throughout development (Aragão et al. 2004). Senegalese sole was used as model to understand how different dietary amino acid profiles could affect protein utilisation and thereby regulate fish larval growth.

Material and methods

The experiment was held at the Ramalhete and LEOA facilities at University of Algarve (Faro, Portugal). From hatching until the end of metamorphosis, larvae were reared in cylindro-conical tanks at 19°C under standard conditions for Senegalese sole rearing. Larvae were fed a co-feeding regime based on live feed (rotifers and enriched *Artemia*) and microbound inert diet from mouth-opening until 51 days after hatching (DAH). After metamorphosis, at 25DAH, when larvae became benthic, they were transferred to flat-bottomed rectangular tanks and reared at 21ºC. Live feed was gradually substituted until complete weaning at 38DAH from when larvae were fed exclusively upon the inert diet. The diets remained the same, though size was increased. The three inert diets were formulated and processed by SPAROS Lda (Faro, Portugal) to ensure consistent ingredients and proximate composition. Only part of the protein fraction was manipulated to result in different AA balances (Table I). Individual dry weight was determined at key developmental stages to monitor growth. The in vivo method of controlled tube-feeding as modified by Rønnestad et al. (2001) was used to

assess the effect of dietary amino acid profile on larval capacity to utilize protein during the most relevant stages throughout metamorphosis: 13, 19, and 25DAH. For this purpose, ^{14}C-labelled model peptides of different molecular weights (1.0KDa and 6.8KDa) were tested to estimate the metabolic budgets of different sized nitrogen forms (including absorption, catabolism, and retention).

Table I. Diet formulations with different AA balances.

Diet	Description
CTRL	Control diet. Based on protein sources that are typically used for larvae, including a commercial fish protein hydrolysate (CTRL) that was encapsulated for preventing leaching losses
PBAL	Partially balanced diet. Same proximate composition as CTRL, except for 4% the encapsulated fish protein hydrolysate that was substituted by encapsulated crystalline free AA (FAA), in order to meet the ideal AA profile defined by (Aragão et al. 2004) for Senegalese sole
WBAL	Well Balanced diet. Same proximate composition as CTRL, except for 8% the encapsulated fish protein hydrolysate that was substituted by encapsulated crystalline FAA, as to make the AA composition meet the ideal AA profile defined by (Aragão et al. 2004), now further corrected for amino acids bioavailability data as determined by (Saavedra et al. 2007).

Results and discussion

The substitution of fish protein hydrolysate by crystalline FAA had a negative effect on larval growth throughout the trial from 2 to 51DAH (Fig. 1). In spite of the remarkable individual variation both between and within replicates in all groups, the larvae co-fed with the control diet clearly performed better than larvae from the other treatments from 13DAH onward. This response to diet was remarkably accentuated with time and along the benthic phase (Fig. 1).

There was no influence of the diet on the absorption of the tracer peptides throughout development, but it is clear that the smaller peptide (1.0KDa) was better absorbed than the 6.8kDa one in all treatments (Fig. 2). This difference and the respective absorption levels remained similar throughout the metamorphosis from 13 to 25DAH (Fig. 2). There was also little influence of diet on retention and catabolism of the tracer peptides throughout development. A difference was noticed only during the metamorphosis climax (19DAH) (Fig.3). This suggests that diets with different amino acid profiles have little effect on larval protein utilization capacity. However there is a shift in the retention capacity of larvae, as until metamorphosis completed there is no difference in the retention of different sized peptides and then the 6.8kDa peptide becomes more efficiently retained than the 1kDa (Fig. 3). This suggests that post-larval metabolism may favour protection of higher molecular size peptides from catabolism, and preferentially use them for growth. This could at least partially explain why the substitution of protein hydrolysate by crystalline FAA in order to better balance die-

tary AA profiles does not improve growth. Another possible explanation for the growth results could be a possible effect of the dietary AA profile on the voluntary feed intake. Larvae fed a diet with an imbalanced AA profile may have increased feed intake as to compensate for AA deficiencies.

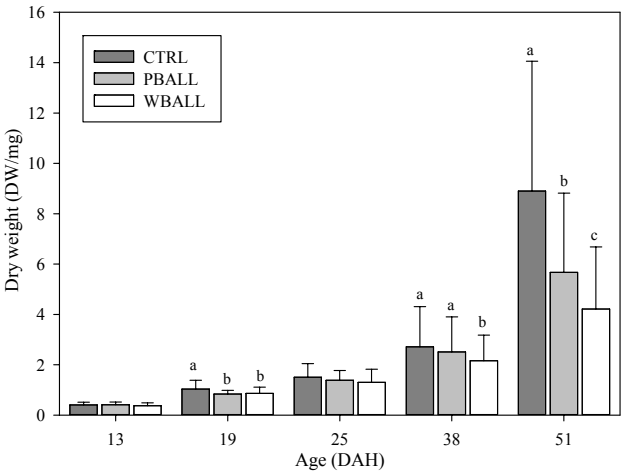


Fig. 1. Sole dry weight during the pelagic (13-25DAH) and benthic phase (38-51DAH) of the experiment. Values are means ± S.D. of treatments replicates. Comparisons between groups fed with different diets were made using one-way ANOVA followed by Tukey-Kramer post-hoc tests (P<0.05).

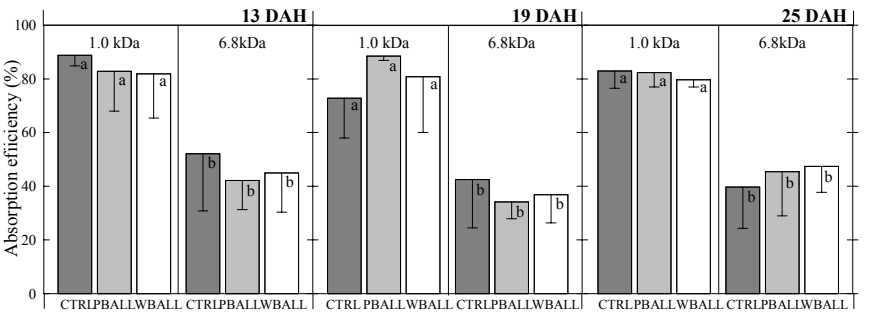


Fig. 2. Absorption efficiency (sum of the % of radiolabel in in body and in the metabolic trap in relation to the total tracer fed) for 1.0kDa and 6.8kDa peptides in sole larvae. Values are means ± SD. Comparisons between groups fed with different diets were made using two-way ANOVA. (P<0.05).

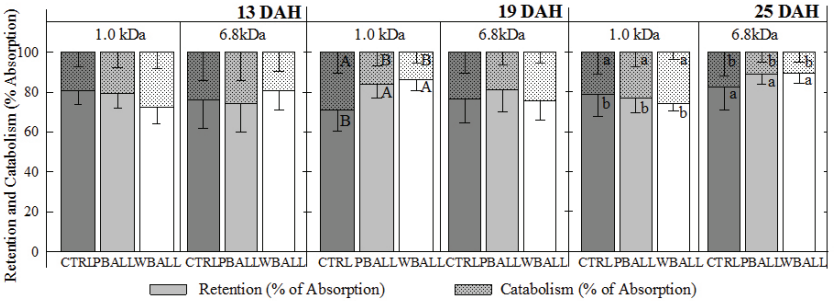


Fig. 3. Retention of 1.0kDa and 6.8kDa peptides (% of radiolabel in the body in relation to absorbed label) and peptide catabolism (% of radiolabel in the metabolic trap in relation to absorbed label) in sole larvae. Values are means±SD. Dissimilar superscript lower-case letters indicate a significant difference ($P<0.05$) between groups, detected by two-way ANOVA. Dissimilar superscript upper-case letters indicate a significant difference ($P<0.05$) between diets, detected by one-way ANOVA followed by Tukey-Kramer post-hoc tests performed within each peptide.

In short, balancing AA profile per se does not affect much AA utilisation and does not improve Senegalese sole larval growth. On the contrary, larvae fed an imbalanced (CTRL group) diet had faster growth. Further studies are needed to ascertain if this is caused by introducing fast-absorbed FAA in the diet and/or by an increase in feed intake to compensate for AA deficiencies.

This work was funded by Project EPISOLE (FCT) [PTDC/MAR/110547/2009] from FCT (Portugal), N. Richard and S. Engrola are supported by grants from FCT (Portugal) SFRH/BPD/23514/2005 and SFRH/BPD/49051/2008.

References

Aragão C., L.E.C. Conceição, H.-J. Fyhn, and M.T. Dinis. 2004. Estimated amino acid requirements during early ontogeny in fish with different life styles: gilthead seabream (Sparus aurata) and Senegalese sole (Solea senegalensis). Aquaculture 242(1-4):589-605.

Conceição L.E.C., L. Ribeiro, S. Engrola, C. Aragão, S. Morais, M. Lacuisse, F. Soares, and M.T. Dinis. 2007. Nutritional physiology during development of Senegalese sole (Solea senegalensis). Aquaculture 268(1-4):64-81.

Rønnestad I., C.R. Rojas-García, L.E.C. Conceição, and S.K. Tonheim. 2001. In vivo studies of digestion and nutrient assimilation in marine fish larvae. Aquaculture 201:161-175.

Saavedra M., M. Beltran, P. Pousão-Ferreira, M.T. Dinis, J. Blasco, and L.E.C. Conceição. 2007. Evaluation of bioavailability of individual amino acids in Diplodus puntazzo larvae: Towards the ideal dietary amino acid profile. Aquaculture 263(1-4):192-198.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

GENE EXPRESSION PROFILE OF *LITOPENAEUS VANNAMEI* JUVENILES FED DIFFERENT PROTEIN SOURCES AND DETECTION OF DIGESTIVE-RELATED GENES BY FUNCTIONAL GENOMICS

J. Carmona-Contreras[1*], D. Tovar-Ramírez[1], R. Civera-Cerecedo[1], M. Rojas-Contreras[2], M.A. Cadena-Roa[2], H. Nolasco-Soria[1], and E. Goytortúa-Bores[1]

[1] Centro de investigaciones Biológicas del Noroeste, La Paz, Baja California Sur, México. *E-mail: jcarmona@cibnor.mx
[2] Universidad Autónoma de Baja California Sur, La Paz, Baja California Sur, México.

Introduction

Food digestion is one of the most important functions in physiology. Analysis of digestive enzyme activity is an easy and reliable methodology for the investigation of the nutritional requirements and feeding ecology of marine invertebrates (Biesiot and Capuzzo, 1990; Fernández-Gimenez et al., 2001).

Penaeids adapt quite well to changes in diet composition by induction of digestive enzymes synthesized and secreted in the hepatopancreas (Le Moullac et al., 1997). Cousin et al. (1996) stated that amylase is one of the most studied digestive enzymes in shrimp and is highly efficient in digesting wheat starch.

It is known that dietary inclusion of red crab meal improve growth and nutrient digestibility in white shrimp *Litopenaeus vannamei* (Goytortúa-Bores et al., 2006). However, the molecular mechanisms involved are unknown. Nutrigenomics is a helpful tool to identify the genes related to feed utilization.

The main objective of this study was to determine the effect of the substitution of fish meal by red crab meal on transcriptomic responses in *L. vannamei*, by using heterologous microarrays,

Materials and methods

A 31-day feeding trial with juvenile shrimp (0.30g mean initial weight) was conducted at the Pichilingue Campus of the Universidad Autónoma de Baja California Sur. Two experimental diets were tested: a control diet, containing fish meal as main protein source, and a diet where fish meal was replaced by red crab meal. Dietary treatments consisted of four replicates (120-l tanks) contain-

ing 30 shrimp per tank. The growth (percent weight gain), survival, feed intake, and feed conversion ratio were determined for each treatment.

At 0 and 31 days of feeding, shrimp in intermolt stage were euthanized and the hepatopancreas was sampled for RNA extraction (Trizol®) and synthesis of cDNAs by using Improm II kit. RNA was extracted and treated with DNase to eliminate the excessive DNA.

The control and experimental cDNAs were hybridized on a heterologous microchip of the fruit fly *Drosophila melanogaster* with the total genome (15 139 genes). The reading was conducted in a microarray reader (Genepix 4100 A) and reader-generated files were processed with the Genepix software. The groups of up-regulated or down-regulated genes data were analyzed by using Genarise software by setting the Z score at a threshold of 1.5.

The validation of overexpressed genes encoding digestive enzymes was carried out through the quantification of the expression of these genes with qPCR, using SYBR® Green BIO-RAD. Specific primers for amylase, lipase, trypsin, and aminopeptidase were designed for qPCR and EF1α (elongation factor) was used as a reference gene.

Results and discussion

The results of the present experiment confirmed that growth performance of shrimp was significantly enhanced by dietary inclusion of red crab meal, as reported in Civera et al. (2000).The weight gain was significantly higher (P≤0.05) in shrimp fed with red crab meal (Fig. 1).

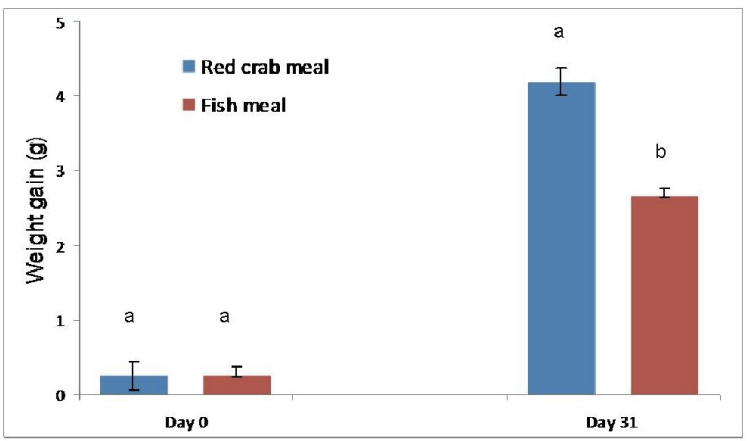


Fig. 1. Growth of *L. vannamei* juveniles fed on diets containing fish meal or red crab meal. Mean values ± S.D. with different letters indicate significant differences (P≤0.05).

A *z* score of 1.5 was obtained with a total of 906 up-regulated genes, from which 366 had no ID, and 536 were found with function and annotation. The search for biological process related to different genes resulted in 38 genes related to proteolysis and 14 genes related to lipid metabolism, which were overexpressed. There were also metabolic processes related enzymes as well as to signaling pathways in shrimp, according to DAVID, The Database for Annotation, Visualization and Integrated Discovery (http://david.abcc.ncifcrf.gov/).

The results related to gene expression (Fig. 2) show that main digestive enzymes aminopeptidase, amylase, lipase and trypsin correlates well with microarray results, and also with the highest growth obtained in shrimps fed red crab meal, compared to those fed fish meal.

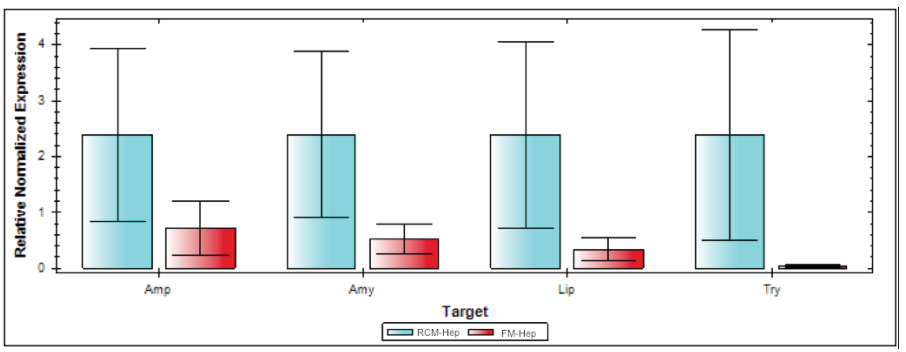


Fig. 2. Relative expression level of aminopeptidase (Amp), amylase (Amy), lipase (Lip), and trypsin (Try) in the hepatopancreas of shrimp fed red crab meal (RCM) or fishmeal (FM).

Nutrigenomic studies shows the importance of hepatopancreas in crustaceans because possess a high gene expression related to catalysis of nutrients and response to both quantity and quality of feeds.

Our study confirms that red crab meal enhances *L. vannamei* juvenile's growth when used to replace fish meal in the diet. A secondary, but most important benefit of red crab meal is that enhance the physiological status, body composition and also accelerates growth of shrimp, because it contain insulin like peptides as well as other functional molecules such as digestive and antioxidant enzymes (Vega-Villasante et al., 2004).

Acknowledgments

We thank Biol. Patricia Hinojosa Baltasar, Biol. Mar. Hever Lastisnere Barragan, M.C Jesús Neftali Gutiérrez Rivera and Dr. Jorge Ramírez for technical assistance, the University of BCS for the facilities and support provided to per-

form the bioassay, and CONACYT for scholarship 166078 granted to Jaime Carmona.

References

Biesiot P.M. and J.M. Capuzzo. 1990. Changes in digestive enzyme activities during early development of the American lobster Homarus americanus Milne Edwards. J Exp Mar BiolEcol 136:107-122

Civera R., E. Goytortúa, S. Rocha, H. Nolasco, F. Vega-Villasante, E. Balart, E., Amador, G. Ponce, G. Colado, G. Lucero, C. Rodríguez, J. Solano, A. Flores-Tom, J. Monroy, and G. Coral. 2000. Uso de la langostilla roja en la nutrición de organismos acuáticos 325-368p. In: Civera-Cerecedo, R., Pérez-Estrada, C. J., Ricque-Marie, D., Cruz-Suárez, L. E. (Eds.). Avances en Nutrición Acuícola IV. Memorias del Cuarto Simposium Internacional de Nutrición Acuícola, 15-18 de Noviembre de 1998. La Paz, B.C.S., México.

Cousin M., G. Cuzon, J. Guillaume, and A. Aquacop. 1996. Digestibility of starch in Penaeusvannamei: in vivo and in vitro study on eight samples of various origin. Aquaculture 140:361-372.

Fernández Gimenez A.V., F.L. García-Carreño, M.A. Navarrete del Toro, and J.L. Fenucci. 2001 Digestive proteinases of red shrimp *Pleoticus muelleri* (Decapoda, Penaeoidea): partial characterization and relationship with molting. Comp. Biochem. Physiol. 130B:331-338.

Goytortúa-Bores E., R. Civera-Cerecedo, S. Rocha-Meza, and A. Green-Yee. 2006. Partial replacement of red crab (*Pleuroncodes planipes*) meal for fish meal in practical diets for the white shrimp *Litopenaeus vannamei*. Effects on growth and *in vivo* digestibility. *Aquaculture* 256 (1-4):414-422.

Le Moullac G., B. Klein, D. Sellos, and A. Van Wormhoudt. 1997. Adaptation of trypsin, chymotrypsin and amylase to casein level and protein source in Penaeus vannamei (Crustacea Decapoda). J. Exp. Mar. Biol. Ecol. 208:107-125.

Vega-Villasante F, H. Nolasco A. Fallarero, and O. Carrillo-Farnes. 2002. Biochemical characterization of crude extract from *Pleuroncodes planipes* (Crustacea: Galatheidae) as potential feed additive, considerations for a new fishery on the Mexico Pacific coast. Hidrobiológica 12(2):119-28.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

INFLUENCE OF FEEDING REGIMES ON THE DIGESTIVE ENZYME PROFILE AND ULTRASTRUCTURE OF DIGESTIVE TRACT OF *CATLA CATLA*

R. Chakrabarti and I.M. Lukram

Aqua Research Lab, Department of Zoology, University of Delhi, Delhi 110 007, India
E-mail: aquaresearchlab@yahoo.co.in

Introduction

The feeding of larvae at early developmental stages with artificial diet is one of the major constraints for larviculture. Poor performance is usually found when the inert diets are fed to larvae from the onset of exogenous feeding (Rosenlund et al*.,* 1997). Lower growth in fish larvae fed with inert diet is related to poor attractiveness and low acceptance of diet; also inadequate digestion and assimilation compared to live food (Kolkovski, 2001). At first feeding, the digestive tract in most fish species contains the enzymes related to metabolism of molecules such as proteins, lipids, and glycogen. Enzyme activity has been observed to be relatively low in larvae compared with adult fish. Each enzyme develops independently during ontogenesis (Chakrabarti and Rathore, 2009). The combined feeding of live food and artificial diet from the start of exogenous feeding may be an alternative strategy. *Catla catla* (catla) is an economically important freshwater carp. Catla is a surface-feeding zooplanktivore. High larval mortality and poor growth have been recorded in this species in absence of live food. Digestive enzymes like amylase, protease, trypsin, chymotrypsin, and lipase were found in first feeding catla, but in less quantity and enzyme activities gradually increased during ontogenesis (Kumar et al*.,* 2000; Rathore et al*.,* 2005). The present investigation attempts to find out the food-mediated responses of various digestive enzymes of catla during early larval development. The effect of different types of food on the ultrastructure of the digestive tract of first feeding catla is also studied.

Materials and methods

Catla larvae (1.0±0.2mg) were cultured in 15-l aquarium in a recirculating system (Sharma and Chakrabarti, 2003). The stocking density was 125 larvae per aquarium. Larvae were fed with six different types of food: (1) live zooplankton for 30d, i.e from day-4 to 34 (LF), (2) artificial food for 30d (AF), (3) mixed food, i.e., 50% live zooplankton and 50% artificial food for 30d (MF), (4) live

zooplankton for initial 12d (i.e., day 4 to12), then shifted to artificial food from 13d onwards (LF-AF-13), (5) live zooplankton for initial 17d (i.e., day 4 to 17) and shifted to artificial food from day 18 (LF-AF-18) and (6) live zooplankton for initial 12d (i.e., day 4 to12) and then shifted to mixed food from day-13 onwards (LF-MF-13). Three replicates were used for each feeding scheme. Artificial diet containing 40% protein was prepared by using fish meal, wheat flour, cod liver oil, vitamin, and mineral premixes.

Fish were harvested after 30d and survival rate and weight of individual fish were recorded. Digestive system from individual fish was collected and pooled. Then pooled sample (100mg) was homogenized with 1ml of chilled distilled water. The homogenate was centrifuged at 10 000×g for 15min at 4°C and the supernatant was used for analysis. Total soluble protein was measured according to Bradford (1976). Digestive enzymes were assayed following standard methods: amylase (Bernfeld, 1955), chitinase and chitinobiase (Gutowska, 2002), total protease (Garcia-Carreno, 1992), trypsin and chymotrypsin (Erlanger et al., 1961); and lipase (Winkler and Stuckman, 1979).

For transmission electron microscopy, proximal, middle, and distal segments of the alimentary canal of larva were fixed separately in 2.5% glutaraldehyde, then post fixed in 1% OsO_4. Tissue was dehydrated in graded acetone series and embedded in araldite. Ultrathin sections were stained with uranyl acetate and lead citrate and observed under transmission electron microscope. All data were analyzed using one-way analysis of variance and Duncan's multiple range test. Statistical significance was accepted at $P<0.05$ level.

Results

Significantly ($P<0.05$) higher survival rate of catla was recorded in the feeding regime MF compared to the others. The average weight of catla was significantly ($P<0.05$) higher LF-MF-13, LF and MF compared to the other regimes. Significantly ($P<0.05$) higher amylase activity was found in fish cultured in LF-AF-18 compared to the others. This group was followed by LF-MF-13, LF-AF-13, AF, MF, and LF. Chitinase and chitinobiase activities were significantly ($P<0.05$) higher in the fish cultured in LF compared to the others. This was followed by MF, LF-AF-18, LF-AF-13, and AF. Protease and trypsin activities were significantly ($P<0.05$) higher in LF-MF-13 compared to others feeding regimes, except MF. There was no significant ($P>0.05$) difference in protease activities between MF and LF-MF-13. Highest chymotrypsin activity was recorded in the fish cultured in LF-MF-13. This group was followed by LF, MF, LF-AF-18, LF-AF-13, and AF. Significantly ($P<0.05$) higher lipase activity was recorded in LF-MF-13 compared to the others. This was followed by MF, LF-AF-18, LF-AF-13, AF, and LF. SDS-PAGE and substrate SDS-PAGE showed the

presence of 14 (14.34-97.57kDa) and 8 (15.26 to 72.88kDa) bands, respectively, in the digestive tissue protein of 34d old catla.

Ultrastructure study showed that the height of microvilli was significantly (P<0.05) higher in artificial diet fed fish. The number of mitochondria was maximum in the proximal segment of fish cultured in LF-MF-13 and middle and distal segments of fish cultured in AF compared to others. MF and LF-MF-13 were conspicuous by the absence of lipid droplet in the proximal and middle segments. Significantly (P<0.05) higher number of goblet cell was observed in LF-MF-13 and this was followed by AF. Pinocytotic channels were found in the distal segment of the fish cultured in LF and LF-MF-13.

Discussion

The effect of various combination of food on the digestive physiology of carp larvae has been recorded in the present study. Higher survival (MF) and average weight (LF-MF-13) of larvae in the mixed feeding scheme shows the importance of live food at the early developmental stage of catla. Presence of higher amount of digestive enzymes like total protease, trypsin, chymotrypsin, and lipase in LF-MF-13 results into better digestion of food in this group compared to the other feeding schemes. Considerable amount of amylase, chitinase, and chitinobiase are also recorded in this group. Ultrastructure study also confirmed the influence of food in the morphology of digestive tract of larva. Co-feeding/mixed feeding strategy at first feeding may be useful for carp larviculture.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

VIBRIO STATIC ACTIVITY IN THE CULTURE OF THE *PICOCHLORUM* SP. S1B CAN BE ATTRIBUTED TO THE BACTERIA ASSOCIATED WITH THE MICROALGA

Y.H. Chang[1], J.C. Kuo[1], and Y.M. Chen[1,2,3*]

[1] Institute of Biotechnology, National Cheng Kung University, Tainan City 701, Taiwan
[2] Center of Bioscience and Biotechnology, National Cheng Kung University, Tainan City 701, Taiwan
[3] Agriculture Biotechnology Research Center, National Cheng Kung University, Tainan 701, Taiwan

Introduction

Microalgae, such as *Nannochloropsis* sp. and *Chlorella* sp., are utilized to make green water systems in fish larval tanks to maintain water quality and provide shield effects; they also retain the nutritional quality of rotifers for fish larvae (Sharifah and Eguchi, 2011). Previously, our research team found an axenic algal strain isolated from the dried-out fish pond in Tainan City, Taiwan. It was named S1b and identified as *Picochlorum* sp. based on both phylogenetic analysis of 18S rDNA and morphological characterization.

According to the results of our previous study, *Picochlorum* sp. S1b could be produced using the ideal composition of amino acids in mixotrophic conditions and supplied the high growth rate rotifers (Chen et al., 2012). Besides, we observed that adjunction of S1b could effectively decrease total vibrio counts in orange-spotted grouper larval tanks. Subsequently, it was demonstrated S1b possesses the vibrio static activity only when cultivated in a non-sterile environment, and we speculated it may be derived from bacteria associated with S1b. Therefore, the purpose of our study to find out the exact mechanism by evaluating the vibrio static with non-axenic S1b.

Materials and methods

Microalgae culture conditions
The microalga was used in this study, S1b, was purified from the dried-out fish pond in southern Taiwan. It was identified as *Picochlorum* sp. based on both phylogenetic analysis of 18S rDNA and morphological characterization (Yang et al., 2010). S1b was cultivated in sterile and non-sterile Walne's medium prepared with seawater in 1-l cylindrical containers. These cultures were incubated

under 27°C, illuminated at 6000lux unilaterally for 12h per day, and aerated at 0.1vvm (air volume flow per unit of liquid volume per minute) with air pre-filtered through 0.22-μm PVDF filter (Millipore, Billerica, MA, USA).

Preparation of S1b crude extracts for its inhibitory effects analysis with V. harveyi and V. campbellii

S1b crude extracts were obtained from axenic cultures of *Picochlorum* sp. S1b. 200ml of algal culture were rinsed with sterile seawater to remove organic contaminants and centrifuged (6000×g, 5min) to collect microalgal cells. Then, these cells were placed in sterile seawater and disrupted with ultrasonication (strength 21%, duty cycle pulse/pause = 1sec/1sec, 40min) at 4°C. Subsequently, products of S1b crude extracts were transferred in marine agar plate inoculating with *V. harveyi* and *V. campbellii* to measure the antibacterial activity by using well-diffusion method (Adwan and Mhanna, 2009).

Antibacterial effects of S1b cultures for V. harveyi and V. campbellii

There are experimental groups for measuring the anti-vibrio effects: (1) non-axenic S1b in Walne's medium with two vibrio strains; (2) S1b extracellular products with two vibrio strains; (3) axenic S1b in Walne's medium with two vibrio strains; and (4) Walne's medium with two vibrio strains (Blank). All of experimental groups were incubated at 25°C, and the numbers of total vibrio count (TVC) were analyzed each day.

Isolation and identification of bacterial strains associated with Picochlorum sp. S1b

We collected non-axenic S1b for different culture days and measured the microfloral composition by ITS-PCR (Internal transcribed spacer PCR) (Gonzalez et al., 2003). The 16S rRNA to 23SrRNA gene was amplified by using forward primer G1 (5'-GAAGTCGTAACAAGG-3') and L1 (5'-CAAGGCATCCACCGT-3'). All PCR products were purified and sequenced using the BLAST program for matches to sequences deposited in GenBank, National Center for Biotechnology Information, USA (www.ncbi.nlm.nih.gov/genbank/index.html).

Results

Picochlorum sp. s1b has the strong inhibitory effect on vibrio attributed to the bacteria associated with the microalga

We selected two pathogenic vibrio species of *V. harveyi* and *V. campbellii* to evaluate the vibrio static with S1b crude extract, S1b extracellular products, axenic, and non-axenic culturing S1b. The crude extract had no significant growth inhibition for vibrio (data not shown), even the S1b extracellular products (Fig. 1B). Only non-axenic culturing S1b possessed the significant vibrio static activ-

ity (Fig. 1A), we speculated this inhibitory phenomenon derived from some bacteria strains in non-axenic culturing S1b.

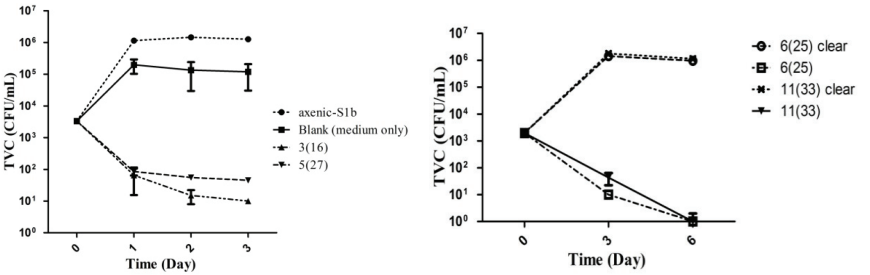


Fig. 1. The change of total counts in different S1b groups co-culture with vibrio. (A) Left. The change of total counts in axenic S1b, two groups of non-axenic S1b, and blank co-culture with vibrio ($3.35\times103CFU.ml^{-1}$) after 3 days. (B) Right. The change of total counts in non-axenic S1b and S1b extracellular products (removed microalga and bacteria with 0.22-μm filter) with vibrio ($1.95\times103CFU.ml^{-1}$) after 6 days. One way ANOVA analysis is used. ($p<0.001$). 3(16), the third batch culture of non-axenic S1b after 16 days. 5(27), the fifth batch culture of non-axenic S1b after 27 days. 6(25), the sixth batch culture of non-axenic S1b and S1b extracellular products after 25 days. 11(33), the eleventh batch culture of non-axenic S1b and S1b extracellular products after 33 days.

The vibrio inhibition of bacteria strains isolated from non-axenic S1b

We isolated 80 bacteria strains from non-sterile cultures of S1b to evaluate the vibrio static activity by co-culturing the bacterial strain with S1b and later with additional pathogenic *V. harveyi* and *V. campbelli*. From the results, two (48 and 50) and four strains (7, 8, 46, and 61) could supress the counts of *V. harveyi* and *V. campbellii* below $10^1CFU.ml^{-1}$, respectively (Table I and II). Through the 16S rDNA alignment, the bacteria belong to the Rhodobacteraceae and Flavobacteriaceae families (data not shown).

Table I. Static activities of *V. harveyi* in target bacterial strains co-cultured with axenic S1b after 6 days.

Strain No.	Incubation time (days)			
	0		6	
	TVC ($CFU.ml^{-1}$)	HBC ($CFU.ml^{-1}$)	TVC ($CFU.ml^{-1}$)	HBC ($CFU.ml^{-1}$)
Blank	$1.51\pm0.05\times10^3$	-	$3.08\pm0.96\times10^6$	-
48	$1.51\pm0.05\times10^3$	$1.88\pm1.52\times10^7$	$6.00\pm0.95\times10^1$	$5.83\pm3.25\times10^6$
50	$1.51\pm0.05\times10^3$	$1.50\pm0.17\times10^6$	$5.00\pm7.07\times10^0$	$7.32\pm7.46\times10^6$

Table II. Static activities of *V. campbellii* in target bacterial strains co-cultured with axenic S1b after 6 days.

Strain No.	Incubation time (days)			
	0		6	
	TVC (CFU.ml^{-1})	HBC (CFU.ml^{-1})	TVC (CFU.ml^{-1})	HBC (CFU.ml^{-1})
Blank	$1.44\pm0.43\times10^{3}$	-	$4.23\pm2.74\times10^{6}$	-
7	$1.44\pm0.43\times10^{3}$	$5.47\pm0.64\times10^{6}$	$1.67\pm2.36\times10^{0}$	$1.72\pm2.02\times10^{7}$
8	$1.44\pm0.43\times10^{3}$	$3.27\pm1.76\times10^{6}$	$0.00\pm0.00\times10^{0}$	$2.82\pm3.40\times10^{6}$
46	$1.44\pm0.43\times10^{3}$	$2.13\pm0.13\times10^{6}$	$4.34\pm0.47\times10^{1}$	$3.76\pm5.04\times10^{7}$
61	$1.44\pm0.43\times10^{3}$	$1.20\pm0.47\times10^{7}$	$0.00\pm0.00\times10^{0}$	$2.89\pm3.42\times10^{7}$

Discussion

According to our investigation, we observed that S1b has the strong inhibitory effect on vibrio only when cultivated in a non-sterile environment. This is a clear indication that the inhibitory effect is not necessarily of S1b, but rather bacteria associated with it. Subsequently, there were 6 bacteria strains were demonstrated possessing the vibrio static activity for *V. campbellii* or *V. harveyi*. These strains belong to Rhodobacteraceae and Flavobacteriaceae families, but the exact mechanism is still unknown. Hence, further study will be required to elucidate the interactions among these six bacterial strains and the *Picochlorum* sp. strain S1b with regard to vibrio static activity, and assess the applicability of these co-cultures as probiotics for the prevention of vibriosis in marine aquaculture.

References

Adwan G. and M. Mhanna. 2009. Synergistic effects of plant extracts and antibiotics on Staphylococcus aureus strains isolated from clinical specimens. Asian Pacific Journal of Tropical *Medicine* 2:46-51.

Chen T.Y., H.Y. Lin, C.C Lin, C.K. Lu, and Y.M. Chen. 2012. *Picochlorum* as an alternative to *Nannochloropsis* for grouper larval rearing. *Aquaculture* 338: 82-88.

Gonzalez N, J. Romero J, and R.T. Espejo. 2003. Comprehensive detection of bacterial populations by PCR amplification of the 16S-23S rRNA spacer region. *Journal of Microbiological Methods* 55: 91-97.

Sharifah E.N. and M. Eguchi. 2011. The Phytoplankton *Nannochloropsis oculata* enhances the ability of Roseobacter Clade bacteria to inhibit the growth of fish pathogen *Vibrio anguillarum*. PLoS ONE 6(10):e26756. doi:10.1371/journal.pone.0026756.

Yang H.L., C.K. Lu, S.F. Chen, and Y.M. Chen. 2010. Isolation and characterization of Taiwanese heterotrophic microalgae: screening of strains for docosahexaenoic acid (DHA) production. *Marine Biotechnology* (NY) 12:173-185.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

TOWARDS THE DEVELOPMENT OF NEW QUALITY INDICES IN JUVENILE FISH – RELATIONSHIP OF CAUDAL-FIN MORPHOLOGY WITH THE THERMAL HISTORY OF JUVENILES

M. Christou, M. Iliopoulou, and G. Koumoundouros

Biology Department, University of Crete, Vasilika Vouton, 70013 Heraklion, Crete, Greece, tel. +30 2810394065, gkoumound@biology.uoc.gr

Introduction

In finfish aquaculture, quality indices aim either to describe or to predict the product quality at certain phases of the rearing process. At the end of the hatchery phase, normal morpho-anatomy of juveniles and intra-population homogeneity of body size are the two most commonly used quality indices (Koumoundouros, 2010; Boglione et al., 2013). Although existing literature clearly suggests that characters like muscle cellularity could be used as predictors of growth rate (e.g., Galloway et al., 1999), currently there is not any such index which is applied at the level of commercial hatcheries.

As a first step towards the development of new quality indices for growth rate, in the present study we examined whether the growth pattern of caudal-fin lepidotrichia could be used to differentiate fish with different thermal histories and thus growth rates. Gilthead seabream (*Sparus aurata*) and zebrafish (*Danio rerio*) were used as test species.

Materials and methods

The effect of water temperature on the morphology of the caudal fin was examined by subjecting the experimental populations to different thermal regimes during the embryonic and larval phase. Selected experimental temperatures were 24, 28, and 32°C for zebrafish and 16, 19, and 22°C for sea bream. Maintenance of zebrafish experimental populations followed Georga and Koumoundouros (2010), with slight modifications. For sea bream, egg incubation and larval rearing were performed as described in Georgakopoulou et al. (2010). All experiments were performed in duplicate.

In zebrafish, 50 individuals were randomly sampled from each experimental population at the end of metamorphosis (11-14mm SL, standard length). In sea bream, 3-4 random samples of 50 individuals each were taken from each popula-

tion during the metamorphosis phase (~10-25mm SL). Sampled fish were anaesthetised, fixed in 5% phosphate buffered formalin and stained for bone and cartilage. Standard length was measured post-staining (from the tip of snout to the base of the middle caudal lepidotrichium).

On each specimen, landmark measurements were taken on the intersegmental joints of three caudal-fin lepidotrichia (Fig. 1). The lengths of the anterior 1-9 segments were estimated from these landmark measurements on each lepidotrichium (Fig. 1). The first upper (M), the ninth upper (U) and the ninth lower (L) lepidotrichium was examined in zebrafish. In sea bream, the examined lepidotrichia were the second upper (U), the second lower (M) and the eighth lower (L). TpsDig2 software was used for the landmark measurements. One-way analysis of variance was used to test the effect of temperature on the length of each segment. The morphometric characters of the caudal fin were subjected to canonical variate analysis and analysis of classification matrix, with the a priori probability of classification to be proportional to the size of each sample.

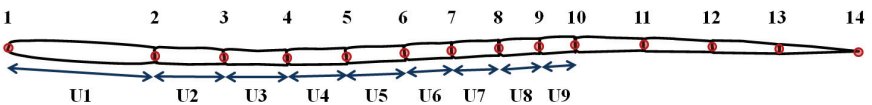


Fig. 1. Landmarks collected on each lepidotrichium. The lengths of the first nine segments are indicated (U1-U9). First landmark was positioned on the anterior tip of the lepidotrichium.

Results and discussion

In zebrafish, the length of the first segment of each lepidotrichium (U1, L1, M1) was significantly correlated with the SL of the fish (Fig. 2). The length of the rest segments was proven to be independent of the SL (data not shown) and to be significantly affected by the water temperature (Fig. 3, only data for the second and third segment of the upper lepidotrichium are shown).

In sea bream, the first segment of all the lepidotrichia followed a bimodal growth with SL, presenting two clear growth zones (Fig. 2). The rest segments of each lepidotrichium followed a continuous pattern of growth with SL (data not shown), similar to what was observed in zebrafish. In all cases, the length of the segments was proven to be independent of the SL (data not shown). ANOVA revealed a significant effect of water temperature on the length of the segments of lepidotrichia. In the case of the first segments, significant differences were shown for both growth zones (Fig. 3).

Canonical variate analysis confirmed the significant effect of temperature on the structure of lepidotrichia for both species (Fig. 4). The reclassification of the in-

dividuals to the initial groups was successful for the 77.4% and 82.7% of the zebrafish and sea bream individuals respectively.

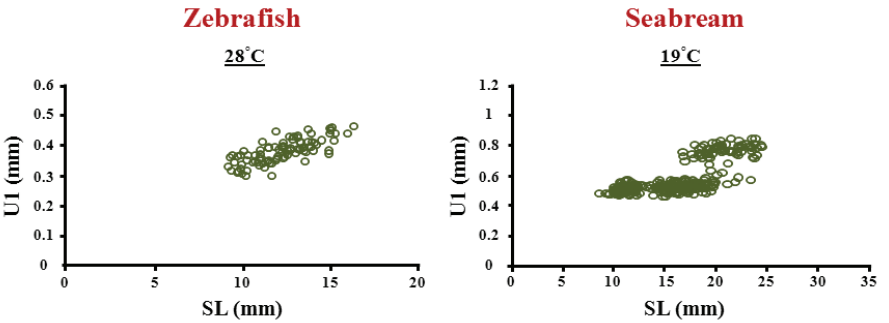


Fig. 2. Relationship of the length of the first segment of U lepidotrichium with the standard length of the fish. Only one temperature for each experiment is shown.

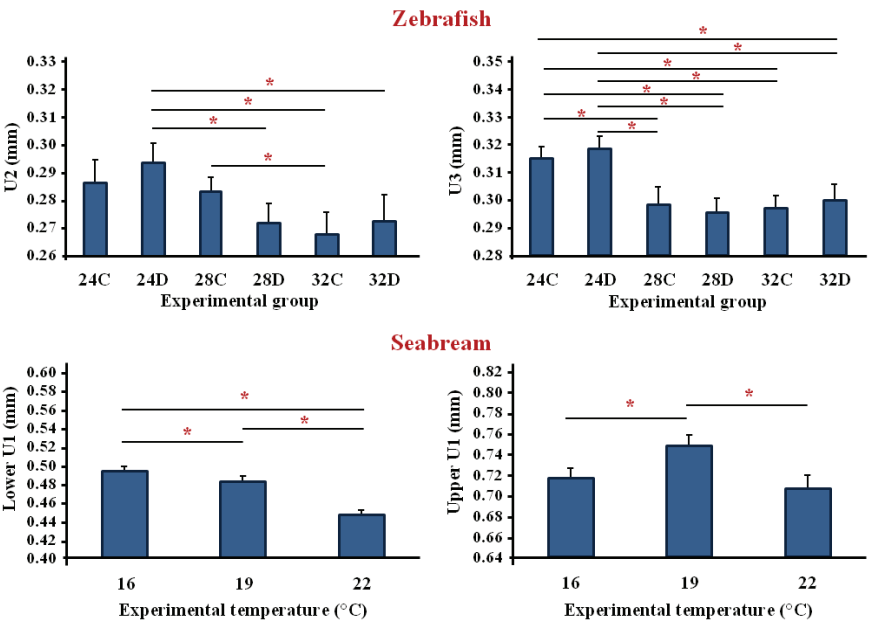


Fig. 3. Effect of water temperature on the length of segments. For zebrafish, only data for the 2nd and 3rd segment of upper lepidotrichium are given. Data given for seabream correspond to the two growth phases of the 1st segment of upper lepidotrichium (Fig. 2). *, $p<0.05$. Error bars equal to 2 SE.

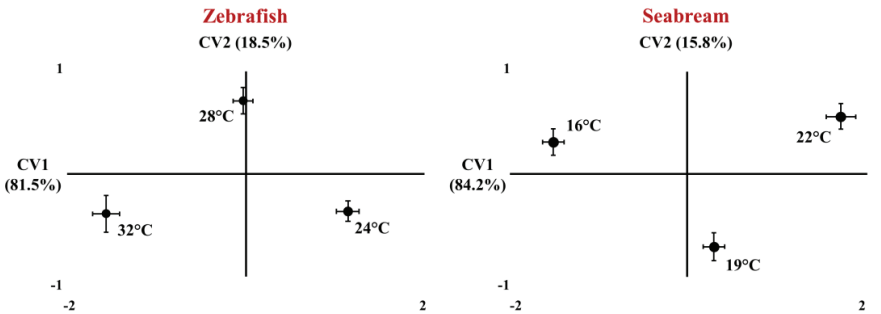


Fig. 4. Distribution of different groups along the canonical variables (CV1, CV2). Error bars equal to 1 SE.

Although our results demonstrate the significant effect of water temperature on the growth pattern of the caudal fin, it is still under question whether differences in the caudal-fin anatomy could serve as an accurate descriptor of growth history of the fish; especially at the intra-population level.

Acknowledgements

This study was partially supported by the program NSRF 2007-2013, «Competitiveness & Entrepreneurship» (call Cooperation I, Project No 09SYN-24-619) of the Ministry of Education, Religious Affairs, Culture and Sports, Greece.

References

Boglione C., E. Gisbert, P. Gavaia, P.E. Witten, M. Moren, S. Fontagné, and G. Koumoundouros. 2013. A review on skeletal anomalies in reared European larvae and juveniles Part 2: Main typologies, occurrences and causative factors. Reviews in Aquaculture, in press.

Galloway T.F., E. Kjorsvik, and H. Kryvi. 1999. Muscle growth and development in Atlantic cod larvae (*Gadus morhua* L.) related to different somatic growth rates. Journal of Experimental Biology 202(15):2111-2120.

Georga I., and G. Koumoundouros. 2010. Thermally induced plasticity of body shape in adult zebrafish *Danio rerio* (Hamilton, 1822). Journal of Morphology 271(11):1319-1327.

Georgakopoulou E., P. Katharios, P. Divanach, and G. Koumoundouros. 2010. Effect of temperature on the development of skeletal deformities in Gilthead seabream (*Sparus aurata* Linnaeus, 1758). Aquaculture 308(1-2):13-19.

Koumoundouros G. 2010. Morpho-anatomical abnormalities in Mediterranean marine aquaculture. In: Recent Advances in Aquaculture Research. Transworld Research Network, Kerala, India. pp. 125-148.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

INVESTIGATING CAUSES OF SKELETAL MALFORMATION IN YELLOWTAIL KINGFISH *SERIOLA LALANDI*

J.M. Cobcroft[1,2*], S.C. Battaglene[1], J.C.G. Biggs[1], and D.S. Fielder[3]

[1] Fisheries, Aquaculture and Coasts Centre, Institute for Marine and Antarctic Studies, University of Tasmania, Private Bag 49, Hobart, Tasmania, 7001, Australia
[2] School of Science, Education and Engineering, University of the Sunshine Coast, Locked Bag 4, Maroochydore DC, Queensland 4558, Australia
[3] New South Wales Department of Primary Industries (Fisheries), Locked Bag 1, Nelson Bay, New South Wales 2315, Australia

Introduction

Hatchery production of yellowtail kingfish, *Seriola lalandi*, in Australia has been hampered by malformations, particularly those to the jaw. The efficiency of hatchery operations is negatively impacted by the costs of larval and nursery rearing of these fish, as well as the labour associated with handsorting out malformed fish prior to transfer to seacages (Cobcroft and Battaglene, 2013). Given the proposed expansion of *S. lalandi* culture in South Australia (SA) from 500 to 3000 tonnes p.a. over 5 years, and in Western Australia (WA), it is vital that factors causing malformations can be understood and managed, and that the proportion of premium quality juveniles can be reliably increased.

Materials and methods

In three experiments, we investigated the effects of temperature (low, 21.5°C and high, 24.5°C), live feed enrichments (S.presso and Algamac), and light intensity (700 and 32 000lux, equivalent to ~10µmol.s^{-1}.m^{-2} and ~450µmol.s^{-1}.m^{-2}, respectively) during larval rearing on jaw and vertebral column malformations in metamorphosed *S. lalandi* (>10mm total length, TL). Fertilised eggs were sourced from captive broodstock in SA for the temperature experiment, and from New South Wales (NSW) Fisheries for the others. Newly-hatched larvae were stocked at 20 larvae.l^{-1} for the temperature experiment, or 3.5 larvae.l^{-1} for the other experiments, into 2000-l tanks and cultured in greenwater (*Tetraselmis* sp. and *Nannochloropsis* sp.; Instant Algae, Reed Mariculture Inc, USA, at 5×10^4 – 1×10^6 cells.ml^{-1}), with 100% exchange daily with ozone-disinfected seawater at 32ppt salinity. Photoperiod was 12h L: 12h D, and light intensity from fluorescent lights was ~700lux at the water surface in the tank centre (except for the light intensity experiment). From 2 days post hatch (dph), larvae

were fed rotifers *Brachionus plicatilis* at $10ml^{-1}$ enriched with Algamac 3050 (Aquafauna Biomarine, CA) or S.presso (INVE Aquaculture, Belgium) according to manufacturers' instructions. Three replicate tanks were used per treatment. The rearing parameter tested in earlier experiments which produced best results for *S. lalandi* performance was adopted as best-practice in subsequent experiments.

Larvae were sampled from each tank at the end of the experiment for growth (n=20 standard length, SL, and n=50 dry weight), n=100 fixed in formalin for malformation assessment, and all remaining larvae were counted to determine final survival. A subsample of n=53–68 larvae per tank from the temperature experiment were cleared and stained with alcian blue and alizarin red using a modified method of Taylor and Van Dyke (1985) to count vertebrae and assess vertebral malformations. Results were analysed by the Student's *t*-test to determine statistical differences between treatments for growth, survival, and jaw and vertebral malformations. Values are presented as mean ± SD and significance was set at $P<0.05$.

Results and discussion

Growth, indicated by final fish size, and survival were significantly higher in larvae fed Algamac-enriched rotifers (Table I). Survival was also significantly higher in fish reared under the higher light intensity. Jaw malformation in the metamorphosed fish was similar to that described by Cobcroft and Battaglene (2013). There were no significant effects of any treatment on the prevalence of jaw deformity in *S. lalandi*, which was low in these experiments compared with values reported from commercial production (10–20% in 2008; Cobcroft and Battaglene, 2013). The current experiments were conducted in NSW and not at commercial hatcheries in SA and WA, and the fertilised eggs sourced from SA for the temperature experiment yielded the highest jaw malformation of all three experiments. However, the larval stocking density was also higher in that experiment which confounds the suggestion that the egg source was an important factor. Consequently, larval stocking density, egg source (genetics), and other factors that are different between facilities (such as water quality, tank colour and light source) remain to be examined in relation to effects on jaw deformity. Tank colour and patterning has been demonstrated to reduce jaw malformation in similar oceanic larvae of striped trumpeter, *Latris lineata*, through a concomitant decrease in hard-surface interactions (walling behaviour) and should be tested with *S. lalandi* (Cobcroft and Battaglene, 2009).

The number of vertebrae in *S. lalandi* reared in different water temperatures ranged from 24-27, due to fusion of vertebrae and additional vertebrae. However, temperature was found to have no significant effect on total vertebrae number, which was 25 in most fish. This finding contradicts the modern inter-

pretation of Jordan's rule which states that cold-water representatives of fishes have a higher number of vertebrae, including intra-species populations (McDowall, 2008). An additional vertebra occurred most often in the pre-haemal region in larvae reared at 21.5°C, agreeing with previous studies that found the pre-haemal region to be susceptible to vertebral malformation (Fernández et al., 2008; Roo et al., 2010). Rearing *S. lalandi* at the lower temperature resulted in a prevalence of vertebral column abnormalities around three times higher (10.8±3.8%) than those at 24.5°C (3.0±2.4%) (P=0.032). These abnormalities included fused caudal vertebrae and caudal vertebrae with supernumerary spines.

Table I. Growth, survival, and jaw malformation of *Seriola lalandi* cultured with different temperature (Temp.), enrichment (Enrich.) and light intensity (Light) levels. Larval age indicates age at the end of the experiment in days post hatch (dph). Values are mean ± SD, and the same superscripts within experiment and parameter indicate treatment levels that are not significantly different (P > 0.05).

Treatment	Level	Larval age (dph)	Standard length (mm)	Dry weight (mg)	Survival (%)	Jaw malformation (%)
Temp. (°C)	21.5	27	10.8±1.4[a]	7.4±2.7[a]	6.2±2.6[a]	13.7±4.5[a]
	24.5	20	11.2±2.4[a]	6.6±3.7[a]	8.1±3.1[a]	7.3±2.5[a]
Enrich.	Algamac	20	13.3±1.2[a]	12.8±1.8[a]	2.5±2.3[a]	2.5±2.1[a]
	S.presso	20	11.8±1.7[b]	6.1±2.4[b]	11.7±5.6[b]	4.3±3.5[a]
Light (lux)	700	18	9.8±0.2[a]	5.1±0.2[a]	4.9±3.6[a]	2.3±1.2[a]
	32 000	18	11.2±0.5[a]	6.1±0.7[a]	11.0±2.3[b]	2.3±1.5[a]

Conclusions

Based on this study, we recommend a rearing temperature of 24.5°C, rotifer enrichment with S.presso, and a high light intensity of 32 000lux for the larval rearing of *S. lalandi* to minimise skeletal abnormalities and to optimise survival and growth. As jaw malformation remains a critical issue in commercial culture, other parameters are under investigation to determine primary causal factors and reduce skeletal malformations.

Acknowledgements

This study was funded by the Australian Seafood CRC (Project 2010/753), which is established and supported under the Australian Government's Cooperative Research Centres Programme. Other investors in the CRC are the Fisheries Research and Development Corporation, Seafood CRC company members, and supporting participants.

References

Cobcroft, J.M. and S.C. Battaglene. 2009. Jaw malformation in striped trumpeter *Latris lineata* larvae linked to walling behaviour and tank colour. Aquaculture 289:274-282.

Cobcroft, J.M. and S.C. Battaglene. 2013. Skeletal malformations in Australian marine finfish hatcheries. Aquaculture 396–399:51-58.

Fernández, I., F. Hontoria, J.B. Ortiz-Delgado, Y. Kotzamanis, A. Estévez, J.L. Zambonino-Infante, and E. Gisbert. 2008. Larval performance and skeletal deformities in farmed gilthead sea bream (*Sparus aurata*) fed with graded levels of Vitamin A enriched rotifers (*Brachionus plicatilis*). Aquaculture 283:102-115.

McDowall, R.M. 2008. Jordan's and other ecogeographical rules, and the vertebral numbers in fishes. Journal of Biogeography 35:501-508.

Roo, F.J., C.M. Hernández-Cruz, J.A. Socorro, H. Fernández-Palacios, and M.S. Izquierdo. 2010. Occurrence of skeletal deformities and osteological development in red porgy *Pagrus pagrus* larvae cultured under different rearing techniques. Journal of Fish Biology 77:1309-1324.

Taylor, W.R., G.C. Van Dyke. 1985. Revised procedures for staining and clearing small fishes and other vertebrates for bone and cartilage study. Cybium 9:107-119.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

INTENSIFICATION OF *LITOPENAEUS VANNAMEI* LARVICULTURE

M.L. Cobo[1], R. Wouters[2], M. Wille[3], S. Sonnenholzner[1], J. Calderón[1], and P. Sorgeloos[3]

[1] Escuela Superior Politécnica del Litoral (ESPOL), Centro Nacional de Acuicultura e Investigaciones Marinas (CENAIM). Campus Gustavo Galindo Velasco, Km 30.5 vía Perimetral, P.O. Box 09-01-5863, Guayaquil, Ecuador
[2] INVE Technologies N.V., Dendermonde, Belgium
[3] Laboratory of Aquaculture & *Artemia* Reference Center, Ghent University, Rozier 44, 9000 Ghent, Belgium

The production of marine crustacean species – dominated by white leg shrimp *Litopenaeus vannamei* – is expected to grow in the coming decades. This growth will require a shift toward more intensive farming practices as well as increasing numbers of good quality larvae from commercial hatcheries. Larval rearing is identified as the weak link in this process, currently already unable to provide constant supply of larvae. Hatcheries applying traditional aquaculture systems (TAS) are facing problems with the degradation of water quality and the occurrence of diseases. The development of recirculating aquaculture systems (RAS) may be very useful for the intensification of larviculture. RAS could increase the larval output, thus enhancing the profitability of hatchery production, while minimizing water discharge into the environment and reducing the risk of pathogen introduction into the culture system. As such, the objective of the present work is to contribute to the development of new culture techniques for the intensive production of *L. vannamei* larvae through the use of RAS.

A first attempt of intensification of larval rearing techniques was made in a series of experiments through the implementation of an experimental RAS. The experiments evaluated the effects of stocking density (750 to 2000 larvae.l^{-1}), feeding strategy (different concentrations of live and artificial food and continuous feeding), and recirculation rate (250 and 1000%.d^{-1}) on the survival and growth of *L. vannamei* larvae. Increasing density led to increased mortality, unless the feed ration was increased to compensate losses through the recirculation system. Our results suggest that increasing water exchange rate does not improve larval survival or growth. The combination of a stocking density of 1000 larvae.l^{-1} and a water exchange rate of 500%.d^{-1} consistently presented a high mean survival and biomass output.

In a first pilot-scale experiment, a simple compact prototype RAS of $1m^3$ was used with an initial stocking density of 1000 larvae.l^{-1}, applying a water exchange rate of 500%.d^{-1}. The high-density culture was compared with a TAS using a stocking density of 100 larvae.l^{-1}. A lower survival (50±7% in RAS versus 62±8% in TAS) was recorded for the intensive larval RAS, probably due to the infestation with filamentous bacteria coming from the live feed, especially rotifers. Dry weights were reduced, but larval output (number of larvae produced per liter) and biomass in RAS was eightfold and fivefold of that in TAS, respectively.

To overcome the problem of fouling, umbrella-stage *Artemia* was evaluated as a food source for the early stages of *L. vannamei* and its optimal feeding concentration for each larval stage was also assessed under traditional culture conditions. Including umbrella-stage *Artemia* in the feeding regime of *L. vannamei* larvae starting from the early Z2 sub-stage yielded good results in terms of growth and survival while filling the size gap between microalgae and *Artemia*. Therefore, this new prey item was also evaluated in the intensive larval RAS. The results show that survival and growth were similar to those obtained in TAS, and higher than those obtained in the experiments where rotifers were used. Survival was comparable to those obtained in TAS (71% in RAS and 74% in TAS). However, the stocking density used in RAS was ten times higher, yielding an average output of 710 larvae.l^{-1} compared to only 74 larvae.l^{-1} in the TAS. Higher dry weights and higher biomass were also obtained. The condition of the larvae was estimated by means of a challenge test with a pathogenic bacterium and by monitoring post-larval performance in a subsequent nursery culture test. These tests indicated that there was no difference in condition between larvae reared intensively in a RAS (including umbrella-stage *Artemia* in the feeding regime) and larvae reared in TAS. Finally, it was calculated that the investment cost and annual production cost for intensive *L. vannamei* larviculture using RAS could be reduced by 25% as compared to the TAS.

In conclusion, the findings of this work demonstrate that the intensification of *L. vannamei* larviculture is feasible through the implementation of RAS, and proved the effectiveness of intensive RAS in producing high quantities of good quality larvae, maximizing biomass, controlling water quality, reducing operating costs, and increasing biosecurity.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EFFECT OF SANOLIFE MIC-F AS LARVAL FISH PROBIOTIC ON HOST STRESS RESISTANCE, HISTOLOGY, AND MICROBIOTA IN SEABREAM

M. Corteel[1], G. Franchi[2], L. Chiappi[2], A. Rekecki[1], T. De Wolf[2], and G. Rombaut[1]

[1] INVE Technologies, Hoogveld 93, 9200 Dendermonde, Belgium
[2] MARICOLTURA DI ROSIGNANO SOLVAY srl, Via P. Gigli (Loc. Lillatro), 57013 Rosignano Solvay (LI), Italy

Introduction

In most, if not all, intensive aquaculture hatchery and rearing facilities, problems with microbial management are encountered. Farmers have relatively few tools to control the microbial condition, for example changing water and using antibiotics. These practices may not guarantee a stable, predictable production, but even in case of the latter may threatening the sustainability of aquaculture.

It is against this background that the importance of microbial management was acknowledged as an important and effective solution. In this view, the use of effective and aquaculture-specific probiotics is one of the practices that can be considered. Probiotic strains colonize the environment and larval fish, excluding (opportunistic) pathogenic microorganisms and stabilizing production.

The aim of the present work was to evaluate the application of a probiotic mixture (Sanolife MIC-F) on the robustness of sea bream larvae.

Materials and methods

Fish test
The effect of Sanolife MIC-F (INVE Aquaculture, Dendermonde, Belgium) on survival, growth, and deformities of sea bream larvae was tested. The test was carried out in triplicate, using nine 1000-l tanks. A standard feeding protocol was applied, using *Nannochloropsis* as greenwater agent, rotifers and *Artemia* as live feed, and different sizes of O.range (INVE Aquaculture, Dendermonde, Belgium) as artificial dry diet. Rotifers were cultured on S.parkle (INVE Aquaculture, Belgium) and rotifers and *Artemia* were enriched with S.presso (INVE Aquaculture, Dendermonde, Belgium). During the trial the temperature, DO and

pH were measured on a daily basis. Swimbladder development, larval length, and survival of the larvae were determined on a weekly basis.

Probiotics
Sanolife MIC-F (INVE Aquaculture, Dendermonde, Belgium) contains $1x10^{10}$ spores per gram. The mixture, composed of three *Bacillus* strains – *Bacillus subtilis, B. licheniformis*, and *B. pumilus* – was mixed with water and administered directly to the larval rearing tank. Two different concentration of MIC-F were tested, i.e., 2.5g MIC-F.m^{-3}.day (MIC 2.5) and 5.0g MIC-F.m^{-3}.day (MIC 5). MIC-F was applied from 2dph onwards, until 30dph.

Osmotic stress test
At 30dph and 46dph, 30 sea bream larvae were randomly taken from the larval fish tanks and transferred to 500-ml beakers filled with seawater adjusted to a salinity of 57g.l^{-1}. The osmotic stress resistance of the fish larvae was evaluated by determining the cumulative mortality for each treatment. The osmotisc stress test lasted for one hour and every three minutes the amount of dead fish was counted. Subsequently, the average cumulative stress index for each treatment was calculated (n=3).

Histological work
A morphometric study of the larval gastrointestinal tract was carried out using image-analysis software.

Microbial community analysis
Bacterial load of the rearing water and larvae was measured on days 9 and 18. Samples of the larval rearing water were taken for bacteriological characterization at weekly intervals. Pyrosequencing was used for microbial community analysis

Results

Fish larviculture
Growth and survival of the fish larvae was similar in all treatments. Principal caudal fin development and swimbladder development was acceptable and similar for all treatments.

Osmotic stress test
The application of MIC-F had a positive effect on the stress resistance of the seabream larvae. The result of the osmotic stress test showed a reduced cumulative mortality of the fish larvae treated with MIC-F (Fig. 1). The probiotic mixture, MIC-F, increased the host stress resistance in both experimental groups at 30dph and 46dph, with the best values obtained in the group MIC 2.5 at 30dph and in group MIC 5 at 46dph.

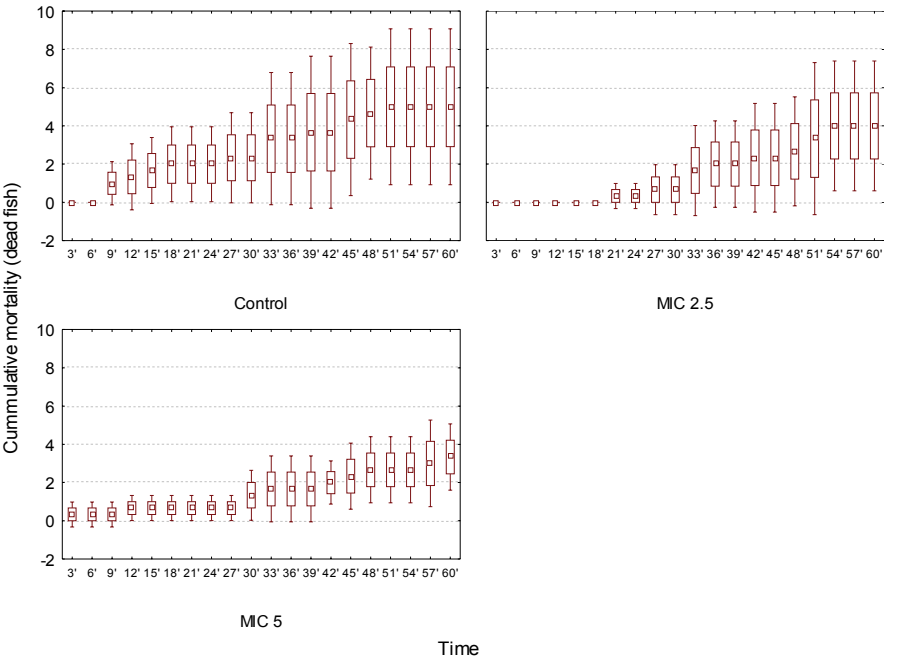


Fig. 1. Results of the osmotic stress test determined at 30dph expressed as the cumulative mortality (n=3) for the three different treatments (control, MIC 2.5, and MIC 5 treatment). Squares represent the average value, bars represent the standard error, whiskers represent 1.96× standard error

Discussion

Aquaculture practices demand intensive productions, causing stress. As a result, methods are sought to increase stress tolerance, one of the solution would be the use of probiotics. The microbial community present in larval fish tanks and in fish larvae consists of different bacterial species. Each of these bacterial species has its own characteristics: some are pathogenic while others are able to promote digestion and absorption of nutrients, increase resistance against pathogens and stress situations, improve welfare, and positively affect growth (Avella et al. 2010, Verschuere et al. 2000). The selected bacterial species present in the probiotic mixture used in the current study are members of the *Bacillus* genus. *Bacillus* sp. have been applied in aquaculture and other areas for many years during which these bacteria have proven their beneficial role on hosts (Moriarty, 1990, 1998; Moriarty and Body, 1995). Bacilli are known to improve the performance of aquaculture practice in terms of survival and growth, to enhance digestive and immune system of the host, and to improve water quality.

In the present study, a trend for an improved robustness of sea bream larvae exposed to MIC was noted. MIC-F applied at a concentration of 2.5 or 5g.m^{-3} in the larval rearing tank from 2-30dph, resulted in larval fish that were more resistant to osmotic stress to which they were exposed at 30dph and 46dph. Moreover, the variability between the animals, cultured in the presence of the probiotic mixture was reduced, indicating the potential of MIC to support a more standardized rearing protocol resulting in more predictable results. These results may suggest treating fish larvae with probiotics wherever conventional aquacultures practices might create stress in animals, such as transport, change in water temperature, and periodic manipulations.

References

Avella M.A., G. Gioacchini, O. Decamp, P. Makridis, C. Bracciatelli, and O. Carnevali. 2010. Application of multi-species of *Bacillus* in sea bream larviculture. Aquaculture 305:12-19.

Moriarty D.J.W. 1990. Interactions of microorganisms and aquatic animals, particularly the nutrional role of the gut flora. Microbiology in Poecilotherms: 217-222.

Moriarty D.J.W. 1998. Control of luminous *Vibrio* species in penaeid aquaculture ponds. Aquaculture 164:351-358.

Moriarty D.J.W. and A.G.C. Body. 1995. Modifying Microbial Ecology in Ponds : the Key to Sustainable Aquacultue. Pages 10. Fish Asia '95 Conference: 2nd Asian Aquaculture and Fisheries Exhibition and Conference. Singapore.

Verschuere L, G. Rombaut, P. Sorgeloos, and W. Verstraete. 2000. Probiotic bacteria as biological control agents in aquaculture. Molecular and Microbial Biological Reviews 64:655-671.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

GENOME-WIDE GENE EXPRESSION ANALYSIS DURING *SOLEA* SP. EMBRYO-LARVAL DEVELOPMENT

X. Cousin[1], M.G. Claros[2], D. Mazurais[3], R. Bautista[2], H. Benzekri[2], M.-L. Bégout[1], M. Ponce[4], P. Armesto[4], J. Zambonino[3], J.V. Planas[5], and M. Manchado[4]

[1] Ifremer Station de La Rochelle; Place Gaby Coll; 17137 L'Houmeau; France
[2] Plataforma Andaluza de Bioinformática – SCBI; Universidad de Málaga; Centro de Bioinnovación/Andalucía Tech; C/ Severo Ochoa 34; Parque Tecnológico de Andalucía; 29590 Campanillas; Spain
[3] Ifremer, LEMAR UMR 6539 - Unit of Functional Physiology of Marine Organisms (PFOM), Technopole Iroise, CS 10070, 29280 Plouzané, France
[4] IFAPA; Centro El Toruño; Ctra Nacional IV; Camino Tiro Pichón s/n; 11500 Puerto de Santa María; Cádiz; Spain
[5] Departament de Fisiologia; Facultat de Biologia; Universitat de Barcelona; Av. Diagonal 645; 08028 Barcelona; Spain

Larvae from *Solea* sp. suffer dramatic tissue remodelling during metamorphosis. Impaired metamorphosis could lead to a loss of individual fitness as well as a decrease in commercial value. There is therefore a need to develop knowledge on those factors that could influence metamorphosis. In the framework of the AQUAGENET network (SUDOE Interreg IVb), a work package is dedicated to the production of both *S. senegalensis* and *S. solea* omics data. In order to provide molecular tools suitable to analyse processes that may disrupt larvae development and metamorphosis, we have produced genomic and transcriptomic resources and data from late embryonic to post-metamorphic stages.

Firstly, genomic data generated using 454 NGS technology from different tissues and conditions were assembled to create a reference transcriptome in *S. senegalensis* with more than 3 million reads and 252 416 identified unigenes with a mean contig length of 336nt. All these data were annotated using AutoFact, Full-LengtherNextand Sam3. Total number of annotated genes was 84 763 including 53 930 descriptions. These data are publicly available in the SoleaDBv3 (www.juntadeandalucia.es/agriculturaypesca/ifapa/soleadb_ifapa/).

This backbone was used as a reference to map RNA-seq data obtained from various samples. Sampling and sequencing analysis have been performed in parallel in *S. senegalensis* and *S. solea* to evaluate mechanisms conservation using samples embryonic, larval, or juvenile stages. We also performed short-term ex-

posure to molecules involved in the retinoic acid (RA) pathway known to regulate several differentiation processes in fish. Libraries were sequenced as 2×100-nt paired-end reads on an Illumina HiSeq 2000 instrument.

Sequencing generated 47 million as average 100-nt read-pairs per library for *S. senegalensis* and 24 million for *S. solea*. Samples were cleaned using SeqTrimNext and mapped using Bowtie2 producing 76% useful paired-end sequences with about 74% of mapped reads for *S. senegalensis* and 62 to 66% for *S. solea*. Statistical analysis was done using Robina and FDR correction of significance to identified differentially expressed genes. At embryonic stage we obtained 256/517 genes up/down-regulated genes in *S. senegalensis* and 225/1275 genes in *S. solea* after atRA exposure. After TTNPB exposure, we obtained 1529/1464 and 2073/4153 regulated genes and 134/118 and 18/217 after DEAB exposure. At larval stages, no samples were obtained for *S. senegalensis* after atRA exposure and for *S. solea* after TTNPB exposure. We obtained 256/200 genes regulated in *S. solea* after atRA 24h-exposure and 1290/1168 after 48h-exposure. We obtained 6377/18 106 genes regulated in *S. senegalensis* after TTNPB 24h-exposure and 1562/354 after 48h-exposure. In the case of DEAB, we observed in most cases an increase in the number of regulated genes for both species after 48h-exposure compared to 24h-exposure: 899/4803 and 3746/3958 for *S. senegalensis* and 93/289 and 1668/2560 for *S. solea*. These genes includes cytochrome P450 26A1, cellular RA/Rol-binding proteins, and short-chain dehydrogenases/reductases known to be involved in the RA pathway, validating our results. Some genes are regulated in some or all stages suggesting different regulation depending on stages. Among these genes we have identified transcription factors such as (*otx1*, *nkx-2.3*, *sox3*, and *sox9*) in embryos and enzymes in larvae.

Using high throughput sequencing technology we obtained transcriptomes of *S. senegalensis* and *S. solea* at embryonic and larval stages. This comprehensive study will allow comparative studies over time and between treatments and provide valuable information on pathways regulated over development and metamorphosis of *Solea* species.

This work was funded by project AQUAGENET (SOE2/P1/ E287) program INTERREG IVB SUDOE.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

STUDIES OF DIGESTIVE PHYSIOLOGY DURING EARLY ONTOGENY OF THE MAYAN CICHLID *CICHLASOMA UROPHTHALMUS*

C.A. Cuenca-Soria[1,2], D.T. Ramírez[3], J.L. Ortiz-Galindo[1], E. Gisbert[4], and C.A. Álvarez-González[5*]

[1] Centro Interdisciplinario de Ciencias Marinas, Av. Instituto Politécnico Nacional s/n, Col. Playa Palo de Sta. Rita, Apdo. Postal 592, La Paz B.C.S. 23096, Mexico
[2] Universidad Juárez Autónoma de Tabasco. División Académica Multidisciplinaria de los Ríos. Carretera Tenosique-Estapilla km 1, Tenosique Tabasco 86901, Mexico
[3] Centro de Investigaciones Biológicas del Noroeste, S. C. Mar Bermejo No. 195, Col. Playa Palo de Santa Rita Apdo. Postal 128; La Paz, BCS 23090, Mexico
[4] Carretera PobleNou km 5.5, P. O. Box 200, 43540 Sant Carles de la Ràpita. Tarragona, Spain
[5] Laboratorio de Acuicultura Tropical UJAT-DACBIOL. Carretera Vhsa-Cárdenas km. 0.5, 86139, Villahermosa, Tabasco Mexico. *alvarez_alfonso@hotmail.com

Introduction

Some of the aspects that should be considered for the design of feeding protocols of fish are the study of the digestive system. The knowledge of the morpho-physiological changes during the initial ontogeny is fundamental to establish the basis for the development of larviculture (Lazo et al., 2011). However, although the development among fishes is quite similar, there are many differences related to the timing of morphological and cytological differentiation during early ontogeny, which depends on several factors such as type of food and feed composition (Zambonino-Infante and Cahu, 2007). On the other hand, the Mayan cichlid, *Cichlasoma urophthalmus*, is an important regional species with a high commercial value and its culture has been recently developed. However, studies on the digestive physiology haven't been conducted. For this reason, the objective of this study was to describe the organogenesis and the main digestive enzymes expression during the initial ontogeny.

Materials and methods

Embryos of *C. urophthalmus* were collected from a natural spawning of broodstock (females: males, 3:1) maintained in the Laboratorio de Acuicultura Tropical, DACBIOL-UJAT, Mexico. At hatching, yolk-sac larvae were transferred into a one 500-l cylindroconical plastic tank for three days until yolk absorption. After this time, larvae were placed in three 100-l cylindroconical tanks (50 lar-

vae.l^{-1}) connected in a close recirculating system (Jiménez et al., 2009). Larvae were feed with *Artemia* nauplii (INVE, Aquaculture Nutrition, Belgium) at apparent satiation three times per day from yolk absorption (six days after hatching, DAH) until 15DAH. At 16DAH, three days of co-feeding, larvae were supplied with trout diet (45% protein and 16% lipid, Nelson and Sons Inc.) until 30DAH. Five larvae per day were taken from each tank and fixed in 4% formalin for histological analysis, from which ten larvae from each sampling day were dehydrated in a graded series of ethanol, embedded in paraffin, and cut into serial sagittal sections (3-5µm thick). Sections were stained by Harris' Haematoxylin and Eosin (HE) for general histomorphological observations, while Periodic Acid-Schiff (PAS) and Alcian Blue (AB) at pH 2.5, 1.0, and 0.5 were used to detect neutral and carboxyl-rich and sulphated glycoconjugates in mucous cells (Pearse, 1985). For the digestive enzymes expression, ten larvae per day per tank were preserved at -80°C in RNAlater until analysis. TRIzol® Reagent (Invitrogen,) was used for total RNA extraction, then 1µg total RNA was reverse-transcribed to cDNA with Improm II (Promega). PCR-purified fragments were cloned according the protocol of Topo TA Cloning kit (Invitrogen, Breda, Netherlands) into TOP 10F′ competent cells, using the pCR 2.1-TOPO as a vector for sequencing. Gene expression was conducted using qPCR and specific primers, for amylase, bile-salt dependent lipase, alkaline phosphatase, and EFα-1 as reference gene. Relative expression was performed in a 7000 Sequence Detection System (Applied Biosystem, Foster City CA, USA), using SYBR Green (Bio-Rad). PCR conditions were start (2min@50°C and 10min@95°C, 1 cycle), denaturalization (15s@95°C), alignment/extension (10min@95°C and 1min@60°C, 40 cycles). Relative expressions were calculated using ΔΔCt method and analyzed with randomized test by REST Software (www.gene-quantification.info).

Results and discussion

Between 3 and 4DAH, the cardiac stomach started to form as a dilatation between the oesophagus and anterior intestine. This area was lined by a pseudostratified columnar epithelium deprived of goblet cells and an incipient mucosal fold separating the future stomach from the intestine was already distinguishable. At 9DAH (4.86±0.09mm SL), the first clusters of cubic cells forming the gastric glands were observed in the mid-posterior region of the developing stomach. At 11DAH (5.75±0.36mm SL), the first mucous cells secreting neutral glycoconjugates (PAS-positive) were visible in the gastric mucosa, whereas at 14DAH (6.35±0.17mm SL), their number and PAS-positive reactivity had remarkably increased with regards to younger ages. At this age, some moderate folding of the gastric mucosa was observed in the cardiac (anterior) stomach, whereas no remarkable changes were observed until 19DAH (7.02±0.25mm SL) when three different regions were clearly distinguished in the stomach: the cardias, fundus and pylorus. At 2DAH, the intestinal mucosa started to form incipi-

ent folds and the first goblet cells appeared in the intestinal epithelium among enterocytes. Most of the intestinal goblet cells were stained in magenta (PAS-positive), whereas some contained neutral and acidic (carboxylated and sulphated) glycoproteins. At this age, the posterior region of the intestine started to differentiate into the rectum, as the absence of folding of the mucosa and flattening of enterocytes indicated. At 4DAH (3.94±0.03mm SL), the intestine bent and the intestinal valve separating the anterior and mid intestinal regions from the posterior one was clearly visible. Coinciding with the onset of exogenous feeding between 5 and 6DAH, the first lipid vacuoles were observed within the enterocytes in both anterior and posterior regions of the intestine. Goblet cells were mainly stained in purple, indicating that they content was a mixture of neutral and acidic glycoproteins, while a few of them only contained neutral or acidic glycoproteins. Eosinophilic supranuclear inclusion bodies in enterocytes from the anterior intestine were detected between 5 and 10DAH. Between 6 and 8DAH (4.52±0.14mm SL), several histomorphological differences were distinguishable between the anterior and posterior intestinal segments. In particular, lipid vacuoles were more abundant in the anterior than the posterior intestine, indicating that this region was the primary site of lipid absorption in the intestine. In addition, mucosal folds were larger and more numerous in the posterior than anterior intestinal regions and goblet cells were three times more abundant in the posterior than in the anterior intestine. Folding of the intestinal mucosa remarkably increased between 11 and 14DAH, coinciding with the full development of gastric glands in the stomach. At hatching, the liver was already developed in *C. urophthalmus* larvae and it appeared as a lobular mass running along the entire abdominal cavity until almost the anal pore. Hepatic tissue was arranged along sinusoids and consisted of polyhedral hepatocytes with centrally located nuclei, reduced eosinophilic cytoplasm, and few and small lipid inclusions. Biliary ducts were already visible between 2 and 3DAH and were lined by short and ciliated columnar epithelium with basal nuclei occupying most part of the cytoplasm. At 4DAH, hepatocytes started to accumulate a larger quantity of lipids as the increase in diameter of fat vacuoles indicated (16.8±2.2μm). Coinciding with the onset of exogenous feeding, lipid and glycogen (PAS-positive) deposits progressively increased within hepatocytes, while after 16DAH (6.61±0.40mm SL), lipid vacuoles occupied most part of the cytoplasm, displacing the nucleus to the periphery of the hepatocyte, which resulted in a decrease in the glycogen (PAS-positive) storage in the liver. Same early development has been detected in other cichlid such as tilapia *Oreochromis niloticus* and bay snook *Petenia splendida* (Morrison et al., 2001; Treviño et al., 2011), also pepsin biochemistry was detected at 13DAH in Mayan cichlid (López-Ramírez et al., 2011), which agreed with the synchronic appear of the gastric cells (5.75±0.36mm SL) and its maturation at 19DAH (7.02±0.25mm SL), also total enterocyte development is reached between 11 and 14DAH, maximizing the digestion and absorption of nutrients (Gisbert et al., 2004).

The amylase and bile salt dependent lipase expressions were early detected at 3DAH and gradually increase until 30DAH. However, alkaline phosphatase was detected at yolk absorption (6DAH) and decreased at 14DAH when enterocytes were mature. Our results showed that the expression of digestive enzymes were early detected, increasing at 11DAH, with the maximum expression at 19DAH, except for alkaline phosphatase. Instead, the similarity analysis showed that genes of *C. urophthalmus* are very conservative compared with other fish.

References

Gisbert E., R.H. Piedrahita, and D.E. Conklin. 2004. Ontogenetic development of the digestive system in California halibut (*Paralichthys californicus*) with notes on feeding practices. Aquaculture 232:455-470.

Jiménez-Martínez L.D., C.A. Alvarez-González, W.M. Contreras-Sánchez, G. Márquez-Couturier, L. Arias-Rodríguez, and J.L. Almeida-Madrigal. 2009. Evaluation of larval growth and survival in Mexican mojarra *Cichlasoma urophthalmus* and bay snook *Petenia splendida* under different initial stocking densities. J. World Aquac. Soc. 40:753–761.

Lazo J.P., M.J. Darias, and E. Gisbert. 2011. Ontogeny of the digestive trac, In: Larval Fish Nutrition, First Edition. Edited by Holt G. J. John Wiley & Sons, Inc. Published by John Wiley & Sons, Inc.

López-Ramírez G., C.A. Cuenca-Soria, C.A. Álvarez-González, D. Tovar-Ramírez, J.L. Ortiz-Galindo, N. Perales-García, G. Márquez-Couturier, L. Arias-Rodríguez, J.R. Indy, W.M. Contreras-Sánchez, E. Gisbert, and F.J. Moyano. 2011. Development of digestive enzymes in larvae of Mayan cichlid *Cichlasoma urophthalmus*. Fish Physiol. Biochem. 37:197-208.

Morrison C.M., T. Miyake, and J.R. Wright. 2001. Histological study of the development of the embryo and early larva of *Oreochromis niloticus* (Pisces: Cichlidae). Journal of Morphology 247(2):172-195.

Pearse A.G.E., 1985. Histochemistry. Theoretical and applied, Vol. 2. Analytic Technology, Churchill Livingston, New York.

Treviño L., C.A. Alvarez-González, N. Perales-García, L. Arévalo-Galán, A. Uscanga-Martıínez, G. Márquez-Couturier, I. Fernández, and E. Gisbert, 2011. A histological study of the organogenesis of the digestive system in bay snook *Petenia splendid* Günther (1862), from hatching to the juvenile stage. J. Applied Ichthyol. 27: 73-82.

Zambonino-Infante J.L., and C.L. Cahu. 2007. Dietary modulation of some digestive enzymes and metabolic processes in developing marine fish, applications to diet formulation. Aquaculture 268(1-4):98-105.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

FISH LARVAL PERFORMANCE FED WITH COPEPODS (*ACARTIA GRANI*) AND THE DINOFLAGELLATE (*OXYRRHIS MARINA*) AS SUPPLEMENT: THE CASE OF DUSKY GROUPER (*EPINEPHELUS MARGINATUS*)

M.E. Cunha[1], M. Gamboa[1], S. Castanho[1], H. Quental-Ferreira[1], L. Ribeiro[1], F. Soares[1], P. Gavaia[2], M. Mateus[1], P. Moura[1], N. Cordeiro[3], R. Gonçalves[3], and P. Pousão-Ferreira[1]

[1] IPMA, Aquaculture Research Station, Av. 5 de Outubro s/n, 8700-305 Olhão, Portugal
[2] CCMAR - Centre of Marine Sciences, University of Algarve, Campus de Gambelas, 8005-139 Faro. Portugal
[3] Competence Centre for Exact Sciences and Engineering,, University of Madeira, Campus da Penteada 9000-390 Funchal, Portugal.

Introduction

Early life stages of dusky grouper, *Epinephelus marginatus* (Lowe 1834) are still a bottleneck in larval rearing due to their high mortality. Diet and low disturbance of the water column during larval rearing affect survival because the species is fragile and needs small and nutritious live feed (Cunha et al., 2009). Copepods are the natural food items in the wild possessing 2 to 3 times more highly unsaturated fatty acids than rotifers. The use of large tanks like the ones used in semi-intensive systems promotes stability of the water column and reduces interference with larvae.

The aim of this work is to compare the effect of the introduction of copepods in the diet of dusky grouper larvae and the use of the semi-intensive systems on their development and quality.

Materials and methods

Oocytes from hormonally induced spawning of captive females were fertilized with milt from fluent males, both kept at indoor tanks (10.6m^3) at 22.5±0.5°C. The eggs were incubated directly in the tanks used for comparison of the effect of diets, volumes, and densities on larval development and quality (Table 1). The parameters used in the comparisons were growth (length and weight), survival, activity of digestive enzymes, and skeletal malformations

Two types of diets were compared. One was the generalized diet use in aquaculture composed by small rotifers *(Brachionus* sp.), artemia (*Artemia* sp.) and dry feed, and the second by a mixed diet of small rotifers (*Brachionus* sp.) and copepod (*Paracartia grani*) nauplii followed by artemia (*Artemia* sp.) and dry feed. These comparisons were carried out on two large volume ($18m^3$) tanks at low larva (3 larvae.l^{-1}) densities and named as semi-intensive system (SIS) with and without copepods (Fig.1). *Acartia grani* eggs were obtained from an adult population fed on a mixture of *Rhodomonas salina* and *Oxyrrhis marina*. Rotifers and *Artemia* instar II/III nauplii were enriched to increase levels of protein and the dry feed contained also high levels of protein.

Table I. Experimental settings for dusky grouper egg incubation and larval rearing. (I. – *Isochrysis*; N. – *Nannochloropsis*; O. – *Oxyrrhis*; B. – *Brachionus*; A. – *Artemia*; P. - *Paracartia*).

Rearing Systems	**Intensive**	**Semi-intensive without copepods**	**Semi-intensive with copepods**
Location	Indoor	Outdoor	Outdoor
Volume (m^3)	1.5	18	18
Dissolved Oxygen ($mg.l^{-1}$)	5.5±0.90	5.9±1.42	5.7±1.35
Temperature (ºC)	23.3±0.87	22.9±1.01	22.9±1.01
Photoperiod	Artificial (14L/10D)	Natural (15L/9D)	Natural (15L/9D)
Phytoplankton	*I. galbana* + *N. oculata*	*I. galbana* + *N. oculata*	*I. galbana* + *N. oculata* + *O. marina*
Zooplankton ($N.ml^{-1}$)	5 *B.* spp. + 1 *A.* sp.	5 *B.* spp. + 1 *A.* sp.	5 *B.* spp. + 1 *A.* sp. + 2 *P. grani*
Larval Density ($N.l^{-1}$)	40	3	3

The effect of larval density/rearing volume was studied comparing the results obtained with the $1.5m^3$ and $18m^3$ tanks using the same rotifers *(Brachionus plicatilis*), artemia (*Artemia* sp.), and dry feed diet. The treatment in the smaller volume tank is referred as intensive system (IS).

10 to 25 larvae were sampled for growth (dry weight and total length) at 2, 6, 11, 16, 20, 25, 30, 62 days after hatch (DAH). Trypsin and pepsin activities were determined in 10 larvae at 30 and 62DAH. Skeletal malformations were analysed at 62DAH (n=60) using radiography, with Kodak X-ray DXS System for 4000 capture the images with software Carestream DXS.

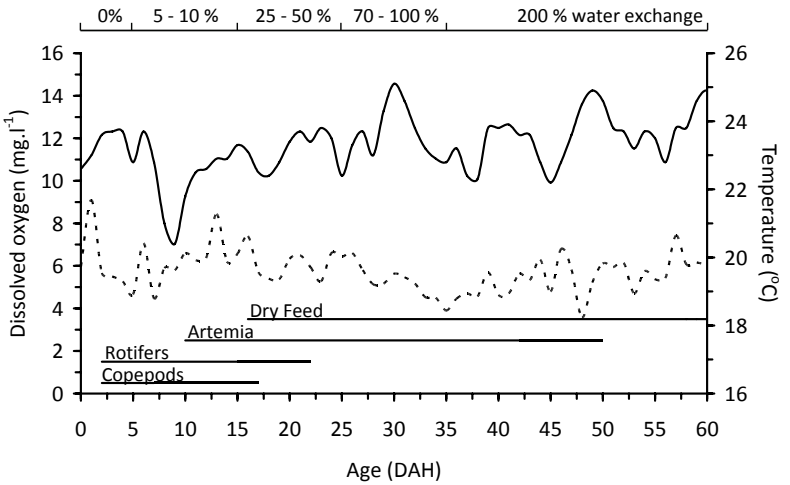


Fig 1. Daily water exchange, mean dissolved oxygen (dashed line) and temperature (solid line) in the rearing tanks, and feeding schedule during the experimental period.

Analyses of variance (ANOVA) were used to compare treatments and data was log normalized when necessary.

Results and discussion

Comparison between the effect of diets (SIS with and without copepods) show that, at the end of the live feed period (30DAH), fish were significantly longer ($P>0.001$) in SIS fed with copepods (Table II). No significant differences were obtained in relation to weight. At the end of the experiment (62DAH) when fish was fed only with dry food for 1 month there were significant differences in fish length and weight with the fish from SIS without copepods showing significantly larger and heavier benthic juveniles. Comparison between the effect of larval densities/rearing volume (IS and SIS without copepods) showed no significant differences in fish length and weigh at 30DAH. However at 62DAH the fish were significantly larger and heavier in the SIS without copepods.

At 30DAH dusky grouper from semi-intensive system without copepods exhibited significant ($P<0.001$) higher values of trypsin activity and lower values of pepsin activity when compared to larvae from other systems. At 62DAH no differences were observed among treatments. Differences at 30DAH reflect the delay of the larvae from the semi-intensive system without copepods, more dependent on pancreatic enzymes whereas the other treatments exhibited already an acidic digestion.

Table II. Mean and standard deviation of main growth parameters and enzymes, final number of harvest fish and degree of total skeletal malformation in dusky grouper juveniles.

Rearing systems:	Intensive		Semi-intensive without copepods		Semi-intensive with copepods	
Age (DAH):	30	62	30	62	30	62
Length (mm)	9.9± 4.51	42.2^{a}±5.51	10.3^{b}±5.98	50.4^{ab}±5.78	11.6^{b}±4.97	46.1^{b}±7.93
Weight (g)	0.04±0.20	1.08^{a}±0.32	0.05±0.23	1.80^{ab}±0.60	0.05±0.23	1.43^{b}±0.69
Trypsin (mU.mg prot^{-1})	0.1^{a}±0.03	0.2±0.16	2.3^{ab}±0.22	0.1±0.07	0.7^{b}±0.49	0.1±0.02
Pepsin (mU.mg prot^{-1})	0.3±0.15	1.3±0.24	0.1±0.03	1.2±0.15	0.3±0.23	1.4±0.39
Final harvest (number)	175		170		351	
Malformations (%)	43		40		23	

Superscript notations denote significant differences between comparisons: a – Intensive system and Semi-intensive without copepods; b – Semi-intensive without and with copepods)

Number of harvest fish at the end of the experiment was similar between the intensive and semi-intensive systems without copepods but at the semi-intensive systems with copepods survival was two times higher. The higher final density in SIS with copepods probably explains the smaller mean size of fish when compared with SIS without copepods due to the aggressive and territorial behaviour of grouper.

Skeletal malformations occurred with lower incidence in the semi-intensive systems fed with copepods and were almost two times lower than in the semi-intensive without copepods.

Conclusions

The semi-intensive systems where the larvae were fed with copepods seem to be the most suitable system in dusky grouper larval production, since there was higher survival, lower rate of malformations, with no significant differences in growth. In general, juveniles were of better quality.

References

Cunha M.E, H. Quental, A. Barradas, P. Pousão-Ferreira, E. Cabrita, and S. Engrola. 2009. Scientia Marina 73S1:201-212.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

CONTROL OF BACTERIAL DISEASE IN CULTURES OF MARINE LARVAE AND LIVE FEED ORGANISMS BY A PROBIOTIC BACTERIUM

P. D'Alvise[1], S. Lillebø[2], H. Wergeland[2], K.F. Nielsen[1], Ø. Bergh[3], and L. Gram[1]

[1] Technical University of Denmark, Department of Systems Biology, Søltofts Plads bldg. 221, DK-2800 Kgs. Lyngby
[2] University of Bergen, Department of Biology, Postboks 7803, NO-5020 Bergen
[3] Institute for Marine Research, Postboks 1870, NO-5020 Bergen

Bacterial infections are a major problem for marine larviculture that limit reliability and cost-efficiency of juvenile production, and constrains introduction of new species and breeding programs. The main source of pathogenic bacteria is live feed cultures, since opportunistic pathogenic bacteria thrive well in the rapidly increasing nutrient concentrations.

The probiotic bacterium *Phaeobacter gallaeciensis* antagonizes many species of fish-pathogenic bacteria and was capable of reducing the concentrations of pathogenic *Vibrio* spp. in gnotobiotic experimental cultures representing the larviculture food chain (microalgae, rotifers, *Artemia*).

In a challenge trial with cod larvae infected with *Vibrio anguillarum*, the probiotic bacterium reduced the cumulative larval mortality to 12.5±2%, which was below the level of the unchallenged control (34.7±9.8%). Untreated challenged larvae reached 100% accumulated mortality within 3 days. *P. gallaeciensis* mutants deficient in production of the antibacterial compound tropodithietic acid (TDA) did not provide the same protective effect (68.8±30.4%) as the wild type, indicating that TDA production was likely the main mechanism of action.

P. gallaeciensis, which occurs naturally in coastal waters and is part of the normal microbiota of fish and mollusc larvae cultures, could be applied preventively in cultures of marine fish larvae and live feed to reduce the incidence of bacterial infections.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

THE FUNCTION OF WAX ESTERS IN LARVAL FISH TRANSITION FROM ENDOGENOUS TO EXOGENOUS NUTRITION – ARE FRESHWATER FISH THE EXCEPTION OR THE RULE?

K. Dabrowski[1], M. Korzeniowska[2], T. Farmer[3], S. Ludsin[3], and E. Marschall[3]

[1] School of Environment and Natural Resources, The Ohio State University, Columbus, OH 43210, USA
[2] Department of Animal Products Technology and Quality Management, Wroclaw University of Environmental and Life Sciences, Wroclaw, Poland
[3] Department of Ecology, Evolution and Organismal Biology, Ohio State University, Columbus, OH 43210, USA

Ideal larval diets are those that match the nutrient profiles and concentration of the yolk sac and the exogenous organisms. Fish larvae have a limited ability to biosynthesize phospholipids de novo but can exchange fatty acids within and between phospholipids (PL) and triglycerides (TG). Dietary PL can, in principal, be utilized (assimilated) unchanged by larval fish. However, waxes have not been previously considered in larval fish nutrition despite the fact that they are found in a wide range of marine copepods and are transferred through the food chain to fish, accumulated in eggs, and become energy/nutrient sources in several species of fish at the larval stage. In some fish, waxes constitute up to 70 % of egg dry matter (or 91-97% of neutral lipid content). Previous reviews of the role of lipids in larval fish nutrition did not mention their presence, function or importance. It remains unknown why the deposition of wax esters is favored over triglycerides in the body (skin, muscle, swim bladder) and consequently the eggs in some fish.

The acquisition and function of waxes in selected species of freshwaters fish remains a mystery, as the deposited wax esters are not of food origin like in marine fish, their synthesis pathways are little studied and the utilization in larval stages is unknown. It has been deduced that fatty alcohols of waxes are synthesized *de novo* from acetate in oocytes after vitellogenin cleavage based on the absence of waxes in blood plasma of mature striped bass and acetate incorporation into fatty alcohols in rainbow trout recrudescent ovary.

For the experimental fish and feeding trial, we collected live yellow perch from Lake Erie during April-May 2011 and domesticated perch were purchased from a commercial fish operation during September 2010. Fish were housed in large fibreglass tanks in an indoor wet lab facility at the Aquatic Ecology Laboratory,

OSU. Domesticated fish were believed to be age-1 at the time of purchase. All individuals experienced natural, seasonal variation in both temperature and photoperiod and were fed according to one of two treatments (maintenance or *ad libitum*). During October 2011 through June 2012, we conducted a controlled laboratory experiment to quantify the effects of 1) winter duration (i.e., 50, 80, or 110 days <5°C) and 2) body condition (i.e., high or low summer feeding rates) at the start of winter on ovarian development, gamete production and quality, female spawning success, and egg hatching success as well as the quality of eggs and larvae from both domestic and wild fish.

For biochemical analysis, sample preparation of the fish ovaries for lipid analysis followed existing methods. We extracted total lipids by methanol-chloroform or hexane and followed with separation of waxes (WE) from NL class using dichloromethane:hexane (1:2), (WE-1) and then dichloromethane to isolate free fatty acids (FFA). All extracts were subjected to methanolysis and GC separation.

At the inception, we confirmed information that yellow perch exhibit an unusual composition of ovarian lipids. We postulate that the synthesis of wax esters de novo takes place in perch ovaries. The mobilization of wax esters contained most of all in the oil globule of the embryo/larvae proceeds through direct trophoblastic exocytosis and subsequent entry into circulation. Therefore, the digestive lipolytic enzyme (wax esters hydrolase) is not of relevance in the process of the utilization of yolk sac reserves. Furthermore, timely provision of wax esters (hydrophobic lipids) to larval fish may facilitate inflation of the swim bladder by preventing inner surface adherence and collapse. Liquid waxes (with unsaturated alcohols) will diminish transepidermal water movement (loss in marine fish), prevent UV damage and microbial entrance.

The results obtained thus far indicate that a unique pathway of synthesis, deposition, and mobilization of the wax esters contained in the oil globule of yellow perch exists, and may/is likely to involve exocytosis of microparticles into circulation that would make the intraluminal hydrolysis rate of secondary importance. However, a significant decline of wax esters that parallels the first exogenous feeding suggests that the role of waxes in the starter diet formulations needs to be explored.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

DIGITAL IMAGE ANALYSIS TO AID BROODSTOCK MANAGEMENT AND EGG QUALITY ASSESSMENT

A. Davie[1], E. Leclerq[1], M.V. Medeiros[1], C. Talbot[2], and H. Migaud[1]

[1] Institute of Aquaculture ,University of Stirling, Stirlingshire, FK9 4LA, UK
[2] Aquaculture Research Services, Fort William, Scotland, PH33 7PB, UK

Digital image analysis (DIA) is a powerful real-time analysis methodology, however its use is somewhat limited in aquaculture. While it has been incorporated in research methodologies for a range of studies to capture parameters like growth and development of larvae and quantify prey densities for larval rearing regimes, the translation of the methods into farm-effective tools and subsequent industry adoption is limited. We present two case studies in which we have developed accurate DIA methods to support hatchery management decision-making processes to showcase how the methods could be more widely used.

Ballan wrasse (*Labrus bergylta*) are protogynous hermaphrodites with males representing approximately 10% of the population. However unlike most other species, they display no apparent external sexual dimorphism. This makes identification of males problematic and thus the establishment of spawning harems of appropriate sex ratios challenging. A dataset of >400 Ballan wrasse sourced from the UK and Norway was compiled where each individual had been digitally photographed and gender confirmed by histological examination of gonads. Two established DIA methods (Geometric morphometrics and Truss network analysis) were employed to explore the variation in bodyform in relation to gender. Of the two, Truss network analysis proved the most accurate giving a gender prediction accuracy of ~80%. However, it became apparent that if weight of the animal was integrated with the body form analysis then gender prediction accuracy could be improved further still. At its conclusion, this study validated a simple analytical formula incorporating weight and morphometric parameters that predicted gender within the study population with 91% accuracy. This method could be used on farm, generating real-time results allowing accurate and informed broodstock management.

Accurate, early stage predictors of egg quality that forecast subsequent fate (i.e. hatch rate/survivability) are vital for efficient hatchery operation. Blastomere morphology analysis has been widely reported as such an accurate methodology in a range of species. The method entails scoring the 8-cell blastomere on 5

morphological parameters based on the symmetry of the cell division. However, the method is time consuming and as it is reliant on subjective scoring criteria it means the methods accuracy can be questioned when performed by multiple operatives. Working with Atlantic halibut (*Hippoglossus hippoglossus*) embryos, we reverse engineered a DIA methodology whereby from a digital photograph of the embryo, the 8-cell blastomere is derived into a grid representation using a truss network analysis method which took 15 landmark points and extrapolated them into 74 separate measurements per individual blastomere. From a study population of 200 halibut embryos in which we had a digital photograph of the blastomere and a record of whether the embryos hatched or not, we subsequently developed a quadratic discriminant analysis which predicted embryo fate with >80%. Per each batch of eggs, this whole process from imaging to analysis takes in the region of one hour once an operator is accustomed to the process, thus this method can provide reliable hatch predictions within 24 hours of embryos being fertilised. In the case of halibut, hatching occurs approximately 14 days post-fertilisation, thus this method is a useful tool in informing the choice of which batches to stock and thus optimising the infrastructure utilisation in the hatchery.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

THE APPLICATION OF ECOLOGICAL THEORY FOR MICROBIAL CONTROL IN AQUACULTURE

P. De Schryver

Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Rozier 44, 9000 Gent, Belgium. Tel. +3292643754, Fax. +3292644193
(peter.deschryver@ugent.be)

Research efforts of recent years indicate that the viability of the larval stages of animals reared in aquaculture systems is determined by both the environmental and the intestinal microflora. However, the detrimental host-microbe interactions resulting in unpredictable larval growth and mortality and the role of the host associated microflora for the animals' health are poorly understood. Ecologically oriented literature suggests that the compositional status of the intestinal microflora, comprising species richness, species abundances, and dynamics of change, may play a determining factor for larval viability. Aquaculture, therefore, would benefit highly from knowledge on how the microbial community composition and organization affect the viability of the host. To address this issue, ecological theory approaches should be integrated with aquaculture-oriented studies. By considering the intestine of individual larvae as the ecological system under investigation, it will be possible to elucidate biodiversity-functionality relationships for the intestinal microbial community. The availability of gnotobiotic research models for aquatic animals such as zebrafish, Atlantic cod, European sea bass, and *Artemia franciscana* will prove to be of high value. As an example, a functionality of relevance for aquaculture is the avoidance of pathogenic invasions in the gut. The overall target in this respect will be to find the characteristics of the intestinal microflora that is more protective as a unit towards invaders. This goal can then be subdivided into several research questions:

- Is the presence of individual traits more important than the status of the community?
- Do microbial species richness and abundances matter in determining the invasion of a pathogen?
- Is a dynamic or a stable microbial community more resistant towards invasion?
- etc.

The improved knowledge on gut microflora composition and functioning in terms of pathogenic invasiveness may contribute considerably to the development of new prevention and treatment methodologies against pathogenic infections in aquaculture.

Knowing the importance of the compositional status is one thing, but actively steering for it is another matter. The three dimensional water matrix in aquaculture systems in which feed is added and faecal matter and nutrients are excreted supports in situ microbial growth and makes that aquatic animals live in an environment with an exceptionally high microbial load. This living environment suggests that the host-associated intestinal microflora is unlikely to exist independently from the environmental microflora, a situation uncommon for land bred animals. The close interactions between host, feed, and environmental microflora make that some unique ecological factors may play in the shaping of the intestinal microbial community. The application of microbial ecological theory will be essential in the understanding of and explaining these interactions, and is a prerequisite to go beyond making situation-bound statements that provide few insights. Only based on theory-driven predictions will it be possible to actively steer for a desired intestinal microflora in aquatic animals to make maximal use of the potential it comprises as a natural defence barrier against pathogenic invasions.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

THE DEVELOPMENT OF NEW ENRICHMENT PRODUCTS AND STRATEGIES FOR LIVE FEED IN FISH HATCHERIES

T. De Wolf[1], P. Cecconi[1], S. Lenzi[1], I. Labaere[2], and G. Rombaut[2]

[1] MARICOLTURA DI ROSIGNANO SOLVAY srl, Via P. Gigli (Loc. Lillatro), 57016 Rosignano Solvay (LI), Italy
[2] NVE Technologies, Hoogveld 93, 9200 Dendermonde, Belgium

Introduction

The development of enrichment products for live prey organisms such as rotifers and *Artemia* was a major breakthrough for aquaculture. The polyunsaturated fatty acids (ARA, EPA, DHA) have been shown to be essential in the diet of marine fish larvae. Deficiency may lead to inferior growth, malformation, pigmentation problems, disruption of maturational processes (Zambonino Infante and Cahu, 2001), diseases, and mortality.

The purpose of this work was to develop new enrichment products and strategies for live feed which targets a fast enrichment of rotifers and *Artemia*, aiming for adequate HUFA levels, a high DHA/EPA ratio, and a significant incorporation of marine phospholipids and other functional components (e.g. astaxanthine).

Special attention is given to marine phospholipids as it is known that the pancreatic maturation process and production of enzymes in many fish species are triggered by their specific substrates. Maturation of the pancreatic secretory process takes place during the first month of life when live prey organisms are fed as the major compound of the complete feed. Lipolytic enzymes of pancreas (lipase and phospholipase) increase with development of marine fish larvae (Izquierdo et al., 2000), and are stimulated by the increase of their respective substrates, triglycerides, and phospholipids in the diet (Zambonino Infante and Cahu, 2001). Inadequate levels may result in an uncompleted maturation process and may have irreversible effects on larval fish development. An improved maturation of the pancreatic secretory process may favor the weaning process.

Materials and methods

Live prey enrichment
Rotifers were cultured on S.parkle (INVE Aquaculture, Dendermonde, Belgium), rinsed and enriched at a density of 1000R.ml^{-1} with 170ppm of the ex-

perimental enrichment product containing high HUFA and high marine phospholipid levels (EP). *Artemia* (EG-type, INVE Aquaculture, Dendermonde, Belgium) were hatched during 24h, harvested and enriched at 400npl.ml^{-1} with 600ppm EP, divided in 2 dosages.

Samples of live food were taken at regular time intervals to determine lipid composition. Lipids were extracted and fatty acid methyl esters (FAME) and phospholipid concentration were analyzed using GC and HPLC, respectively.

Fish test
The use of EP as live food enrichment product for rotifers and *Artemia* was evaluated during the larval rearing of Sea bream. The test was carried out in duplicate using larval rearing tanks of 6000 l. Two tanks were fed on live food enriched with classic enrichment products (DHA Protein Selco for rotifers and Easy DHA Selco for *Artemia*, INVE Aquaculture, Dendermonde, Belgium) and 2 tanks were fed on EP enriched live food. Rotifers were fed from 4 to 30 days post-hatch (dph), while enriched *Artemia* were fed starting from 22dph. The larval development was compared during the first 35dph.

Results

Live prey enrichment
The enrichment kinetic of the new experimental product administered to rotifers and *Artemia* was determined. The fatty acid profile and the phospholipid content in the live food were analyzed. The FAME and phospholipid content of the rotifers and *Artemia* nauplii enriched according to the fast protocol are presented in Table I. The (n-3) fatty acid enrichment occurs very fast. In rotifers, after only 1h of enrichment sum(n-3) HUFA levels of more than 30mg.g^{-1} DW are obtained with DHA/EPA>2. In *Artemia*, levels of more than 40 mg (n-3) HUFA.g^{-1} DW were obtained after 14h of enrichment, with a DHA/EPA ratio of >1.5. In rotifers, no increase in phospholipid (PL) content could be demonstrated, while the PL content of the *Artemia* nauplii increased by more than 34% to a level of ± 7% of DW.

Fish test
Sea bream larval growth and development was similar up to around 20dph. Between 20 and 35dph, a faster development was observed with the EP enriched live food compared to the classic enriched live food: larvae were growing faster and flexion of the notochord was achieved earlier.

Discussion

New products and strategies are explored in marine fish larviculture to enrich live feed (rotifers and *Artemia*) much faster and with specific components able to trigger specific processes in larval fish, ensuring an improved development of

the larvae. The presence of lipids and phospholipids at early stages triggers the pancreatic maturation, resulting in secretion of lipases and phospholipases. The timing of the maturation process and thus the availability of lipids and phospholipids is crucial for the larva so that optimal development of the larva can occur. This process, which takes place during the first month, is extremely important for the subsequent weaning process when dry feeds are fed to the larvae.

Table I. Fatty acid composition and phospholipid content of short-term enriched rotifers (Rot) and *Artemia* (Art), expressed as mean ($mg.g^{-1}$ dw) and as percentage of original level in non-enriched live food.

Live food	Enr time (h)	ARA 20:4(n-6) ($mg.g^{-1}DW$)	EPA 20:5(n-3) ($mg.g^{-1}DW$)	DHA 22:6(n-3) ($mg.g^{-1}DW$)	FAME Sum n-3 ($mg.g^{-1}DW$)	PL ($mg.g^{-1}DW$)	FAME %	PL %
Rot	0	0.88	3.93	3.66	10.31	28.0	100	100
Rot	1	1.86	10.31	22.08	37.16	25.2	360	90
Rot	2	1.85	10.94	22.76	38.63	19.8	375	71
Rot	3	2.00	11.97	24.85	42.10	18.4	408	66
Rot	4	2.23	13.36	28.43	47.69	14.4	462	51
Art	0	0.47	1.53	0.14	6.4	58.6	100	100
Art	6	1.22	6.73	10.04	22.51	63.6	120	109
Art	10	1.64	9.84	15.29	31.06	79.9	122	136
Art	14	2.29	14.35	22.61	43.51	66.2	134	113

In the present study, new enrichment strategies were tested on rotifers and *Artemia*, resulting in a fast (n-3) HUFA enrichment. The phospholipid enrichment could be only demonstrated in the *Artemia*, while in the enriched rotifers, the PL content decreased, as has already been observed (Rainuzzo et al., 1994). The enrichment period known for current enrichment products was significantly reduced. Rotifers and *Artemia* could be enriched within 1h and 10-14h, respectively, whereas normally more time is needed (6h and 18-24h, respectively. This advantage is offering a flexible tool for the live food producers in the hatcheries.

References

Izquierdo M., J. Socorro, L. Arantzamendi, and C. Hernández-Cruz. 2000. Recent advances in lipid nutrition in fish larvae. Fish physiology and biochemistry 22: 97-107.

Rainuzzo J.R., K.I. Reitan, and Y. Olsen. 1994. Effect of short- and long-term lipid enrichment on total lipids, lipid class and fatty acid composition in rotifers. Aquaculture International 2:19-32.

Zambonino Infante J and C. Cahu. 2001. Ontogeny of the gastrointestinal tract of marine fish larvae. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 130:477-487.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EFFECTS OF DIETARY *DUNALIELLA SALINA* EXTRACT AND HIGHLY UNSATURATED FATTY ACIDS ON THE FECUNDITY AND LIPID CONTENT OF POND-REARED *PENAEUS JAPONICUS* BROOD-STOCK

N. El-Bermawi

Alexandria University, Animal and fish production Dep., 17 Ezbet saad Street Somoha, 9000 Alexandria

Abstract

Five basic diets containing fresh squid meat and trash fish were supplemented with different amount of *Dunaleilla salina* extract (DSE) and highly unsaturated fatty acids (HUFA). Supplemented diets were fed to pond-reared *Penaeus japonicus* broodstock. Diet A was solely squid and trash fish. Diets B1 and B2 were supplemented with 400 and 600mg DSE.kg^{-1} diet, respectively. Diets C1 and C2 were supplemented with HUFA 5 and 10g.kg^{-1} and 400mg.kg^{-1} DSE, respectively. The results showed that the group fed diet C2 had the best reproductive performance in all experimental groups. It had the highest proportion of spawns (73.5%) and egg production per female (589.0) than all the other experimental groups. The fatty acid composition strongly affected fecundity and stress tolerance of broodstock. The results showed that both HUFA and β-carotene DSE may play role in stress tolerance and reproductive performance

Introduction

Lipids are important components of diets, both as energy and essential fatty acids sources, which fish need for basic functions, including growth, reproduction, and maintenance of healthy tissues (Sargent et al., 1989; Parpoura and Alexis, 2001). The fatty acids composition of lipids has been shown to affect the reproductive performance of prawns (Harrison 1990). The effect of highly unsaturated fatty acids (HUFA) on ovarian development spawning rate and tissue composition in prawns have been well documented in *Penaeus chinensis* (Xu et al. 1994), *P. japonicus* (Alava *et al*., 1989), *P. monodon* (Millamena 1989; Marsden et al. 1997), *Litopenaeus vannamei* (Cahu et al.,1994; Wouters et al., 2001), and *P. indicus* (Cahu et al., 1995)

Materials and methods

Tested diets and feeding processing

Five tested diets were formulated to have fresh basic ingredients (Squid and trash fish). Four diets out of this five were supplemented with different amount of *Dunaleilla salina* extract (DSE; El-Max Salines Company Alexandria) and highly unsaturated fatty acids (HUFA). Diets B1 and B2 were supplemented with 400 and 600mg DSE.kg^{-1} diet, respectively. Diets C1 and C2 were supplemented with HUFA 5 and 10g.kg^{-1} and 400mg.kg^{-1} DSE, respectively. Diets were fed to *(P. monodon*) broodstock for 30 days. 20 prawns were distributed into 5-m^3 tanks. Male:female ratio was 1:1. Ovarian maturation of each female was monitored daily by external examination. Lipid was extracted according to Folch et al (1957). Fatty acids were determined by gas chromatography. Hepatopancreas and ovary of 10 wild maturate females were collected and stored for analysis. At the end of the experiment hepatopancreas and ovary of the tested females were dissected. Eggs were collected for biochemical analysis.

Table I. Composition of the experimental diets

Ingredient	Control	B1	B2	C1	C2
Squid and trash fish (1:1) (g)	950	950	950	950	950
DSE (mg)		400	600	400	400
HUFA(g)				5	10
Kelp powder (g)	50	50	50	50	50
Proximate analysis					
Crude protein(% of dry matter)	63.9	64.4	63.7	65.4	65.7
Crude lipid (% of dry matter)	5.1	4.7	4.2	8.2	9.5
Ash (% of dry matter)	16.4	14.4	16.3	14.7	14.8

Results and discussion

Table II. Effect of different diets on reproductive performance of pond-reared *P. monodon* broodstock

	Control	B1	B2	C1	C2
Carapace length (mm)	44.9±0.8^{a}	0.6^{b}±45.3	0.8^{b}±45.1	0.6^{b}±44.8	0.6^{b}±45.4
Body weight (g)	88.7±2.8	2.4±87.9	2.5±88.5	2.6±88.8	2.2±89.1
Spawning rate (Spawns.female^{-1}.day)	0.002^{a}±0.022	0.001^{c}±0.039	0.003^{ab}±0.029	0.002^{bc}±0.032	0.003^{d}±0.047
Latency period (Days)	0.5^{a}±11.3	0.6^{b}±9.6	0.4^{b}±11.1	0.6^{b}±11.2	0.3^{b}±9.7
Proportion of spawns (%)	4.3^{a}±32.8	1.8^{b} ±58.5	3.2^{b}±50. 0	1.9^{b}±53.7	3.3^{c}±71.5
Egg production per female	35.6^{a}±493.0	33.2^{ab}±542.7	8.2^{a}±517.5	8.6^{a}±500.2	20.0^{b}±588.0
Survival (%)	11±61.0	5.5±84.0	3.5±75.4	6.3±65.2	6.6±75.5

Reproductive performance

Effect of fresh diet supplemented with different levels of DSE and HUFA is shown Table II. Prawn body weight, carapace length, and survival % of females were not significantly different. The proportion of spawns for the control

(32.8%) was significantly lower than other groups fed the tested diets. The spawning rates for females fed diets B1 and C2were higher than groups fed diets control, B2 and C1. The latency period of spawns longer for group's control, B2 and C1 than C2. Fatty acids composition of hepatopencreas (Table III) was correlated with broodstock diets. The 18:0 and 18:2n-6 was significantly lower for the broodstock diets than for the hepatopencreas. The results showed that the fatty acid compositions of hepatopencreas were strongly affected by the broodstock diet and fatty acid conversions were observed among the hepatopencreas. Several authors (Castille and Lawrece 19 89; Teshima, Kanazawa, Koshio and Horinouchi 1989; Han et al., 2008) reported a decrease in total or specific lipid in hepatopencreas during maturation, and assumed that they were transferred to the ovary.

Table III. Effect of different diets on fatty acids composition of hepatopencreas of *P. monodon* broodstock

Fatty acids	control	B1	B2	C1	C2
14:0	0.1	0.3	0.4	0.2	0.7
14:1n-5	Nd	Nd	nd	nd	nd
15:0	Nd	Nd	nd	nd	nd
15:1n-5	Nd	Nd	nd	nd	nd
16:0	6.2	15.4	14.1	10.3	12.1
16:1n-7	0.4	9.6	3.9	3.7	10.3
17:0	Nd	nd	nd	nd	nd
17:1n-7	Nd	nd	nd	nd	nd
18:0	2.9	3.7	5.1	5.4	3.1
18:1n-9	2.6	4.2	4.4	2.9	5.9
18:1n-7	Nd	nd	nd	nd	Nd
18:2n-6	2.2	11.0	12.7	10.6	17.9
18:3n-6	Nd	nd	nd	nd	Nd
18:1n-9	Nd	nd	nd	nd	Nd
18:3n-3	0.6	0.3	1.1	1.3	nd
18:4n-3	Nd	Nd	Nd	Nd	Nd
19:0	Nd	Nd	Nd	Nd	Nd
19:1n-9	Nd	Nd	Nd	Nd	Nd
20:1n-9	Nd	Nd	Nd	Nd	Nd
20:2n-6	5.7	3.1	2.3	2.3	2.9
20:3n-6	Nd	nd	nd	nd	nd
20:4n-6	5.0	9.6	8.4	7.9	10.6
20:5n-6	1.3	1.8	0.3	2.7	0.9
20:3n-3	Nd	Nd	nd	nd	Nd
20:4n-3	Nd	Nd	nd	nd	nd
20:5n-3	11.1	15.4	19.0	18.2	11.9
21:5n-3	Nd	nd	nd	nd	nd
22:6n-3	60.6	23.4	24.8	30.5	18.4
22:5n-3	1.9	1.3	1.5	0.3	2.9
22.0	1.1	0.6	1.0	2.3	1.5
22:1n-11	nd	nd	nd	1.2	nd
Total n-3	73.4	40.3	46.3	50.3	34.1
Total n-6	14.2	14.2	25.6	23.5	32.3
DHA/EPA	5.9	1.5	1.3	1.7	1.6

References

Castillo F.L. and L.A. Lawrence. 1989. Relation between maturation and biochemical composition of the gonads and digestive glands of the shrimps Penaeus oztecus Ives and P. setiferus. Journal of Crustacean Biology 9:202-211.

Huang H-H, S.-G. Jiang, H.-Z. Lin, F.-L. Zhou, and L. Ye. 2008. Effect of dietary highly unsaturated fatty acids and astaxanthin on the fecundity and lipid content of pond-reared Penaeus monodon (Fabricius) broodstock. Aquaculture Research 39:240-251.

Teshima S., A. Kanazawa, S. Koshio, and K. Horinouchi. 1989. Lipid metabolism of the prawn Penaeus japonicas during maturation: variation in lipid profiles of the overy and hepatopencreas. Comparative Biochemistry and physiology 92B:45-49.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

LINKING WEANING SUCCESS TO LARVAL DIGESTIVE CAPACITY USING RADIOLABELLED PEPTIDE FRACTIONS

S. Engrola[1*], N. Richard[1], A.F. Lopes[1], W. Pinto[2], and L.E.C. Conceição[2]

[1] CCMAR/CIMAR, Centro de Ciências do Mar, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal ([*]E-mail: sengrola@ualg.pt)
[2] SPAROS Lda, CRIA- Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

Introduction

Fast growth is essential for marine fish larvae; yet live prey alone are unable to sustain growth at later stages. In order to solve this problem *Artemia* replacement strategies at mouth opening are commonly used to assure higher larval quality. In sole, as in other marine fish species, digestive tract maturation, and consequently protein metabolism, can be positively affected by an *Artemia* replacement feeding strategy at mouth opening (Engrola et al., 2009; Engrola et al., 2010). However, for most marine fish species, inert diets alone from first feeding are unable to sustain growth. Therefore, the complexity of the dietary nitrogen is a key issue to improve marine larval growth (Conceição et al., 2011). Growth – meaning protein accretion – is dependent on how fish ingest absorb and retain protein, i.e., amino acids. Fish digestion is paramount to obtain short-chain peptides and amino acids that may be absorbed to the tissues. The aim of this study was to evaluate how peptide absorption in the intestine may be influenced by different feeding regime.

Materials and methods

Larval rearing
Newly hatched Senegalese sole larvae were reared in 100-l cylindroconical fibreglass tanks in a closed recirculation system with an initial density of 100 larvae.l^{-1} at the Ramalhete facilities (University of Algarve, Faro, Portugal). Two feeding regimes were randomly assigned during the pelagic phase: LF treatment – live feed feeding regime and COM treatment –live feed plus 20% of commercial inert diet (dry matter basis) from mouth opening feeding regime (for further details see Engrola et al., 2010) (Fig. 1). The pelagic rearing lasted 18 days.

Postlarval rearing

After 18 days postlarvae were transfer to flat-bottomed fiberglass tanks and the LF treatment was split in two treatments: LFCOM (co-feeding regime with a commercial inert diet) and LFEXP (co-feeding regime with an experimental inert diet), the COM treatment remained the same. Postlarvae were co-fed with frozen *Artemia* metanauplii and inert diet during two weeks until 35 days after hatching (DAH) when fish was considered weaned and fed exclusively with inert diet. Inert diets were offered to the fish according to the treatment. The benthic rearing lasted until 60DAH.

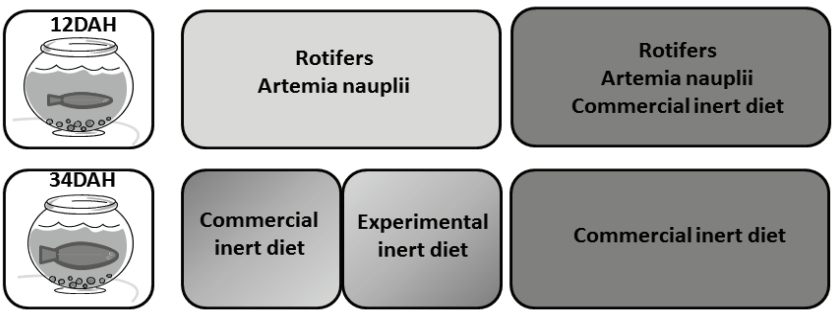


Fig. 1. Schematic design of the experimental feeding regimes.

Peptide ^{14}C labelling and metabolic trials

Radiolabelled peptides and proteins of different molecular weight were produced in vivo through *Artemia* radiolabeling according to the method developed by Richard et al. (2013). Therefore, sole larvae (12DAH) and postlarvae (34DAH) from the different feeding regimes were tube-fed with three radiolabelled peptides (<0.5kDa, 1.0kDa, and 6.8kDa) and then transferred to an incubation vial. The incubation setup was described previously by Rønnestad et al. (2001). Briefly, the incubation setup consisted of sealed vials containing 7.5ml of seawater with gentle air flow, with larvae being placed individually into these vials. The air is forced through a capillary from the incubation vial to a $^{14}CO_2$ trap. After a 24-h incubation period each sole larva was sampled for further analysis.

Results and discussion

Growth

Feeding regimes had a significant effect on larval growth at the end of the pelagic phase (18DAH). Sole that were co-fed with inert diet from mouth opening (COM treatment) were significantly smaller ($P<0.05$) in weight than postlarvae fed exclusively with live prey (LF treatment). At the end of the experiment (60DAH) all fish presented similar dry weight, suggesting that all the feeding regimes were able to sustain a fast growth (Fig. 2). Sole weight at the end of the

experiment in all of the feeding regimes was within the normal range observed in Senegalese sole.

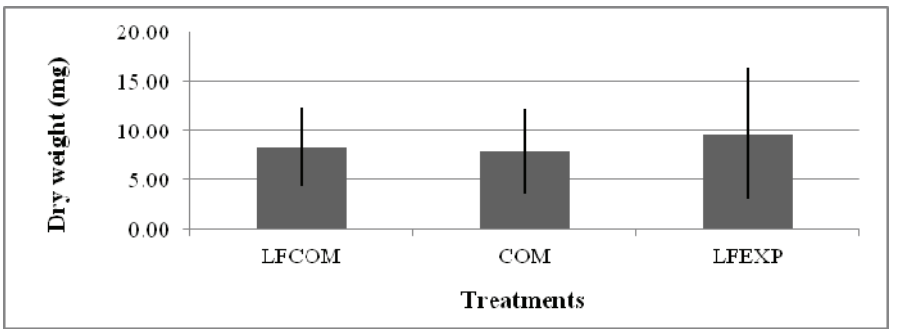


Fig. 2. Sole postlarvae dry weight at the end of the experimental period, 60 days after hatching. Values are means±SD. Absence of different letters indicate no statistical differences (P>0.05, Tukey's test) between treatments at the same age.

Survival at the end of the experiment was not affected by feeding regimes and averaged 62.53±9.40%. These survival rates are two- to threefold higher than previously published values using comparable feeding regimes (Engrola et al., 2009).

Protein utilization

Protein absorption of 12-DAH sole was not affected (P>0.05) by feeding regime (Fig. 3) but was affected by peptide size (P<0.05). Meaning that at this age sole are able to absorb around 80% of the peptide fraction with similar size (highly hydrolysed) of the commercial fish hydrolysates normally incorporated in larval inert diets. However, pre-metamorphic sole larvae seem to have difficulties in digesting and absorbing a relatively small oligopeptide (6.8kDa).

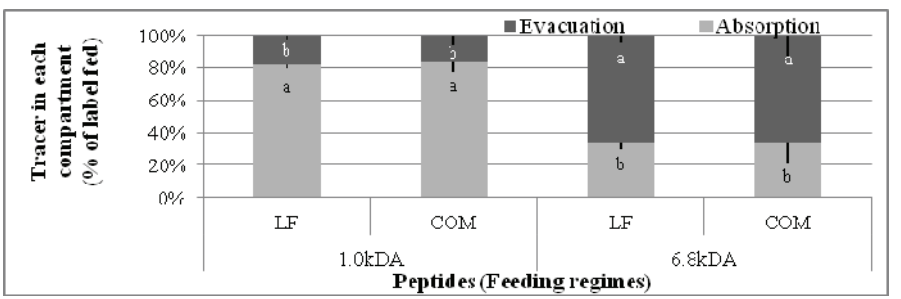


Fig. 3. Protein absorption (% of radiolabel in the larvae and metabolic trap in relation to label fed), and evacuation (% of radiolabel in the seawater in relation to label fed) in sole at 12 days after hatching (DAH), after 24 h of incubation. Values are means ± SD of sole absorption and evacuation (n=6). Different letters indicate statistical differences (P<0.05, Tukey's test) between treatments at the same age.

Conclusion

In conclusion, the methodology applied in the experiment was able to show that the complexity of the dietary nitrogen is a key issue for formulation inert diets for marine fish larvae. However, intestinal peptide absorption was not influenced by the different feeding regimes used in this study, what was also reflected in the similar growth performances obtained.

Acknowledgments

S. Engrola and N. Richard are supported by grants from Fundação para a Ciência e Tecnologia (FCT), Portugal SFRH/BPD/49051/2008 and SFRH/BPD/23514/2005, respectively. This work was funded by Project EPI-SOLE (FCT) [PTDC/MAR/110547/2009] and by project MICALA – QREN I&DT Co-Promoção Nº 13380 (Portugal).

References

Conceição L.E.C., C. Aragão, and I. Rønnestad. 2011. Proteins. In J. Holt (ed.). Larval Fish Nutrition. 1st Ed., John Wiley & Sons, Inc. UK, pp: 83-116.

Engrola S., L. Figueira, L.E.C. Conceição, P.J. Gavaia, L. Ribeiro, and M.T. Dinis. 2009. Co-feeding in Senegalese sole larvae with inert diet from mouth opening promotes growth at weaning. Aquaculture 288:264-272.

Engrola S., M.T. Dinis, and L.E.C. Conceição. 2010. Senegalese sole larvae growth and protein utilization is depressed when co-fed high levels of inert diet and Artemia since first feeding. Aquacult. Nutr. 16:457-465.

Richard N., S. Engrola, P. Palma, W. Pinto, D. Simes, and L.E.C. Conceição. 2013. Assessment of protein digestive capacity and utilisation during ontogeny of Senegalese sole: a tracer study using in vivo produced radiolabelled peptide fractions. Larvi'13 – Fish & Shellfish Larviculture Symposium, Ghent, Belgium, pp.

Rønnestad I., C.R. Rojas-García, S.K. Tonheim, and L.E.C. Conceição. 2001. In vivo studies of digestion and nutrient assimilation in marine fish larvae. Aquaculture 201:161-175.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

FATTY ACID PROFILE IN EGGS AND NEWLY HATCHED PARA-LARVAE OF *OCTOPUS VULGARIS* COLLECTED FROM THE WILD, AND AFTER 1-5 DAYS STARVATION

J. Estefanell*, J. Socorro, B. Ramírez, M. Izquierdo, and J. Roo

Grupo de Investigación en Acuicultura, Universidad de Las Palmas de Gran Canaria & Instituto Universitario de Sanidad Animal y Seguridad Alimentaria, Trasmontaña s/n, 35416, Arucas, Las Palmas, Canary Islands, Spain. Phone number: 34 928 459736. *E-mail: juan.estefanell@giaqua.org.

Introduction

The common octopus *O. vulgaris* is a promising candidate to diversify marine farming (Estefanell et al., 2012), however, low survival of the paralarvae after the planktonic phase still constrains industrial rearing of this species (Iglesias et al., 2007). To date, best growth and survival were obtained when *Artemia* was complemented with crab zoeas (Iglesias et al., 2007), implying that nutrition is the main factor affecting biological performance of early stages in this species. *Artemia* seems to be deficient in n-3 HUFA in comparison with zoeas and newly hatched paralarvae (Navarro and Villanueva, 2000). Little is known regarding the broodstock diet for *O. vulgaris*. Best paralarvae quality have been obtained from specimens fed on crab or tattler rather than sardine (Quintana, 2009). Also, the initial biochemical profile of wild paralarvae of this species is unknown, since to our knowledge all available data was obtained from hatchlings maintained under lab conditions, with broodstock being fed on different fresh diets.

In this study we collected wild egg masses and newly hatched paralarvae at sea, to obtain information regarding the fatty acid profile of the broodstock natural diet. Secondly, we compared the fatty acid profile among wild eggs, newly hatched wild paralarvae, and those newly hatched paralarvae under lab conditions whose broodstock were fed on low price fish species commonly used during the grow out phase (Estefanell et al., 2012). Finally, we performed a five-day starving trial on newly hatched paralarvae obtained under lab conditions to study the lipid and fatty acid utilization.

Materials and methods

Samples - Wild eggs (3 strings per female, n=6) and wild paralarvae (n=2) were collected at 10-15 m depth in coastal Arguineguín (Las Palmas, Canary Islands).

Paralarvae obtained under lab conditions - One female (1440g) and two males (1651±55g) were kept in 2-m^3 rectangular tanks under open flow throw sea water conditions (100% per hour) and natural photoperiod (March-April 2012). The tank was provided with six dens (PVC tubes of 160mm diameter and 50cm length) and covered with a shadowing net. The octopi were fed ad libitum once a day (6 times.$week^{-1}$) with fresh bogue *Boops boops* provided by fishermen as trash species (Estefanell et al., 2013). Spawning occurred naturally after two months in captivity.

Experimental design - 6 groups of 150 paralarvae were kept unfed for 1 to 5 days post-hatching in PVC tubes (110mm diameter and 45 cm length), enclosed with net of 125µm in one end. These tubes were placed inside a circular 500-l tank, so the open end would be off the water. Only live paralarvae were taken for the following determinations.

Dry weight determinations - For each group and day post-hatching, 10 paralarvae were randomly selected. The dry weight was determined by drying them at 105°C until constant weight.

Biochemical analysis - Moisture was determined after drying the sample in an oven at 105°C to constant weight AOAC (1997) and crude lipid was extracted following the method described by Folch et al. (1957). Fatty acids methyl esters from total lipids were extracted by transmethylation as described by Christie (1982) and separated by gas chromatography according to Izquierdo et al. (1992). Samples of unfed paralarvae at 5 days post-hatching and wild paralarvae were insufficient to calculate the moisture content.

Statistical analysis – All data, presented as mean±standard deviation were submitted to a one-way ANOVA. Significant differences were considered when $P<0.05$.

Results and discussion

The lipid content was 11.5-13.9DW in the samples analyzed (Table I), higher than those observed in adults of *O. vulgaris* (Estefanell et al., 2013). This underlines the importance of the lipid fraction in early stages (Navarro and Villanueva, 2000; 2003), probably associated to reserves in the digestive gland or to main component of the visual and nervous system. Important deviations in the fatty acid profile were observed between wild and reared paralarvae, especially in the ARA, EPA, and DHA content (Table I). This suggests the inadequacy of low price trash species, such as fisheries and aquaculture byproducts (Estefanell et al., 2013), as a single diet for *O. vulgaris* broodstock. Indeed, the deficiency or imbalance of DHA, EPA, and ARA in broodstock diets induced low larval survival in several marine fish species (Fernández Palacios et al.,

2011). In addition, relatively low hatching weight was observed in this study (Table II), which again suggests low paralarvae quality, lower than in other studies where the broodstock were fed on diets containing crustaceans (Navarro and Villanueva, 2003; Quintana, 2009).

Table I: Fatty acid profile (%) in wild eggs, wild hatchlings (0dah), and in hatchlings obtained under lab conditions (lab).

	Eggs (wild)	**Hatchlings (wild)**	**Hatchlings (lab)**
Lipid (% dw)	11.5±0.7	-	13.9±1.1
16:0	27.1±2.2^{b}	19.9±0.2^{a}	18.9±0.1^{a}
18:1 n-9	7.1±0.7^{a}	9.7±0.0^{b}	9.8±0.0^{b}
20:4 n-6	13.0±2.0^{b}	11.5±0.4^{b}	5.3±0.1^{a}
20:5 n-3	7.6±0.9^{a}	11.2±0.1^{b}	14.4±0.1^{c}
22:6 n-3	16.8±1.6^{a}	16.0±0.4^{a}	28.6±0.2^{b}

Table II: Dry weight (g), survival (%), lipid (% dw) and fatty acid profile (%) in unfed paralarvae obtained under lab conditions at 0-5 days after hatching.

	0dah	**1dah**	**2dah**	**3dah**	**4dah**	**5dah**
Dry weight (g)	0.23±0.01^{e}	0.20±0.01^{d}	0.18±0.01^{c}	0.16±0.01^{b}	0.14±0.01^{a}	0.13±0.01^{a}
Survival (%)	100%	100%	100%	100%	98%	65%
Lipids (% dw)	13.9±1.1	13.1±0.5	13.0±0.5	13.4±0.3	13.9±0.1	-
16:0	18.9±0.1^{e}	18.4±0.2^{d}	17.2±0.1^{c}	16.6±0.1^{b}	15.6±0.1^{a}	15.3
18:1 n-9	9.8±0.0^{a}	10.3±0.2^{b}	10.7±0.2^{c}	10.9±0.1^{cd}	11.3±0.1^{d}	11.2
20:4 n-6	5.3±0.1^{b}	4.6±0.1^{a}	5.4±0.2^{b}	5.4±0.1^{b}	5.4±0.2^{b}	5.3
20:5 n-3	14.4±0.1^{a}	14.4±0.6^{a}	15.2±0.3^{ab}	15.5±0.2^{b}	15.7±0.3^{b}	14.6
22:6 n-3	28.6±0.2	28.1±0.3	28.9±0.4	28.8±0.2	28.6±0.5	27.8

The decrease in dry weight in the paralarvae was due to starvation, and no cannibalism was observed until day 5 (Table II). In relative terms, no decrease in lipid content was observed up to 4 days after hatching (Table II), which suggests that both lipids and proteins were used as energy sources. The use of lipids from digestive gland and proteins form muscle tissues were already reported in starved adults of this *O. vulgaris* (García Garrido et al., 2010; 2012). Regarding the fatty acid profile, ARA and DHA remained constant (Table II), suggesting the essentiality of these fatty acids. In contrast, a decrease in 16:0 and an increase in 18:1n-9 and EPA were observed in the paralarvae from 0 to 4 days after hatching (Table II). Similar findings were observed in starved *Sepia officinalis*, with retention of ARA and DHA and a decrease in 16:0, although no increase in 18:1n-9 and EPA was observed (Sykes et al., 2012).

References

A.O.A.C. 1997. Official Methods of Analysis. Association of Official Analytical Chemists, Washington.

Christie W.W. 1982. Lipids Analysis. Pergamon Press, Oxford

Estefanell J., J. Roo, R. Guirao, J.M. Afonso, H. Fernández Palacios, M. Izquierdo, and J. Socorro. 2013. Efficient utilization of dietary lipids in *Octopus vulgaris* (Cuvier 1797) fed fresh and agglutinated moist diets based on aquaculture by products and low price trash species. Aquaculture Research 44:93-105.

Estefanell J., J. Roo, R. Guirao, M. Izquierdo, and J. Socorro. 2012. Benthic cages *versus* floating cages in *Octopus vulgaris*: biological performance and biochemical composition feeding on *Boops boops* discarded from fish farms. Aquacultural Engineering 49:46-52.

Fernández-Palacios H., M.S. Izquierdo, B. Norberg, and K. Hamre. 2011. Effects of broodstock diet on eggs and larvae. In: (Holt, J., ed) Larval Fish Nutrition, Wiley-Blackwell, John Wiley and Sons Publisher Editor. pp 153-183. ISBN: 978-0-8138-1792-7

Folch J., M. Lees, and G.H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497-509.

García Garrido S., I. Hachero Cruzado, D. Garrido, C. Rosas, and P. Domingues. 2010. Lipid composition of the mantle and digestive gland of *Octopus vulgaris* juveniles (Cuvier, 1797) exposed to prolonged starvation. Aquaculture International 18:1223-1241.

García Garrido S., I. Hachero Cruzado, C. Rosas, and P. Domingues. 2012. Protein and amino acid composition from the mantle of juvenile *Octopus vulgaris* exposed to prolonged starvation. Aquaculture Research. DOI:10.1111/j.1365-2109.2012.03180.x

Iglesias J., F.J. Sánchez, J.G.F. Bersano, J.F. Carrasco, J. Dhont, L. Fuentes, F. Linares, J.L. Muñoz, S. Okumura, J. Roo, T. Van Der Meeren, E.A.G., and R. Villanueva. 2007. Rearing of *Octopus vulgaris* paralarvae: present status, bottlenecks and trends. Aquaculture 266:1-15.

Izquierdo M.S., T. Arakawa, T. Takeuchi, R. Haroun, and T. Watanabe. 1992. Effect of n-3 HUFA levels in Artemia on growth of larval japanese flounder (*Paralichthys olivaceous*). Aquaculture 105:73-82.

Navarro J.C. and R. Villanueva. 2000. Lipid and fatty acid composition of early stages of cephalopods: an approach to their lipid requirements. Aquaculture 183:61-177.

Navarro J.C. and R. Villanueva. 2003. The fatty acid composition of *Octopus vulgaris* paralarvae reared with live and inert food: deviation from their natural fatty acid profile. Aquaculture 219:613-631.

Quintana D. 2009. Valoración de los requerimientos nutricionales de reproductores de pulpo común (*Octopus vulgaris*). PhD thesis. University of La Laguna (Spain)

Sykes AV, E. Almansa, R. Martin, C. Rodríguez, A. Lorenzo, and J.P. Andrade. 2012. Lipid characterization of starved and grass shrimp-fed cuttlefish (*Sepia officinalis*, l.), during the first 7 days after hatching. CIAC symposium, Florianopolis (Brasil).

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

VITAMIN A AND K, TWO FAT-SOLUBLE VITAMINS REQUIRED FOR HARMONIC FISH LARVAL DEVELOPMENT

I. Fernández[1], E. Gisbert[2], and P. Gavaia[1]

[1] Developmental and Evolutionary Gene Expression Lab, Centro de Ciências do Mar (CCMAR), Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal. e-mail: ivmonzon@ualg.pt
[2] Intitut de Recerca i Tecnologies Agroalimentaries (IRTA), Centre de Sant Carles de la Ràpita, Crta. Del Poble Nou s/n km 5.5, 43540 Sant Carles de la Ràpita, Tarragona, Spain

Aquaculture will be the main worldwide source for animal proteins in the near future. To reach that role, fish farmers need to overcome several industrial bottlenecks such as the quantity and quality of larval production. Fry quality in terms of pigmentation and skeletal development deeply relies on larval protocols, genetic background, and nutrition among other factors. Optimal nutritional requirements for harmonic larval development on fat-soluble vitamins (vitamin A, E, D, and K) are still not known. In recent years, research has focused on unveiling the role of vitamins A (VA) and K (VK) during larval development. On one hand, it is well known that retinoic acid (RA), the main active VA metabolite, plays a key role in morphogenesis and cell proliferation and differentiation, determining growth, body patterning, and the development of the nervous system, limbs, and skeletal development. On the other hand, VK is recognized to act as a coenzyme for γ-glutamyl carboxylase, which performs the conversion of Glu to Gla residues in VK-dependent proteins. Through this metabolic role, VK controls blood clotting, but also seems to be involved in inflammation, growth, chemotaxis, apoptosis, and mineralization. Both vitamins are able to activate specific gene transcription through their binding to specific nuclear receptors: RA receptors (RARs) and retinoic X receptors (RXRs) in the case of RA; and pregnane X receptor (PXR) in the case of VK. Since fish are not able to synthesize either VA or VK, they have to obtain them from the diet at the optimum level, but also in the proper chemical form. Interestingly, intestinal flora could also be an important source of VK. Moreover, although both are fat-soluble vitamins, their metabolism is quite different since VK could be recycled through the reaction catalyzed by the VK epoxide reductase, while RA could not be recycled at all. Several commercial, 'new' aquaculture, and research model species as well as different nutritional approaches (bioencapsulation, bath exposure, and microinjection) have been used to study VA and VK metabolism and function in order to establish their optimal dietary requirements in fish. A broad range of

tools and systems have been also applied in order to get further insights about the key role of both vitamins in early development such as in vivo, ex-vivo, and in vitro systems; as well as different biochemical, genomic, and molecular biology tools. All that research work pointed out that larval performance deeply relies on VA and VK dietary intake. In fact, survival, growth, digestive system, immune system, pigmentation pattern, and skeletal development have been shown to be affected in larvae fed with nutritional unbalanced diets on those vitamins. Recent research investigations show the optimum dietary VA and VK dose for proper skeletogenesis seems to be species-specific and depends on the developmental stage and physiological condition of the larvae. In *Paralichthys olivaceus* larvae, safe dietary levels of VA in *Artemia* should be less than 50 000IU.kg^{-1}, in *Dicentrarchus labrax* it should be around 116.55IU.kg^{-1}, meanwhile it must be less than 1.0×10^{8}IU.kg^{-1} in *Sparus aurata* fed with rotifers and less than 42 666IU.kg^{-1} VA in *Solea senegalensis* fed *Artemia* metanauplii. In contrast, less is known about the safe limits for VK. In salmonids, minimum requirements for early juveniles are suggested to be between 0.1 and 2mg.kg^{-1}. Recently, it has been found that *Artemia* supplemented with 250mg.kg^{-1} in their enriching emulsion gives better skeletal development in *S. senegalensis* larvae. Unfortunately, all that basic knowledge on VA and VK physiology in fish for designing nutritionally balanced diets for larval development seems to not have been translated in an effective reduction of skeletal deformities and production costs in marine fish aquaculture. This could be due to the fact that those research works were performed from unifactorial approaches. In this sense, one important and unexplored aspect of both vitamins' physiology is their biological interaction at the nuclear level. It is already known that RXRs perform heterodimers with other nuclear receptors such as RARs, PPARs, VDR, and THRs, but interestingly, they also conform heterodimers with PXR, which at the same time also binds to PPARs. Then, different vitamin levels might induce the formation of different nuclear receptor heterodimers, which in turn lead to a specific gene transcription and downstream signalling pathways during larval development that needs to be revealed. Therefore, further research should be done using multifactorial approaches in fat-soluble vitamins providing a full integrated view of metabolic pathways affected in fish larvae and how both vitamins (and other nutrients) interact at different levels (e.g. nuclear, cellular, tissue, and whole organism).

Present work was partially funded by the Portuguese Foundation for Science and Technology (FCT) through PTDC/MAR/105152/2008 (SpecialK) and PTDC/MAR/105313/2008 (FishCell) projects and the European Commission (ERDF-COMPETE) through PEst-C/MAR/LA0015/2011 project and AIB2010-PT-00313 project (MIMECO, Spain). IF is financed by FCT post-doctoral grant SFRH/BPD/82049/2011.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

INFLUENCE OF THE FORMS AND LEVELS OF DIETARY SELENIUM ON OXIDATIVE STRESS IN RAINBOW TROUT FRY

S. Fontagné-Dicharry[1], S. Godin[2], H. Liu[1,3], P. Antony Jesu Prabhu[1], B. Bouyssiere[2], M. Bueno[2], P. Tacon[4], F. Médale[1], and S. Kaushik[1]

[1] INRA, UR1067 NuMéA, F-64310 Saint-Pée-sur-Nivelle, France
[2] LCABIE UMR5254, F-64053 Pau Cedex 09, France
[3] FPE, Institute of Hydrobiology, CAS, 430072 Wuhan, P.R. China
[4] Lesaffre Feed Additives, F-59700 Marcq-en-Barœul, France

Introduction

Selenium (Se) is an essential micronutrient for all vertebrates required as an integral part of diverse Se-containing proteins as selenocysteine, called selenoproteins, with diverse physiological functions and fishmeal usually is a major source of this essential element in aquafeeds. As a component of glutathione peroxidases (GPx), thioredoxin reductases, and methionine sulfoxide reductases, Se plays a pivotal role against oxidative cellular injury and lipid peroxidation. On the other hand, this metalloid may exert toxic effects at levels (or even marginally) above those required and the chemical form of Se is known to strongly affect its bioavailability and its impact on metabolism. A dietary Se requirement varying from 0.15-0.7mg.kg^{-1} diet has been reported for different fish species based on weight gain and GPx activity (NRC, 2011). Fish meal and plant ingredients contain variable Se levels and so the objective of the study was to assess the impact of dietary Se supplementation on the antioxidant status of rainbow trout (*Oncorhynchus mykiss*) fry fed fish meal or plant-based feeds.

Materials and methods

Triplicate groups of first-feeding swim-up fry (initial body weight: 91±4mg) were reared at 17°C and fed for 12 weeks either plant (P-diets) or fish meal-based (F-diets) diets with a total Se content of 0.5 or 1.2mg.kg^{-1} respectively. These diets were either non-supplemented or supplemented with 0.3mg Se.kg^{-1} supplied as sodium selenite (SeS-diets) or Se-yeast (SeY-diets). Whole fry samples were taken on day 0 and from each tank at the end of the feeding trial after 16h of fasting. Fish were anaesthetised with benzocaine before wet weight determination and frozen in liquid nitrogen. The total Se concentration in diets and whole fry was measured by inductively coupled plasma-mass spectrometry according to Bierla et al. (2008). Analyses of antioxidant enzyme activities, gene

expression, lipid peroxidation products and fatty acid profiles were determined as previously described (Fontagné et al., 2008).

Results and discussion

Growth (final body weight: 8.1±0.8g) and survival (86±7%) of rainbow trout fry were not significantly (P>0.05) affected by dietary Se sources and levels suggesting that no deficient or toxic dietary Se level was tested in the present study.

Compared to fish fed the non-supplemented diets (Se0), whole-body Se was raised by both Se sources and to a greater extent by Se-yeast (Fig. 1A) highlighting the higher retention of the organic form of Se for rainbow trout fry probably related to the higher bioavailability reported by Rider et al. (2010). Compared to initial first-feeding swim-up fry, the whole-body Se content was reduced in rainbow trout fry fed plant-based diet supplemented with sodium selenite PSeS (227±9 vs. 200±5ng.g^{-1}).

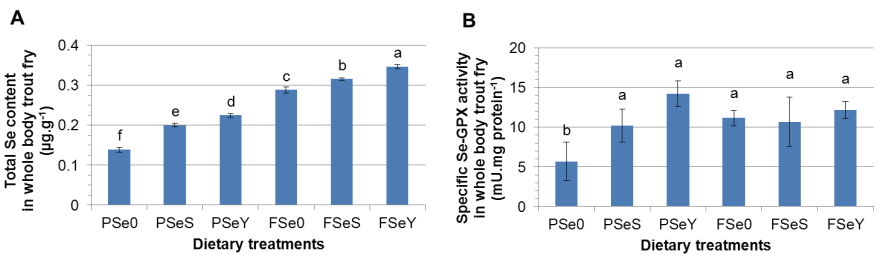


Fig. 1. Whole-body Se (A) and specific Se-dependent GPx activity (B) of rainbow trout fry fed the plant (P) or fish meal-based (F) diets supplemented or not (Se0) with 0.3mg Se.kg^{-1} diet supplied as sodium selenite (SeS) or Se-yeast (SeY). Values are means±SD of 3 rearing tanks. Different superscripts indicate significant differences (P<0.05).

Whole-body Se-dependent GPx activity was significantly lower in fish fed the non-supplemented plant-based diet PSe0 (Fig. 1B) suggesting that the dietary Se level required to maintain maximal whole-body GPx activity is higher than 0.5mg.kg^{-1} when fed a completely plant based diet and thus higher than the supplemental inorganic Se level of 0.38mg.kg^{-1} diet determined by Hilton et al. (1980) to be adequate for rainbow trout juveniles. However, according to these authors, the Se requirement may decline as the fish grows and so these results may indicate a higher Se requirement for rainbow trout fry stages compared to juveniles. According to two-way ANOVA (diet basis and Se supplementation), whole-body Se-dependent GPx activity was enhanced by dietary Se-yeast compared to sodium selenite. The other antioxidant enzyme activities (catalase, superoxide dismutase, Se-independent GPx, glutathione reductase, and glu-

tathione-S-transferase) were not significantly affected by dietary Se sources and levels.

Gene expression profiles were correlated to activity levels of antioxidant enzymes with a significant decreased expression of some Se-GPx (GPx1b1, GPx1b2 and GPx4a1 as described by Pacitti et al., 2013) in rainbow trout fry fed the non-supplemented plant-based diet (PSe0) and no effect on the expression of genes coding for the other antioxidant enzymes. Expression of selenoprotein P was also significantly reduced in the PSe0 group. On the other hand, gene expression profiles of the other selenoproteins GPx1a, GPx4a2, GPx4b, and the methionine sulfoxide reductases were not affected by dietary Se sources and levels and expression of thioredoxine reductase 1 was even decreased in the FSeS and FSeY groups with the highest whole-body Se contents.

The oxidative status as measured by peroxide value, conjugated dienes, anisidine value and lipid-soluble fluorescent products of whole rainbow trout fry as well as the fatty acid profiles were not significantly affected by dietary Se sources and levels contrary to the antioxidant status. This lack of effect of supplemental Se on these parameters may indicate that the decrease of the antioxidant status noticed in the group fed the non-supplemented plant-based diet PSe0 is not great enough to provoke oxidative stress in the low stressful conditions used in the present study (e.g., low stocking density) as also suggested by the observations of Küçükbay et al. (2009).

Conclusions

These results highlight the superiority of organic form of Se to fulfil the dietary Se requirement of rainbow trout fry and the necessity to supplement plant-based diets.

Acknowledgements

The authors wish to thank F. Terrier, F. Sandres and Y. Hontang for the care of fish and M. Cluzeaud, L. Larroquet, and V. Véron for technical assistance.

References

Bierla K., M. Dernovics, V. Vacchina, J. Szpunar, G. Bertin, and R. Lobinski. 2008. Determination of selenocysteine and selenomethionine in edible animal tissues by 2D size-exclusion reversed-phase HPLC-ICP MS following carbamidomethylation and proteolytic extraction. Analytical and Bioanalytical Chemistry 390:1789-1798.

Fontagné S., E. Lataillade, J. Brèque, and S. Kaushik. 2008. Lipid peroxidative stress and antioxidant defence status during ontogeny of rainbow trout (*Oncorhynchus mykiss*). British Journal of Nutrition 100:102-111.

Hilton J.W., P.V. Hodson, and S.J. Slinger. 1980. The requirement and toxicity of selenium in rainbow trout (*Salmo gairdneri*). Journal of Nutrition 110:2527-2535.

Küçükbay F.Z., H. Yazlak, I. Karaca, N. Sahin, M. Tuzcu, M.N. Cakmak, and K. Sahin. 2009. The effects of dietary organic or inorganic selenium in rainbow trout (*Oncorhynchus mykiss*) under crowding conditions. Aquaculture Nutrition 15:569-576.

NRC 2011. Nutrient requirements of fish and shrimp. The National Academies Press, Washington, DC. 376p.

Pacitti D., T. Wang, M.M. Page, S.A.M. Martin, J. Sweetman, J. Feldmann, and C.J. Secombes. 2013. Characterization of cytosolic glutathione peroxidase and phospholipid-hydroperoxide glutathione peroxidase genes in rainbow trout (*Oncorhynchus mykiss*) and their modulation by in vitro selenium exposure. Aquatic Toxicology 130-131:97-111.

Rider S.A., S.J. Davies, A.N. Jha, R. Clough, and J.W. Sweetman. 2010. Bioavailability of co-supplemented organic and inorganic zinc and selenium sources in a white fishmeal-based rainbow trout (*Oncorhynchus mykiss*) diet. Journal of Animal Physiology and Animal Nutrition 94:99-110.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

LESSONS LEARNED FROM GNOTOBIOTIC SYSTEMS ON THE EFFECT OF BACTERIA ON GROWTH, SURVIVAL, AND GENE EXPRESSION IN MARINE LARVAE

T. Forberg and O. Vadstein

Norwegian University of Science and Technology, Department of Biotechnology, Sem Sælands veg 6-8, N7491, Norway

Through the use of gnotobiotic systems (i.e., free of, or containing only known, bacteria), the contributions of bacteria on their vertebrate hosts have been confirmed. The commensal microbiota (all bacteria normally present in the gut, excluding pathogens) not only outnumbers the host cells, but represents a myriad of biochemical pathways and activities that can alter the gut environmental conditions and also directly influence host cells. For humans it has been estimated that the genes of the microbiota outnumber the human genes by a factor higher than 300.

Germ-free mammals often show metabolic deficiencies such as reduced digestion and vitamin synthesis. Some of the differences observed may be due to the lack of bacterial enzymes, but the commensal microflora also directly affect host-regulation of metabolic enzymes. Several germ-free mammals, and also germ-free zebrafish (*Danio rerio* L.), have been found to have less developed intestines, and lower rates of intestinal cell proliferation.

The earliest gnotobiotic systems for marine larvae were developed for salmon (*Salmo salar*), halibut (*Hippoglossus hippoglossus*), and turbot (*Scophthalmus maximus*). However, not all of these describe a system for feeding of the larvae, and the methods used to verify the gnotobiotic state were mainly culture-based.

Recently, gnotobiotic systems allowing for feeding of the larvae has been developed for seabass (*Dicentrarchus labrax*) and cod (*Gadus morhua*) The gnotobiotic statuses of these systems are verified not only by culture-based methods, which is paramount as most environmental bacteria will not grow on standard media.

While germ-free zebrafish are smaller and less developed than conventional larvae (with undefined microbiota), no such effect was seen on the growth of cod and sea bass. A morphological analysis of sea bass larvae revealed that the germ-free larvae were larger, and also had a greater gut/total body volume ratio.

In gnotobiotic cod larvae, gene-expression studies have identified 10 genes whose expression is regulated by bacteria. These 10 host-response genes are involved in processes such as immune response and nutrient uptake, and represent a first glance at the role bacteria may play in the gut of cod larvae.

The effect of bacteria on host-response genes in both zebrafish and cod seem to be species-specific, illustrating that which bacteria are present may be far more important than how many they are.

Gnotobiotic studies will improve our knowledge on the functionality of host-microbe interactions. Fish as model species extend the phylogenetic and evolutionary perspective of host-microbe interactions of vertebrates, which today is biased towards mammals. The completion of genome sequences for marine fish species combined with new methods for high throughput gene expression analysis means that the amount of information gained by such systems will most likely dramatically increase in the years to come. A major challenge will be to bridge the gap between gene expression data and biological variables such as growth and survival. Increased knowledge into host-microbial interactions can lead to better strategies for microbial control during larval rearing, with the aim of improving both survival and growth of the larvae.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

CORRELATION OF SADDLEBACK SYNDROME WITH DEFORMITIES OF THE PELVIC FINS AND LATERAL LINE IN EUROPEAN SEA BASS, *DICENTRARCHUS LABRAX*

S. Fragkoulis, P. Kokkinias, and G. Koumoundouros*

Biology Department, University of Crete, Vasilika Vouton, 70013 Heraklion, Crete, Greece, tel. +30 2810394065, *gkoumound@biology.uoc.gr

Introduction

Despite the intensive research of the last decade, morpho-anatomical abnormalities continue nowadays to be a significant problem for finfish aquaculture. This is especially because of the high variation of the frequency of deformities during the production process, as well as due to the appearance of new deformity types (Koumoundouros, 2010; Boglione et al., 2013).

Saddleback syndrome is a rare deformity (<1%), however, with significant effects on the anatomy of dorsal fin and on the external morphology of fish. Anatomically, saddleback syndrome is characterized by the partial to complete lack of rays, accompanied by severe abnormalities of the respective pterygiophores (Koumoundouros, 2010). In the present study we describe a new type of saddleback syndrome which is highly correlated with abnormalities of the pelvic fins and of the lateral line.

Materials and methods

Samples were taken from a population of sea bass juveniles (70-90mm standard length, SL; 145-150 days post hatch) during routine quality control in a commercial hatchery. Juveniles were anaesthetised and examined for the presence of saddleback syndrome. Subsequently, a random sample of 50 normal and 50 deformed individuals was taken. Both sides of the body were photographed to examine the lateral line in each sampled individual. Finally, the juveniles were x-rayed and examined for the presence of skeletal deformities (Koumoudouros et al., 2000). To study the ontogeny of the new type of saddleback syndrome, larval samples from the records of the laboratory were examined. Larvae were double stained with Alcian-blue and Alizarin-red (Park & Kim, 1984).

G-test was applied to test the significance of the differences between groups (Sokal and Rolhf, 1981).

Results and discussion

In the examined population, the 12% of the fish presented a new type of saddle-back syndrome (SBS). This deformity is mainly characterised by the loss of fin rays on the pterygiophores Prx3-Prx7 (92% of the cases), which rarely expands at the first two pterygiophores (8% of the cases; Fig. 1). Unlikely to the saddle-back types which have been recorded so far (Koumoundouros, 2010), this new type neither correlates with deformities of the pterygiophores, nor with deformities of the caudal fin. However, it is highly correlated with a partial or total lack of pelvic-fin rays (in the 66% of the fish with SBS vs. 0% of the fish without SBS, Figs. 1,2).

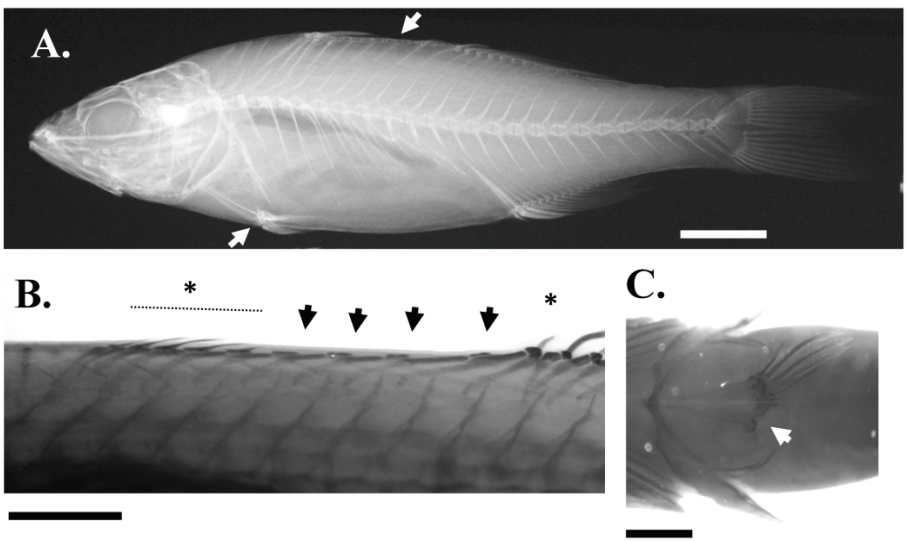


Fig. 1. Anatomy of SBS in a sea bass juvenile (A) and larva (B). Missing rays are indicated with arrows. Asterisks in B indicate atrophied rays. C. Lack of rays and spine of the right pelvic fin (arrow). Scale bars equal to 10mm (A) or 1mm (B, C).

The examination of external morphology of the two groups showed that both SBS and non-SBS juveniles presented a high frequency of abnormalities of the lateral line, mainly in the form of bilaterally or unilaterally lacking sectors (Fig. 3). Statistics revealed that the abnormality of the lateral line was significantly more frequent in the SBS than in the non-SBS fish (62% vs. 42%, $p<0.05$, Fig. 2). Other types of lateral line abnormalities consisted of bilaterally double-overlapping lateral line (Fig. 3C-3C', 10%-16%), and to a less extent of discontinuous or dorso-ventrally curved lateral line. Similar abnormalities of the lateral line have been previously documented to develop at very high frequencies in

reared *Sparus aurata*, with however still unknown causative factors (Carillo et al., 2001).

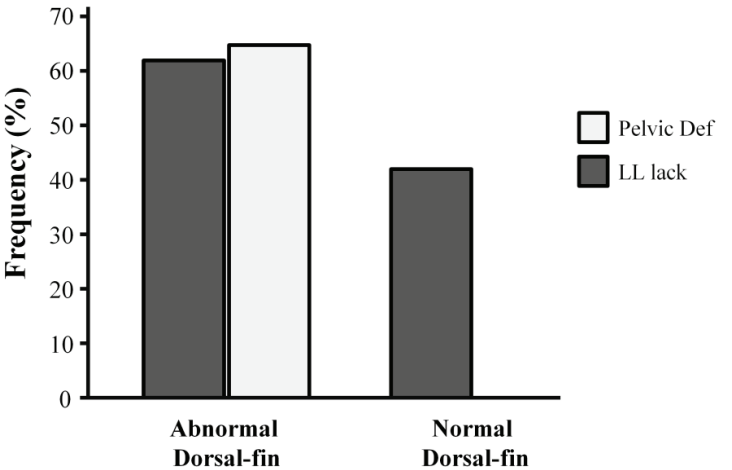


Fig. 2. Frequency of the pelvic-fin deformities and of the abnormalities of the lateral line (*LL*) in the juveniles with normal and abnormal dorsal fin.

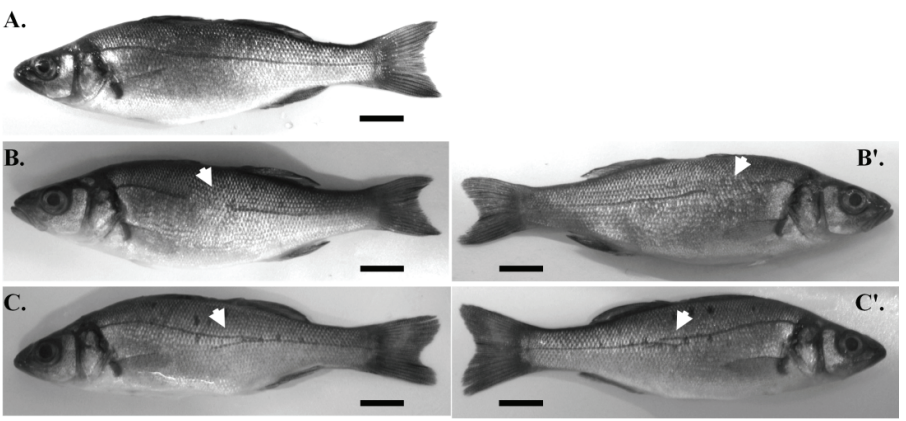


Fig. 3. Abnormalities of the lateral line in the studied juveniles. A. Normal fish. B-B', bilateral lack of a part of the lateral line. C-C', bilaterally double-overlapping lateral line. Scale bars equal to 10mm.

In the present study, we document a highly correlated development of three different morpho-anatomical abnormalities. Although bibliographically rare, such high correlations between deformities of different structures, anatomically and/or ontogenetically, have been attributed to common causative factors acting during specific ontogenetic stages (Koumoundouros et al., 2001; 2002). Such a hypothesis could explain the strong correlation of SBS with pelvic-fin deformities in *D. labrax* (present study), since dorsal and pelvic fins develop during

overlapping periods (unpublished data). The high frequency of the abnormalities of the lateral line in the non-SBS juveniles could be explained by the fact that scales develop later than the dorsal and pelvic fins (unpublished data).

Acknowledgements

This study was supported by the program NSRF 2007-2013, «Competitiveness & Entrepreneurship» (call Cooperation I, Project No 09SYN-24-619) of the Ministry of Education, Religious Affairs, Culture and Sports, Greece.

References

Boglione C., E. Gisbert, P. Gavaia, P.E. Witten, M. Moren, S. Fontagné, and G. Koumoundouros. 2013. A review on skeletal anomalies in reared European larvae and juveniles Part 2: Main typologies, occurrences and causative factors. Reviews in Aquaculture, in press.

Carrillo J., G. Koumoundouros, P. Divanach, and J. Martinez. 2001. Morphological malformations of the lateral line in reared gilthead sea bream (*Sparus aurata* L. 1758). Aquaculture 192:281-290.

Koumoundouros G. 2010. Morpho-anatomical abnormalities in Mediterranean marine aquaculture. In: Recent Advances in Aquaculture Research. Transworld Research Network, Kerala, India. pp. 125-148.

Koumoundouros G., P. Divanach, A. Savaki, and M. Kentouri. 2000. Effects of three preservation methods on the evolution of swimbladder radiographic appearance in sea bass and sea bream juveniles. Aquaculture 182:17-25.

Koumoundouros G., P. Divanach, and M. Kentouri. 2001. The effect of rearing conditions on development of saddleback syndrome and caudal fin deformities in *Dentex dentex* (L.). Aquaculture 200:285-304.

Koumoundouros G., E. Maingot, P. Divanach, and M. Kentouri. 2002. Kyphosis in reared sea bass (*Dicentrarchus labrax* L.): ontogeny and effects on mortality. Aquaculture 209:49-58.

Park E.H. and D.S. Kim. 1984. A procedure for staining cartilage and bone of whole vertebrate larvae while rendering all other tissues transparent. Stain Technology 59: 269-272.

Sokal R.R. and F.J. Rohlf. 1981. Biometry. Freeman, San Francisco, CA.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EXPRESSION AND ACTIVITY OF THREE DIGESTIVE PROTEASES IN LARVAE OF THE TOTOABA (*TOTOABA MACDONALDI*)

M.A. Galaviz[1*], L.M. López[1], A. García Gasca[2], E. Gisbert[3], A.C. Álvarez[4], G. Sandoval[1], and C.D. True[1]

[1] Universidad Autónoma de Baja California (UABC), Facultad de Ciencias Marinas. PO Box 76, Ensenada B.C. 22860, México. E-mail: mgalaviz@uabc.edu.mx
[2] Centro de Investigación en Alimentación y Desarrollo, Unidad Mazatlán, Avenida Sábalo Cerritos s/n, Mazatlán, Sinaloa 82010, México
[3] Institut de Recercai Tecnologia Agroalimentàries. IRTA Sant Carles de la Ràpita, Tarragona, Spain
[4] Laboratorio de Acuicultura Tropical DACBIOL-UJAT, Carr. Vhsa-Cárdenas Km 0.5, Bosques de Saloya, Villahermosa, Tabasco, México

Introduction

Knowledge of ontogeny of digestive enzyme development during larval stages is of value for establishing appropriate feeding and weaning routines for target aquaculture species (Zambonino-Infante and Cahu, 2001). *T. macdonaldi* is an endemic fish from the Gulf of California, Mexico, and is considered one of the largest members of the Sciaenidae family. This species has been included in the list of endangered species (CITES, 2005) and important efforts have focused on restocking through reproduction in captivity (True et al., 1997); however, larval rearing of this species is still under development with variable results. Therefore, the present study was aimed to provide insight into totoaba's larval digestive physiology by measuring gene expression and enzymatic activity of three major digestive proteases: trypsin, chymotrypsin, and pepsin and relating these data with larval feeding protocols in order to understand the mechanisms triggering enzyme production and feed digestion.

Materials and methods

Fertilized eggs were obtained from captive broodstock held in the marine finfish hatchery of the Facultad de Ciencias Marinas, Universidad Autónoma de Baja California located in the city of Ensenada, in north-western Mexico. The eggs were produced by wild-caught brood fish maintained in two separate groups of ten fish in equal sex ratios. Broodstock were matured using photothermal control and were further induced to tank spawn using GnRHa. Fertile eggs were treated with 100ppm formalin for 30min and stocked at a density of 100 eggs.l^{-1} in

cone-bottom tanks with 24°C seawater recirculated at a rate of 1.5-2 $l.min^{-1}$ through a fluidized bead biofilter and further UV-sterilized. Larvae hatched at ~20h post-fertilization. Yolk-sac larvae were stocked at a density of 30 $ind.l^{-1}$ in ten 100-l experimental tanks. Beginning at 4dph (days post hatch), larvae were fed three times per day (08:00, 12:00, and 18:00h) starting with rotifers (*Brachionus plicatillis*) from 4-20dph, followed by *Artemia* metanauplii (Salt Creek Inc., Salt Lake City, UT, USA) enriched with lipid emulsion (Bio-Marine Algamac 3050™) at a concentration of $0.6g.l^{-1}$. *Artemia* nauplii were supplied at a concentration of 5 nauplii ml^{-1} from 18-34dph. At 30dph live feed was decreased and a combination of enriched *Artemia* metanauplii and formulated diet (Otohime Japanese Marine Weaning Diet, Red Mariculture; protein 52.1%, lipid 16.3%, ash 11.2%, particle size 200-1410µm) was supplied. The weaning period was complete at 34dph, when live food was no longer supplemented and larvae were fed the microdiet from 34-40dph (end of the trial).

Totoaba larvae (*n*=50 to 100 depending on their size) were randomly sampled from the rearing tanks using a 200-µm dip net. Sampling was conducted 1h after feeding to evaluate the production of digestive enzymes and its relation to feeding regime. Larvae were collected daily from hatching to 6dph, then every two days until 20dph and thereafter every fourth day until the end of the study at 40dph. After sampling, larvae were sacrificed by anaesthetic overdose (tricaine methanesulfonate-MS 222), rinsed with distilled water to remove the excess of salts and stored at -70ºC until further use. Additional samples (n=30 larvae) were collected daily for measuring larval size in total length. The quantification of digestive proteases was conducted using spectrophotometric methods and the quantification of gene expression of the selected digestive enzymes was performed according to García-Gasca et al. (2006) and Galaviz et al. (2011).

Results and discussion

Totoaba macdonaldi larvae showed an exponential growth in total length from hatching until the end of the study at 40dph (Fig. 1).

During the present study the expression and activity of pancreatic enzymes trypsin and chymotrypsin were detected at the moment of hatching and increased gradually during larval development. These activities were independent of exogenous food intake since the larvae were not fed before 4dph. Before yolk absorption, most of the trypsin and chymotrypsin were inactive (trypsinogen, chymotrypsinogen), becoming active after mouth opening. The expression and activity of trypsin and chymotrypsin prior to the first feeding suggest that the activity of this enzyme is derived from genetically preprogramed expression and not by the first exogenous feeding (Zambonino-Infante and Cahu, 2001; Galaviz et al., 2011) (Fig. 2).

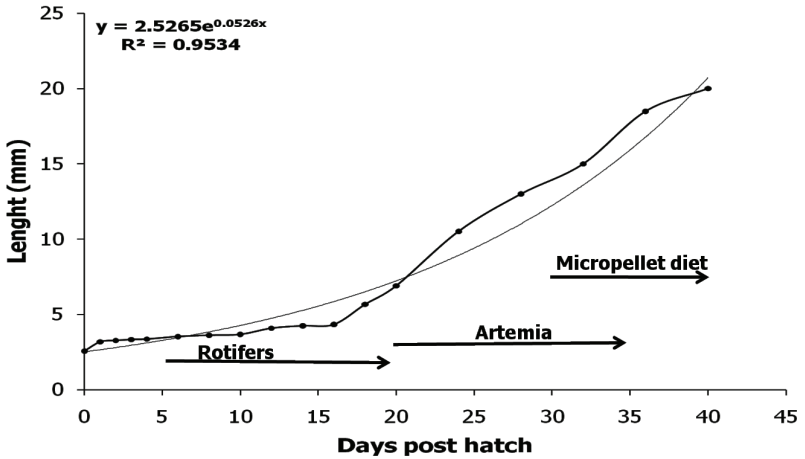


Fig.1. Feeding and growth (length) of *T. macdonaldi* during culture experimental conditions.

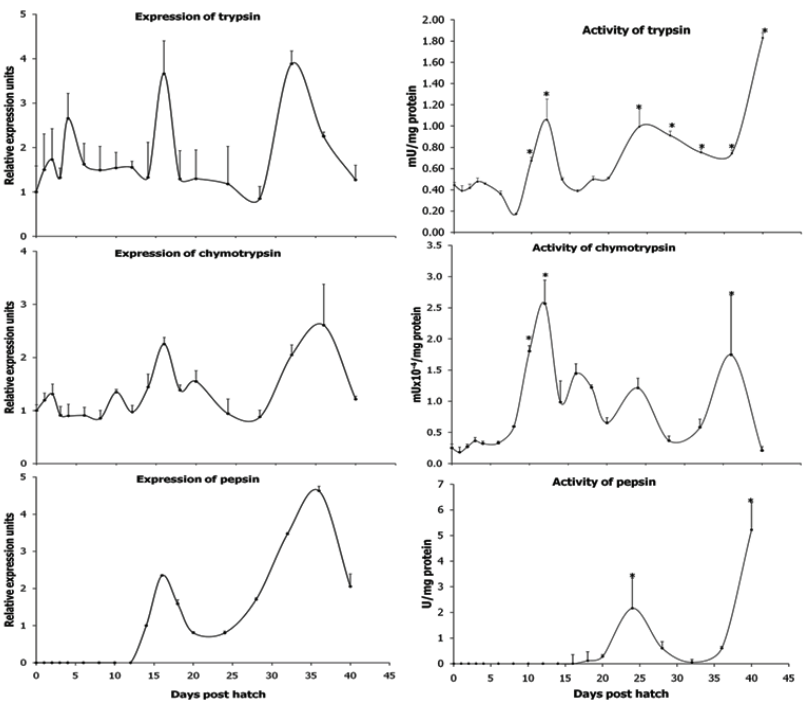


Fig. 2. Expression (relative expression units) and activity (specific activity) of trypsin (top), chymotrypsin (middle), and pepsin (bottom) during larviculture of *T. macdonaldi.*

The presence of gastric glands indicates functional development of the stomach and is considered as the end of the larval period and the onset of the juvenile period in teleosts. Functional development of gastric glands in totoaba larvae has not been studied; however, in the white sea bass larvae, a closely-related scianid-species, gastric glands begin to differentiate at 16dph and the acid protease (pepsin) activity has been detected as early as 10dph (Galaviz et al., 2011). In contrast, totoaba larvae pepsinogen gene expression was identified as early as 16dph and reached maximum expression level at 36dph. Pepsin specific activity in totoaba was detected at low levels between 18 and 20dph, observing a significant increase at 24dph. Therefore, it is likely that totoaba larvae develop a full functional stomach between day 20 and 24, thereby indicating the end of the larval stage of this species (Fig. 2).

In totoaba larvae, the digestive system is fully developed and functional between 20-24dph, as demonstrated by molecular observations and the biochemical quantification of three major proteases, trypsin, chymotrypsin and pepsin. Based on these results, the onset of the weaning period (currently performed between 28-32dph) could be performed earlier, almost certainly between 20- 24dph.

Acknowledgements

This work was supported by (CONACyT) of México (SNI-COMPL-2008-90950) and scholarship number 164557, and internal project by Autonomous University of Baja California (UABC) Mexico. Thanks to R. Hernandez and D. Rodarte for technical assistance. This work was supported by CONACYT and UABC, México.

References

Galaviz M., A. García-Gasca, M. Drawbridge, C.A. Álvarez-González, and L. López. 2011. Ontogeny of the digestive tract and enzymatic activity in white seabass, *Atractoscion nobilis*, larvae. Aquaculture 318:162–168.

García-Gasca A., M. Galaviz, J.N. Gutiérrez, and A. García-Ortega. 2006. Development of the digestive tract, trypsin activity and gene expression in eggs and larvae of the bullseye puffer fish Sphoeroides annulatus. Aquaculture 256:366–376.

True C.D., A. Silva-Loera, and N. Castro-Castro. 1997. Acquisition of Broodstock of *Totoaba macdonaldi*: Field Handling, Decompression, and Prophylaxis of an Endangered Species. The Progressive Fish Culturist 59, 246-248.

Zambonino-Infante J.L. and C.L. Cahu. 2001. Ontogeny of the gastrointestinal tract of marine fish larvae. Comp. Biochem. Physiol. C 130:477–487.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

FIRST FEEDING REGIMES FOR LONG-SNOUT SEAHORSE *HIPPOCAMPUS REIDI* LARVAE

J. García-Manchón[1], J. Socorro-Cruz[2], Á. Segade Botella[2], F. Otero-Ferrer[2], A. Mesa[2], and L. Molina Domínguez[2]

[1] Acuario de Zaragoza. Av. Ranillas, S/N. 50015. Zaragoza (Spain)
[2] Grupo de Investigación en Acuicultura (Instituto Canario de Ciencias Marinas & Universidad de Las Palmas de Gran Canaria) Apdo. 56, 35200 Telde (Las Palmas)

Abstract

Seahorses are endangered species included in Appendix II of CITES from 2002 due to the progressive regression of wild populations. This study focused in *Hippocampus reidi*, one of the species with highest interest in trade, showing an increasing demand in the last decades. This study was conducted during 28 days to compare the effects of different time enrichment (0, 24, and 48h) for *Artemia* using a commercial product (Easy-Selco DHA INVE Aquaculture, Dendermonde, Belgium). Results showed no significant differences in growth between treatments until 21DAB, after which animals fed for 48h one enriched *Artemia* showed significantly better growth. Cumulative average survival during the first 14DAB was significantly higher in 0h treatment, while from 15DAB to the end of the experiment, no significantly differences were observed.

Introduction

Seahorses have been considered endangered species due the progressive regression of wild populations, constituting a typical example of a natural species' mismanagement. For these reasons, all seahorse species have been included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2002) which was implemented in May 2004. Seahorse aquaculture initiatives are a recent phenomenon in different countries attempting to improve their conservation status, and it represents a relatively new industry despite the significant improvements made over the last decade (Koldewey and Martin-Schmidt, 2010).

On the other hand, the presence of seahorses in public aquaria is very common. The popularity of these species is related to its look, attractiveness, and unusual reproductive behaviour. Public aquaria not only provide entertainment and pleasure to visitors, but also increase knowledge of environmental problems.

Syngnathids have the potential to act as powerful icons for sustainable use in public aquaria. Moreover, these institutions can be adopting environmental friendly and sustainable procedures, among them the development of technology for the rearing of ornamental species (Calado et al., 2003).

Feeding and nutrition are some of the initial challenges for seahorse aquaculture, especially for newborns in the first few weeks. Food used as first feeding for cultured seahorses includes wild live copepods or other zooplanktonic animals, as well as rotifers or *Artemia salina,* the most commonly live prey used in aquaculture. These diets provided different degrees of success, in terms of survival and growth, depending on seahorse size and species (Otero–Ferrer et al., 2010). The success of marine larval rearing is greatly influenced by first-feeding regimes and the nutritional quality of starter diets (Izquierdo et al. 2001). Because of these reasons, the nutritional enrichment of live prey organisms is a common practice in aquaculture, including seahorse aquaculture. However, for commercial operations, the cost and effectiveness of the use of commercial enrichment products must be taken into account.

The present study focused on *H. reidi*. The feeding trial was conducted during a period of 28 days using the batches obtained from stocked animals. In this work, the effects on survival rate and growth related on the use of different *Artemia* enrichment time with a commercial enrichment product were studied.

Materials and methods

Immediately after birth, 900 larvae obtained from stocked animals (size ranged from 8-10cm) were randomly selected and equally divided into nine 30-l square glass aquaria. The rearing tanks were supplied with flow-through seawater (salinity, 1025g.l^{-1}; T^a, 25°C; O_2, 7mg.l^{-1}). Light was provided by fluorescent tubes (Sera blue sky, 36W, 12 000°K, Germany) and the photoperiod was 12L:12D.

The larvae were feed on *Artemia* (Specialty Cysts MC 450, Inves, Acuazul, Cádiz, España), using three different treatments: unenriched, or enriched for 24 and 48h (treatments 0, 24, and 48, respectively) with a commercial product Easy DHA Selco® (INVE, Belgium). Artemia was provided twice daily (9:00 and 14:00h) at a rate of 0.5 *Artemia*.ml^{-1}. During the trial, mortality was measured daily. At days 0, 7, 14, 21, and 28 days after birth (DAB), seahorses were measured for height (HT) using a "profile projector" (Mytuyo PJ-A3000, Japan) according with Lourie (2003).

For statistical analysis, normality and homogeneity of the variables were tested using Kolmogorov-Smirnov's and Levene's tests, respectively (Zar, 1996). Comparative studies regarding size were analyzed using one way ANOVA test (Zar, 1996) using Tukey's test for multiple comparisons. Survival was analyzed

by a loglinear test. All the results were processed and analyzed using SPSS Statistical Software System ver. 17.0. (SPSS Chicago, Illinois, 1999).

Results and discussion

From the moment that seahorses were expelled from the paternal pouch, hunting behaviour was observed and can be described as active and voracious. Since first feeding, visual inspection during feeding periods showed a fully orange gut due to *Artemia* ingestion in all the treatments. This live prey is commonly used for seahorse aquaculture; however, it shows a low nutritional content and in some cases is poorly digested by newborn seahorses (Payne and Rippindale, 2000), affecting growth and survival.

In this trial, from 0 to 21DAB samplings, no significantly differences ($P \geq 0.05$) in HT were observed between treatments. At 28DAB, the positive role of the *Artemia* enrichment became evident, showing a significantly better growth ($P \geq 0.001$) when used; no significant differences were observed regarding 24 and 48h enrichment time, however an increased value for 48h enrichment was observed (Table I).

Table I. Larval height (mm, HT, mean ± standard deviation, SD) evolution during the trial for the three treatments.

	0DAB	**7DAB**	**14DAB**	**21DAB**	**28DAB**
Artemia 0h	7.58±0.28	10.78±1.25	14.91±2.07	19.58±2.44	22.72±3.72[a]
Artemia 24h	7.58±0.28	10.41±1.61	14.20±2.88	20.26±3.49	24.55±3.24[ab]
Artemia 48h	7.58±0.28	10.15±1.42	12.99±2.91	22.79±2.06	26.34±2.61[b]

Values in columns with different superscripts are significantly different ($P \geq 0.001$).

Until 14DAB, average survival was significantly lower (13.33±4.02) in seahorses fed on 48h enriched *Artemia* compared with those fed on other treatments ($P \geq 0.001$), while from 15DAB to the end of experiment, no significant differences were observed ($P \geq 0.05$) between the three treatments (Fig. 1).

Similar survival results were observed by Olivotto et al. (2008) for the same species at 21DAB, when enriched *Artemia* nauplii were used as first feeding. In that case, a commercial enrichment product based on algae dried cells was used.

The present results suggest the possibility of using unenriched *Artemia* for *H. reidi* larval feeding during the first two weeks followed by enriched prey. This breeding protocol could be a useful option to decrease production costs in this species.

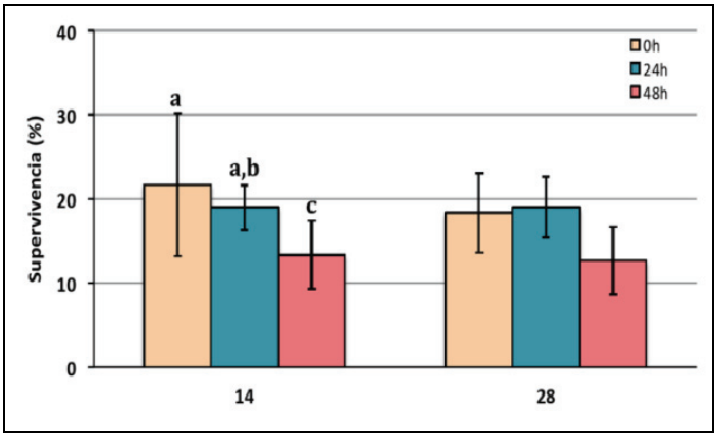


Fig. 1. *H. reidi* survival differences between feeding treatments used for *Artemia* (unenriched, 24 and 48h enrichment time) during the experimental period. Columns showing letters are significantly different (P≥0.001).

References

Koldewey H.J. and K.M. Martin-Smith. 2010. A global review of seahorse aquaculture. Aquaculture 302:131-152.

Calado R., L. Narciso, R. Araújo, and J. Lin. 2003. Overview of marine ornamental Aquaculture. p.219-230. In: Marine Ornamental species: Collection, Culture & Conservation. J.C. Cato and C.L. Brown (Eds.) Iowa State Press, USA, 470 p.

Izquierdo M.S., A. Tandler, M. Salhi, and S. Kolkovsky. 2001. Influence of dietary polar lipids' quantity and quality on ingestion and assimilation of labelled fatty acids by larval gilthead seabream. Aquaculture Nutrition 7:153-160.

Lourie S.A. 2003. Measuring seahorses. Project Seahorse Technical Report No.4, Version 1.0. Project Seahorse, Fisheries Centre, University of British Columbia. 15 pp.

Otero-Ferrer F, L. Molina, J. Socorro, R. Herrera, H. Fernandez-Palacios, and M.S. Izquierdo. 2010. Effects of first feeding on survival and growth of Short-snouted seahorse juveniles, *Hippocampus hippocampus*, (Linnaeus, 1758). Aquaculture research, DOI 10.111/j.1365-2109.2010.02505.

Olivotto I., M.A. Avella , G. Sampaolesi , C.C. Piccinetti, P. Navarro Ruiz, and O. Carnevali. 2008. Breeding and rearing the longsnout seahorse *Hippocampus reidi*: Rearing and feeding studiesI. Aquaculture 283.p 92–96

Payne M.F. and R.J. Rippingale. 2000. Rearing West Australian seahorse, *Hippocampus subelongatus*, juveniles on copepod nauplii and enriched *Artemia*. Aquaculture 188:353-361.

Zar J.H. 1996. Biostatistical analysis, 3rd ed., Upper Saddle River, NJ: Prentice–578 Hall.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

THERMALLY-INDUCED PHENOTYPIC PLASTICITY IN GILTHEAD SEA BREAM

E. Georgakopoulou[1], M. Loizides[1], M. Christou[1], M. Iliopoulou[1], I. Papadakis[2], P. Katharios[2], P. Divanach[2], and G. Koumoundouros[1*]

[1] Biology Department, University of Crete, Vasilika Vouton, 70013 Heraklion, Crete, Greece, tel. +30 2810394065, *E-mail: gkoumound@biology.uoc.gr
[2] Hellenic Centre for Marine Research, 71003 Gournes Pediados, Crete, Greece

Introduction

Water temperature has major effects on fish ontogeny. It affects not only the ontogenetic timing, but it also determines characters like sex, muscle cellularity, skeleton anatomy and body shape (e.g. Fuiman et al., 1998; Koumoundouros et al., 2009; Georga and Koumoundouros, 2010). It is therefore a crucial environmental factor for the function and survival of fish populations in nature, as well as for the quality of the reared fish.

Gilthead sea bream (*Sparus aurata* L.) is an important species for the European aquaculture. However, and despite the great importance of water temperature for the ontogeny of fish, relative literature is scarce for this species (Polo et al., 1991; Georgakopoulou et al., 2010). The present study examined the effects of water temperature during the embryonic and larval phase on the ontogenetic scaling and on the juvenile body-shape of *S. aurata*.

Materials and methods

Eggs of *S. aurata* were randomly distributed to twelve different populations. During the autotrophic and exotrophic phases, three different temperatures (16, 19, and 22°C) were applied either constantly throughout the studied period, or in various combinations with each other (G16, G19, G22, G16–19, G19–22, G22–19, Fig. 1). Egg incubation and larval rearing were performed as it is described in Georgakopoulou et al. (2010).

For the examination of temperature effects on the ontogenetic scaling, a random sample of 50 individuals was taken from each population every 5-7 days. Sampled fish were anaesthetised, fixed in 5% phosphate buffered formalin and stained for bone and cartilage. Standard length (SL) was measured post-staining (from the tip of snout to the base of the middle caudal lepidotrichium). The ef-

fect of temperature on the ontogenetic rate of *S. aurata* was studied by estimating the SL_{50} at which different events of fin development and squamation take place (Koumoundouros et al., 2001).

Geometric morphometrics were used to study the effect of water temperature on the juvenile body-shape (33-37mm SL). At the juvenile stage, a random sample of 20-24 fish per population was x-rayed and subjected to geometric morphometric analysis (Fig. 2) (e.g. Georga and Koumoundouros, 2010). Only individuals without any skeletal deformity were included in the analysis.

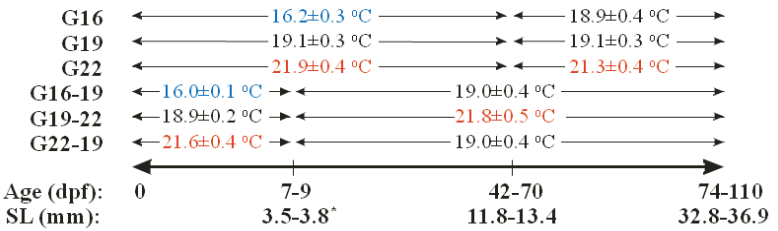


Fig. 1. Thermal treatments of the present study. Modified from Georgakopoulou et al. (2010).

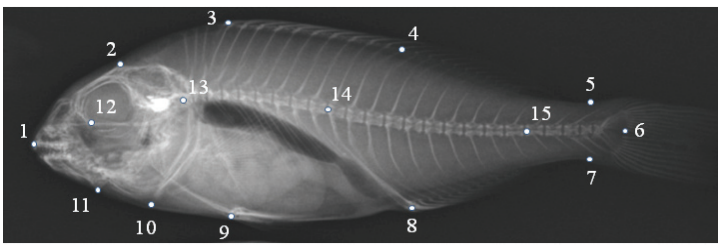


Fig. 2. Landmarks collected for the shape analysis in the x-rayed juveniles.

Results and discussion

The ontogeny of *S. aurata* showed high plasticity with respect to the SL_{50} of all characters studied (Fig. 3). Compared with the G16 regime, elevated water temperature during the embryonic and larval phases (G22) resulted in an 11% average decrease of the SL at which the ontogeny of fins and scales took place. As it was indicated by the distinct and non-overlapping confidence intervals, in most of the characters studied, the differences of the SL_{50} between the G16 and G22 conditions were proven significant (Fig. 3). In accordance to our results, previous studies also show that elevated temperature generally accelerates the differentiation rate somewhat more than the growth rate of fish, leading to smaller larval sizes at certain ontogenetic events (Fuiman et al., 1998; Koumoundouros et al., 2001).

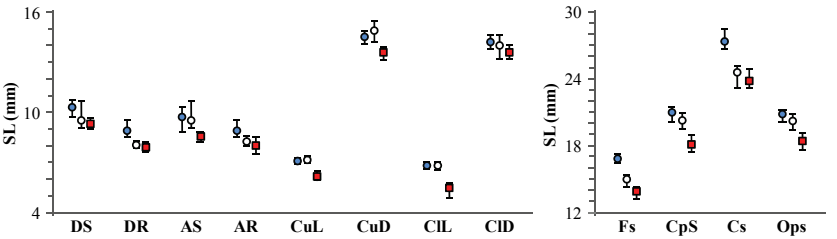


Fig. 3. Timing of selected ontogenetic events with respect to body size (SL, standard length). Closed circles, G16 treatment. Open circles, G19 treatment. Closed squares, G22 treatment. SL_{50} and 95% confidence intervals are given. DS, DR, full complement of the dorsal-fin spines and lepidotrichia respectively. AS, AR, full complement of the anal-fin spines and lepidotrichia respectively. CuL, CuD, full complement of the upper caudal lepidotrichia and dermatotrichia respectively. ClL, ClD, full complement of the lower caudal lepidotrichia and dermatotrichia respectively. Fs, first development of scales on the side of the body. CpS, full squamation of the caudal peduncle. Cs, full squamation of the body with development of scales on the dorsal post-cranial area. Ops, appearance of scales on the operculum.

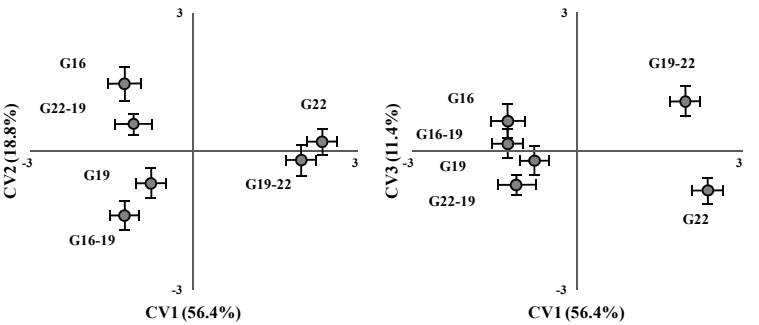


Fig. 4. Effect of water temperature on the scores of canonical variate analysis (CV1, CV2, CV3). Means±2SE of the canonical scores are given. Numbers in brackets equal to the percentage of shape variance explained along each canonical axis.

Geometric morphometric analysis revealed a significant effect of water temperature on the body shape of *S. aurata* juveniles (Wilk's λ=0.0630, $p<0.001$). Shape differences were mainly expressed along the first canonical variable, discriminating G22 and G19-22 groups from the rest of the groups (Fig. 4). Compared with the rest, G22 and G19-22 groups were characterised by an anterior-dorsal transposition of the base of supraoccipital (landmark 2) and of basioccipital bones (landmark 13), a ventral displacement of the upper jaw (landmark 1), a posterior transposition of the dorsal and anal fins (landmarks 3, 4, 8) and a ventral transposition of the pelvic fins (landmark 9) (Fig. 5). As the analysis included only fish with normally developed skeleton, any intermediate effects of skeletal deformities on body shape should be excluded. Future research efforts

should examine whether this variation of juvenile body-shape is maintained during the growth of the fish.

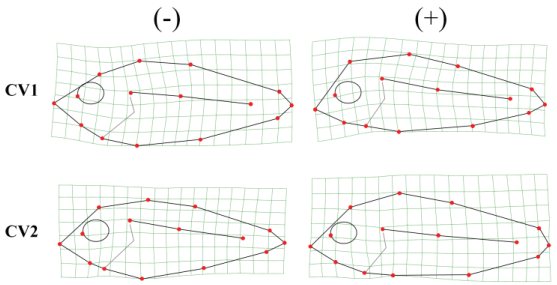


Fig. 5. Spline diagrams demonstrating the components of shape change relative to the extreme values (-, +, X3) of the canonical axes (CV1–CV2). The descriptions of the landmarks are given in Figure 2.

Acknowledgements

This study was partially supported by the program NSRF 2007-2013, «Competitiveness & Entrepreneurship» (call Cooperation I, Project No 09SYN-24-619) of the Ministry of Education, Religious Affairs, Culture and Sports, Greece.

References

Fuiman L.A., K.R. Polling, and D.M. Higgs. 1998. Quantifying developmental progress for comparative studies of larval fish. Copeia 1998(3):602-611.

Georga I., and G. Koumoundouros. 2010. Thermally induced plasticity of body shape in adult zebrafish *Danio rerio* (Hamilton, 1822). Journal of Morphology 271(11):1319–1327.

Georgakopoulou E., P. Katharios, P. Divanach, and G. Koumoundouros. 2010. Effect of temperature on the development of skeletal deformities in Gilthead seabream (*Sparus aurata* Linnaeus, 1758). Aquaculture 308(1-2):13–19.

Koumoundouros G., C. Ashton, D.G. Sfakianakis, P. Divanach, M. Kentouri, N. Anthwal, and N.C. Stickland. 2009. Thermally induced phenotypic plasticity of swimming performance in European sea bass [*Dicentrarchus labrax* (Linnaeus 1758)] juveniles. Journal of Fish Biology 74(6):1309–1322.

Koumoundouros G., P. Divanach, L. Anezaki, and M. Kentouri. 2001. Temperature-induced ontogenetic plasticity in sea bass (*Dicentrarchus labrax*). Marine Biology 139(5):817-830.

Polo A., M. Yufera, and E. Pascual. 1991. Effects of temperature on egg and larval development on *Sparus aurata* L. Aquaculture 92:367–375.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

LARVAL DIET DETERMINES JUVENILE AND ADULT PHENOTYPE IN ZEBRAFISH (*DANIO RERIO*)

A.N. Georgiou[1,2], M. Christou[1], M. Sxoinaraki[1], P. Kokkinias[1], and G. Koumoundouros[1*]

[1] Biology Department, University of Crete, Vasilika Vouton, 70013 Heraklion, Crete, Greece, tel. +30 2810394065, *gkoumound@biology.uoc.gr
[2] Biology Department, University of Patras, 26500 Rio, Greece

Introduction

Nutritional conditions during the larval stage determine to a large extent the growth, survival, and normal development of fish larvae, as well as the phenotype of juveniles (e.g., Carvalho et al., 2006; Kjørsvik et al., 2009; Mazurais et al., 2009; Kaushik et al., 2011). Nutritionally-induced phenotypic variation either results from abnormal ontogenetic deviations (i.e. skeletal deformities) or from normal variations of ontogeny (e.g., Mazurais et al., 2009; Georga et al., 2011). In the present study we examined whether larval diet determines the body shape of juvenile and adult zebrafish (*Danio rerio*).

Materials and methods

Eight larval populations of zebrafish were subjected in tetraplicates to two different nutritional treatments up to 33 days post fertilization (dpf). In the "AR" treatment, larvae were fed on *Artemia* nauplii during the entire experimental period (AF, INVE SA, <33dpf) and then on inert diet (Cichlid Omni Flakes, Ocean Nutricion, >34dpf). In the "C" treatment, larvae were fed on a combination of Paramecium (4-25dpf), *Artemia* nauplii (AF, INVE SA, 16-33dpf), and inert diet (Cichlid Omni Flakes, Ocean Nutrition, >26dpf). Egg incubation and larval rearing were performed at 28°C following the methodology of Georga and Koumoundouros (2010).

To study the effect of larval diet on the juvenile and adult body shapes, geometric morphometric analysis was performed on ~50 juveniles (33dpf old) and approximately 30 female and male fish per replicate (two replicates, 150dpf old). All the sampled individuals were photographed from the left lateral view. On the image of each fish a total of 15 landmarks were scored using tpsDig2 software. Geometric morphometric analysis followed Georga and Koumoundouros (2010).

Only individuals without morpho-anatomical abnormalities were included in the study.

To estimate growth rates, two random samples of 30 and 50 fish were taken from each population at 15dpf and at 33dpf, respectively. The standard length (SL) of each individual was measured on its digital photograph by means of tpsDig2. Furthermore, juvenile samples (33dpf) were stained for bone and cartilage and examined for the presence of skeletal deformities. Survival rates were estimated at 15 and 33dpf, after counting all the individuals.

Results and discussion

Larval diet affected the growth rate of the fish up to 15dpf, with the AR condition presenting significantly higher growth rate ($0.053\pm0.006.d^{-1}$) than the C condition (0.053 ± 0.006 vs. $0.033\pm0.004.d^{-1}$, $p<0.05$, Mann-Whitney U test). No significant differences were found between the growth rates of the two conditions at 33dpf (0.045 ± 0.005 vs $0.033\pm0.006.d^{-1}$). Similarly, no differences were observed in the survival rates between the two regimes (55-85% and 51-78% at 15 and 33dpf, respectively).

Larval diet had a significant effect on the body-shape of *D. rerio* juveniles (Wilk's $\lambda=0.569$, $p<0.001$). Shape differences between the two experimental conditions were mainly expressed along the first canonical axis for the r2 replicate and along the third canonical axis for the r4 replicate (Fig. 1). The second canonical axis expressed mainly differences between the two replicates, probably indicating that more factors except the larval diet affected the shape of juveniles.

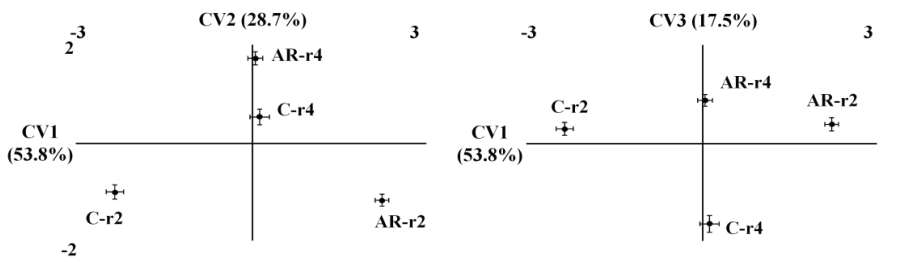


Fig. 1. Distribution of different groups (AR, C) and replicates (r2, r4) along the canonical axes (CV1, CV2, CV3). Numbers in brackets equal to the percentage of shape variance explained along each canonical axis. Error bars equal to 1 SE.

The significant effect of larval diet on the body-shape was evident also in adults, both for females (Wilk's $\lambda=0.412$, $p<0.001$) and males (Wilks-$\lambda=0.847$, $p<0.001$) (Fig. 2). In both sexes, the shape differences between the two regimes mainly concerned the gill cover and the pectoral and pelvic fins, as well as the mouth (Fig. 2). These differences cannot be attributed to differences in the de-

velopment of skeletal deformities between the two regimes, since the latter concerned only lacks of processes of the haemal vertebrae ($p<0.01$, G-test, Fig. 3). Observed shape variation between the different nutritional regimes could be attributed to the direct effect of certain nutrients on the development of skeleton, and/or to the different swimming activity of the larvae when attempting to catch preys of different swimming mode.

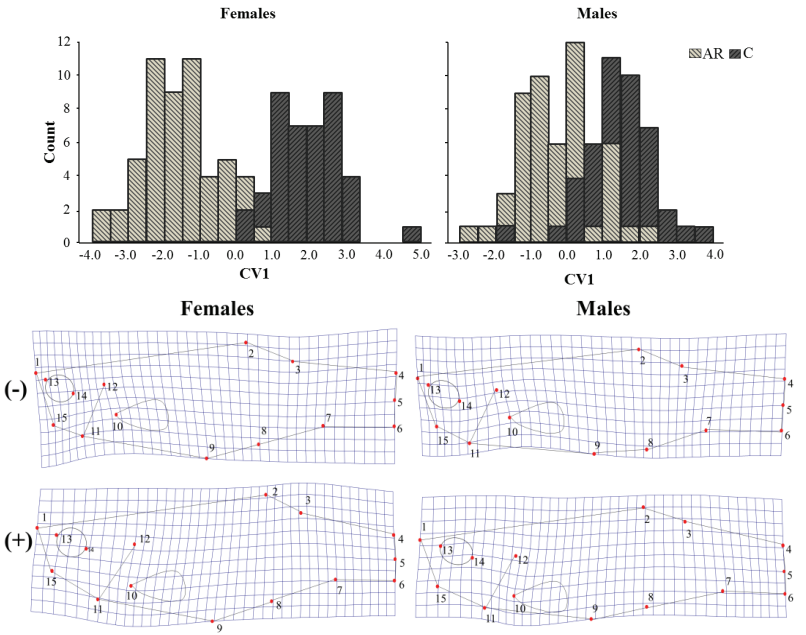


Fig. 2. Top. Distribution of different groups ("AR" Artemia, "C" Control) along the canonical variable (CV1). Bottom. Splines diagrams demonstrating the componets of shape variation relatively to the extreme values (-, +, ×3) of the canonical axis (CV1). Analysis includes 20-27 individuals per sex and replicate.

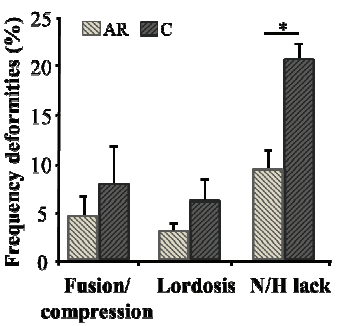


Fig. 3. Effect of larval diet on the development of skeletal deformities. Asterisk indicates significant difference between the two regimes ($p<0.01$, Mann Whitney U-test).

Acknowledgements

This study was funded by the program NSRF 2007-2013, «Competitiveness & Entrepreneurship» (call Cooperation I, Project No 09SYN-24-619) of the Ministry of Education, Religious Affairs, Culture and Sports, Greece. Mrs Georgiou holds a scholarship from European Union (European Social Fund – ESF) and Greek national funds through the Operational Program "Education and Lifelong Learning" of the NSRF (Heracleitus II).

References

Carvalho A.P., L. Arauj, and M.M. Santos. 2006. Rearing zebrafish (*Danio rerio*) larvae without live food: evaluation of a commercial, a practical and a purified starter diet on larval performance. Aquaculture Research 37(11):1107-1111.

Georga I. and G. Koumoundouros. 2010. Thermally induced plasticity of body shape in adult zebrafish *Danio rerio* (Hamilton, 1822). Journal of Morphology 271(11):1319-1327.

Georga I., N. Glynatsi., A. Baltzois, D. Karamanos, D. Mazurais, M.J. Darias, C. Cahu, J. Zambonino-Infante, and G. Koumoundouros. 2011. Effect of vitamin A on the skeletal morphogenesis of European sea bass, *Dicentrarchus labrax* (Linnaeus, 1758). Aquaculture Research 42(5):684-692.

Kaushik S., I. Georga, and G. Koumoundouros. 2011. Growth and body composition of zebrafish (*Danio rerio*) larvae fed a compound feed from first feeding onwards: towards implications on nutrient requirements. Zebrafish, 8(2):87-95.

Kjørsvik E., C. Olsen, P. Wold, K. Hoehne-Reitan, C.L. Cahu, J. Rainuzzo, A.I. Olsen, G. Øie, and Y. Olsen. 2009. Comparison of dietary phospholipids and neutral lipids on skeletal development and fatty acid composition in Atlantic cod (*Gadus morhua*). Aquaculture 294(3-4):246-255.

Mazurais D., N. Glynatsi, M.J. Darias, S. Christodoulopoulou, C.L. Cahu, J.L. Zambonino-Infante, and Koumoundouros G. 2009. Optimal levels of dietary vitamin A for reduced deformity incidence during development of European sea bass larvae (*Dicentrarchus labrax*) depend on malformation type. Aquaculture 294(3-4):262-270.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EXPRESSION OF SKELETAL MYOSIN LIGHT CHAIN 2 IN GILTHEAD SEA BREAM (*SPARUS AURATA*, L.): REGULATION AND CORRELATION TO GROWTH MARKERS

S. Georgiou[1], E. Sarropoulou[2], D.M. Power[3], H. Alami-Durante[4], Z. Mamuris[1], and K.A. Moutou[1]

[1] Department of Biochemistry & Biotechnology, University of Thessaly, Greece
[2] Institute of Marine Biology & Genetics, Hellenic Centre for Marine Research, Greece
[3] Centre of Marine Sciences, CCMar, Portugal, [4]Pôle d'Hydrobiologie, INRA, France

In gilthead sea bream skeletal muscle, two isoforms of myosin light chain 2 (MLC2) are expressed. MLC2A is encoded by three transcripts produced through alternative polyadenylation signal selection. 3' UTR regulation of transcript stability and translation was studied in cell cultures using reporter luciferase constructs. Data indicated that the three transcripts are subject to differential regulation according to 3' UTR length and significant regulatory interactions between transcripts were detected.

Gene organization of the two isoforms and the syntenic relationships between sea bream, zebrafish, fugu, tetraodon, medaka, three-spined stickleback, and Atlantic cod were studied. MLC2A and MLC2B genes contain five and six exons respectively, in all organisms examined. Exon organization is slightly different between species, probably indicating different evolutionary trajectories between the two MLC2 isoforms.

White muscle development and growth processes occur through hyperplastic and hypertrophic events driven by several regulatory and hormonal factors. White muscle cellularity and gene expression profiles were studied in 5 to 60 days post-hatch (dph) sea bream larvae along with the cellularity of developing muscle. The two isoforms of MLC2 marked the different developmental stages with MLC2A being the major isoform expressed throughout early development to metamorphosis. Significant increases in MLC2A expression marked the hyperplastic phase of the developing muscle, accompanied by an increase in myogenin expression. MLC2A transcripts exhibited a distinct expression pattern during development that was highly correlated to the cellularity of the developing muscle. The transition to hypertrophy was marked by a significant increase of MLC2B and collagen Ia expression. Hierarchical gene clustering revealed two gene clusters: cluster A included the muscle structural genes (MLC2A, MLC2B,

collagen 1a) and myostatin while cluster B gathered the regulatory factors (myogenin, IGFI, follistatin, MRF4).

The effect of rearing conditions to MLC2 isoform plasticity was determined in (a) larvae and juvenile sea bream outcoming from the same egg batch and raised under intensive versus mesocosm conditions and (b) juvenile sea bream reared under commercial aquaculture practices subjected to size-grading with sorters of increasing diameter. In the first experiment, larvae raised in mesocosm outperformed in growth and MLC isoform expression those raised under intensive conditions and this was evident even six months after transfer to sea cages. In the second experiment, size heterogeneity increased with increasing age (55-117dph) in all graded groups. The ratio of MLC2A/MLC2B expression was tightly correlated to size (R= -0.707) at all size sortings performed.

Growth hormone treatment effects were investigated in both MLC2 isoforms gene expression and growth hormone receptors I and II gene expression in white muscle and liver. Juvenile gilthead sea bream (37g) received intraperitoneal injections with ovine growth hormone (oGH, 0.1-10μg.g^{-1} body weight) and expression of target genes was measured on 1, 2, 4, and 7 days following injection. Expression levels of MLC2A and MLC2B were significantly elevated in the white muscle of sea bream on days 1 and 7, respectively, following GH administration. The effect was not dose-dependent. On the contrary, GH did not elicit a significant effect on the expression of growth hormone receptors.

Overall, these data support the validity of MLC2 as a molecular marker of growth potential in sea bream. MLC2A isoform marks the hyperplastic activity of early developmental stages to metamorphosis and offers an insight to the growth potential of later stages. MLC2B is the predominant adult isoform in sea bream muscle and a candidate marker of muscle hypertrophy.

This research has been co-financed by the European Union (European Social Fund – ESF) and Greek national funds through the Operational Program "Education and Lifelong Learning" of the National Strategic Reference Framework (NSRF) - Research Funding Program: Heracleitus II. Investing in knowledge society through the European Social Fund.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

GENOMICS IN BIVALVE AQUACULTURE

M. Gerdol[1], G. De Moro[1], V. Torboli[1], C. Manfrin[1], U. Rosani[2], P. Venier[2], and A. Pallavicini[1]

[1] Department of Life Sciences, University of Trieste, Via L. Giorgieri 5, 34127 Trieste (TS), Italy
[2] Department of Biology, University of Padua, Via U. Bassi, 58/B, 35121, Padova (PD), Italy

Introduction

Molecular studies of non-model organisms, including bivalve molluscs, have always been complicated by a substantial lack of genetic and genomic knowledge. Whole-transcriptome gene expression studies, which can solve many biological questions of great interest in the aquaculture field, including the identification of molecular markers of disease and transcriptional signatures of various stress factors, have been based for a long time on cDNA microarrays. Despite its feasibility, this technology was still based on pre-existing genetic information and heavily affected by intrinsic limitations.

This gap is progressively being filled by the availability of new tools, based on the most recent advances in the field of sequencing technologies, such as RNA-seq. In fact, the advent of Next Generation Sequencing (NGS) has quickly led to the sequencing of the oyster genome and of several diverse transcriptomes, including some of great interest for aquaculture management, such as larval developmental stages and adult organisms subject to different environmental stressors.

Deep RNA sequencing also disclosed the sequences of several thousand novel genes in bivalve farmed species, permitting to increase our knowledge of complex molecular pathways, and offering new insights on reproduction, development and the immune response to pathogens and diseases. This information could potentially provide useful information for improving many aspects of broodstock management and disease monitoring.

As a key example, here we present the most recent goals achieved in the genomic study of the Mediterranean mussel *Mytilus galloprovincialis* through the long-lasting collaboration between the Universities of Trieste and Padova.

Materials and methods

Different mussel tissues obtained from *M. galloprovincialis* individuals from the Northern Adriatic Sea subject to different environmental stressors were collected and used for total RNA extraction. RNA-seq libraries were prepared according to the manufacturer's protocols and sequenced using a 2×100 base pairs paired-end sequencing strategy on an Illumina HiSeq2000 platform.

Raw sequencing reads were trimmed for adapter removal and by quality and subsequently de novo assembled using different algorithms (namely Trinity and the CLC Genomics Workbench assembler), in order to obtain a complete overview of the different possible splicing isoforms and to solve the assembly of problematic contigs. Sequence redundancy was reduced with CDHit and resulting contigs were annotated with BLAST2GO and assigned Interpro domains and Gene Ontology terms falling into the 3 main categories, namely Biological Process, Molecular Function and Cellular Component.

Reads obtained from specific samples were separately mapped on the high quality transcriptome assembly, obtaining digital read counts for each single transcript, which were used for gene expression analysis aimed at the analysis of the effects of environmental stressors. Differential expression was concluded with appropriate statistical tests (Kal's Z-test or Baggerly's test) and the observed effects were interpreted based on over- or under-representation of annotations in the subsets of differentially expressed genes with hypergeometric tests.

The de novo assembly was mined looking for transcripts of interest possibly involved in the innate immune response to pathogens by bioinformatics methods based on Hidden Markov Models profile analysis, combined presence of annotated functional domains, specific sequence patterns or predefined chemo-physical properties.

Results and discussion

The de novo assembly of *M. galloprovincialis* can rely on the existence of about 300 million Illumina sequencing reads, in addition to several rounds of 454 Life Sciences RNA-seq and the pre-existing data included in Mytibase. Overall, this impressive amount of sequence data led to the assembly of about 110K contigs, a remarkable transcriptomic resource which massively improved our genetic knowledge of this organism with a fundamental importance both as a commodity and as a tool for biomonitoring.

As an example of the potential of RNA-seq for gene expression studies we present here the results of s study performed to evaluate the consequences of paralytic shellfish toxin accumulation (produced by *Alexandrium minutum*) in the digestive gland, and to identify potential molecular markers of contamination.

RNA-seq permitted the simultaneous monitoring of over 20K transcripts over 5 days, revealing that PSP only had a little effect on mussels, which are generally classified as organisms refractory to this class of toxins and which, for this reason, don't modify their feeding behavior during harmful algal blooms, thus accumulating toxins to concentrations which are harmful to human. Nevertheless, the identification of a limited number of candidate responsive transcripts (28), despite not clarifying the molecular mechanisms linked to toxin accumulation, resistance and biomodification, is still appealing in the light of the necessity to identify alternative, efficient and fast biomonitoring tools based on molecular markers. Further molecular studies will be needed in order to clarify the involvement in paralytic shellfish poisoning of several of these transcripts, which don't show any similarity with other known sequences (Gerdol et al., 2013).

Concerning the identification of molecules which may be involved in response to pathogens, we first described the astonishing C1q gene family expansion event which occurred in bivalves, leading to the generation of more than 300 different lectin-like genes in these marine organisms (Gerdol et al., 2010). The role of these proteins and of other abundant lectin-like molecules (C-type lectins, FREPs, etc.) in the recognition of pathogens still remain to be fully understood. More recently, we contributed to the identification of 23 Toll-like receptors, and provided the first lines of evidence about the existence of definite Toll signaling pathway in bivalves leading to the transcriptional activation of antimicrobial peptides (AMP) and possibly other genes involved in innate immunity (Toubiana et al., 2013).

Another main topic of our research is represented by the antimicrobial peptides, which are extremely abundant and diversified in mussels. Thanks to our bioinformatics approach, we identified two novel families (big defensins and mytimacins; Gerdol et al., 2012) and another cysteine-rich family sharing a remarkable similarity to several toxins found in cone snails, spiders and scorpions, which in mussel may be involved in the response to eukaryotic parasites.

Despite the great advances in bivalve genomics accomplished over the past few years, our knowledge of most molecular pathways involved in the response to environmental stress and pathogens still remain to be completely unveiled. Yet, the potential of these new technologies and the contribution they have already given to bivalve genomics is undeniable and certainly new resources will be allocated in the near future to these studies due to the potential application in aquaculture management.

References

Gerdol M., C. Manfrin, G. De Moro, A. Figueras, B. Novoa, P. Venier, and A. Pallavicini. 2011. The C1q domain containing proteins of the Mediterranean

mussel *Mytilus galloprovincialis*: A widespread and diverse family of immune-related molecules. Developmental & Comparative Immunology 35:635-643.

Gerdol M., G. De Moro, C. Manfrin, P. Venier, and A. Pallavicini. 2012. Big defensins and mytimacins, new AMP families of the Mediterranean mussel *Mytilus galloprovincialis*. Developmental & Comparative Immunology 36:390-399.

Gerdol M., G. De Moro, C. Manfrin, A. Milandri, E. Riccardi, A. Beran, P. Venier, and A. Pallavicini. 2013. RNA-seq and de novo digestive gland transcriptome assembly of the mussel *Mytilus galloprovincialis* provide insights on mussel response to paralytic shellfish poisoning. Submitted.

Toubiana M., M. Gerdol, U. Rosani, A. Pallavicini, P. Venier, and P. Roch. 2013. Toll-like receptors and MyD88 adaptors in *Mytilus*: Complete cds and gene expression levels. Developmental & Comparative Immunology 40:158-166.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EARLY-FEEDING EXPOSURE TO A PLANT-BASED DIET IMPROVES ITS FUTURE ACCEPTANCE AND UTILIZATION IN RAINBOW TROUT

I. Geurden[1], P. Borchert[2], M. Balasubramanian[1], J. Schrama[2], M. Dupont-Nivet[3], E. Quillet[3], S. Kaushik[1], P. Panserat[1], and F. Médale[1]

[1] INRA, UR1067 NUMEA Nutrition, Métabolisme et Aquaculture, Pôle d'Hydrobiologie INRA, 64310 Saint Pée-sur-Nivelle, France
[2] Aquaculture and Fisheries Group, Wageningen Institute of Animal Sciences (WIAS), Wageningen University, P.O. Box 338, 6700 AH Wageningen, The Netherlands
[3] INRA, UMR1313 GABI Génétique Animale et Biologie Intégrative, 78350 Jouy-en-Josas, France

Introduction

In mammals it is now widely accepted that early nutritional events may permanently alter the organism's physiology and metabolism in preparation for their predicted environment. If the prediction is correct, then the metabolism will be matched to the future nutritional environment, increasing its evolutionary fitness (Lucas et al., 1998; Gluckman and Hanson, 2008). In fish, only few studies explored the concept of early nutritional programming. These showed that early exposure to dietary factors such as high carbohydrate content (Geurden et al., 2007) or changes in fatty acid profile (Vagner et al., 2007) induced some persistent metabolic adaptations, at least at a molecular level. The present study explores the potential to improve, via early feed exposure, the poor growth response frequently seen in rainbow trout fed high levels of plant ingredients.

Materials and methods

Rainbow trout (*Oncorhynchus mykiss*) swim-up fry were fed for the first 3 weeks of exogenous feeding (see Fig. 1) either (i) with a plant-based diet (diet V) containing a blend of plant oils (palm, linseed, rapeseed) and plant-protein sources (wheat gluten, corn gluten, soybean meal, dehulled peas, white lupin) or (ii) with an isoproteic diet containing fishmeal and fish oil as sole protein and fat source (diet M). After a 7-month common rearing period on the control diet M, both groups (M- and V-fish) were challenged to feed the plant-based diet V (Fig. 1) during which voluntary feed intake (FI), growth, and nutrient utilization were monitored. We used three (isogenic) families of rainbow trout in order to test whether the potential early nutritional history effect is family-dependent.

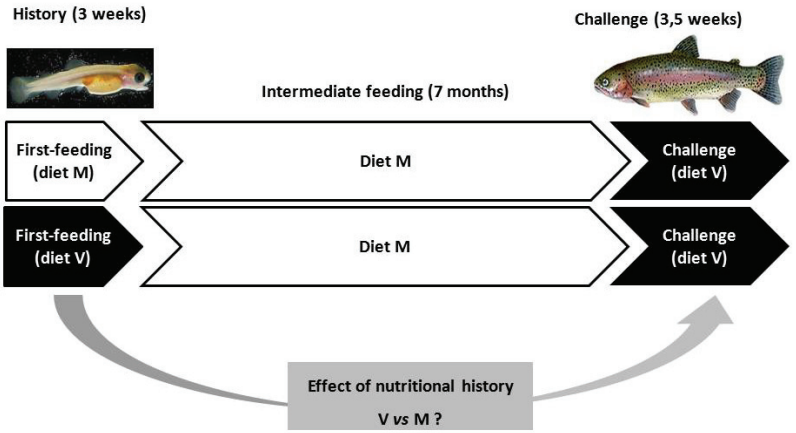


Fig. 1. Experimental feeding design

Results

The body weight (BW) of the trout at the end of the 3-wk first-feeding period was significantly affected by the diet (M-fish>V-fish). The body weight at the end of 7-month common M-rearing (start V-challenge, ~30-40g, data not shown) was not anymore affected by the nutritional history ($P>0.05$), but showed a significant family effect (F1<F2<F3).

The results of the V-challenge (averaged over the three families) show a 42% higher growth rate in trout juveniles of nutritional history V compared to M (Fig. 2A). FI drastically dropped in all groups challenged to eat diet V (data not shown). Key to the present study is the finding that voluntary FI was higher in V- compared to that in M-trout which had never been confronted before with diet V. This effect was more pronounced at the start of the challenge. Cumulated over the V-challenge and depending on the family, V-exposed fish consumed 20 to 70% more than M-fish (Fig. 2B). Besides the effects on FI, V-fish also utilized diet V more efficiently than M-fish, as reflected by the on average 18% higher feed efficiency (Fig. 2C). This was associated with better retention efficiencies for both lipid and protein being, averaged over the three families, respectively 36 and 11% higher in V- compared to M-fish. The positive effect of early V-history on later performances was consistent for all three families (no nutritional history x family interaction, $P>0.05$).

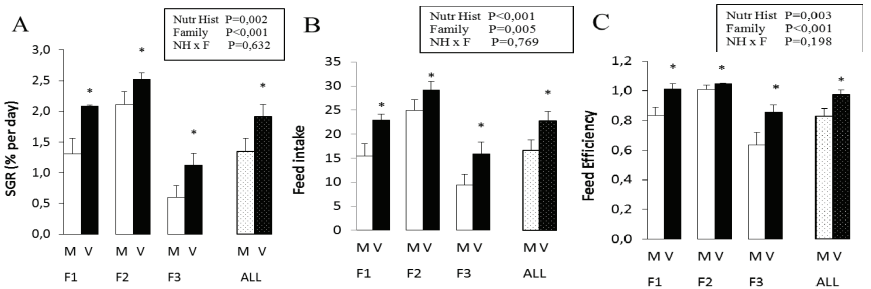


Fig. 2. A. Specific growth rate (SGR, %BW d^{-1}), B. Feed intake (mg $kgBW^{-0.8}$ d^{-1}) and C. Feed efficiency (BW gain per dry matter intake) of the trout during the 25-day V-challenge according to early nutritional history (M or V) and family (F1, F2, F3). Values are means ±SE (n=4). ALL represent the effect of nutritional history (M or V) averaged over the three families. The significance of the main effects and interaction (2-way ANOVA) is added in the figure, * indicates a significant effect of nutritional history (V>M, $p<0.05$).

Discussion

The positive nutritional V-history effect on FI implies that the V-fish were able to 'recognize' diet V, and this almost 7 months after the early exposure. In mammals, early flavor experiences are important in establishing life-long food flavor acceptances (Beauchamp and Mennella, 2009). Knowledge on the development of flavor preferences in fish is scarce. Of specific interest, although not directly related with the development of later feed flavor acceptance, is the susceptibility of the salmonid olfactory system to imprinting, helping adult salmon to find their way back to the natal streams. L-amino acids, derived from a variety of living organisms in and near streams, have been suggested to function as guiding substances for salmonids homing (Shoji et al., 2003). In our study, specific compounds released in the water from diet V during the trout's early-life exposure perhaps provoked an olfactory imprinting responsible for the reduced neophobia and higher FI later in life.

Besides FI, also feed utilization efficiency was higher in V- relative to M-fish. Although actual FI was not monitored during the early feed exposure, it is likely that V-fry at early feeding consumed less than M-fry. In mammals, early-life exposure to a nutrient-limited environment has been reported program obesity and hyperphagia later in life (Gluckman and Hanson, 2008). We therefore conceived the possibility that a general early 'malnutrition' effect, resulting in over-compensation, might explain the superior FI and/or feed utilization of the V-fish during the V-challenge. However, no such effects were seen with diet M during the 7-month intermediate rearing. This finding points toward a directed diet V response in the juvenile V-fish rather than to an overall compensatory sign of malnutrition.

In summary, our study shows that an early short term exposure of trout fry to a plant based diet improves its acceptance and utilization when given later in life. Further studies need to evaluate the persistency of the observed effects and to determine the mechanisms which mediate the positive effects set by the early life diet V exposure.

References

Beauchamp G.K. and J.A. Mennella. 2009. Early flavor learning and its impact on later feeding behavior. Journal of Pediatric Gastroenterology and Nutrition 48, S25–S30.

Geurden I., M. Aramendi, J. Zambonino-Infante, and S. Panserat. 2007. Early feeding of carnivorous rainbow trout (*Oncorhynchus mykiss*) with a hyperglucidic diet during a short period: effect on dietary glucose utilization in juveniles. American Journal of Physiology 292:R2275–R2283.

Gluckman P.D., and M.A. Hanson. 2008. Developmental and epigenetic pathways to obesity: an evolutionary-developmental perspective. International Journal of Obesity 32:S62–S71.

Lucas A., 1998. Programming by Early Nutrition: An Experimental Approach. Journal of Nutrition 128:401S–406S.

Shoji T., Y. Yamamoto, D. Nishikawa, K. Kurihara, and H. Ueda. 2003. Amino acids in stream water are essential for salmon homing migration. Fish Physiology and Biochemistry 28:249–251.

Vagner M., J.L. Zambonino Infante, J.H. Robin, and J. Person-Le Ruyet. 2007. Is it possible to influence European sea bass (*Dicentrarchus labrax*) juvenile metabolism by a nutritional conditioning during larval stage? Aquaculture 267:165–174.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

SURFACE AREA ESTIMATION OF THE GUT SEGMENTS OF *ARTEMIA FRANCISCANA* NAUPLII FED WITH MNN9 YEAST VS. WILD TYPE YEAST

R.A.Y.S.A. Gunasekara[1*], C. Casteleyn[2], P. Bossier[3], and W. Van den Broeck[1]

[1] Department of Morphology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820, Merelbeke, Belgium (former institution) *Email: Rangalle.Gunasekara@UGent.be
[2] Department of Applied Veterinary Morphology, University of Antwerp, Campus Drie Eiken, D.U.015, Universiteitsplein 1, 2610 Wilrijk
[3] Laboratory of Aquaculture and Artemia Reference Center, Ghent University, Rozier 44, 9000 Ghent, Belgium

Introduction

The brine shrimp, *Artemia*, is one of the most important live feeds for commercial production of fish and shellfish larvae (Sorgeloos et al., 1986). In the present study, surface areas of the gut segments of *Artemia* fed with mnn9 and wild type yeast were estimated using stereological tools.

Materials and methods

Artemia cysts were decapsulated and cultured axenically for six days and fed with mnn9 and wild type yeast together with dead *Aeromonas hydrophila* LVS3. Live nauplii were sampled on day 2, day 4 and 6. They were fixed in a fixative containing 80ml of 100% ethyl alcohol, 15ml of 40% formaldehyde and 5ml of acetic acid for 5min, dehydrated in a graded series of alcohols and embedded in paraffin wax. 5-µm thick sections of 6 nauplii per group were stained with Haematoxylin/Eosin for stereological examination.

Lengths of the mid- (MGL) and hindgut (HGL) of each nauplius were calculated on histological sections by multiplying the total number of sections (*n*) in which the mid- or hindgut were present with the thickness of the sections (*t*).

The volumes of the mid- (MGV) and hindguts (HGV) of 6 nauplii per treatment were estimated based on histological sections by applying the Cavalieri method as performed by Casteleyn et al. (2007). Ten sections of each mid- and hindgut of serially sectioned specimen, separated by a fixed interval *(T)*, were selected randomly by randomizing the position of the first section within the interval T.

A point grid with a known fixed area associated with each point (*a/p*) was uniform randomly placed on the histological section of interest using the software Cell*F (Olympus Belgium) (Fig. 1a). The gut epithelium together with its brush border, when present and the underlying muscle layer were taken into account. The grid points (*p*) which hit these tissues were counted and the total area of the tissues per section (*Ai*: area of the *i*th section) were calculated by multiplying the area per point (*a/p*) by the total number of points counted per section (*Pi*: number of points hitting the tissues of the digestive tract on the *i*th section).

$$A_i = \frac{a}{p} \cdot P_i$$

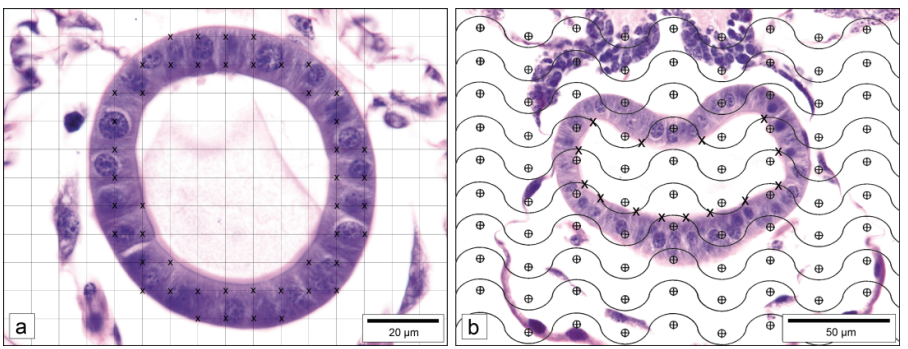


Fig. 1. Transverse histological section through the midgut of an *Artemia* nauplius showing the, points of the grid hitting the tissue of interest that are counted (crosses) (a). The squares represent the area associated to each grid point. Intersections of the cycloidal grid (wavy line) in the tissue of interest that are counted (crosses) (b).

Then, the total volume (*V*) of the digestive tract of the examined nauplius was calculated by multiplying the sum of all areas by the section interval (*T*).

$$V = T \cdot \sum_{i=1}^{m} A_i$$

The precision of the volume estimation of each mid- and hindgut was obtained by calculating the coefficient of error (CE) for the Cavalieri method developed by Gundersen and Jensen (1987). The lower the value, the more precise the volume estimation is. To estimate CE, three sums (a, b, and c) were first calculated:

$$a = \sum_{i=1}^{m} A_i \cdot A_i \qquad b = \sum_{i=1}^{m-1} A_i \cdot A_{i+1} \qquad c = \sum_{i=1}^{m-2} A_i \cdot A_{i+2}$$

Using a, b, and c, it is possible to calculate the CE, as shown in the following equation.

$$CE(V) = \frac{1}{\sum_{i=1}^{m} A_i} \cdot \sqrt{\left[\frac{1}{12}(3a + c - 4b)\right]}$$

The surface area of the mid- (MGV) and hindguts (HGV) of 6 nauplii per treatment were estimated based on histological sections by applying the Cavalieri method. Ten sections of each mid- and hindgut of a serially sectioned specimen, separated by a fixed interval, were selected randomly by randomizing the position of the first section. An appropriate cycloidal grid was superimposed on the histological sections and intersections of the epithelial surface with the cycloidal grid were counted (Fig. 1b).

The surface density was calculated as follows.

$$S_V(Y, ref) = \frac{2 \cdot \sum_{i=1}^{n} I_i}{l/p \cdot \sum_{i=1}^{n} P_i}$$

Where *1/p* is the length of the cycloid associated with each grid point (inherent to the grid used), I_i is the number of intersections of the cycloidal grid and the epithelial surface, and P_i is the number of points hitting the reference space (Howard and Reed, 1998), which in this case was the midgut or hindgut wall (Van Ginneken et al., 2002). The surface area $S(Y)$ was calculated by multiplying the volume with the surface density. $S(Y) = V(ref) \cdot Sv(Y, ref)$

The CE which predicts the precisions of the measurements was calculated using the formula described by Howard and Reed (1998).

$$CE(S) = \sqrt{\frac{k}{k-1}\left(\frac{\sum I^2}{\sum I \cdot \sum I} + \frac{\sum P^2}{\sum P \cdot \sum P} - 2 \cdot \frac{\sum I \cdot P}{\sum I \cdot \sum P}\right)}$$

k is the number of sections examined per mid/ hindgut.

Results and discussion

The ratio between the midgut surface area and the individual length were significantly higher at days 2, 4, and 6 in the group that was fed mnn9 yeast

whereas the ratios between the hindgut surface area and the individual length were not significantly different on days 4 and 6 (Table 1). Further studies are needed to confirm the results and to find explanations for these observations.

Table I. Ratio between the surface area and individual length (μm^2:µm) (n=4-6) of gnotobiotic brine shrimp nauplii fed daily with dead LVS3 bacteria and either wild type yeast (WT) or mnn9 yeast (mnn9).

	Surface area.Individual length^{-1} (µm)	
	WT	**mnn9**
	MGS:IL	**MGS:IL**
Day 2	176.9±37.2[a]	176.9±37.2[b]
Day 4	395.9±3.9[a]	1015.5±29.7[b]
Day 6	465.5±56.1[a]	1641.9±55.6[b]
	HGS:IL	**HGS:IL**
Day 2	20.9±3.4[a]	176.9±37.2[b]
Day 4	25.5±1.9[a]	41.1±6.8[a]
Day 6	26.2±12.2[a]	82.7±25.9[a]

Conclusions

The mutant yeast strain, mnn9, sustains increased midgut surface area under the described culture conditions.

Acknowledgements

This research is financed by the BOF, Ghent University (grant number B/ 07289/02; 05B01906). The authors acknowledge the technical assistance of L. De Bels and B. Depauw.

References

Casteleyn C., W. Van den Broeck, and P. Simoens .2007. Histological characteristics and stereological volume assessment of the ovine tonsils. Veterinary Immunology. Immunopathology 120:124–135

Gundersen H.J.G. and E.B. Jensen .1987. The efficiency of systematic sampling in stereology and its prediction. Journal of Microscopy 147:229–263

Howard C.V. and M.G. Reed. 1998. Unbiased stereology. Springer – Verlag New York Inc. and BIOS Scientific Publishers Limited, New York, p. 246.

Sorgeloos P., P. Lavens, P. Lèger, W. Tackaert, and D. Versichele .1986. Manual for the culture and use of brine shrimp *Artemia* in aquaculture, p. 318. *Artemia* Reference Center, Faculty of Agriculture, State University of Ghent, Ghent, Belgium

Van Ginneken, C., F. Van Meir, S. Sys, and A. Weyns .2002. Stereologic characteristics of pig small intestine during normal development. Digestive Diseases and Sciences 47:868–878

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

DOMESTICATION OF MAHSEER (*TOR SORO*) IN INDONESIA

R. Gustiano[1], E.K. Kontara[2], H. Wahyuningsih[3,4], J. Subagja[1], S. Asih[1], and A. Saputra[1]

[1] Research and Development Institute for Freshwater Aquaculture, Jl. sempur No. 1, Bogor 16154, Indonesia.
[2] Central Research Institute for Aquaculture, Jl. Raya Pasar Minggu, Jakarta 12540, Indonesia
[3] Post Graduate School, Bogor Agricultural University, Darmaga Campus, Bogor 16680, Indonesia
[4] Department Biology, Faculty of Science, University of North Sumatera, Jl. Bioteknologi No. 1, Medan 20155, Indonesia

Abstract

Mahseer is an economically important fish in the world with a distribution from the Himalayan Mountains up to Southeast Asia. One species of Masheer is *Tor soro* in the Indonesian Archipelago. For a long time, *T. soro* has been collected from the wild and now is becoming rare. In addition, the degradation of its habitat is another problem. Hence, it is urgent that this species is saved from extinction. Domestication of *T. soro* from North Sumatra has been conducted since 1996. This paper reports the results of domestication of *T. soro* starting from collecting live specimens, genetic and morphometric characterizations, evaluation to breeding, and growing out. In 2010, domestication was successful to produce a second generation of young broodstocks and now the breeding technology is being shared with several provinces where *T. soro* exist.

Introduction

There are 24 species of Mahseer reported in the world. Mahseer has a distribution from the Himalayan Mountains to Southeast Asia. One species of Masheer is *T. soro*, an economically important species in Indonesia. For a long time, *T. soro* has been collected from the wild, but now is becoming rare. The degradation of its habitat is also another problem reducing the population rapidly due to the loss of breeding and nursery grounds. Therefore, it is very urgent to save this species from extinction. Efforts to domesticate *T. soro* have been conducted since 1996 in North Sumatra in order to save this species. This paper reports the domestication of *T. soro* collected from several rivers in North Sumatra for culture in Indonesia. The domestication process started from collecting live speci-

mens, genetic and morphometric characterizations, evaluation up to breeding and growout. Breeding activities including biological aspects such as gonad maturation, hormonal implant, induce breeding and natural spawning, and larval rearing were observed. For growout, development was followed to maturity.

Materials and methods

Total of 445 live specimens of North Sumatera sized 500-800g were collected from Samosir, Ambarita, Tarutung, Aek Sirambe, Asahan, and Bahorok in North Sumatera. Identification of *T. soro* was done following key provided by Kottelat et al. (1993). Genetic variabilities were examined by Random Amplified Polymorphism DNA (RAPD). Primers used for analysis were OPC-1 and OPC-2. At the Research Installation for Germ Plasm in Bogor, fish were reared in a 72-m^2 concrete pond equiped with sand and stone at the bottom. The pond has a turbidity less than 30JTU and water flow ~300-450 $l.m^{-}1$. Fish were fed 1.5-2.0% body weight per day with commercial feed containing 24-27% protein given three times daily. Parameters observed included oocyte diameter and blood chemistry carried out every month during a year. The effect of Human Chorionic Gonadotropin (HCG) implantation on gonadal development, final maturation, and spawning was conducted. Effect of pregnant mare serum gonadotropin (PMSG) and estradiol-17β (E_2) on gonadal development in immature females was also observed. In artificial fertilization, priming injection on female using 500$IU.kg^{-1}$ HCG was done. After 24 hours, the second injection of 0.6$ml.kg^{-1}$ ovaprim hormone was applied for ovulation. The second injection was carried out two times within a 6-h interval, with the dosage divided into 40% the first time and another 60% the second time. The male was injected using 0.3$ml.kg^{-1}$ ovaprim hormone at the second female injection time. Artificial fertilization followed Woynarovich (1980).

Results and discussion

The genus *Tor* is identified with several characteristics as follow (Kottelat et al., 1993): upper lip more or less covered by a pendulous rostral fold; abdomen in front of pelvic origin rounded or flat, not compressed into a sharp keel if a keel present (it is restricted to behind the pelvis); no symphysal knob on lower jaw; 5-8½ branched anal rays; no such spine; lateral line absent, incomplete, or ending at the middle of the caudal base; head pores isolated, not forming dense parallel rows; 5-6½ branched anal rays; mouth subterminal; last simple anal ray either weak or bony but posterior edge not serrated; 18-30½ branched dorsal rays; lips fringed. Key identification for *T. soro* are anal rays less high than dorsal rays and no median lobe on lower lip. The genetic analysis showed that only OPC-01 primer produced polymorphism DNA. Heterozygosity (0.008-0.1250) and percentage of polymorphic locus value (22-33%) indicated that genetic variation of *T. soro* from North Sumatera was low. RAPD analysis showed that

no significant difference among population was observed. Nugroho et al. (2006) reported similar result that two of 20 RAPD primers worked. However, there was a significant difference among the population in West Java. It seems that the population of *T. soro* from North Sumatera was more homogeneous since they come from the same river or branches of the same river.

T. soro were acclimatised to the pond environment at the research installation. Twenty young broodstock were then used for the founder of domesticated population. Natural spawn could produce 1-2cm fry. The fry was transferred into aquaria for rearing up to 5cm. Afterward, the fry would be transferred into a pond for growing out. After 18 months, the fry could reach sizes varied from 400-700g as young broodstock. Male mature at size 300g while female mature above 700g. Data showed that *T. soro* is a partial spawning species indicated by various size of oocyte and oogonia. Within a year, the peaks of oocyte diameter above average size were in July and in November. Two months afterward the oocyte diameter decreased significantly due to atresia. The results obtained in this study confirm the finding reported by Wahyuningsih (2012) in which two peaks of ovarian maturity took place in June 2009 and September 2009. The hormonal analysis showed that the estradiol-17β concentration was high in July 2009 ($0.9\pm0.80 ng.ml^{-1}$), then decreased significantly in August 2009 ($0.2\pm0.16 ng.ml^{-1}$), and increased until achieving the highest concentration in March 2010. The highest estradiol-17β concentration corresponds to the peak of vitellogenesis towards maturation. Blood plasma chemistry was low in June 2009 as follows: total protein $3.99\pm0.4 g.dl^{-1}$, cholesterol $0.13\pm0.4 g.dl^{-1}$, tryglis-eride 0.1 ± 0.0 $g.dl^{-1}$ and occured at the time of maximum size oocyte development. The concentration of low glucose was in September 2009 ($0.04\pm0.0 g.dl^{-1}$) when the fish ovulated, then this condition increased gradually up to a maximum in February 2010 ($0.12 \pm0.0 g.dl^{-1}$).

Hormonal treatment using HCG showed a significant difference among treatment observed at days 25 and 50 after implantation. A dosage of $500IU.kg^{-1}$ body weight produced the biggest oocyte with average diameter 3.07 ± 0.31mm, and followed by control, 300IU and 400IU with average diameter 2.11 ± 0.53mm, 1.97 ± 0.55mm and 0.87 ± 0.50mm, respectively. Successful spawning rate of *T. soro* treated with $500IU.kg^{-1}$ body weight hormone pellet reached 100% at latency time of 25-27h, incubation temperature of 22-24°C, and 90.19% normal larvae development. Hormonal study of the combination effect of PMSG and E_2 on gonadal development in immature female showed that in all treatments, vitellogenesis occurred. Some treatments were two months faster than others. The gonadal development was significantly influenced by PMSG induction (4IU PMSG) compared to a combination of PMSG and E_2. These results confirm the study reported by Subagja and Gustiano (2009). Both studies showed HCG and PMSG triggered gonad development outside the natural spawning seasons. Finally, SDS PAGE result showed that the molecular weight of the vitellogenin in

T. soro was 153kDa. There was a strong correlation observed with low total protein, cholesterol, trygeliserides, and the high glucose concentration with the spawning activity of the fish observed. For artificial spawning, female ovulated ten to fourteen hours after the second injection at a water temperature of 21-25°C. Since the first week, larvae were fed with *Artemia* nauplii, then continued with tubifex worms.

References

Asih S., E. Nugroho, A.H. Kristanto, and Mulyasari. 2008. Determination of genetic variation of *Tor soro* from North Sumatera and West Java using random amplified polymorphism DNA (RAPD) methods. Jurnal Riset Akuakultur 3:91-97.

Kottelat M., A.J. Whitten, S.N. Kartikasari, and S. Wirjoadmodjo. 1993. Freshwater fishes of western Indonesia and Sulawesi. Periplus Edition Ltd, Jakarta, Indonesia. 293 p.

Nugroho E., J. Subagja., S. Asih, and T. Kurniasih. 2006. Genetic divergence of *Tor soro* analyzed by mitochondri D-loop and random amplified polymorphism DNA (RAPD). Jurnal Riset Akuakultur 1:211-217.

Subagja J and R. Gustiano. 2006. The effect of HCG implantation on the eggs development, final maturation, and spawning rate of *Tor soro*. Jurnal Riset Akuakultur 1:219-225.

Wahyuningsih H. 2012. Artificial induction on gonadal development of *Tor soro*. Disertation. Post Graduate School, Bogor Agricultural University, Indonesia. 94 p.

Woynarovich E and L. Horvath. 1980. The artificial propagation of warm-water finfishes-a manual for extension. FAO Fish. Tech. Pap. 201: 201p.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

CONTENT OF ESSENTIAL FATTY ACIDS IN CULTIVATED *ACARTIA TONSA* NAUPLII FED A DHA-DEFICIENT *TETRASELMIS* SP. CONCENTRATE

A. Hagemann[1], G. Øie, Y. Attramadal[1], R. Veiseth[2], and J.O. Evjemo[1]

[1] SINTEF Fisheries & Aquaculture AS, SINTEF Sealab, Brattørkaia 17C, N-7010 Trondheim, Norway
[2] Stiftelsen Atlanterhavsparken, Tueneset, N-6006 Ålesund, Norway

Introduction

In the marine food web copepods are the natural prey organism for a number of marine larvae and believed to be the ideal first feeding organisms in the future aquaculture industry. Today eggs from *Acartia tonsa* Dana (Copepoda: Calanoida) are produced intensively and newly hatched nauplii and later developmental stages are superior alternatives to traditional live feed organisms such as rotifers (*Brachionus* sp.) and brine shrimp (*Artemia* sp.). This is due to the copepods high levels of essential n-3 highly unsaturated fatty acids (HUFA), such as docosahexaenoic acid (22:6n-3; DHA) and eicosapentaenoic acid (20:5n-3; EPA), predominantly found in the phospholipids. In addition to the n-6 HUFA arachidonic acid (20:4n-6; ARA), these HUFA's are all important for successful growth and development of marine fish larvae (Bell et al., 2003). The main challenge for marine hatcheries in implementing on site intensive controlled production of copepods is the necessity of culturing live microalgae. Algae production is both labor-intensive and space-demanding, thus algal concentrates and pastes are cost-efficient alternatives to live algae production.

A. tonsa is a fastidious species which until now has been difficult to produce on commercial microalgae pastes or concentrates. A preliminary trial has shown that nauplii grow and develop normally when fed a concentrate based on *Tetraselmis* sp. (BlueBioTech GmbH, Germany) with a low DHA content ($<0.5 mg.g^{-1}$).

The objective of this study was to determine if the stage IV nauplii and the stage IV copepodids produced on *Tetraselmis* concentrate were suitable as live feed organisms with respect to their fatty acid composition.

Materials and methods

The experiment was conducted at SINTEF Sealab, Trondheim, Norway. Intensively produced *A. tonsa* eggs (several batches <2 weeks old) were mixed together and sampled for fatty acids (FA). The eggs (n=72 000 000) were hatched in normoxic, aerated seawater (480 l, 25ppt, 23±0.5°C). After 48 hours the newly hatched nauplii were sampled for FA, and distributed into six plastic tanks (100 l) filled with sand-filtered (20µm), protein-skimmed, and aerated seawater (90 l, 25ppt, 23±0.5°C), and fed either *Rhodomonas baltica* or *Tetraselmis* sp. concentrate.

The dietary effects of the two monoalgal treatments were evaluated at 4 and 10 days post-hatch (dph) for developmental stage and fatty acid composition. The two different treatments were run in triplicates; the cryptophyte *R. baltica* obtained from a culture kept in a logarithmic growth phase, and a concentrate of *Tetraselmis* sp. (BlueBioTech GmbH, Germany). All copepod cultures were fed algae two times per day at a concentration of 1500µg carbon l^{-1}. The samples for FA were taken from the copepods >16h after last feeding.

Results and discussion

The experiment showed that *A. tonsa* was produced successfully on the *Tetraselmis* sp. concentrate. There were no differences in the distribution of developmental stages between the treatments at 4 and 10 dph. The absolute (A) and relative (B) content of FA are shown in Fig. 1. In *A. tonsa* eggs the total lipid content was 18% of the dry weight (DW) and a sum of DHA and EPA was close to 25% of total fatty acids. In newly hatched nauplii, the lipid content was 14% of DW and the DHA and EPA content was 16 and 11% of total FA, respectively.

Both the stage IV nauplii and the stage IV copepodites had a lower total lipid content when fed *Tetraselmis* (Fig. 1A). However, in relative terms the nauplii had a higher content of both DHA and EPA compared to the group fed *R. baltica*. In the copepodid stage IV the total lipid content was close to 11% for the group fed *R. baltica* and 8% for the group fed *Tetraselmis*. In these groups the sum of DHA and EPA was 45 and 40% of total FA, respectively, however in the group fed *Tetraselmis* the DHA content was 13% compared to 31% in the group fed *R. baltica*. The content of ARA was relatively low in the eggs (0.04% of total FA), increased to 1% in the newly hatched nauplii, and 1.3-1.6% in the stage IV naulpii. In the copepodid stage the ARA content was 0.08% for the group fed *R. baltica* and 0.13% for the group fed *Tetraselmis*.

The stage IV nauplii fed *R. baltica* showed a total fatty acid content almost twice as high compared to those fed *Tetraselmis*, with significant differences in the fraction of other n-3 content (in particular 18:3n-3 and 18:4n-3). However, nau-

plii produced on the *Tetraselmis* concentrate showed a DHA content close to 10mg.g^{-1} DW, a DHA:EPA ratio of 1.6:1, and an EPA:ARA ratio of 8:1, which suggest that the nutritional quality with respect to EFAs is sufficient for use as live feed for first-feeding marine larval fish. The copepodid stages had a lower DHA content then the naupliar stages. Since *Tetraselmis* sediment to the bottom of the tanks at a higher rate than *R. baltica* this might have caused low concentration of food when the growth of the copepods is at a high rate.

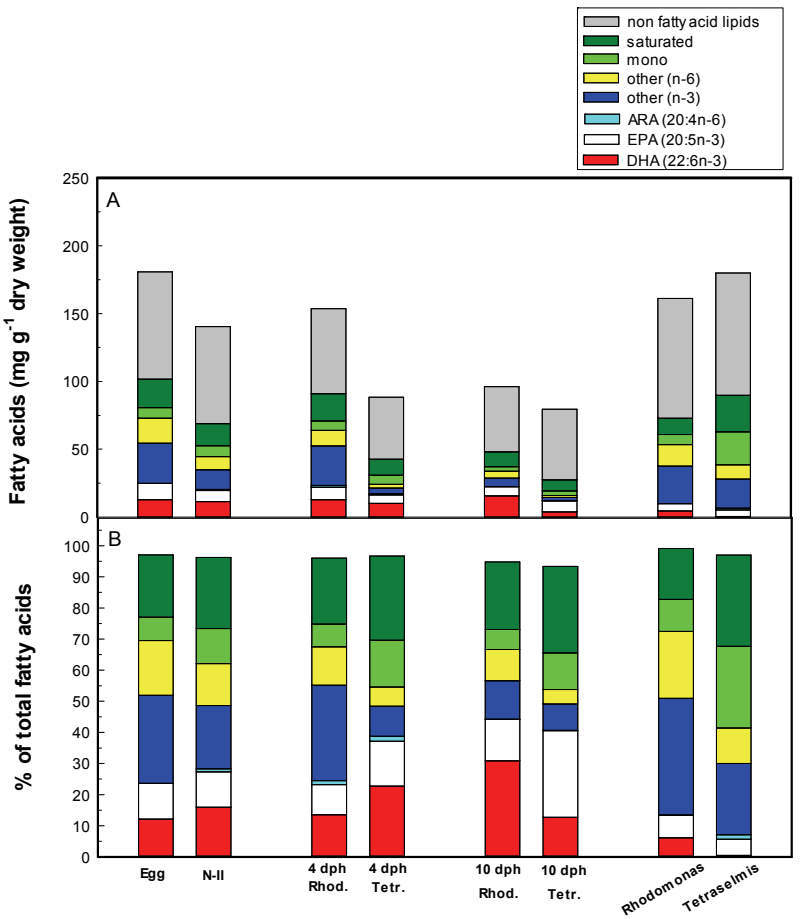


Fig. 1. Fatty acid profiles of *A. tonsa* eggs, newly hatched nauplii (N-I and N-II), stage IV nauplii (4 dph) and stage IV copepodids (10dph) fed *R. baltica* (Rhod.) or *Tetraselmis* (Tetr.) and the algal diets *R. baltica* and *Tetraselmis*, shown as A; quantitative content (mg.g^{-1} DW) and (B) relative distribution (% of total fatty acids) of important essential fatty acids and groups of FA. The total length of each bar in (A) represents the total lipid content of the samples.

Conclusions

The results show that *A. tonsa* nauplii can be produced intensively on a concentrate of *Tetraselmis*. Even if the total content of essential fatty acids is lower compared to copepods produced on live *R. baltica*, the amount of DHA and EPA is still at a satisfactory level and in the range of wild populations of different copepod species (Evjemo and Olsen, 1997; Evjemo et al., 2003).

The stage IV nauplii are similar to rotifers (*Brachionus* sp.) in size (180µm), thus this should be of interest to hatcheries who want to replace rotifer production with copepod production without the need to cultivate live microalgae. For the copepodites, it should be assessed through further studies whether the low fatty acid content reflect a suboptimal feeding regime or an inferior algal diet.

References

Bell J.G., L.A. McEvoy, A. Estevez, R.J. Shields, and J.R. Sargent. 2003. Optimising lipid nutrition in first-feeding flatfish larvae. *Aquaculture* 227:211-220.

Evjemo J.O. and Y. Olsen. 1997. Lipid and fatty acid content in cultivated live feed organisms compared to marine copepods. *Hydrobiologia* 358:159-162.

Evjemo J.O., K.I. Reitan, and Y. Olsen. 2003. Copepods as live food organisms in the larval rearing of halibut larvae (Hippoglossus hippoglossus L.) with special emphasis on the nutritional value. *Aquaculture,* 227, 191-210.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EURYHALINE ROTIFER *PROALES SIMILIS* AS INITIAL LIVE FOOD FOR REARING FISH LARVAE WITH SMALL MOUTHS

A. Hagiwara[1], S. Wullur[2], N. Hirai[3], and Y. Sakakura[1]

[1] Graduate School of Fisheries Science and Environmental Studies, Nagasaki University, Nagasaki 852-8521, Japan
[2] Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado 95115, Indonesia
[3] National Research Institute of Aquaculture, Aquaculture Systems Division, Kagoshima 899-7101, Japan

The SS-type rotifer *Brachionus rotundiformis* is a common initial food for rearing fish larvae with small mouths, such as groupers. There are commercially important fish species, however, whose mouth size at larval stage are too small to feed SS-type rotifers. In July 2004, we collected a small (mean±SD; body length 82.7±10.9µm and body width 40.5±6.4µm, n=480), smooth and flexible (lorica lacking body) rotifer, identified morphologically as *Proales similis*, from an estuary (salinity 2ppt, temperature 27°C) in Ishigaki Island, Okinawa, Japan.

When *P. similis* females were cultured individually in *Nannochloropsis oculata* suspension (5.8µg dry weight.ml^{-1}) at temperature 25°C and salinity 2 to 25ppt, they produced first offspring on 2.5 to 2.8 days after hatch, and their reproductive period ranged from 2.9 to 3.4 days, and they produced 4.3 to 7.8 offspring. In batch culture (*N. oculata* suspension at 5.8µg dry weight.ml^{-1}), *P. similis* showed higher growth (density=517.6-1027.0 ind.ml^{-1}; r=0.68-0.81.day^{-1}) at temperatures from 25 to 35°C (at 2ppt) and population growth was suppressed at temperatures 15 to 20°C. Among salinities tested (2-30ppt, at 25°C), it demonstrated higher growth at salinities from 2 to 15ppt (density=361.7-497.9ind.ml^{-1}; r = 0.73-0.78.day^{-1}). Population density of *P. similis* was not significantly different between fresh *N. oculata* and *C. vulgaris* (Chlorella V-12®, 1.2-28.8µg dry weight.ml^{-1}, at 25°C and 25ppt), but was higher than *B. rotundiformis* cultured at the same food conditions after 8 days culture period. Population density of *P. similis* cultured in 2-l containers fed *N. oculata* at 28.8µg dry weight.ml^{-1} (25°C and 25ppt) increased exponentially from 25 to 2400ind.ml^{-1} (r = 0.42.day^{-1}) after 11 days.

Total lipid per wet weight of *P. similis* fed by *N. oculata* and Super Fresh Chlorella V-12® was 2.4 and 2.6%, respectively. The compositions of eicosapentaenoic acid (EPA, 20:5*n*-3), docosahexaenoic acid (DHA, 22:6*n*-3), and arachi-

donic acid (AA, 20:4*n*-6) in total lipid of *P. similis* cultured by *N. oculata* were 23.2, 0.0, and 5.3%, respectively, while these were 11.0, 17.5, and 0.5% respectively, when Super Fresh Chlorella V-12® was fed. The composition in *B. rotundiformis* fed Super Fresh Chlorella V-12® was 5.8, 6.1. and 1.2%, respectively. The ratios of DHA/EPA in two rotifer species fed Super Fresh Chlorella V-12® were 1.59 and 1.05 for *P. similis* and *B. rotundiformis*, respectively.

Seven-band grouper *Epinephelus septemfasciatus* larvae cultured in 100-l rearing tanks were fed with *B. rotundiformis* and *P. similis*. Mouth size of the larvae at first feeding on 4DAH was 180±20μm (n=10). On this day, the larvae showed higher selectivity against *P. similis* than *B. rotundiformis* (Chesson's selectivity index, 0.7 and 0.3, respectively). The larvae digested and utilized *P. similis* as energy resource as they grew, and survived (2.7, 6.4, and 14.3% for *P. similis*, *B. rotundiformis* and mixture of two rotifers, respectively) till the end of experiment (day 10). Co-feeding of *P. similis* with *B. rotundiformis* improved larval initial growth and survival. Feeding incidence of rusty angelfish *Centropyge ferrugata* larvae (mouth size 160±16μm, n=10) on 4 to 6DAH was 20 to 60% for *P. similis*, while it was 0 to 20% for those fed other small zooplanktons (*B. rotundiformis*, *Keratella* sp. cf. *sinensis* and nauplii of copepod *P. nana*). Growth of the larvae among treatments on 5 or 6DAH was not significantly different but survival was higher in the larvae fed *P. similis* (18.5-38.0%) than those in other treatments (0-11.5%). Mouth size of Japanese eel *Anguilla japonica* larvae is large among fish (521±28μm, n=10), but they have difficulty to ingest large and solid food items because of their special esophageal characteristics; narrow without mucus cells. In this experiment, feeding of the larvae on slurry diet (based on shark spiny dogfish *Squalus acanthias* egg yolk), a primary food source for the larvae, was compared to six-minute zooplankton species (*P. similis*, *Synchaeta* sp. cf. *cecilia*, *B. rotundiformis*, *Keratella* sp. cf. *sinensis*, *B. angularis* and nauplii of copepod *Paracyclopina nana*). The zooplanktons were harvested using plankton net, kept at temperature 4°C and fed as paste (nonliving) diet. Feeding incidence (percentage of larvae with food in gut) of the larvae fed slurry diet (26.7-100.0%) and *P. similis* (20.0-46.7%) during the experiment was significantly higher than the other zooplanktons tested (0-6.7%). The ingested slurry diet (20.3-68.9%) and *P. similis* (1.8-37.2%) appeared in larval foregut and mid-hindgut, while it remained in the foregut (0-3.4%) for rotifers *B. rotundiformis*, *Keratella* sp., and *B. angularis*. Preliminary rearing on the larvae using slurry diet, nonliving and living *P. similis* as food source showed 62.8, 37.2, and 0.8% survivals, respectively on 13DAH, while those larvae stocked without feeding in other rearing tanks died on 11DAH. Larvae of humphead wrasse *Cheilinus undulatus* also preferred *P. similis* better than SS-type during first 7 days after hatching. We produced 537 juveniles at 50DAH (survival rate 10.7%), suggesting that examined rearing method was reproducible for the seed production of humphead wrasse.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

ONTOGENY OF THE REDOX BALANCE IN RELATION TO ORGANOGENESIS IN ATLANTIC COD (*GADUS MORHUA*) LARVAE

K. Hamre*, S. Penglase, J. Rasinger, and P.A. Olsvik

National Institute of Nutrition and Seafood Research (NIFES). PO Box 2029, 5817 Bergen, Norway. (*E-mail: kha@nifes.no)

A cell's redox potential is related to its fate. Proliferating cells are the most reduced, differentiating cells are more oxidized, while apoptosis is accompanied by an even more oxidized environment and necrosis by the most oxidized environment. Glutathione (GSH) is considered the most important cellular redox buffer and the average potential of a cell/organism can be calculated from the concentrations of reduced and oxidised GSH. In the present study, total and oxidized glutathione were measured in developing cod larvae, together with activities of antioxidant enzymes and expression of genes involved in redox metabolism. Triplicate groups of cod larvae at different stages of development (3, 6, 10, 13, 20, 34, 48, and 62 days post-hatch (dph)) were sampled from the facility of Cod Culture Norway near Bergen in western Norway. For RNA extraction the whole larvae were pooled and homogenized in Trizol and frozen on dry ice. For analyses of GSH and antioxidant enzymes, larvae were flash frozen in liquid nitrogen. All samples were stored at -80°C until analyses. RNA was extracted and expression of 20 genes associated with redox metabolism and stress response were analyzed using RT-PCR. Four antioxidant enzymes and total and oxidized glutathione were analyzed using commercial kits.

Both for gene expression and for enzyme activities, PCA plots showed that the different sampling days were well grouped and separated from one another. Moreover, early development (3-20dph) was separated from later stages (34-62dph). The concentration of total GSH increased from 3-13dph, was stable from 13-48dph, and then decreased. The concentration of oxidized glutathione (GSSG) comprised in the order of 1% of the total GSH concentration and was stable throughout the sampling series. The average whole-body redox potential was around -200mV just before hatching and decreased to -260mV at 20dph, where after it was stable until 48dph. This was accompanied by changes in expression of a number of genes involved in redox balance and -signalling and activities of antioxidant enzymes. It is hypothesized that the period before 20dph is the most active period of larval organogenesis and differentiation of cells, while after day 20, the formed structures are mainly growing (i.e., proliferation).

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

COMBATING SOME OF THE CRUCIAL BOTTLENECKS FOR CALANOID COPEPOD CULTIVATION FOR LIVE FEED

B.W. Hansen[1], G. Drillet[2], and P.M. Jepsen[1]

[1] Dep. of Environmental, Social and Spatial Change, Roskilde University, Denmark
[2] DHI Water and Environment, Singapore

Introduction

IMPAQ is a multi-disciplinary international Research Alliance that, in collaboration with DenSelect, aims at developing a sustainable live feed in terms of calanoid copepods. The aim is to supply Danish and international aquaculture fish hatcheries with a live feed item that can be used to produce high value fish larvae. IMPAQ has facilitated a unique indoor Recirculated Aquaculture System (RAS) for production (Roskilde University) of the calanoid copepod *Acartia tonsa* (Dana). The biological requirements and tolerance of *A. tonsa* are challenging, as well as technical design of cultivation systems for optimal copepod rearing (copepod density, water quality, fecundity) and egg physiology and storage (Hansen et al., 2010). The ultimate aim is to produce, harvest, rinse ,and store vast amounts of copepod eggs to be sent to marine fish farmers as live feed for fish larvae.

Materials and methods

We investigated the tolerance of nauplii and adults to ammonia in a dose vs. response experiment with mortality as the end-point. Density of copepods in sustainable cultures was tested from 10 to >5000 ind.l^{-1} and survival and fecundity were the end-points. Two egg storage techniques were developed and documented: (1) incubate vast amounts of eggs in anoxic seawater at 3°C and cold store for up to one year with monitoring hatching success approximately every 3 months; (2) incubate vast amount of eggs in freshwater and store at room temperature 17°C and 25°C, and monitor hatching success after transfer to 32ppt seawater.

Results

We have determined a recommended ammonia concentration under which culture should be operated. Keep NH_3 concentration below NOEC 30µg $NH_3.l^{-1}$ and

477µg $NH_3.l^{-1}$ for nauplii and adult copepods, respectively, and monitor pH regularly (Fig. 1).

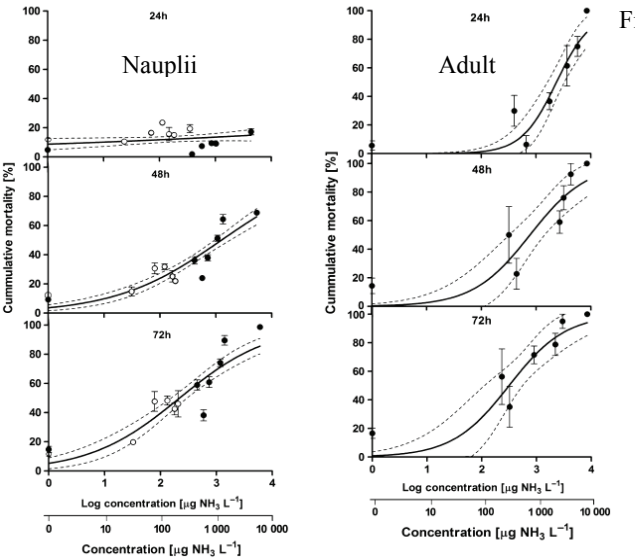


Fig. 1. Nauplii and adult *A. tonsa* tolerance to NH_3 (Jepsen et al., 2013).

Even at a density of >5000 ind.l^{-1}, no adverse effect on mortality was observed confirming that attaining this high density in cultures is possible. However, total maximum egg harvest was achieved at ~2500 ind.l^{-1}. The optimal egg production was ~12 000 eggs.l^{-1}. Hence, egg cannibalism, physical obstructions, and food limitations limit the fecundity and determine the practical culture densities (Drillet et al., 2013 submitted; Fig. 2).

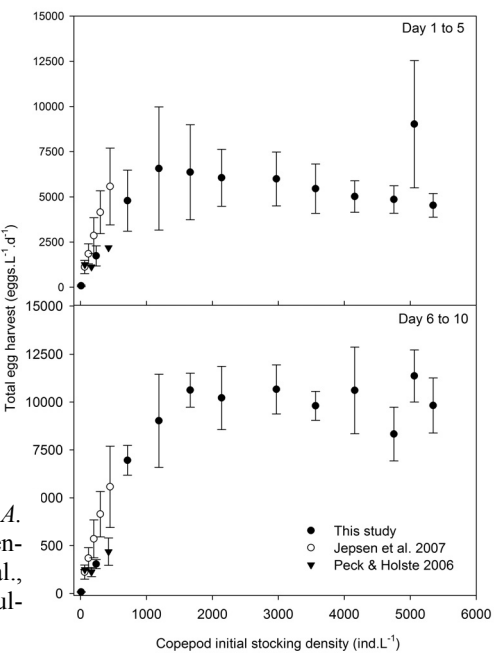


Fig. 2. Total eggs harvested l^{-1} of *A. tonsa* at different stocking densities (ind l^{-1}) (Drillet et al., 2013 submitted to Aquaculture).

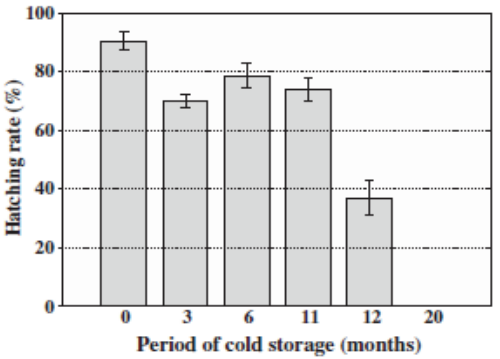


Fig. 3. Hatching rate of cold stored eggs is high for up to one year of storage (Drillet et al., 2006).

With our egg storage techniques, it is possible to cold store eggs for up to one year with high viability (Fig. 3) (Drillet et al., 2006) and even a month at room temperature with a modest but constant viability (Fig. 4) (Højgaard et al., 2008).

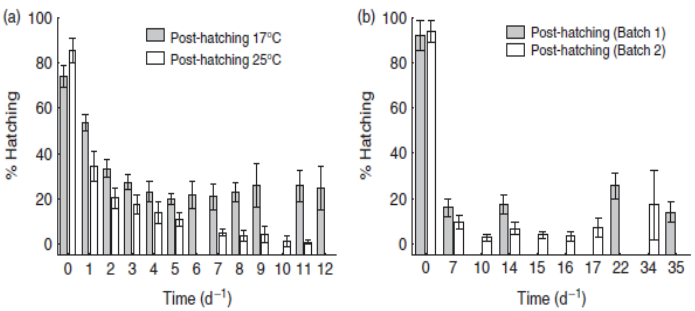


Fig. 4. Left: Show hatching success of eggs that has been stored in freshwater and then transferred to seawater, at 17 and 25°C. Right: Show hatching success of two different egg batches stored at 17°C (Højgaard et al., 2008).

This enables us now to create an egg bank and send out eggs by request to end users that can hatch them and feed the nauplii to fish larvae.

Discussion

IMPAQ has, after 2.5 years of research activities (halfway into the program), formulated recommendations for procedures for optimization of copepod rearing in indoor RAS for the benefit of the aquaculture industry. Results from intensive copepod cultivation systems:

- New cultivation methodology (tanks, harvest, cleaning, density determination, etc.) has been explored and evaluated.
- Abiotic (water quality) and biotic (density) parameters has been explored and limitations as well as recommendations formulated.
- Egg production limitations as a function of stocking density have been documented.
- Reliable egg storage techniques have been developed.

Understanding the physiology of copepods, when stocked at high density, is seen as crucial for intensifying copepod production. Technical solutions such as continuous separation of eggs from swimming stages in the water column, recirculation, as well as the continuous provision of food are major issues that must be solved to improve the volume-specific egg harvest yields in intensive copepod cultures. This will be focal points in a new Danish research consortia COMA which aim at up scaling copepod production.

References

Drillet G., N.O.G. Jørgensen, et al. 2006. Biochemical and technical observations supporting the use of copepods as live feed organisms in marine larviculture. *Aquaculture Research* 37(8):756-772.

Drillet G., M. Rais, et al. (Submitted). Total egg harvest by the calanoid copepod Acartia tonsa (Dana) in intensive culture – effects of high stocking densities and water quality on daily egg production and egg quality. *Aquaculture*.

Hansen B.W., G. Drillet, et al. 2010 Temperature effect on hatching of copepod eggs: Does acclimatization matters? *J. Plankton Res* .2:305-315.

Højgaard J.K., P.M. Jepsen, et al. 2008. Salinity-induced quiescence in eggs of the calanoid copepod Acartia tonsa (Dana): a simple method for egg storage. *Aquaculture Research* 39(8):828-836.

Jepsen P.M., N. Andersen, et al. 2007. Effects of adult stocking density on egg production and viability in cultures of the calanoid copepod Acartia tonsa (Dana). *Aquaculture Research* 38(7):764-772.

Jepsen P.M., C.V.B. Andersen, et al. 2013. Tolerance of un-ionized ammonia in live feed cultures of the calanoid copepod Acartia tonsa Dana. *Aquaculture Research* Online 1-12, doi:10.1111/are.12190.

Peck M.A. and L. Holste .2006. Effects of salinity, photoperiod and adult stocking density on egg production and egg hatching success in Acartia tonsa (Calanoida : Copepoda): Optimizing intensive. *Aquaculture* 255(1-4):341-350.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EFFECTS OF PROBIOTICS ON POMPANO (*TRACHINOTUS CAROLINUS*), COMMON SNOOK (*CENTROPOMUS UNDECIMALIS*), AND RED DRUM (*SCIAENOPS OCELLATUS*) LARVAE

M.R. Hauville[1,2*], J.L. Zambonino-Infante[3], H. Migaud[1], J.G. Bell[1], and K.L. Main[2]

[1] Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland, UK ([*]Corresponding author: marionhauville@gmail.com)
[2] Mote Marine Laboratory, Center for Marine and Freshwater Aquaculture Research, 34240 Sarasota, Florida, USA
[3] Ifremer, Functional Physiology of Marine Organisms Unit, UMR 6539, 29200 Plouzané, France

Introduction:

Fish gastrointestinal microbiota is highly influenced by the external environment due to the constant ingestion of surrounding water (Gatesoupe, 1999). Over the past decade, several studies have demonstrated multiple benefits of probiotic supplementations including antimicrobial activity, competitive exclusion of pathogenic bacteria, stimulation of the immune system, and improvement of the gastrointestinal structure and digestion (Kiron, 2012). The future larval gut is sterile until hatching, therefore the digestive tract colonization is determined by the environment and live-feed ingested. After hatching the digestive system is immature and the immune system is incomplete leading to a high vulnerability to microbiota-associated disorders. Probiotic supplementations, by promoting the establishment of a healthy microflora, are especially appropriate during larval stages (Ringø and Gatesoupe, 1998).

This experiment aimed at studying the effects of a commercial mix of *Bacillus* spp. on the early larval stages of some of Florida's high-value marine food fish (Florida pompano and red drum) and stock enhancement species (snook) with a focus on larvae performances and enzyme activities.

Materials and methods:

The experimental set-up included three identical independent systems. Each system was comprised of four 100-l tanks. Within each system the water was recirculating from the tanks to the sump/biofilter and back to the tanks through a 25-watt UV light. Temperature was maintained at 28±1°C for snook and red drum

and 27±1°C for pompano. Photoperiod was maintained at 12h Dark: 12hLight, salinity at 35±1g.l^{-1}; pH at 8±0.5, and dissolved oxygen at 6±2mg.l^{-1}.

Larvae were fed rotifers twice a day at a final density of 5 rotifers.ml^{-1}. In addition, a microdiet product (Gemma micro, Skretting, France) was delivered twice a day in between rotifer feedings. Trials were completed at the end of the rotifer-feeding period for pompano (9 days post-hatch, dph) and snook (12dph). The red drum trial was extended until 21dph due to the lack of significant difference in growth at the end of the rotifer-feeding period.

Three treatments were tested in quadruplicate (block design) for all trials. Each independent experimental system was assigned a treatment to avoid probiotic cross contamination. The first treatment (Control) was fed rotifers enriched with Algamac 3050 (Aquafauna Bio-marine Inc, USA). The second treatment (Probiotics) was fed rotifers enriched with Algamac 3050 and a mix of *Bacillus* spp. (0.5g.l^{-1} of enrichment, Sanolife MIC-F, INVE Technologies, Belgium). The third treatment (Probiotics+) was fed according to treatment 2 and in addition, probiotics (5g.m^{-3}) were added daily directly to the tank water.

Larvae growth was monitored by the measurement of 10 larvae from each tank at several sampling points during the trials depending on the species.

At the end of the trials, 50 larvae from each tank were preserved at -70°C for enzyme analyses. Trypsin, amylase, alkaline phosphatase, and leucine–alanine peptidase activities were assayed according to Zambonino Infante et al., (1997).

At the end of the pompano trial, 25 larvae from each tank were preserved at 4°C and bacterial analyses were performed the next day. Larvae were rinsed three times with sterile seawater then ground using a PowerSoil® DNA isolation kit (MO-BIO Laboratories, Inc., USA). Serial dilutions of the homogenates were then plated on marine agar and TCBS media. The Petri dishes were incubated at 22°C and the number of colony-forming units was counted 48h after plating.

Results and discussion:

No significant difference in survival from hatching was observed between treatments regardless of the species, with an average of 2.43±0.7% for snook, 7.62±1.92% for pompano, and 9.89±0.76% for red drum.

At the end of the pompano trial, standard lengths of larvae from the Probiotics and Probiotics+ treatments were significantly greater than for the control larvae being 4.34±0.10mm, 4.22±0.07mm, and 3.89±0.09mm, respectively. Likewise, at the end of the snook trial, standard lengths of larvae from the Probiotics and Probiotics+ treatments were significantly greater than for the control larvae being 3.69±0.02mm, 3.60±0.03mm, and 3.29±0.03mm, respectively. However, at

the end of the red drum trial, no significant difference was observed between treatments with an average standard length of 5.44±0.07mm.

Results from microbiological analyses on pompano larvae showed significantly higher counts of colony-forming units (CFU) per larva on the marine agar media for the larvae fed the probiotics supplementation (38.10^3±8.10^3CFU for Probiotics and 18.10^3±22.10^3CFU for Probiotics+) compared to the larvae from the control treatment (10^3±6.10^2CFU). These higher counts of heterotrophic bacteria reflect the successful colonization of the probiotics in the larvae digestive system. Numbers of presumptive *Vibrio* were low and not significantly different between treatments with an average of 60±10CFU per larva. Therefore, it was assumed that pathogenic bacteria were not a major concern in the experimental system. Microbiological analyses were not performed for the other species.

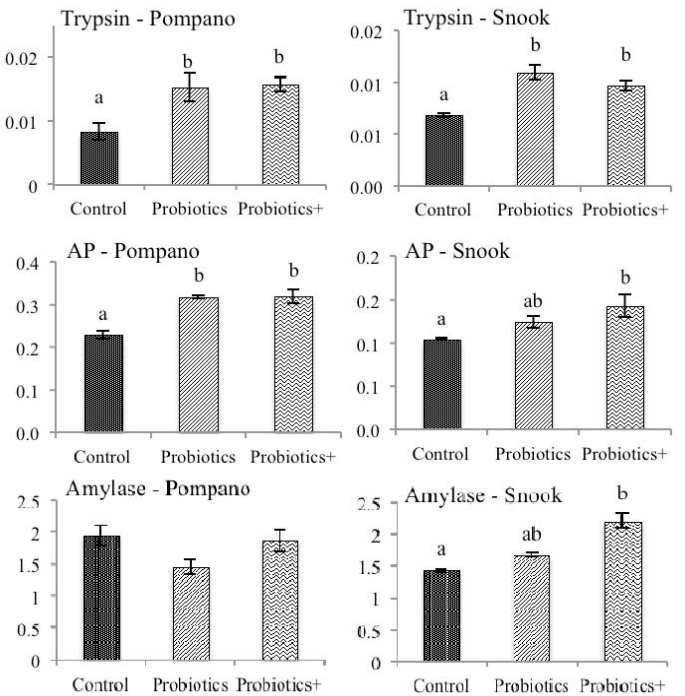


Fig. 1. Specific activities (U.mg^{-1} of protein) of trypsin, amylase, and alkaline phosphatase (AP) for either snook or pompano at the end of the trial (n=3 for snook, n=4 for pompano). Letters indicate significant differences, Tukey's test, $p<0.05$.

Results from enzyme analyses did not show any significant differences in enzyme activities between treatments for red drum. For all species, no significant differences were observed in the specific activity of leucine-alanine peptidase.

However, for both pompano and snook, trypsin specific activity was significantly higher in larvae fed the Probiotic and the Probiotics+ treatment compared to the control larvae. Similarly, alkaline phosphatase activity was significantly higher for the pompano larvae fed the Probiotic and Probiotics+ treatments and for the snook larvae fed the Probiotics+ treatment compared to the control larvae. In addition, amylase specific activity was significantly higher in the snook larvae fed the Probiotics+ treatment compared to the control larvae.

Overall, the probiotic supplementation improved growth and digestive maturation in pompano and snook larvae while no effect was noticed on red drum larvae development. Pompano and snook larvae are not as robust as red drum larvae and seem to benefit more from the supplementation. However, no negative effect was observed on red drum and the probiotic supplementation might have had positive effects on aspects other than growth and enzyme activities such as disease resistance or general welfare.

A probiotic supplementation appears recommended during early stages of larval rearing for pompano and snook. Adding the probiotic supplementation to both the live feed and the tank water appeared to provide optimal effects.

These results are consistent with previous studies that observed an increase in growth and enzyme activities when *Bacillus* spp. supplementation was provided to common carp (Wang and Zirong, 2006), Pacific white shrimp (Wang, 2007) or sea bream (Avella et al., 2010).

References:

Avella M.A., G. Gioacchini, O. Decamp, P. Makridis, C. Bracciatelli, and O. Carnevali. 2010. Application of multi-species of Bacillus in sea bream larviculture. Aquaculture 305:12–19.

Gatesoupe F.J., 1999. The use of probiotics in aquaculture. Aquaculture 180, 147–165.

Kiron V. 2012. Fish immune system and its nutritional modulation for preventive health care. Animal Feed Science and Technology 173, 111–133.

Ringø E. and F.-J. Gatesoupe. 1998. Lactic acid bacteria in fish□: a review. Aquaculture 160:177–201.

Wang Y.-B. 2007. Effect of probiotics on growth performance and digestive enzyme activity of the shrimp Penaeus vannamei. Aquaculture 269, 259–264.

Wang Y.-B. and X. Zirong. 2006. Effect of probiotics for common carp (Cyprinus carpio) based on growth performance and digestive enzyme activities. Animal Feed Science and Technology 127:283–292.

Zambonino Infante J.L., C.L. Cahu, and A. Peres. 1997. Partial substitution of di- and tripeptides for native proteins in sea bass diet improves Dicentrarchus labrax larval development. The Journal of nutrition 127:608–14.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

MICROPARTICULATE ENRICHMENT OF ROTIFER LIVE DIETS WITH TAURINE AND NUTRITIONAL EFFECTS ON NORTHERN ROCK SOLE (*LEPIDOPSETTA POLYXYSTRA*) LARVAE

M. Hawkyard[1], B. Laurel[2], Y. Barr[3], and C. Langdon[1]

[1] Coastal Oregon Marine Experiment Station and Department of Fisheries and Wildlife, Oregon State University, Newport, OR 97365 USA
[2] Alaska Fisheries Science Center, Fish Behavioral Ecology Lab, Newport, OR 97365 USA
[3] Nofima, NO-6600, Sunndalsora, Norway

Several water-soluble micronutrients, such as taurine, are highly concentrated in copepods but nearly absent in rotifers. Enrichment of rotifers with water-soluble nutrients is often accomplished by dissolution in the rotifer culture medium, which may result in low uptake and high wastage of nutrients. A potentially more efficient way to deliver water-soluble nutrients is to encapsulate them in water-insoluble microparticles prior to rotifer enrichment. We evaluated two such microparticles – wax spray beads (WSB) and liposomes – in their ability to enrich rotifers and deliver water-soluble taurine to cold-water marine fish larvae.

Digital images and video analyses indicated that rotifers were able to breakdown WSB and liposomes resulting in the release of encapsulated materials into the rotifer gut and body. However, particle breakdown was more apparent when rotifers were fed liposomes than when rotifers were fed WSB. Enrichment of rotifers with taurine-WSB (333mg lipid.l^{-1}) resulted in 4000µg taurine g^{-1} DW of rotifers whereas enrichment of rotifers with taurine-liposomes (250mg lipid l^{-1}) resulted in 15 000 µg taurine g^{-1} DW in enriched rotifers. Obtaining similar concentrations of taurine in rotifers (4000µg.g^{-1} DW and 15 000µg.g^{-1} DW) using aqueous solutions required 4g taurine.l^{-1} and 15g taurine.l^{-1}, respectively. These results show that the microparticulate enrichment methods used in this study obtained similar taurine concentrations in rotifers when compared to those enriched with aqueous solutions of taurine with a 60-fold (liposomes) to 80-fold (WSB) reduction in taurine usage.

Two growth trials were conducted to evaluate the effects of taurine-enriched rotifers on the growth and development of northern rock sole (*Lepidopsetta polyxystra*) larvae. In the first trial, northern rock sole larvae were fed rotifers enriched with either: 1a) WSB containing taurine, 2a) an aqueous taurine solution with a concentration of 50mg taurine.l^{-1}, 3a) an aqueous taurine solution with a

concentration of 4g taurine.l^{-1} or 4a) no additional taurine. In the second trial, northern rock sole larvae were fed rotifers enriched with either: 1b) liposomes containing taurine, 2b) aqueous taurine solutions or 3b) no additional taurine.

At the end of trial 1 (week 7), larval standard lengths (SL) and dry weights (DW) were significantly higher when larvae were fed rotifers enriched with either taurine-WSB or 4g taurine.l^{-1} when compared to larvae fed unenriched rotifers. Larval DW and SL were not significantly different when larvae were fed rotifers enriched with 50mg taurine.l^{-1} when compared to the control larvae. In addition, larvae were more developed, measured in discreet states of flexion, with increasing concentrations of taurine. Trial 2 was ongoing at the time this abstract was submitted and the full results will be presented at Larvi 2013. Preliminary results suggest that rock sole larvae respond positively to liposome-delivered taurine.

The results of this research indicate that high concentrations of taurine can be obtained in rotifers using microparticulate enrichment techniques, with lower nutrient usage when compared to immersion of rotifers in aqueous solutions. The results of the growth trials indicate that northern rock sole larvae benefit from taurine concentrations beyond those provided from rotifers that are not enriched with taurine. This is one of the first studies to demonstrate positive growth effects in marine fish larvae as a result of the enrichment of rotifers with a water-soluble nutrient via microparticles. Microparticulate enrichment of water-soluble substances may greatly reduce the quantities, and therefore costs, of water-soluble nutrients needed for the enrichment of live prey. These methods may have further advantages, such as, improvement of water-quality and reduction of bacterial pathogens during enrichment of live prey.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

IS SINKING MORTALITY IN SOUTHERN BLUEFIN TUNA LARVAE CAUSED BY HIGH LIGHT INTENSITY?

P.I. Hilder[1], J.M. Cobcroft[1,2], N.S. Hart[3], S.P. Colin[3], and S.C. Battaglene[1]

[1] Institute for Marine and Antarctic Studies, University of Tasmania, Nubeena Crescent, Taroona, Tasmania 7053, Australia
[2] School of Science, Education and Engineering, University of the Sunshine Coast, Locked Bag 4, Maroochydore DC, Queensland 4558, Australia
[3] School of Animal Biology and The Oceans Institute, University of Western Australia, Crawley, WA 6009, Australia

Introduction

The southern bluefin tuna (*Thunnus maccoyii*) aquaculture industry in Australia is based on fattening wild-caught juveniles and industry development is constrained by a quota system. Land-based aquaculture (from egg) is now being investigated to increase production and the scope of the industry. *T. maccoyii* experience major mortality during the first two weeks of culture – up to >95% of the cohort – and high larval mortality is not uncommon among cultured tuna species (Margulies, 1997). In general, marine fish larvae are visual feeders and, therefore, require light to feed and avoid predators; consequently, light has been identified as an important factor affecting survival. Larvae generally possess a pure cone retina (simplex retina), which presumably limits visually guided feeding to conditions of relatively high light intensity. However, unlike the majority of marine fish larvae, the culture of *T. maccoyii* under high ambient light intensities may not be conducive to early survival. Cultured tuna larvae are often documented to "sink" to the tank base where the subsequent exposure to high detritus and bacterial loads and hard surfaces are thought to result in major mortality (Tanaka et al., 2009). We hypothesise that sinking mortality in *T. maccoyii* is due to the larvae actively migrating away from areas of high light intensity.

Materials and methods

Embryos were supplied by Clean Seas Tuna Ltd, South Australia. Short-term (4h), small scale (3 l) feeding trials were conducted on 3, 6, and 9 days post-hatching (dph) larvae to assess the effect of light intensity on feeding and mortality, and on 3-dph larvae to assess the effect of blue, red, or white light on feeding. In order to identify the spectral sensitivity of the visual pigments, retinal samples were collected for microspectrophotometry. This involved dark-

adapting larvae (3 to 30dph) for a 2-h period prior to euthanasia and removal of the retina. Samples were placed in a cryopreservation medium, frozen and transferred to the University of Western Australia for microspectrophotometric analysis. Larvae collected for histological analysis (3 to 30dph) were preserved in 5% glutaraldehyde, embedded in methacrylate resin, sectioned at 2µm then stained with Lee's Methylene Blue-Basic Fuchsin.

Results

Light intensity experiments revealed there was significantly less feeding and greater mortality as light intensity and age increased (Fig. 1).

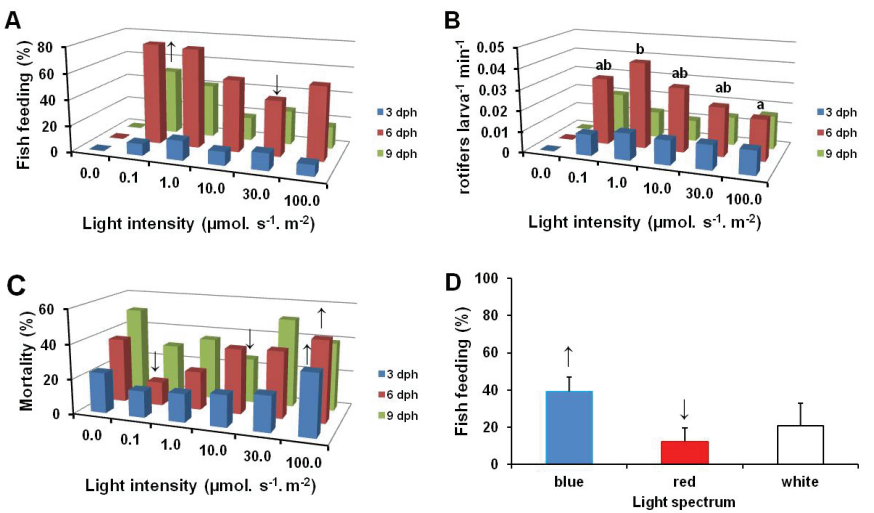


Fig. 1 The response of *Thunnus maccoyii* to different light intensities (A) the proportion of larvae feeding (B) the intensity of larvae feeding (C) mortality experienced and (D) the proportion of 4dph larvae feeding under different coloured lights. The arrows indicate treatments with significantly more (↑) or less (↓) feeding ($P<0.05$) mean+sd, n=4.

Microspectrophotometry of twin cone visual pigments revealed sensitivity in the blue-green spectral region (494nm, 507nm, and 524nm). This concurred with spectral sensitivity feeding experiments which found that larvae fed significantly better under blue light compared to white or red light matched for equal quantal intensity (Fig 1D). Histology of the retina revealed large single cones at first-feeding (2µm), low convergence of cones to ganglion cells throughout larval development (~1:1), and morphological specialisations supporting sensitivity in the ventral retinal region. Migration of retinal pigment epithelium was observed at 12dph.

Discussion

Sinking behaviour in *T. maccoyii* is commonly observed during the first two weeks of culture (Woolley et al., 2013). In the culture environment, this brings the larvae into prolonged contact with the base of the tank. This has a number of negative impacts that include physical abrasion as larvae come in contact with the hard tank surfaces, exposure to high bacterial load (including pathogenic bacteria), and sub-optimal water quality. Body density affects the distribution of the larvae in the water column and is affected by a number of factors including, body composition, swimbladder inflation, gut fullness, and the density of the surrounding waters. The body density of *T. maccoyii* is denser than the surrounding waters – i.e., negatively buoyant – regardless of swimbladder inflation (Woolley et al., 2013). In order to suspend the negatively buoyant larvae in the water column, increased turbulence has been employed, as is the practice in Pacific bluefin tuna (*T. orientalis*) culture (Tanaka et al., 2005). While increased turbulence may negate sinking mortality during the early life history, significant mortality is still experienced with increasing development (Woolley et al., 2013). The retinal adaptations and behavioural responses displayed by *T. maccoyii* would strongly suggest not only a preference for low light but also an aversion to high light conditions which may result in active benthic migration to avoid such. The ongoing sinking mortality observed in *T. maccoyii* may be a result of increased swimming performance associated with improved skeletal and musculature development of the larvae, permitting active choice by the larvae of their position in the water column. Other pelagic fish larvae have displayed behaviour associated with benthic migration; however, this is commonly observed at metamorphosis as larvae make the transition from pelagic waters to the settlement on rocky reefs. This is not the case in *T. maccoyii* as active benthic migration can be seen as early as 3dph. Larvae displayed increased feeding and survival, particularly at 6dph in response to low light. Preference for feeding under blue light observed in behavioural experiments and blue-green spectral sensitivity demonstrated with microspectrophotometry would allow feeding in deeper waters and conditions at dawn and dusk (or moonlight) characterised by maximum light transmission in the blue-green region of the spectrum. The relatively high photon capture afforded by well-matched visual pigment spectral sensitivities would enhance both visual acuity and absolute sensitivity (Poling and Fuiman, 1998). The identification of retinal pigment epithelial migration as early as 12dph indicated an early developmental need to control the amount of light reaching the retina. In addition, the area of specialisation observed in the ventral retinal region indicates higher acuity in the dorsal visual field. This suggests larvae in deeper waters associated with lower light intensities can detect prey and predators best in the visual plane above the larvae.

The observation of light sensitivity in *T. maccoyii* suggests active migration away from strong surface light sources. A multipronged approach may be re-

quired to reduce sinking mortality utilising strategies adopted from our study and proven methods from the literature including low light intensity rearing, maximising swimbladder inflation to increase buoyancy, feeding strategies to control gut fullness and turbulence levels to maintain larvae in the water column.

References

Margulies D. 1997. Development of the visual system and inferred performance capabilities of larval and early juvenile scombrids. Marine and Freshwater Behaviour and Physiology 30:75-98.

Poling K.R. and L.A. Fuiman. 1998. Sensory development and its relation to habitat change in three species of sciaenids. Brain Behavior and Evolution 52:270-284.

Tanaka Y., K. Kumon, A. Nishi, T. Eba, H. Nikaido, and S. Shiozawa. Status of the sinking of hatchery-reared larval Pacific bluefin tuna on the mass culture tank with different aeration design. Aquaculture Science 54:587-593.

Woolley L.D., S. Fielder, and J.G. Qin. 2013. Swimbladder inflation associated with body density change and larval survival in southern bluefin tuna *Thunnus maccoyii*. Aquaculture International. DOI 10.1007/s10499013-9626-9.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EFFECT OF HEAT SHOCK PROTEIN 70 (HSP70) HOMOLOGUE DNAK ON GENE EXPRESSION OF PROPHENOLOXIDASE AND TRANSGLUTAMINASE IN HAEMOCYTES OF *LITOPENAEUS VANNAMEI*

B. Hu, P. Bossier, and P. Sorgeloos

Laboratory of aquaculture and Artemia reference center, Ghent University, 9000 Ghent, Belgium

Introduction

Litopenaeus vannamei is one of the most important aquaculture species in the world. Like other crustaceans, it does not have an acquired immunity; instead they have an innate immune system called non-specific immunity. This kind of immunity consists of melanization by activating the prophenoloxidase-activating system (proPO-AS), hemolymph coagulation, phagocytosis, encapsulation of foreign particles, antimicrobial peptide production, and cell agglutination (Soderhall, 1998; Cerenius, 2011). Heat shock proteins (HSPs) are abundant and ubiquitous in both eukaryotes and prokaryotes, performing a multitude of housekeeping and cytoprotective functions. Increasing evidence suggests that HSPs play an important role in regulating the early innate immune response (Srivastava, 2002). In higher eukaryotes, HSP70 stimulates innate immune cells – e.g., dendritic cells, macrophages, monocytes – producing several pro-inflammatory cytokines via certain receptors and signal transaction pathway. In crustaceans, both heterologous and homologous HSP70 were found to successfully improve the PO activity and shield the *Artemia* from *Vibrio* infection. It still remains unknown, however, how DnaK affects *L. vannamei* immunity (Baruah, 2010). In our study, we aimed at evaluating the HSP70 homologue DnaK immuno-stimulant effect on *L. vannamei* via gene expression of two immune-related genes: prophenoloxidase and transglutaminase. The result showed that the DnaK is able to dramatically up-regulate the transglutaminase type 2 and prophenoloxidase type 1 within 12h post-injection. These findings indicate that HSP70 can be explored to fight infectious disease in *L. vannamei*.

Materials and methods

Recombinant DnaK production
The *E. coli* strain E_{native} that expresses Dnak with a hexahistidine-tag were grown at 37°C in Luria-Bertani (LB). Overproduction of DnaK was induced by adding

L-arabinose ($0.5mg.ml^{-1}$). After induction, the bacteria were centrifuged at 2200×G for 15min and suspended in sterile Dulbecco's Phosphate-Buffered Saline (DPBS) (no calcium, no magnesium, Gibco®, Invitrogen). Bacteria were homogenized and subsequently centrifuged at 2200×G for 1 min at 4°C. The supernatant protein was taken for DnaK isolation. For DnaK isolation, Dynabeads® (Dynabeads® His-Tag Isolation & Pull down, Invitrogen) were used according to recommended protocol. The elution buffer was exchanged to DPBS using the Amicon Ultra Centrifugal Filter (Millipore) according to the manual.

Experimental animal

Specific pathogen-free (SPF) *L. vannamei* for experimental purpose were housed individually in covered 10-l aquaria filled with Instant Ocean® seawater prepared with distilled water at a salinity of $35g.l^{-1}$, provided with constant aeration, and maintained at 27±1°C by air heaters. Shrimp were acclimated under the latter conditions for 3 days before any subsequent treatment. Feeding was skipped for 24h prior to the injection.

Recombinant DnaK treatment

Two doses of recombinant DnaK protein were injected to shrimp: high dose (5 µg) and low dose (0.05µg) per animal. They were dissolved in 100µl Dulbecco's Phosphate-Buffered Saline (DPBS) (no calcium and no magnesium, Gibco®, Invitrogen) and then intramuscularly injected on the tail. As control, the blank DPBS was injected. The hemolymph was collected at 5 times post-injection: 1, 3, 6, 9, and 12 hours post injection (hpi). Three animals from each treatment were sacrificed at every time point.

Total RNA isolation and cDNA synthesis

Haemocytes were spun down from the hemolymph by centrifuge. Total RNA was extracted from haemocytes using RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Total RNA was treated with RNase-free DNase (Fermentas) to remove genomic DNA contamination, after which the RNA was quantified spectrophotometrically (NanoDrop Technologies, Wilmington, DE, USA). RNA quality was confirmed by electrophoresis. First strand cDNA was synthesized from 0.5µg total RNA using the RevertAid™ 6H minus First strand cDNA synthesis kit (Fermentas, Germany) according to the manufacturer's instructions.

Quantitative real-time PCR (RT-qPCR) analysis

The immune-related genes, prophenoloxidase, and transglutaminase mRNA expression in *L. vannamei* was analyzed by RT-PCR using a pair of specific primers designed on the basis of several *L. vannamei* cDNA sequences. Real-time PCR was performed in an ABI StepOne Real Time System thermal cycler (Applied Biosystem). Dissociation curve analysis in the real-time PCR was performed for each gene to check for the amplification of untargeted fragments.

Data acquisition was performed with the StepOne software (v2.2.2, Applied Biosystem) at the end of each elongation step. The expression of the target genes was normalized to the endogenous control gene β-actin then the relative expression was calculated as $2^{-\Delta\Delta Ct}$ (Livak, 2001).

Statistics
Data were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni post-test using statistic software GraphPad Prism Programs (GraphPad Prism, San Diego, CA) to determine significance. Values were considered significantly different if $p<0.01$.

Results and discussion

The prophenoloxidase-activating system (proPO-AS), present in the open circulatory system of crustaceans and other invertebrate, is regarded as the most important component of the innate immunity and plays a vital role in defense against pathogens. Here, the immune stimulating function of recombinant DnaK was evaluated via this vital parameter (Fig.1A, B). The prophnoloxidase (proPO) 1 expression was not significant different from the control. However, upon injection of DnaK (Fig. 1B), both high-dose (5μg) and low-dose (0.05μg) of DnaK up-regulated proPO 2 gene expression 5-fold and 4-fold respectively during the first three hours ($p<0.001$). Hereafter, proPO 2 expression decreased.

Gene expression of two transglutaminase (TGase) genes were also analyzed by qPCR. As shown in Fig. 1C, within 12 hours, the expression of TGase 1 in *L. vannamei* injected with either a high dose or low dose of DnaK is different relative to the control. High dose of DnaK exhibited a highly significant ($p<0.01$) 6-fold increase in the relative abundance of TGase 1 mRNA transcript at 1hpi, after which it declined. In contrast, the low dose of DnaK up regulated the TGase expression 60-fold at 6hpi ($p<0.001$). The TGase 2 expression fluctuated within 12hpi. The low dose of DnaK induced significant higher TGase 2 expression at 1hpi and 12hpi (2-fold, $p<0.05$).

Those results suggested that the microbial HSP70 is able to boost the immunity of *L. vannamei* through clotting system and proPO-AS. It offers an opportunity that primes the immune system of *L. vannamei* using microbial HSP70 homologue DnaK and eventually helps *L. vannamei* fight against pathogens.

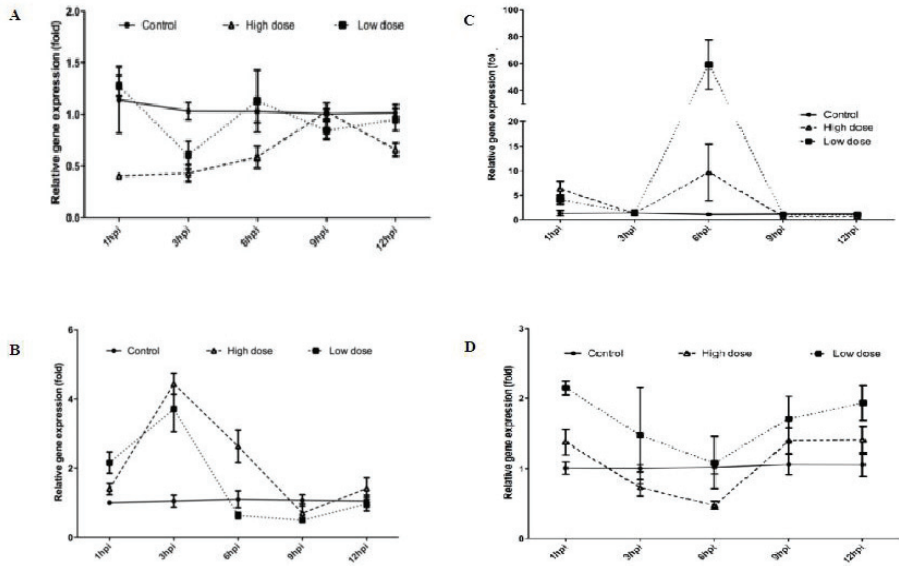


Fig. 1. In vivo expression of prophenoloxidase 1 (A), prophenoloxidase 2 (B), transglutaminase 1 (C), transglutaminase 2 (D) of *L. vannamei* injected with recombinant DnaK. Control group was injected with endotoxin-tested phosphate saline (DPBS). The haemocytes were sampled at 1, 3, 6, 9, and 12h post-injection. The mRNA expression was analysis by quantitive real time PCR. Two doses of DnaK were given to *L. vannamei*: high-dose (5μg per shrimp) and low-dose (0.05μg per shrimp). Bars indicated standard error.

References

Baruah K, J. Ranjan, P. Sorgeloos, and P. Bossier. 2010. Efficacy of heterologous and homologous heat shock protein 70s as protective agents to *Artemia franciscana* challenged with *Vibrio campbellii*. Fish and Shellfish Immunol 29:733–739.

Cerenius L. and K. Söderhäll. 2011. Coagulation in Invertebrates. J Innate Immun 3:3–8.

Livak K.J.,and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT Method. Methods:25:402–8.

Soderhall K. and L. Cerenius. (1998) Role of the prophenoloxidase-activating system in invertebrate immunity. Curr Opin Immunol 10(2):23-28.

Srivastava P. 2002. Roles of heat-shock proteins in innate and adaptive immunity. Nat Rev Immunol 2:185-194. doi:10.1038/nri749.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

MATURATION AND SPAWNING INDUCTION IN HAWAIIAN OPIHI *CELLANA* SPP. BY HORMONE GNRH

N.T. Hua and H. Ako

Department of Molecular Biosciences and Bioengineering, University of Hawaii at Manoa, 1955 East West Road, #218 Honolulu, HI, 96822, USA

Abstract

Gonadotropin releasing hormone (GnRH) is a neuropeptide hormone which plays an important role in control of the reproduction among vertebrates and some work is showing that it is active in invertebrate species. We matured and spawned the Hawaiian opihi *Cellana* spp. by using salmon GnRH analogue. In the first experiment on maturation, twelve adult opihi (3.18±0.23cm, shell length) were injected weekly with salmon GnRHa at dose of 250ng.g^{-1}body weight (BW) for a 6-week period. Gonad development was assessed using gonadosomatic index (GSI) and histological techniques. GSI incorporated with histological analysis showed that the gonads reached full maturation after 4 weeks. GSI increased significantly (31%) in comparison to the control group of saline solution injection did not produce any maturity in opihi. For the spawning trial, eight ripe opihi were administered a single injection at dose of 1000ng.g^{-1} BW. The results showed that an average of 33% of the animal spawned in 4-6h after hormone injection. Fertilized eggs developed through embryo and to the complete larval stage of veligers. Our preliminary findings in this study provide a new aspect of hormone controlling reproduction of opihi, which could be applied in opihi aquaculture.

Introduction

Opihi are endemic limpets of the Hawaiian Islands. Our aim was able to produce seed for a future commercial hatchery of Hawaiian opihi. The ability to mature opihi will not only benefit hatchery management but also successful aquaculture of this species in the future. Relying on natural broodstock sources is often difficult due to the uncertain ripeness of animals to be spawned. Moreover, it is dangerous to collect ripe broodstock from the wild for spawning due to rough water conditions; many people have died while collecting opihi for food. Wild broodstock are sometimes not stable and do not produce good quality larvae and larval metamorphosis (Corpuz, 1981). Different forms of GnRH analogue have been widely used in the induction of maturation and spawning of various aquatic spe-

cies. It was reported that mammalian GnRHa and octopus GnRHa were effective in controlling reproduction in abalone, *Haliotis asinia* (Nuurai et al., 2010). In this study we attempted to mature opihi in captivity by using hormone salmon GnRHa working with naturally matured broodstock for our spawning trial.

Materials and methods

Maturation studies of opihi were conducted using OvaRH which is a synthetic salmon GnRH analog (Des-Gly10-6Ala-LHRH-ethylaminde) (Syndel Laboratories Ltd. Canada). Adult opihi were collected from Oahu Island and held in tanks in which biofilms were grown in moving seawater in the Laboratory, University of Hawaii at Manoa, Hawaii. Seawater (35‰) was obtained from the Waikiki aquarium. Twelve opihi (9.17±3.17g) were tested and each was weighed for BW and tagged for individual identification. Each opihi received a total of 6 injections at 7-day intervals at dose of 250ng.g^{-1}BW. This dose was chosen because it was reported to be adequate for abalone (Nuurai et al., 2010). The control was run with saline injection. Prior to beginning the experiment, several animals were sacrificed to obtain initial GSI and histological assessment of gametogensis. At least 2-3 animals were sacrificed weekly and gonads immediately fixed in 10% formalin for histological examination at Pathology Laboratory, Queen Hospital, Hawaii. The GSI was examined based on the formula:

$$GSI = \frac{GW}{BW} x100$$ where GW=gonad weight; BW=soft body weight.

Sexually mature opihi were selected for spawning trials. Each individual received a single dose of 1000ng.g^{-1}BW at 10:00pm. Opihi were injected with saline solution instead of hormone served as the control. After injection, the animals were placed into the spawning jars (3 l). The jars were lined by plastic and supplied with aeration. The duration of spawning and number of spawned opihi were observed. Spawning parameters such as fecundity, fertilization rate, and hatching rate were determined in this study. The embryonic and larval developments were observed continuously under a compound microscope.

Results and discussion

The gonads were rapidly developed after the third injection (Fig. 1). GSI of opihi increased significantly after two weeks and over the 4-weeks period compared to the control. Sections of gonads showed proliferative stages in maturation when treated with hormone (Fig. 2).

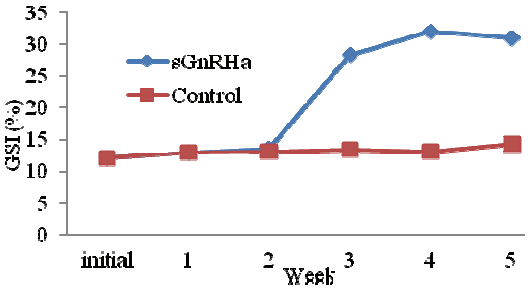


Fig. 1. Gonadosomatic growth of opihi by hormone treatment.

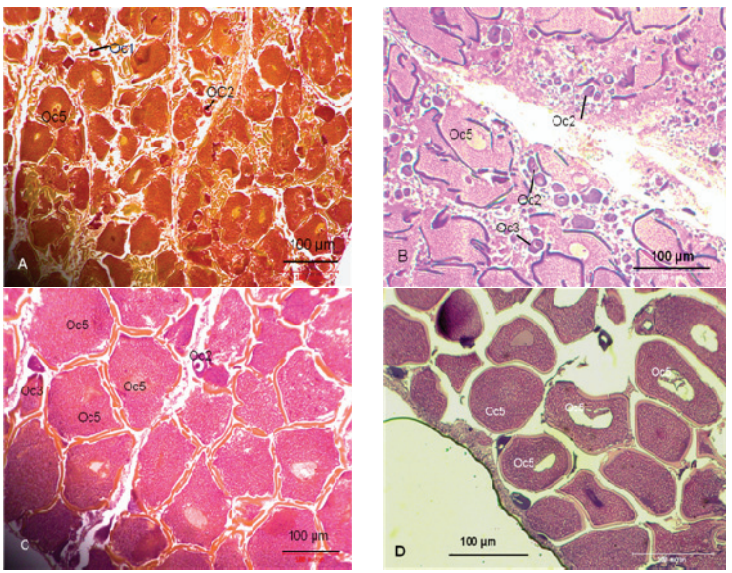


Fig. 2. The effect of hormones on gonad maturation (haematoxylin-eosin staining); (A) initial week of injection showing a proliferative stage with several stage I (Oc1), II oocytes (Oc2). (B) Control group without hormone injection after 5 week. (C) Hormone treatment after 4 weeks and (D) Gonad maturation stage after 5-week injections with 250ng.g^{-1}BW, showing fully mature stage V oocytes (Oc5).

The OvaRH also induced gamete release in ripe opihi (Table I). The average percentage of spawned opihi was 33% and no spawning occurred in the control group. Spawned eggs after fertilization were normal embryos and developed to complete free-swimming veligers within the water column. It was noticed that the induction of spawning of opihi by sGnRHa was effective only in ripe opihi. A low (33%) percentage number of opihi spawned in this study may reflect involvement of a level of unripe animals.

Table I. Induction of spawning on opihi by using sGnRHa.

Trial	n	No. of spawn	% spawn	No. eggs	Fecundity (egg.g^{-1}BW)	Fertilization rate (%)	Hatching rate (%)
1	8	3 (2F, 1M)	38	69 440	11 573	32±5.6	53±12.5
2	8	3 (M)	38				
3	8	2 (M)	25				
Control	5	0	0				

Notes: n, number of tested animal; F, female; M, male

Our hope is to mature opihi and these results show the possibility of the existence of a form of GnRH involved in regulating reproduction in opihi. Similarly, the maturation and spawning of abalone were stimulated by using mammalian GnRHa and octopus GnRHa (Nuurai et al., 2010). It has been reported that GnRH is involved in the control of reproductive processes of gastropod species (Zhang et al., 2000). Other studies reported the presence of GnRH immunoreactivity in the central nervous system and gonadal tissues of gastropods (Zhang et al., 2008). Our preliminary findings provide a new aspect of hormone control of reproduction of opihi, which could be applied to opihi aquaculture.

Acknowledgments

The authors wish to thank the Centre for Tropical and Subtropical Aquaculture and the Vietnam Education Foundation for financial support and Dr. Clyde Tamaru for useful discussion.

References

Corpuz G.C. 1981. Laboratory of *Cellana exarata* reeve (Gastropoda: prosobranchia, Patellidae). Aquaculture 24:219-231.

Nuurai P., A. Engsusophon, T. Poomtong, P. Sretarugsa, P. Hanna, P. Sobhon, and C. Wanichanon. 2010. Stimulatory Effects of Egg-Laying Hormone and Gonadotropin-Releasing Hormone on Reproduction of the Tropical Abalone, *Haliotis asinina* Linnaeus. Journal of shellfish Research 29 (3):627-635.

Zhang L., J.A. Tello, W. Zhang, and S. Tsai. 2008. Molecular cloning, expression pattern, and immunocytochemical localization of a gonadotropin-releasing hormone-like molecule in the gastropod mollusk, *Aplysia californica*. General and Comparative Endocrinology 156:201-209.

Zhang L., N.L. Wayne, N.M. Sherwood, H.R. Postigo, and P.S. Tsai. 2000. Biological and immunological characterization of multiple GnRH in an opisthobranch mollusk, *Aplysia californica*. General and Comparative Endocrinology 188:77-89.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

PROMOTING OF BACTERIA GROWTH BY MANIPULATING CARBON/NITROGEN RATIO AND USE AS MICROALGAE SUBSTITUTION FOR FILTER FEEDERS: A DEMONSTRATION ON *ARTEMIA* CULTURE

Huynh Thanh Toi[1,2], Nguyen Van Hoa[2], P. Bossier[1], P. Sorgeloos[1], and G. Van Stappen[1]

[1] Laboratory of Aquaculture & *Artemia* Reference Center, Rozier 44, B-9000, Gent, Belgium
[2] College of Aquaculture and Fisheries, Cantho University, 2/3 street, Ninhkieu district, Cantho City, Vietnam

Introduction

Among live food organisms, *Artemia* (nauplii and adults) are extensively used for the larval stage of fish and crustaceans because they satisfy nutritional requirements, are convenient to use, and are readily available (Sorgeloos et al., 2001). Outdoor and indoor *Artemia* mass production has been applied in nearly all hatcheries. Thanks to its particular biological characteristics, yeast, microalgae, and low-nutrient diets from agriculture by-products (e.g., rice, bran, soybean meal, animal waste (Anh et al., 2009)) are offered to *Artemia.* With the drive to commercialize aquaculture, efforts have been made to increase production per culture unit, reduce production costs, and reduce nitrogen load from aquaculture. In recent years, the reduction of production costs has been done in retaining more nitrogen from foods in the host animals via heterotrophic bacteria/biofloc techniques (Avnimelech, 1999). The size of biofloc is between 0.1 to a few mm (Avnimelech, 2011) and it can serve as a high natural protein for target animals (Hari et al., 2004; Nootong et al., 2011). In addition, stimulating bacterial growth in *Artemia* cultures has been proven to increase *Artemia* biomass production (Toi et al., 2013), but the use of bacteria as food substitute remains unclear. Hence, we investigate manipulating the C:N ratio to convert nitrogen excreted from *Artemia* waste to bacterial biomass, and use as substitute foods for *Artemia* culture to produce *Artemia* biomass for environmently friendly aquaculture with low production cost.

Materials and methods

Non-viable *Tetraselmis* sp. microalgae (Instant algae 3600; Reed Mariculture Inc. USA) were offered to *Artemia* (*A. franciscana)* in four different feeding re-

gimes: standard feeding (SF1) or ad libitum feeding, half of standard feeding (SF1/2), one third of SF1 feeding regime (SF1/3), and one fourth of SF1 feeding regimes (SF1/4). Nitrogen waste from *Artemia* per day was converted to bacterial cells by stimulating growth. Sucrose was used as a carbon source to manipulate carbon:nitrogen (C:N) ratio (Avnimelech, 1999) to convert nitrogen waste from *Artemia* in each treatment to bacterial biomass. The experiment was run in 15d at 28.0±0.5°C. Each treatment was conducted with three replications. 1-l conical glass tubes were used and each tube was filled with 800ml of 0.2-µm filtered Instant Ocean seawater (FIOSW) at 33g.l^{-1} salinity (Naegel, 1999). *Artemia* nauplii instar I were stocked at 2 nauplii.ml^{-1}.

At the end day of study periods, the uneaten food in the *Artemia* gut was purged by feeding *Artemia* with 20µm cellulose particles (Sigma). Final survival and total biomass (TBP) (g.l^{-1}) was obtained after cellulose-treated *Artemia*. Afterwards, individual length (IL) of *Artemia* was determined according to a methodology described by Marques et al. (2004). To assess the utilization of heterotrophic bacteria, the fatty acids of *Artemia* biomass were analyzed to compare with fatty acids in microalgae and bacteria (grown on sucrose and nitrogen). The methodology of FAME analysis was modified from Lepage and Roy (1984).

Survival, individual length, biomass production, and fatty acid data were evaluated by one-way analysis of variance (ANOVA), followed by Tukey´s honest significant difference (HSD) test (Statistica 7.0 software for windows) employed at 0.05 probability level.

Results and discussions

The result of survival showed that the *Artemia* in stimulated treatments was significantly increased when compared to corresponding control treatments ($P<0.05$) in all feeding regimes, except for stimulated SF1/3 treatment was not significantly increased ($P>0.05$) when compared to its corresponding non-stimulated treatment.

The IL of *Artemia* cultures was only increased in stimulated treatment where *Artemia* received SF1 and SF1/2 feeding regimes, while the addition of sucrose reduced the body length of *Artemia* in SF1/3 and SF1/4 treatments. The decrease of IL was related to high survival of *Artemia* and lack of food supply. The increase/decrease of *Artemia* body length was not significant ($P>0.05$), except *Artemia* in stimulated SF1/3 treatment ($P<0.05$). Moreover, the TBP was significantly improved in all stimulated treatments when compared to corresponding control treatments ($P<0.05$). Especially, the addition of sucrose to the lower feeding regimes SF1/2 and SF1/3 was more or less equal to that obtained in solely algae-fed *Artemia* at SF1 (the control 1). Bacteria have been reported as the direct foods for *Artemia* (Gorospe et al., 1996). Moreover, bacteria may con-

tribute digestive enzymes to degrade algae in *Artemia* (Intriago and Jones, 1993). The results of TBP in our study indicate that *Artemia* receives more supplemental nutrients from bacteria in conditions of low algae supply.

Table I: The final survival (%), IL (mm), and TBP in wet weight ($g.l^{-1}$), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acid (PUFA) composition ($mg.g^{-1}$ DW) of *Artemia* fed on different algal paste rations and stimulation bacterial growth by sucrose. The values are mean ± standard deviation (n=3). Different superscript letters in the same column denote significant differences ($P<0.05$). SF denotes for standard feeding regime, S10 denotes for sucrose addition to produce C:N ratio 10.

Treatment	Survival (%)	Individual length (mm)	Biomass production ($g.l^{-1}$ WW)	MUFA ($mg.g^{-1}$ DW)	PUFA ($mg.g^{-1}$ DW)
Tetraselmis sp.				19.7±0.8	57.1±2.6
Bacteria				37.7	2.7
SF1 (control 1)	36.4 ± 12.7^{bc}	7.0 ± 1.6^{ab}	2.8 ± 0.6^{cdef}	23.3 ± 1.9^{bc}	27.5 ± 1.2^{b}
SF1+S10	62.4 ± 25.8^{cd}	7.9 ± 1.7^{c}	4.6 ± 1.7^{ef}	26.0 ± 2.1^{c}	26.4 ± 1.9^{ab}
SF1/2 (control 2)	41.1 ± 13.4^{bcd}	6.7 ± 1.1^{abc}	2.1 ± 0.2^{d}	21.1 ± 0.2^{abc}	23.9 ± 1.2^{ab}
SF1/2+S10	75.9 ± 5.3^{d}	6.9 ± 1.1^{abc}	3.5 ± 0.7^{f}	23.6 ± 1.9^{bc}	23.4 ± 0.9^{ab}
SF1/3 (control 3)	22.7 ± 4.1^{ab}	7.2 ± 1.4^{bc}	1.2 ± 0.3^{ab}	20.3 ± 1.2^{ab}	21.8 ± 2.1^{ab}
SF1/3+S10	61.1 ± 11.6^{cd}	5.9 ± 1.1^{a}	2.7 ± 0.3^{d}	25.6 ± 2.5^{c}	19.7 ± 1.3^{a}
SF1/4 (control 4)	14.4 ± 8.1^{a}	6.4 ± 1.3^{ab}	0.6 ± 0.3^{a}	19.5 ± 0.2^{a}	23.2 ± 1.5^{ab}
SF1/4+S10	42.0 ± 7.6^{bcd}	6.1 ± 1.3^{ab}	1.4 ± 0.3^{b}	23.7 ± 0.6^{c}	20.2 ± 0.6^{ab}

The difference of fatty acids composition between algae and bacteria can be used as the fatty biomarkers (Chamberlain et al., 2005) to assess the bacteria assimilation by *Artemia* (Intriago and Jones, 1993). The total monounsaturated fatty acids (MUFA) ($mg.g^{-1}$ DW) of *Artemia* in stimulated treatment are evidently higher than control treatments. Particularly, the MUFA of *Artemia* in stimulated treatments receiving SF1/3 and SF1/4 was significantly higher ($P<0.05$) than obtained in corresponding non-stimulated treatments. MUFA is the major component fatty acid in bacteria, whereas this fatty acid is low in microalgae. The increase MUFA in *Artemia* in stimulated treatments may be derived from bacteria. The level of PUFA and its fraction was not significantly different between stimulated treatments and non-stimulated treatments ($P>0.05$).

Conclusions

The stimulation of bacteria growth in *Artemia* by using a carbon source improves the survival and total biomass production when lacking a microalgae supply. Particularly, the biomass production of stimulated treatment receiving SF1/3 algae feeding regimes nearly equals to that obtained in solely microalgae fed *Artemia* at SF1 feeding regimes. The result of MUFA in *Artemia* indicates that *Artemia* consumes more bacteria when lacking a microalgae supply.

References

Anh N.T.N., N. Van Hoa, G. Van Stappen, and P. Sorgeloos. 2009. Effect of different supplemental feeds on proximate composition and *Artemia* biomass production in salt ponds. Aquaculture 286(3-4):217-225.

Avnimelech Y. 1999. Carbon/nitrogen ratio as a control element in aquaculture systems. Aquaculture 176(3-4):227-235.

Avnimelech Y. 2011. Tilapia Production Using Biofloc Technology, Saving Water, Waste Recycling Improves Economics. Global aquaculture Advocate, May/June 2011: pp.66-68.

Brown M.R., S.M. Barrett, J.K. Volkman, S.P. Nearhos, J.A. Nell, and G.L. Allan. 1996. Biochemical composition of new yeasts and bacteria evaluated as food for bivalve aquaculture. Aquaculture 143(3-4):341-360.

Chamberlain P.M., I.D. Bull, H.I.J. Black, P. Ineson, and R.P. Evershed. 2005. Fatty acid composition and change in Collembola fed differing diets: identification of trophic biomarkers. Soil Biology and Biochemistry 37(9):1608-1624.

Gorospe J.N., K. Nakamura, M. Abe, and S. Higashi. 1996. Nutritional Contribution of Pseudomonas sp. in *Artemia* culture. Fisheries Science 62(6):914-918.

Hari B., B. Madhusoodana Kurup, J.T. Varghese, J.W. Schrama, and M.C.J. Verdegem. 2004. Effects of carbohydrate addition on production in extensive shrimp culture systems. Aquaculture 241(1-4):179-194.

Intriago P. and D.A. Jones. 1993. Bacteria as food for *Artemia*. Aquaculture 113(1-2):115-127.

Lepage G. and C.C. Roy. 1984. Improved recovery of fatty acid through direct transesterification without prior extraction or purification. Journal of Lipid Research 25(12):1391-6.

Marques A., J.-M. François, J. Dhont, P. Bossier, and P. Sorgeloos. 2004. Influence of yeast quality on performance of gnotobiotically grown *Artemia*. Journal of Experimental Marine Biology and Ecology 310(2):247-264.

Naegel L.C.A. 1999. Controlled production of *Artemia* biomass using an inert commercial diet, compared with the microalgae Chaetoceros. Aquacultural Engineering 21(1):49-59.

Nootong K., P. Pavasant, and S. Powtongsook. 2011. Effects of Organic Carbon Addition in Controlling Inorganic Nitrogen Concentrations in a Biofloc System. Journal of the World Aquaculture Society 42(3):339-346.

Sorgeloos P., P. Dhert, and P. Candreva. 2001. Use of the brine shrimp, *Artemia* spp., in marine fish larviculture. Aquaculture 200(1-2):147-159.

Toi H. T., P. Boeckx, P. Sorgeloos, P. Bossier, and G. Van Stappen (2013). Bacteria contribute to *Artemia* nutrition in algae-limited conditions: A laboratory study. Aquaculture 388-391(0):1-7.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

NANOPARTICLES AS A NOVEL DELIVERY SYSTEM FOR VITAMIN C ADMINISTRATION IN AQUACULTURE

E. Jiménez-Fernández[1], E. Zuasti[1], A. Ruyra[2], N. Roher[2], C. Infante[1], and C. Fernández-Díaz[1]*

[1] IFAPA Centro El Toruño, Junta de Andalucía. Camino Tiro pichón s/n, Apdo 16, 11500 El Puerto de Santa María (Cádiz), Spain
[2] Institute of Biotechnology and Biomedicine, Dep. Biologia Cellular, Immunologia i Fisiologia Animal, Universitat Autònoma de Barcelona, 08193 Barcelona, Spain.

Studies on specific vitamin C (ascorbic acid) requirements are scarce in marine fish larvae and juveniles due to its poor retention in inert foods. The application of nanotechnology offers the unique opportunity to achieve more effective procedures in order to supply unstable and/or hydrosoluble micronutrients. The aim of this study was to adapt and assess the potential of a chitosan-based nanoencapsulation system as a tool to deliver ascorbic acid to marine organisms under aquaculture conditions. For this purpose, in vitro performance of these nanoparticles (NPs) was evaluated in a zebrafish liver cell-line (ZFL) and in vivo studies were carried out in juvenile sole (*Solea senegalensis*) and rotifers (*Brachionus plicatilis*).

In this study, NPs were prepared by the ionotropic gelation technique. The particle size and zeta potential were analysed using a Zetasizer Nano ZS. The ascorbic acid release from ascorbic acid-loaded NPs (AA-NPs) was evaluated in 30ppt seawater at 20ºC during 2h. To analyze the ascorbic acid content, the samples were processed using high performance liquid chromatography (HPLC). The results showed that these NPs are suitable to trap hydrosoluble ascorbic acid with encapsulation efficiency (EE) of 20%. In addition, AA-NPs have nanometric size (200-250nm), are positive charged (30-35mV), and are stable in seawater with a total release of ascorbic acid lower than 7% after 2h of incubation.

The potential cytotoxicity of the NPs was evaluated in ZFL cells using the MTT assay. Briefly, ZFL cells were deprived in basal medium for 3h and treated with 0, 0.1, 0.25, 0.5, 1, 2.5, 3.5, and 5.0mg.ml^{-1} of NPs solution for 24h. Finally, MTT was added to the cells and cell viability was expressed as a percentage of the control. Cell viability did not decrease up to a NPs concentration of 2.5mg.ml^{-1} (relative cell viability>90%); it decreased for values close to 40% at higher NPs concentrations. Thus, to avoid potential side-toxic effects, these NPs should be used at doses below 2.5mg.ml^{-1}. The NPs uptake was analysed in ZFL

cells by cytometry and confocal laser microscopy. Time course and dose-response experiments were performed using fluorescein isothiocyanate labelled NPs (FITC-NPs). The time-course analysis was done at 1, 3, 6, 12, 16, 24, and 48h post-treatment and the dose-response assays were done at 6h using different FITC-NPs doses (0.25, 0.5, 1, and 2.5mg.ml^{-1}). The in vitro endocytosis assays with ZFL cells showed a maximum uptake after 6h of incubation and a dose–dependent increase of fluorescence intensity directly proportional to the FITC-NPs concentration. Confocal-laser microscopy imaging was performed at non-toxic doses and at optimized incubation time in order to determinate whether these NPs were efficiently endocytosed. Our results showed the presence of FITC-NPs agglomerates in intracellular compartments. The antioxidant effect of AA-NPs was also analysed in ZFL cell extracts. Lipopolysaccharide (LPS) at a final concentration of 10µg.ml^{-1} was added to ZFL cells to induce oxidative stress. We have determined the effects of AA-NPs on the antioxidant intracellular levels using an antioxidant assay kit (Sigma). The evaluation of the total antioxidant capacity of the ascorbic acid-treated cells showed a statistically significant increase in the antioxidant capacity respect to the control (74.60±12.12 and 16.75±5.01µM Trolox; $p<0.05$ to AA-NPs and non-loaded-NPs respectively).

We also carried out in vivo studies to evaluate the NPs ability to penetrate fish intestinal epithelium. For that, juvenile sole were anaesthetized and FITC-NPs (10µg.µl^{-1}) were oral administered using a syringe. After 2h, each intestine was fixed, permeabilized, stained and analyzed by confocal laser-scanning microscope. We have demonstrated that these NPs are able to penetrate through intestinal epithelium in juvenile sole as detected by confocal-laser microscope. To perform the rotifers enrichment with AA-NPs, rotifers were placed in seawater (20ºC) at 1000 rotifers.ml^{-1} under constant light conditions. After 24h of starvation, 50mg of AA-loaded or non-loaded NPs were provided for 2h. Control groups fed with free AA were maintained under the same experimental conditions. The AA-NPs were efficiently ingested by rotifers, increasing up to twofold their ascorbic acid levels in comparison to control groups.

Overall these results show that chitosan NPs are a suitable tool to trap hydrosoluble compounds such as ascorbic acid by forming positively charged complexes, in a nanosize range, being highly stable in seawater. In vitro assays demonstrated that these NPs exhibit the ability to enter the cells, deliver the ascorbic acid and increase the total antioxidant capacity in cells. In vivo assays show that these NPs are able to penetrate through the intestinal epithelium in juvenile sole. Finally, rotifers feed with AA-NPs present an increase in its endogenous ascorbic acid content making them suitable for larvae prey. Consequently, the NPs developed in the present work might represent an interesting tool for oral administration of active compounds in aquaculture.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

BACTERIOPHAGE APPLICATION AS A MANAGEMENT STRATEGY IN SHRIMP HATCHERIES

I. Karunasagar, S.K. Girisha, M.N. Venugopal, and M. Biswajit

UNESCO MIRCEN for Marine Biotechnology, Department of Fishery Microbiology, Karnataka Veterinary, Animal & Fisheries Sciences University, College of Fisheries, Mangalore-575002, India

Development of antibiotic-resistant microorganisms in the environment necessitates the use of alternate strategies that control bacterial pathogens without disturbing the natural flora of the environment. Since antibiotic resistance is a major problem in aquaculture, bacteriophages can be used to control the pathogenic bacteria in conjunction with other eco-friendly methods such as use of immunostimulants, probiotics, etc. Bacteriophages, like bacteria, are ubiquitous in nature and are found in the different aquatic environments such as freshwater, estuarine and marine waters. Prevalence of bacteriophages acting on *Vibrio* spp. was studied since vibrios are significant pathogens of larvae in shrimp hatcheries. Water samples from shrimp hatcheries and farms were analyzed for the presence of phages specific for vibrios. A phage specific for *V. fischeri,* five specific for *V. alginolyticus*, and seven for *V. harveyi* were isolated from shrimp farm and hatchery water.

Oysters are known to harbour *V. parahaemolyticus* and *V. vulnificus*. Hence, oyster samples were also analyzed for the presence of vibriophages. A phage specific for *V. parahaemolyticus* and *V.vulnificus* could be isolated.

Experiments were done in microcosms to see whether phages can be used to reduce bacterial load. Results showed that they were able to reduce the bacterial load to significant level. However, the host range of these phages was narrow. Since chitin is present in shrimp hatcheries, a small amount of chitin was also added to the microcosm to see whether it had effect on the phage in reducing the bacterial load. Reduction in bacterial load in the microcosm with chitin was much higher compared to the one without chitin.

Storage ability of phages was studied by storing them under different temperatures and estimating the titer values at different days up to one month. Results showed that phages have good storage stability. The titer was not reduced at 0ºC, -20ºC, and even at room temperature at 1, 5, 15, and 30 days of storage.

Vibrios like *V. harveyi* can survive in hatchery environments by forming biofilms. Therefore, we also studied the biofilm formation on high density polyethylene (HDPE) surfaces and the effectiveness of bacteriophage for biofilm control. Results showed that the effectiveness of bacteriophage treatment on *V. harveyi* biofilm was dose-dependent. Treatment of phage at 10µl, 100µl, and 1000µl to the biofilm on the HDPE surface led to count reductions of 10^1, 10^2, and 10^3 cells, respectively, when enumerated at 18h. These results suggest that bacteriophages have potential applications in biocontrol of vibrios in larval production systems.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

REPRODUCTIVE CAPACITY OF *HEMICULTER LEUCISCULUS* (BASILEWSKY, 1855) IN UZBEKISTAN

E.E. Khurshut

Institute of Flora and Fauna, Academy of Sciences of Uzbekistan. E-mail: ernest.khurshut@gmail.com

The native range of Korean sawbelly (*Hemiculter leucisculus*) is East Asia: from Far East Russia and Mongolia in the north, through China and Korea, to North Vietnam in the south. It was unintentionally introduced into the Aral Sea Basin, Central Asia in the 1960s along with Asian carps. It is currently widespread in the plains of the region up to the foothills in the drainages of the Amu Darya, Syr Darya, Zarafshan, Qashqadaryo, and Tedzhen rivers.

Material was collected from the Balykchi, Damachi, and Tashkent fish farms, Lake Arnasay, Charvak reservoir (Syr Darya River Basin), Zarafshan River, and the Lower Amu Darya. Gonad samples for histological analysis were preserved in Bouin's solution. For tissue processing, ethanol-butanol dehydration and paraffin wax embedding was used. Tissue sections were stained with Heidenhain's iron haematoxylin. Reproductive capacity was characterized by absolute and relative indices: absolute fecundity (AF), relative fecundity (RF), gonadosomatic index (GSI), and maturity coefficient (GSI_{MC}). The last two similar indices were calculated as the ratio of the gonad weight to the body weight without viscera for GSI and to the total body weight for GSI_{MC}, respectively. Average absolute fecundity was estimated by adding the direct calculation of individual absolute fecundity divided by the sample size.

Females of most sawbelly populations in Uzbekistan mature at the age of 2-3 years and a length of 8-10cm. But in the cold-water Charvak reservoir they mature one year later with a length of 13-15cm. Spawning is fractional and takes place from May to July, but in the Charvak reservoir it starts 3-4 weeks later.

GSI_{MC} ranges between 0.4-21.1 (average 5.6±0.46), and GSI between 0.5-31.2 (7.7±0.65). AF averages 19.9±2.2 thousand eggs (1.7-88.9 thousand eggs) and RF is 583±42 eggs per gram of total body weight (86-2075 $eggs.g^{-1}$).

Relationship between the ovarian mass (Wg) and the standard length (SL) or the body weight (Ws) is described by a power regression:

$$Wg = 21.88 \times Ws^{1.328} \ (r=0.853); \ Wg = 0.000002 \times SL^{4.174} \ (r=0.871).$$

A wide variation of the gonad weight of mature females is caused by seasonal changes in the gonads and the peculiarities of reproductive cycles, namely the asynchronous oocyte development and fractional spawning. Mature females with low GSI releasing the eggs batch, as well as females of the same age with high GSI when the next batch is developed, are present during the spawning period. Gonad weight and all related indexes vary less at first maturation.

AF depends on the age, body size, and weight of females. Relationship between absolute fecundity and body length is described by the power equation:

$$AF = 0.0126 \times SL^{2.878} \ (r=0.790).$$

Two to three size classes of oocytes were recorded in gonads. Diameters of oocytes of the two senior batches are highly correlated (r = 0.883). The average diameter of oocytes of the elder batch reaches the maximum in July and then decreases. Size of oocytes of the senior batch determines the degree of gonadal maturity. It correlates with GSI (r=0.704) and GSI_{MC} (r=0.735).

The diameter of oocytes of the elder generation significantly changes with age. Middle-aged females (3-4 years old) have larger eggs than the young and old females.

Females overwinter with the gonads in the third stage of maturity. Vitellogenesis starts in early spring. Vacuoles are visible on the cytoplasm periphery. The nucleus diameter slightly increased (0.75±0.04mm). Multiple nucleoli are located on its periphery. By May, ovaries contain oocytes at different stages of cytoplasmic transformation: previtellogenesis, vacuolization, and trophoplasmic growth. In the most developed oocytes, yolk accumulation begins. First yolk granules appear on the cytoplasm periphery between the vacuoles, and then in the middle zone, gradually filling the entire cell. Vacuoles coalesce into larger droplets to form the cortical alveoli on the cytoplasm periphery.

During spawning, oocytes of different developmental phases forming different batches are visible in ovaries. After spawning, the gonads are again in the II stage, containing previtellogenic oocytes of 0.93±0.02mm diameter with relatively large nuclei (0.62±0.01mm).

The changes occurring in the gonads during the year can be seen in the dynamics of GSI and GSI_{MC}, peaking in July and falling to a minimum in November. Analysis of variance (ANOVA) showed a significant ($P<0.01$) influence of the month on the indexes.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

DEVELOPMENT OF MATURATION DIET FOR PENAEID SHRIMP USING HERBAL EXTRACTS

S. Kolkovski and J. Kolkovski

Nutrakol Pty Ltd, 27 Korella st. Mullaloo, 6027, Western Australia, info@nutrakol.com

Introduction

In recent years, the shrimp aquaculture industry has shifted to utilising genetically improved and domesticated broodstock. Specific Pathogen Free (SPF) broodstock are becoming the industry standard for *Penaeus vanemei.* Other species such as *P. monodon* have been domesticated and many of the producers are using either domesticated broodstock or genetically improved broodstock.

Currently, wild-caught and cultured organisms (squid, polychaetes), coupled with a variety of nutritional boosters, are the basis for standard broodstock diet used by industry. As in any fresh feed, the nutritional profile may fluctuate due to the source, time, and handling of the food. Even more importantly, the fresh feed may act as a vector for transferring pathogens and infecting broodstock.

Considering the importance and the cost of the spawners, a maturation diet that can successfully replace fresh diet is highly sought after. The advantage of formulated diet is the ability to incorporate additives that can support the immune system, hormonal and digestive system, and gonadal development. Most importantly, formulated feed will eliminate the risk of pathogen infection.

Phytotherapy – i.e., the use of herbal extracts in naturopathy for humans – has been known for thousands of years. In some countries such as China, India, and many others, phytotherapy is considered mainstream, while in Western medicine, naturopathy has increased in recognition.

Different medicinal plants and herbs and/or combinations are known to contain anti-bacterial, anti-fungal, physiological system supportive (immune system, digestive system,), and hormonal balancing properties, among others. However, in aquaculture, especially Western intensive culture, herbal remedies and additives are still considered to be anecdotal.

Maturation diet for penaeid shrimp was developed to answer these issues. The small pellets (5mm long, 3mm diameter) have 37% moisture and more than 24h stability in water.

Several herbal extracts were included in the diet to boost the immune system, hormonal cycle, and gonadal development. Table I shows some of the herbal extracts used in shrimp broodstock diets.

Table I. Herbal extracts used in broodstock diets.

Botanical name	Distribution	Reference
Cinnamonum zeylanicum	India, Sri Lanka	Kolkovski et al., 2010
Elettaria cardomomum	India, Burma, Sri Lanka	Kolkovski et al., 2010
Eugenia caryophyllata	India, Sri Lanka	Kolkovski et al., 2010
Mesua ferrea	India, Nicobar Islands Burma, Andaman	Kolkovski et al., 2010
Asparagus racemous	India	Citrasu, 2009
Mucuna pruriens	Tropics	Citrasu, 2009
Witania somnifera	India	Citrasu, 2009

Materials and methods

A large experiment was conducted independently by one of the largest shrimp producers in the world. The company compared the maturation diet to their standard feed, which includes squid (7% BW), polychaetes (7% BW), and nutritional additive booster (0.5%). The diet was given as the sole feed to satiation. Table II shows the chemical analysis of the different feeds.

Table II. Chemical analysis of feeds.

Diet	Moist %	Protein %	Fat, %	Fiber %	Ash %	NFE %	Cholesterol $mg.kg^{-1}$	Carotenoids $mg.kg^{-1}$
Polychaete (avg)	84.95	10.8	1.89	0.30	1.06	1.79	128.5	
Squid	82.80	15.90	1.21	0.42	0.94	1.28	191	
Booster	7.25	54	12.00	0.95	13.4	12.40	824	605
Nutrafeed	38.00	31.90	7.43	2.06	6.84	13.77	480	9000

Four hundred prawn broodstock were used in the experiment. Two hundred white shrimp *L. vannamei* were fed control diet (squid, polychaetes, and nutritional booster) or NutraFeed diet. The experiment was conducted over 124 days. The animals were acclimated for 2 weeks prior to ablation procedure. The broodstock animals were kept in identical tanks and environmental conditions. Growth, mortalities, spawning events, fecundity (number of eggs), hatching rates, and number of nauplii were determined.

Results

Broodstock fed with the maturation diet demonstrated significant performance improvements (up to 40%, Table III) compared to animals fed with traditional feeds (fresh/frozen organisms). Spawning events increased by almost 30% and

total number of nauplii produced increased by over 46%. The maturation diet also improved the broodstock survival by 44% compared to animal fed on traditional feed.

Table III. Spawning results for *P. vannamei* broodstock fed different diets.

Treatment	%Mort per day[1]	Total Spawns[2]	Eggs per Female[3]	Nauplii per Female[4]	Total Nauplii
Standard	0.09%	602	179 364	154 252	92 860 000
NutraFeed	0.05%	849	186 266	160,188	136 000 000
Difference	44.4%	29.1%	3.7%	3.7%	46.5%

[1]Percentage broodstock died (out of 200 initial number) each day (average), [2]Number of spawning events for all the tank, [3]Average number of eggs for each female, [4]Average number of nauplii for each female, [5]Total number of nauplii produced in each treatment for the duration of the experiment (124 days).

Conclusions

A maturation diet for penaeid shrimp containing herbal extracts can play an important role in shrimp broodstock nutrition. The benefits are not only improving the physiological condition of the broodstock but also resulting in better spawning performances over multiple spawning events, better eggs and larval quality, and higher stress resistance resulting in better survival.

While a formulated diet can significantly improve broodstock performances when used as a booster, complete replacement of fresh and frozen feeds can be achieved; reducing or eliminating health risks and achieving stable a nutritional profile over time.

References

Citrasu T. 2009. Herbal biomedicines: a new opportunity for aquaculture industry. Aquaculture International 18:3.

Kolkovski S., J. Kolkovski, M. Babu, and T. Citarasu. 2010. Development of maturation diet for black tiger shrimp *penaeus monodon* using herbal extracts and other natural ingredients. Word Aquaculture Society symposium proceedings, San Diego, CA.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

ASSOCIATED EFFECTS OF BACTERIA ON *OCTOPUS TETRICUS* LARVAE REARING

S. Kolkovski, J. King, N. Watts, M. Natale, J. Bannister, and F. Stephens

Department of Fisheries, Western Australia, Marine Research Laboratories, PO Box 20, North Beach, Western Australia 6920, Australia

Introduction

For several years, octopus aquaculture has gained significant attention around the world. Spain, Italy, Greece, Mexico, Chile, Australia, and several other countries have R&D projects aimed at closing the octopus life cycle. However, to date, there has been no significant breakthrough in this area. *Octopus tetricus* is endemic to Western Australia and very similar in its life stages, growth, and other parameters to the Mediterranean species *O. vulgaris*. These two species have a fairly long pelagic stage of larvae ranging between 45-60 days. Most of the environmental factors to sustain optimal growth are still unknown, as well as, the nutritional requirements of these species.

During the past two years, a project looking at the hatchery stages of *O. tetricus* has achieved a significant amount of information on the environmental conditions (light, temperature, tank hydrodynamics), food (size, feeding routing, etc) and nutritional requirements. Different systems were developed to assist with continuous live feed supply.

One of the problems with octopus larvae is the bacterial growth in tanks as a result of a unique feeding method, which leaves the dead *Artemia* in the water after their internal liquids are 'sucked out'. Disinfecting the water (UV) did not improve survival since the *Artemia* 'leftovers' remained in the tank. After analysis of water and larvae samples, it was discovered that larvae were becoming encased in a 'mat' of filamentous bacteria. A ten-day course of antibiotic therapy (99% Oxytetracycline hydrochloride) was employed in an attempt to eradicate the bacterial load and improve paralarva survival. Paralarvae were histologically sampled to examine their development and their gut condition was also investigated.

Materials and methods

Larvae for this trial were hatched from eggs that were spawned by wild-caught female octopi. Eggs were incubated for 35 days prior to hatching. Larvae were reared in a flow-through (filtered seawater, 21°C) system comprised of seven 40-l cylindrical tanks. Tanks were operated as upwelling with a 24h photoperiod (450-600lux).

Paralarvae were fed to satiation with 14-day-old enriched *Artemia* (Octopus enrichment, Nutrakol Pty Ltd). Both treatments were fed a semi-moist microdiet 220-500µm (Nutrakol Pty Ltd) and live *Portunus pelagicus* larvae from 7dph onwards before each *Artemia* feeding event. A 10-day course of antibiotics was given to 4 tanks and 3 tanks were used as control without antibiotics. Oxytetracycline was dissolved in the water and water flow was stopped for 60min. Every second day, the larvae were individually removed from the rearing tanks to new, clean tanks using a pipette.

Results

While survival in the first 3 weeks was higher in the antibiotic treatment, at the trial completion (3), the antibiotic treatment did not result in higher paralarvae survival with an average of 0.3%±0.29 and 1.41±1.4% survival in the antibiotic and control treatment, respectively (Fig. 1). There was a steep decrease in survival across both treatments from 21dph onward.

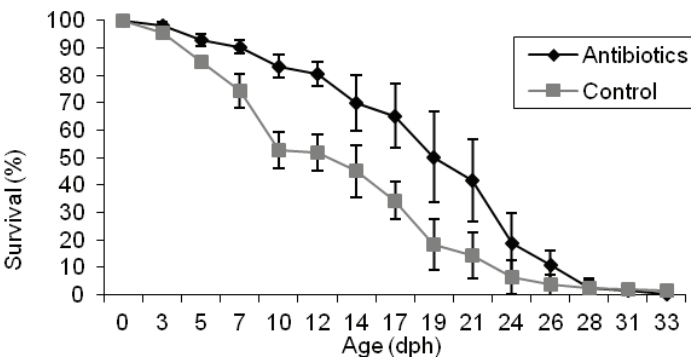


Fig. 1: Percent survival of larvae in tanks with or without antibiotic treatment across trial duration.

Throughout the trial, samples of larvae from the control treatment (1) and from the antibiotic treatment (2) were taken for histological analysis and routine weekly health testing. It was found that some larvae from Treatment 2 had filamentous bacteria present in the connective tissue and the skin (Fig. 2). The de-

velopment of haemocytes was also found in Treatment 2; haemocytes are specialised cells that play a role in the immune system of invertebrates. The presence of haemocytes suggests that larvae are developing some immune response to bacteria. The digestive gland of larvae from treatment 1 samples appeared to be better developed in comparison to treatment 2, where the gut enterocyte cells appeared irregular in size and unstructured within the gut. Vacuoles within a number of larvae were found to have accumulated undigested material within lysosomes, indicating that the digestive system is not functioning properly and food is not being efficiently broken down.

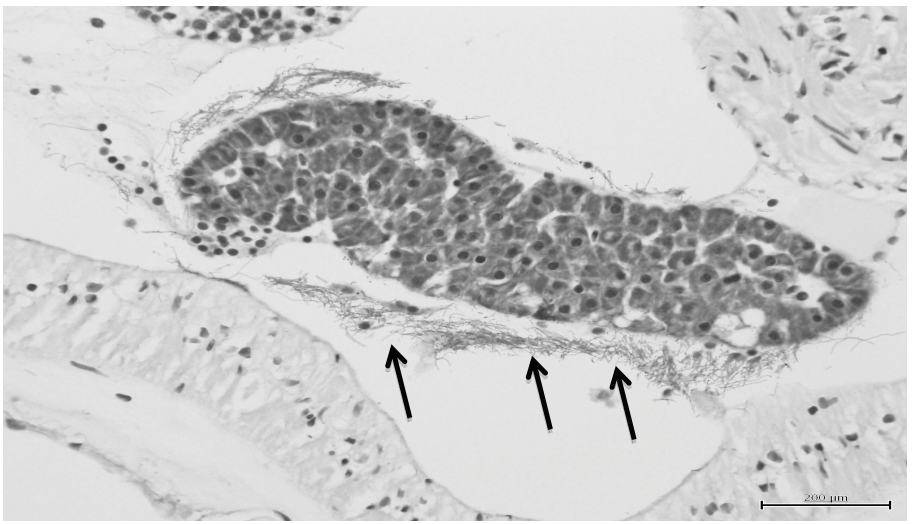


Fig. 2: Mat of filamentous bacteria in connective tissue and dermis of 19-dph larvae.

Toward the end of the trial, several larvae were 'swollen' and filled with fluid between the muscle tissue and the skin (Fig. 3). This fluid is most likely haemolymph that has leaked out due to breakdown of the gut. This fluid accumulation has caused the body to swell. A healthy specimen with normal gut structure would normally be able to reabsorb this fluid.

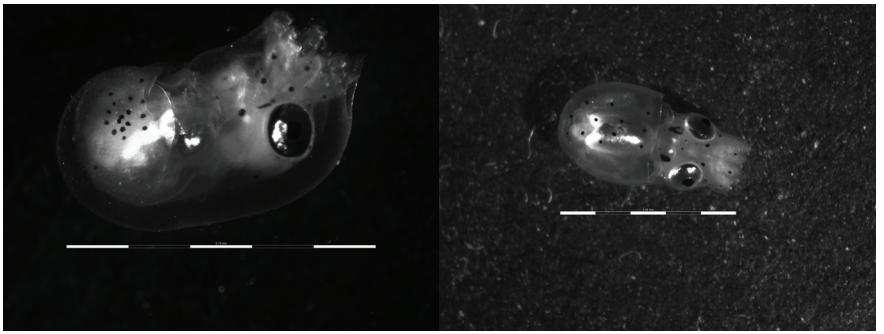


Fig. 3: Larvae with dilated haemolymph channel at 23dph (left) and normal larvae at 33dph (right)

Conclusions

Antibiotics did not aid in reducing bacteria or improving survival. The gut condition was also found to be poorer in paralarvae that received antibiotic treatment. This suggests that paralarvae survival after 14-21dph is affected by nutrition more so than bacteria. These results are in agreement with other publications on *O. vulgaris* (Iglesias and Fuentes, 2013)

References

Iglesias J. and L. Fuentes. 2013. Research on the production of hatchery-reared juveniles of cephalopods with special reference to the common octopus (Octopus vulgaris). P.374-403. In: Advances in aquaculture hatchery technology. Allan G. and Burnell G. (EDs). Woodhead Publishing, Oxford, Cambridge, Philadelphia.645p.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

THE EFFECT OF pH ON THE FERTILIZABILITY OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) EGGS STORED IN A CHILLED STATE

M. Komrakova, U. Kuembet, and W. Holtz

Department of Animal Science, Georg-August-University, Albrecht-Thaer-Weg 3, 37075 Goettingen, Germany

Abstract

To verify the observation that fertilizability of stored rainbow trout eggs is affected by deviations in pH (Komrakova and Holtz, 2011) freshly collected eggs were immersed in coelomic fluid adjusted to pH 6, 7, 8, 9, or 10 (3 replicates each) and stored at 2°C. At 0, 1, 24, 48, and 96h, pH was measured and eggs were inseminated with cryopreserved semen. Egg development until the eyed stage proceeded normally in all cases. However hatching rates, amounting to 70% in controls (pH 8, SEM 0.02), were reduced to 50% in samples exposed to pH 7 or pH 9 and to 40% and 30% in samples exposed to pH 6 and pH 10, respectively. In conclusion, when eggs are subjected to coelomic fluid with deviating pH, the damage afflicted upon the eggs will not become evident until they are due to hatch.

Introduction

In salmonids, the composition of coelomic fluid is optimally suited for storing eggs and prolonging their fertilizability during natural spawning and artificial fertilization conditions (Lahnsteiner et al., 1995). Coelomic fluid of rainbow trout has a pH of 8.3-8.4 (Lahnsteiner et al., 1995; Komrakova et al., 2009; 2011). pH declines if eggs are overripe or stored in vitro (Komrakova et al., 2009), whereas in vivo an accumulation of aspartate aminotransferase, protein, and esterified and non-esterified fatty acids is said to be responsible for the decrease in pH (Lansteiner, 2000). During in vitro storage, the pH change is associated with an accumulation of carbon dioxide resulting from respiration of stored eggs (Komrakova et al., 2009). Low pH during post-ovulatory oocyte ageing is considered an indicator of poor egg quality (Lahnsteiner et al., 1999; Aegerter and Jalabert, 2004), the mechanism of action being unknown (Lahnsteiner et al., 1999; Komrakova et al., 2009). The aim of the present investigation was to study the effect of pH on the developmental potential of rainbow trout eggs stored in a chilled state.

Materials and methods

At the Aquaculture facility of the Goettingen University Experimental Farm Relliehausen, 3 to 5-year-old rainbow trout spawners were stripped during the spawning season. Strippings from several spawners were pooled. Coelomic fluid was separated from eggs with the aid of a sieve and portions of coelomic fluid were adjusted to pH 6, 7, 8, 9, and 10, respectively, by addition of 1M NaOH or 1M HCl. One portion remained at the original pH of 8.3. Eggs were distributed four layers deep (on average 160 eggs) to capped photofilm vials (i.d. 29mm, height 50mm) and 5ml of the pH-adjusted coelomic fluid was added. After 1, 24, 48, and 96 hours of storage at +2°C, pH in coelomic fluid was measured and eggs were inseminated with uniform frozen-thawed semen (8×10^6 spermatozoa.egg^{-1}) pooled from several males and cryopreserved as 0.1-ml pellets as described in Holtz (1993). Eggs were incubated in a vertical flow incubator (Veco, Horgen, Switzerland) at water temperature of 10°C until hatching. Treatments were conducted with three replicates.

Statistical analysis was performed using SAS (Version 9.3). Changes in percentage of eyed eggs and hatched fry were analyzed using two-way analysis of variance (ANOVA, F-test) with duration of storage and pH as fixed effects. Differences between individual means were assessed by Scheffé-test ($P<0.05$).

Results and discussion

As shown in Fig. 1A, the original differences in pH of coelomic fluid among groups set by addition of HCl or NaOH, was less extreme after addition of the eggs. Thereafter, it remained constant until the end of the experiment. It may be assumed that the change in pH is partly a dilution effect, and, according to Czihak et al. (1979) partly due to the capacity of unfertilized and fertilized trout eggs to regulate the pH of the surrounding medium, probably by releasing buffering ions.

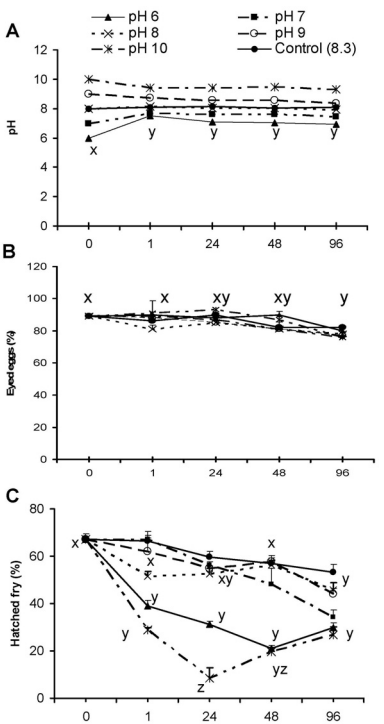


Fig. 1. pH (A), percent eyed eggs (B) and percent hatched fry (C) in unfertilized rainbow trout eggs immersed in coelomic fluid of different pH (control, 6, 7, 8, 9, and 10) stored at 2°C for 1, 24, 48 and 96 hours. (xyz: means with different letters in eggs stored at pH 6,7,9, and 10 differ between hours of storage; C: pH 7 and 9 shown with the same letters).

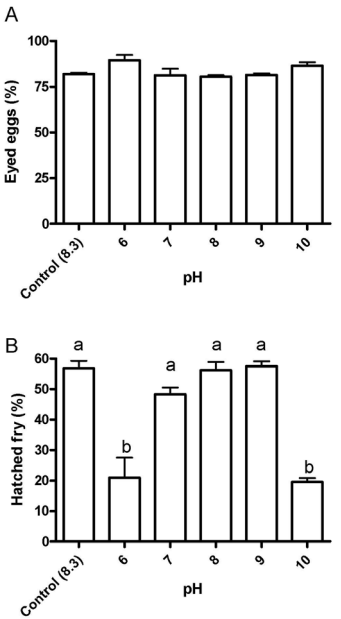


Fig .2. Percent eyed eggs (A) and hatched fry (B) in eggs stored in coelomic fluid at pH 6, 7, 8, 9, 10 and control (pH 8.3) for 48 hours. (A: differences are not significant; B: means with different letters differ ($P<0.05$).

Up to the eyed egg-stage the overall effect of storage time was non-significant (Fig. 1B, Fig. 2A). Only if focusing on samples in which the pH deviated from the physiological level of 8.3 in either direction a significant decrease in percent eyed eggs by between 11-15% after 96 hours of storage was recorded ($P<0.05$). In contrast to the unspectacular differences at the eyed stage, at hatching differences among groups were prominent (Fig. 1C, Fig. 2B). The most spectacular decline in hatching rate was encountered in eggs exposed to pH 6 and pH 10, perceivable after as little as one hour. Revealingly, no correlation was found between percentage of eyed and percentage of hatched eggs ($r = -0.0098$, $P=0.934$). The pH of coelomic fluid did not correlate with either parameter ($r=0.13$, $P=0.27$ for pH and eyed eggs; $r=0.14$, $P=0.23$ for pH and hatched eggs). In most studies high correlations were observed between the numbers of eyed eggs and hatched fry. Storage conditions, including different gas atmospheres, number of layers and duration of storage had no effect on further development of eyed eggs (Babiak and Dabrowski, 2003; Komrakova et al., 2009) and it is generally assumed that embryos reaching the eyed stage will continue developing with only minor losses. In many cases the eyed stage was taken as index for fertilizing capacity of eggs (Komrakova et al., 2009). Studies on the effect of pollutants on fish egg and larva development showed that in water of low pH (5.2) embryos of rainbow trout are prevented from hatching and that hatching activity is immediately resumed when the pH is increased. The impairment of the hatching process at low pH seems to occur due to inhibition of the enzyme chorionase, the activity of which is optimal at pH 8.5 (Hoar and Randall, 1988).

In conclusion, the present study verifies the interpretation of previous studies (Komrakova et al., 2009; 2011) suggesting that during chilled storage of rainbow trout eggs in hermetically closed containers it is the change in pH occurring as a result of carbon dioxide accumulation that is responsible for the detrimental ef-

fect on fry production. Apparently embryonic development appears to proceed normally, whereas subsequent steps of development are impaired.

References

Aegerter S. and B. Jalabert. 2004. Effects of post-ovulatory oocyte ageing and temperature on egg quality and on the occurrence of triploid fry in rainbow trout, *Oncorhynchus mykiss*. Aquaculture 231:59-71.

Babiak I. and K. Dabrowski. 2003. Refrigeration of rainbow trout gametes and embryos. Journal of Experimental Zoology 300A:140-151.

Czihak G., R. Peter, B. Puschendorf, and H. Grunicke. 1979. Some data on the basic metabolism of trout eggs. Journal of Fish Biology 15:185-193.

Hoar W.S. and D.J. Randall. 1988. Fish Physiology. Volume XI. The Physiology of developing fish. Part A. Eggs ad Larvae. Academic Press INC (London) LTD.

Holtz W. 1993. Cryopreservation of rainbow trout (Oncorhynchus mykiss) sperm: practical recommendations. Aquaculture 110:97-100.

Komrakova M. and W. Holtz. 2009. Factors responsible for successful chilled storage of unfertilized rainbow trout (Oncorhynchus mykiss) eggs. Aquaculture 286:156-163.

Komrakova M. and W. Holtz. 2011. Storage of unfertilized rainbow trout (Oncorhynchus mykiss) eggs in sealed polyethylene (PE) bags. Aquaculture 313:65-72.

Lahnsteiner F., T. Weismann, and R.A. Patzner. 1995. Composition of the ovarian fluid in 4 salmonid species: *Oncorhynchus mykiss, Salmo trutte f lacustris, Salvelinus alpinus* and *Hucho hucho*. Reproduction Nutrition Development 35:465-474.

Lahnsteiner F., T. Weismann, and R.A. Patzner. 1999. Physiological and biochemical parameters for egg quality determination in lake trout, Salmo trutta lacustris. Fish Physiology and Biochemistry 20:375-388

Lahnsteiner F. 2000. Morphological, physiological and biochemical parameters characterizing the over-ripening of rainbow trout eggs. Fish Physiology and Biochemistry 23:107-118.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

THE EFFECT OF DIFFERENT PHYTOPLANKTON SPECIES AND COMMERCIAL ENRICHMENT PRODUCTS ON THE FATTY ACID PROFILE, ENZYME ACTIVITY, AND OVERALL CONDITION OF THE ROTIFER *BRACHIONUS PLICATILIS*

V. Kostopoulou[1], A. Tsopelakos[2], E. Zogopoulou[2], H. Miliou[2], P. Divanach[1], and P. Katharios[1]

[1] Institute of Aquaculture, Hellenic Centre for Marine Research, P.O. Box 2214, Heraklion, 71 003 Crete, Greece
[2] Department of Applied Hydrobiology, Faculty of Animal Production and Aquaculture, Agricultural University of Athens, Iera Odos 75, 118 55 Athens, Greece

Introduction

The efficacy of *Chlorella minutissima* to enrich rotifers (*Brachionus plicatilis*) was assessed in comparison with non-enriched rotifers (fed on baker's yeast), other phytoplankton species (*Nannochloropsis* sp.), and commercially available products.

Materials and methods

All experiments were conducted at the Institute of Aquaculture (HCMR) in Crete, Greece. Rotifers (*Brachionus plicatilis* s.l.) were mass-cultured with Sparkle (INVE, Belgium) at an initial density of 300ind ml^{-1}. Following culture, enrichment took place at the same initial density and a culture volume of 50 l (T=24-26°C, S=25ppt, O_2=7.94±0.42mg.l^{-1}, pH=7.97±0.03). All cultures were subjected to natural sunlight (0-200lux) and supplied with air (phytoplankton treatments) or oxygen (commercial products) (Table I).

Sampling took place at the beginning and end of enrichment. Rotifer population profile was studied in terms of egg female ratio (EF = no. of eggs:no. of females) and swimming speed (area covered in specified time interval). Rotifer enzymatic activity was assayed using the semi-quantitative micromethod APIZYM (Biomerieux Hellas). Lipids were extracted according to Folch et al. (1957). Fatty acid methyl esters were prepared as described by Christie (1982) and separated by gas chromatography (GC Agilent 6890).

One-way analysis of variance (ANOVA) and Tukey's HSD multiple range test were performed to detect significant differences ($P<0.05$) between experimental

parameters, with the exception of swimming speed, which was analyzed with Kruskal-Wallis (Statgraphics, Statistical Graphics Corp., Herndon, VA, USA).

Table I. Description of experimental treatments (n=3). Commercial enrichment products were supplied as per manufacturer's directions.

Treat-ment	Enrichment	Duration	Concentration	Dose
Chlorella	*Chlorella minutissima*	24h	20×10^{6}cells.ml^{-1}	1
Nanno	*Nannochloropsis* sp.	24h	20×10^{6}cells.ml^{-1}	1
Yeast	*Saccharomyces cereviciae*	24h	1g.10^{-6}rotifer	1
Omega	Omega (Foresee Management)	6h	0.9g.10^{-6}rotifer	2
Pepper	Red Pepper (Bernaqua)	6h	300g.m^{-3}	2
DHA	DHA Protein Selco (INVE)	6h	250g.m^{-3}	2
Spresso	Selco Spresso (INVE)	6h	350g.m^{-3}	2
Algamac	Algamac3050 (Pacific Trading)	8h	0.3g.10^{-6}rotifer	1

Results

EF ratio and swimming speed showed significant differences between treatments (Table II). EF ratio was lower in Omega and Spresso and higher in Nanno ($P<0.001$). Swimming speed was higher during culture (Sparkle) and lower in Spresso ($P<0.001$).

Regarding rotifer enzymatic activity, phytoplankton-enriched rotifers had fewer enzymes compared to those enriched with commercial products (Table III).Of the commercial products, most enzymes were present in Algamac, DHA and yeast, and less in Pepper. As for phytoplankton, Chlorella had most enzymes and Nanno, less. Lipase and β-glucuronidase were absent from all rotifers.

As for lipid analysis, rotifers enriched on Omega, Spresso and Chlorella had the highest DHA content, compared to the rest (Table IV).

Table II. EF ratio and swimming speed (mean±SE) in treatments. Means of the same column having a different letter in superscript are significantly different ($P<0.001$).

Treatment	EF ratio	Swimming speed (mm.min^{-1})
Sparkle	0.30 ± 0.02^{b}	44.4 ± 3.2^{d}
Chlorella	0.28 ± 0.01^{b}	38.8 ± 2.1^{bcd}
Nanno	0.45 ± 0.01^{c}	35.3 ± 1.8^{bc}
Yeast	0.31 ± 0.01^{b}	40.7 ± 3.0^{abcd}
Omega	0.19 ± 0.01^{a}	36.7 ± 1.5^{bc}
Pepper	0.34 ± 0.02^{b}	35.0 ± 1.3^{bc}
DHA	0.32 ± 0.01^{b}	31.8 ± 1.2^{ab}
Spresso	0.19 ± 0.01^{a}	29.3 ± 1.3^{a}
Algamac	0.31 ± 0.01^{b}	33.9 ± 1.2^{b}

Table III. Rotifer enzymatic activity using APIZYM. Presence or absence is marked by (+) and (-) signs respectively and intensity of reaction is scaled from 1-5. Lipase and β-glucuronidase were omitted because they were absent from all rotifers.

Enzymes	Sparkle	Chlorella	Nanno	Yeast	Omega	Pepper	DHA	Spresso	Algamac
Alkaline phosphatase	+++	+++	++	+++++	++++	++	+++++	++++	+++++
Esterase	+	++	+	++	+	-	+	+	++
Esterase lipase	+	+	+	++	+	-	+	+	++
Leucine arylamidase	++	+++	+	++++	++	-	++	++++	+++++
Valine arylamidase	++	+	-	++++	++	+	++	++++	+++++
Cystine arylamidase	-	-	-	+	-	-	+	-	+
Trypsin	-	-	-	+	-	++	+	-	+
α-chymotrypsin	+	-	-	++	+	-	+	++	+
Acid phosphatase	++	+++	+	+++++	++++	+	+++	+++	+++
Naphthol-AS-BI-phosphohydrolase	+	+	+	+++++	++	+	+++	++	+++
α-galactosidase	+	+	-	+++	+	+	+	+	+
β-galactosidase	+++	+	-	++++	++	++	++	+++	+++
α-glucosidase	+++	++	-	++++	++	++	+++	+++	+++
β-glucosidase	+	+	-	+++	+	+	+	+	+
N-acetyl-β-glucosaminidase	++	+	-	++++	+	+	++	++	++
α-mannosidase	+	-	-	+++	+	+	+	+	+
α-fucosidase	+	-	-	++++	+	+	+	++	++

Table IV. Mean±SE of selected fatty acid composition (% total fatty acids) in rotifers of experimental treatments.

	Algamac	Yeast	Nanno	Chlorella	DHA	Spresso	Pepper	Omega
20:2n6	0.60±0.00	0.66±0.19	0.48±0.03	0.42±0.13	0.46±0.28	1.50±1.25	0.21±0.09	0.28±0.07
20:4n6	1.14±0.05	1.11±0.35	0.94±0.06	0.50±0.03	0.38±0.08	5.78±0.55	1.05±0.19	1.67±1.27
20:4n3	0.25±0.07	0.30±0.10	0.10±0.01	0.56±0.17	0.60±0.35	0.15±0.05	0.20±0.02	0.79±0.53
20:5n3	1.14±0.32	5.56±0.67	2.38±0.61	2.54±0.49	2.36±1.19	4.09±1.34	0.55±0.11	5.98±2.56
22:5n3	0.50±0.03	0.15±0.01	0.41±0.06	0.21±0.01	0.52±0.40	0.88±0.68	1.22±0.00	0.96±0.82
22:6n3	5.63±0.01	6.44±1.15	5.20±0.64	11.19±1.47	8.83±3.12	15.66±2.74	5.94±0.28	17.68±6.08

Discussion

EF ratio and swimming speed are used as predictors of the status of rotifer cultures. If one considers the levels of the cultured rotifers as a baseline (i.e., Sparkle), then the treatments below those levels can be considered as having reduced performance. According to the results, Spresso and Omega were on the lower range of both indices. On the other hand, Chlorella and yeast showed a better overall condition. The rest of the treatments did not show a clear trend. It should be noted however that the duration of the enrichment is too short to allow for definite conclusions.

The presence of enzymes in enriched rotifers is considered advantageous, since it has been argued that enzymes from live feed activate the fish larval digestion process (Kolkovski, 2001). In that respect, Algamac, DHA, and yeast yielded the richest enzymatic profile in rotifers.

The lipid profile is one of the most important parameters for rotifer enrichment. According to the results of the present study, *Chlorella minutissima* can be used

to enrich rotifers, as it yielded comparable results to most commercial products tested.

Acknowledgements

This work was part of the research project BIOEXPLORE (National Strategic Reference Framework NSRF 2007-2013, National Action: Cooperation, General Secretariat of Research and Technology).

References

Christie W.W. 1982. Lipid Analysis. Pergamon, Oxford. 207p.

Folch J., N. Lees, and G.H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry 226:497–509.

Kolkovski S. 2001. Digestive enzymes in fish larvae and juveniles – implications and applications to formulated diets. Aquaculture 200:181-201.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

CULTURE SYSTEMS IN THE COASTAL COMPLEXES WHICH ARE USED IN THE PROCESS OF RED KING CRAB ARTIFICIAL REPRODUCTION IN RUSSIA

N.P. Kovatcheva, R.R. Borisov, A.V. Parshin-Chudin, R.O. Lebedev, and D.S. Pechonkin

Russian Federal Research Institute of Fisheries and Oceanography (VNIRO), Moscow 107140, Russia

Introduction

The red king crab is one of the most important invertebrate species fished in Russian waters. In recent years, a long-term restoration program has been conducted for this species in the waters of the Barents Sea and the Sea of Japan (Russia). An artificial reproduction program in the offshore complexes aims to recover natural stocks by catching females with eggs from the sea and storing in tanks; receiving larvae and growing to the viable juvenile stage in the control conditions; and releasing juveniles in the natural environment.

An experimental module for red king crab cultivation was installed based on specifications developed by scientists of VNIRO, Moscow (Kovatcheva et al., 2006; Kovatcheva, 2008; Kovatcheva et al., 2012).

The tank module integrates technical construction and includes all necessary technology required for holding red king crab juveniles. Installation of numerous tanks allows increasing number of juveniles available for release. The module includes tanks for holding females and larvae hatching; tanks for larvae, post-larvae (glaucothoe) and juvenile rearing; a water supply system without thermoregulation and recirculation water system with thermoregulation, ultraviolet sterilization, and mechanical filtration.

The tank module (Fig. 1) is based on 1 million red king crab larvae that will guarantee production of 300-500 000 juveniles for release into the sea.

Materials and methods

Capture and transportation of red king crab females
It is best to start artificial reproduction with the capture of vigorous females when the eggs are at late zoeal stage of embryogenesis (about 300 days old).

Female crabs may be collected by scuba divers or captured by standard king crab pots (rectangular or conical) set at a depth from 10-30m. The females should be caught in late March or early April in the North Pacific Ocean and no later than the end of February in the Barents Sea, i.e., several weeks before hatching. Female crabs may be transported in insulated containers. If shipping time is less than 10h, the crabs can be kept without water (Zagorsky, 2011).

Keeping of females and larvae receiving

The water temperature in the female holding tanks should be adjusted so that it is the same as in the transportation container (1.0-3.0°C).

Using different light regimes in hatching increases efficiency of larval collection. The tank holding females is covered compared to the larvae collector (Fig.1-1.1). A collection procedure is based on the positively phototaxis nature of larvae (Fig.1-1.2). Collected larvae are transferred into new rearing tanks at the same temperature as in the holding tank (usually around 4°C). Once hatching has occurred, the females can be released back to where they were caught.

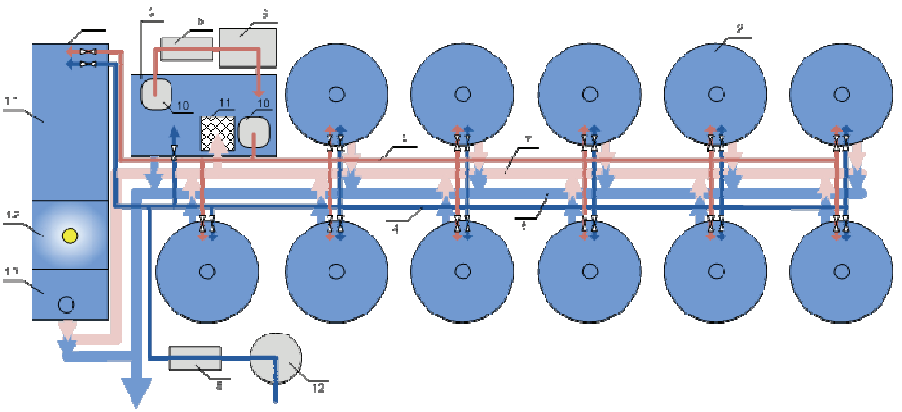


Fig. 1. Scheme of the experimental module in the coastal complex for red king crab artificial reproduction. 1: Holding tank for females and hatching larvae: 1.1: Chamber for females. 1.2: Chamber for larvae concentrate after hatching. 1.3: Technical chamber. 2: Tanks for larvae, glaucothoe, and juvenile rearing. 3: Reservoir. 4: Marine water pipe without temperature control. 5: Water pipe of the recirculation system. 6: Bleed manifold. 7: Recirculation collector. 8: UV-sterilizer. 9: Chiller. 10: Pump. 11: Mechanical filter. 12: Sand filter.

Larval culture

The optimal water temperature for larval cultivation is 7-8°C. Tanks for larvae are cylindrical in form for equal water circulation and for avoiding larvae and food settlement on the bottom (Fig. 1-2). The light has to be applied at 12 light, 12h dark. Water exchange during larval rearing has to be not less than two volumes per day.

Post-larvae (glaucothoe) and juveniles culture

Glaucothoes may be reared in the same reservoirs where the larvae were cultured (Figs. 1-2). Morphological and behavioral observations of post-larvae demonstrate that glaucothoes do not feed, but swim actively. During mass culture, early glaucothoes tended to form dense groups in more illuminated areas of the tanks. So, it is recommended to illuminate rearing tanks, especially in cases of mass culture. Glaucothoe settling behavior determines the necessity for placing appropriate substrates into the rearing reservoirs. The substrate should be nontoxic, have texture that allows the glaucothoes to grab and hold easily, and not impede cleaning procedures. After molting to the first juvenile stage, crabs preferred a plastic filament substrate. The juveniles are completely benthic and adult-like.

Juvenile releasing in the natural environment

First stage juveniles may be released at previously selected sites, which should have enough natural shelters and food or prepared artificial substrates (collectors, reefs, etc.). In the last three years, for more effective release of juveniles into the ocean, we used conical cages made with two types of nets – mesh size 6mm and 1mm (Fig. 2).

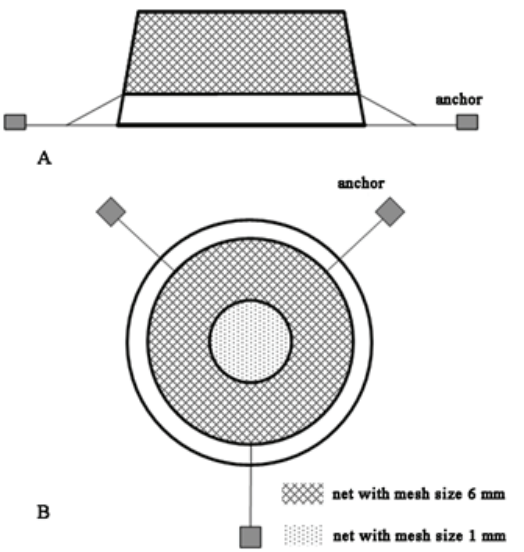


Fig. 2. A schematic of cage placement on the bottom for crab juvenile release. A. – side view; B. – top view.

The cage is provided with three anchors weighing a total of 15kg. Crab juveniles coupled with the substrate settle in the cage before sinking to the bottom.

Conclusion

Artificial reproduction and subsequent culture followed by the release of early juveniles into the ocean are currently being considered as a possible way to aid stock enhancement of red king crab in Russia.

References

Kovatcheva N.P., A.B. Epelbaum, A.V. Kalinin, R.R. Borisov, and R.O. Lebedev. 2006. Early life history stages of the red king crab Paralithodes camtchaticus (Tilesius, 1815). Biology and culture. Moscow, VNIRO Publishing. 240 p. [in English].

Kovatcheva N.P. 2008. Aquaculture of crustacean of the order Decapoda: red king crab (*Paralithodes camtschaticus*) and giant freshwater prawn (*Macrobrachium rosenbergii)*. Moscow, VNIRO Publishing. 240 p. [in Russian].

Kovatcheva N.P., R.R. Borisov, N.V. Kryahova, R.O. Lebedev, A.V. Parshin-Chudin, and M .Y. Nazartceva. 2012. Achievements of artificial reproduction of red king crab (*Paralithodes camtschaticus*) in the Far Eastern and northern basins. Ribnoe hosiaistvo 3:63-67. [in Russian].

Zagorsky I., 2011. The main methods of long term transportation of the red king crab Paralithodes camtschaticus. Ribnoe hoziastvo 5:52-54. [in Russian].

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

DYNAMIC OF EFFICIENCY OF MS-222 AS AN ANAESTHETIC FOR TENCH *TINCA TINCA* (L.) LARVAE

S. Krejszeff[1*], D. Żarski[1], P. Gomułka[2], K. Kupren[1], K. Palińska-Żarska[1], J. Nowosad[1], K. Stańczak[3], and D. Kucharczyk[1]

[1] Department of Lake and River Fisheries, University of Warmia and Mazury, Olsztyn, Poland. [*]E-mail: s.krejszeff@wp.pl
[2] Department of Ichthyology, University of Warmia and Mazury, Olsztyn, Poland
[3] Department of Fish Biology and Pisciculture, University of Warmia and Mazury, Olsztyn, Poland

Introduction

The aquaculture of tench has a long tradition, beginning in the Middle Ages (Steffens, 1995). The growth of consumer demand for tench has been observed since the middle 80s, and since then, global production has been increasing, although only part of this comprises aquaculture production (FAO 2013).

Tench is a species with big potential for aquaculture and is considered as a candidate for diversification of aquaculture production (Gela et al., 2010; García et al., 2011). In order to increase efficiency of tench production, research focused on the following elements: reproduction, larval and juvenile rearing, and market size fish rearing (Kocour et al., 2010; Podhorec et al., 2011). From these, larval rearing is the most important bottleneck because many more factors and phenomena affect the survival and growth rate of larvae than juveniles and adults (Krejszeff et al., 2013).

Optimizing non-lethal methods of measuring body weight and length should facilitate carrying out experiments on larval rearing by lowering the number of specimens and will result in decreasing the size of rearing tanks. This, in turn, allows increasing the number of rearing variants and repetitions. Non-lethal larvae sampling also allows increasing the frequency of sampling. Anesthesia is one of methods allowing for such manipulations.

The aim of this study was to examine the dynamic of efficiency of MS-222 as an anesthetic during larval period of tench.

Materials and methods

During the experimental period (from 4-34DPH) the efficacy of MS-222 was tested at 10-day intervals. The efficacy of MS-222 as an anesthetic for tench larvae was evaluated using the following guidelines (Gilderhus, 1990): an induction time of approximately 3min (criterion 1) and a recovery time of 10min or less (criterion 2). An added criterion (criterion 3) was post-anesthesia survival of 100% of larvae. Experiments were carried out using MS-222 concentrations recommended by the authors as appropriate for the specific DPH. If the tested concentration didn't meet the set criteria, the next tested concentration was higher or lower by 20ppm or 10ppm. Efficacy was tested on 7 larvae per concentration. The exposure duration was set for 15min.

During the experiment period, larvae were reared in an experimental device for spawn incubation and larvae rearing, described by Krejszeff et al. (2010). The rearing was carried out at 27°C (±0.1°C). The photoperiod was set at 12 hours of light and 12 hours of darkness and pH ranged between 8.24 and 8.66.

Results

During each day of a test, it was possible to determine the concentration meeting the criterion I. At 4DPH it was 40ppm, at 14DPH it was 80ppm, at 24DPH and 34DPH it was 90ppm (Table I). It was also observed that the larvae started to recover during exposure at 4DPH at 40ppm, at 14DPH at 60ppm, and at 34DPH at 80ppm.

Table I. Time of induction of anaesthesia (mean±SD, n=7) at tested MS-222 concentrations.

Age	Dose (ppm)						
(DPH)	20	40	50	60	80	90	100
4	>900	193±20	132±22	92±19	-	-	-
14	-	>900	-	295±38	162±27	-	68±17
24	-	-	-	-	>900	160±33	135±42
34	-	-	-	-	313±88	164±22	117±22

Table II. Time of recovery from anaesthesia (mean±SD, n=7) at tested MS-222 concentrations.

Age	Dose (ppm)					
(DPH)	40	50	60	80	90	100
4	59±28	93±15	122±37	-	-	-
14		-	115±16	217±93	-	473±119
24	-	-	-	-	83±28	>600
34	-	-	-	83±27	92±8	>600

During each day of the test, each tested concentration met criterion 2 except concentration 100ppm at 24DPH and 34DPH (Table II). Apart from this, the third criterion wasn't met in the case of 24DPH and 34DPH at 100ppm concentration. The recorded mortality was 100% at 24DPH and 86% at 34DPH.

Discussion

The efficient concentration of anaesthetic in juveniles and adults depends on many factors; not only the species and temperature, but also on the size. The younger fish have less developed scales and a smaller surface area to body weight ratio. Therefore the amount of the anaesthetic getting through the skin of younger fish in relation to the total amount of absorbed anaesthetic is bigger than in older fish (Massee et al., 1995; Myszkowski et al. 2003; Hamačkova et al., 2004). The same correlation has been observed in larvae of tench. With growth, the larval sensitivity was decreasing. Therefore, establishing the effective dose demanded an increase of MS-222 concentration parallel to the growth of fish.

During tests, apart from dynamics of efficacy, the changes in the safety of MS-222 application was observed. At 24DPH and 34DPH, increasing efficient concentration by 11.11% resulted in mortality, while at 4DPH and 14DPH, increasing effective concentration by 50% and 25% didn't have a negative impact.

The observed results show high dynamics of MS-222 efficacy change in tench larvae. It results in the conclusion that application of this anesthetic should be preceded by the evaluation of larval development stage. The additional hindrance is the fact that at the beginning of larval development fish could recover during exposure in the concentration which meets the first criterion, resulting in the necessity to use higher concentrations.

Conclusions

MS-222 application in tench larvae resulted in highly dynamic change of anesthetic efficacy. Therefore, usefulness of the drug is limited. The evaluation of larval development stage is essential before application of MS-222 anaesthetic for tench larvae.

Acknowledgements

This study was partially financed by the project "Innovations in finfish aquaculture with special references to reproduction" (InnovaFish), Operational Programme Sustainable Development of the Fisheries Sector and Coastal Fishing Areas 2007-2013 (OR14-61724-OR1400003/09/10/11).

References

FAO 2013 statistics. http://www.fao.org/fishery/statistics/en

García V., J.D. Celada, J.M. Carral, R. González, Á. González, and M. Sáez-Royuela. 2011. A comparative study of different preparations of decapsulated *Artemia* cysts as food for tench (*Tinca tinca* L.) larvae. Animal Feed Science and Technology 170:72-77.

Gela D., M. Kocour, M. Flajšhans, O. Linhart, and M. Rodina. 2010.Comparison of performance of genome manipulated and standard tench, *Tinca tinca* (L.), groups under pond management conditions. Reviews in Fish Biology and Fisheries 20:301-306.

Gilderhus P.A. 1990. Benzocaine as a fish anesthetic: efficacy and safety for spawning-phase salmon. The Progressive Fish-Culturist 52:189-191.

Hamackova J., A. Lepicoa, P. Kozak, Z. Stupka, J. Kouril, and P. Lepic. 2004. The efficacy of various anaesthetics in tench (*Tinca tinca* L.) related to water temperature. Veterinarni Medicina 49:467-472.

Kocour M., D. Gela, M. Rodina, and M. Flajšhans. 2010. Performance of different tench, *Tinca tinca* (L.), groups under semi-intensive pond conditions: it is worth establishing a coordinated breeding program. Reviews in Fish Biology and Fisheries 20:345-355.

Krejszeff S., D. Żarski, D. Kucharczyk, K. Kupren, K. Targońska, and A. Mamcarz. 2010. An experimental device for eggs incubation and fish larvae rearing under laboratory conditions. Polish Journal of Natural Sciences 25:190-199.

Krejszeff S., D. Żarski, K. Palińska, I. Trąbska, K. Kupren, K. Targońska, M. Bowszys, and D. Kucharczyk. 2013. Procedure for harmless estimation of fish larvae weight. Italian Journal of Animal Science (in press)

Massee K.C., M.B. Rust, R.W. Hardy, and R.R. Stickney. 1995. The effectiveness of tricaine, quinaldine sulfate and metomidate as anesthetics for larval fish. Aquaculture 134:351-359.

Myszkowski L., R. Kamiński, and J. Wolnicki. 2003. Response of juvenile tench *Tinca tinca* (L.) to the anaesthetic 2–phenoxyethanol. Journal of Applied Ichthyology 19:142-145.

Podhorec P., M. Socha, M. Sokolowska-Mikolajczyk, B. Drozd, T. Policar, V. Stejskal, and J. Kouril. 2011. Effective dose of mGnRHa for induction of ovulation in tench (*Tinca tinca* L.). Aquaculture 319:184-187.

Steffens W. 1995. The tench (*Tinca tinca* L.), a neglected pond fish species. Polish Archives of Hydrobiology 42:161-180.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EMERGENCE OF CANNIBALISM IN EUROPEAN PERCID FISH – SIZE HETEROGENEITY OR NATURAL BORN KILLERS CONSEQUENCE?

J. Król[1], Z. Zakęś[2], and P. Kestemont[3]

[1] University of Warmia and Mazury, Warszawska 117A, 10-718 Olsztyn, Poland
[2] Inland Fisheries Institute, Oczapowskiego 10, 10-719 Olsztyn, Poland
[3] University of Namur, 61 Rue de Bruxelles, B-5000 Namur, Belgium

Intracohort cannibalism is considered as an important issue in European percid larviculture (Eurasian perch *Perca fluviatilis* and pikeperch *Sander lucioperca*) that can induce losses exceeding 50% of the initial density during the first two weeks of rearing. There are two theories on what may be a proximate reason for cannibalistic behavior in fish culture. First, cannibalism can be viewed as a cause or consequence of size heterogeneity of a fish stock. Second, some fish are simply "natural born killers" with a greater propensity to exert cannibalism. Nowadays, cannibalism is regarded as an alternative feeding strategy, influenced by environmental factors, while intrinsic factors inducing intracohort cannibalism in percid larviculture have never been examined so far. The complete elimination of cannibalism in larviculture is probably impossible. However, it may be possible to mitigate cannibalistic behaviour between larvae using procedures based on both extrinsic and intrinsic factors, which could affect on the dynamics of cannibalism in carnivorous fish species.

To evaluate the effects of genetic factors (offspring-parents relatedness) on cannibalism occurrence in Eurasian perch, monosex culture was tested as a strategy for reducing aggressive interactions within a cohort of perch larvae (first experiment). All-female stocks were obtained using neomales from a sex-reversed line produced at the Aquaculture Training and Research Center in Tihange (University of Liege, Belgium). For progeny parenthood tests, parents were genetically characterized with five microsatellite markers, originally isolated from yellow perch *P. flavescens*, and available from Genbank. Microsatellites were analyzed using fluorescent capillary electrophoresis device (Beckman Coulter CEQ8000 Genetic Analysis System).

In this first study, any differences in cannibalism rate and its relation to further growth and survival between normal and all-female Eurasian perch larvae were observed. In the other hand some maternal influence, independent of larval size variability, on cannibalism-survival relationship was reported. Although the re-

sults of the present study did not demonstrate a clear evidence of maternity for cannibalistic behavior in Eurasian perch, since cannibalism intensity was imposed by maternal effect during the larval stage not during the early juvenile one, however, this maternal influence on cannibalism during early life stages was found responsible for final survival.

High size variation in a population of larvae could be a primary cause of emergence cannibalism. However, cannibalistic behavior can also have an effect on increasing size heterogeneity. The second experiment was conducted to study effect of different initial weight variations (CV; small – 23%, medium – 48%, and large – 62%) on cannibalism and further quantitative characteristics of pikeperch larvae. Differences between initial CV of larvae probably resulted from different initial densities in the three stocks before experiment.

Despite considerable differences at the onset of the experiment, initial CV had no impact on growth rates and further heterogeneity of pikeperch larvae, which has been confirmed earlier in Eurasian perch and European seabass (*Dicentrarchus labrax*). As expected, the intensity of type I cannibalism was highest in the "small CV" group and higher rate of type II cannibalism was observed in the "large CV" group compared to others. Cannibalism negatively affected the final survival, with a main impact of type II cannibalism.

To date, attempts to mitigate cannibalism in larviculture have focused mainly on environmental and population factors or food quantity and quality with special emphasis on weaning time from live to commercial diets. An alternative could be using external dietary factors that influence cannibalistic behavior. Tryptophan (TRP) is a precursor of serotonin (5-HT) which is known to reduce aggression but also limits food intake in vertebrates, including fishes. Based on those findings, the purpose of third experiment was to investigate effect of dietary TRP supplementation (0.5%, 1%, and 2% of dry diets) on growth-cannibalism-survival dynamics within a cohort of pikeperch larvae. The 5-HT content of each fish was measured with ELISA using a commercial kit (IBL, Germany).

ELISA analysis showed that TRP-supplemented diets were effective in increasing the levels of 5-HT in the brain of pikeperch. TRP supplementation reduced both types of cannibalism compared to the control group, although the reduction did not amount to more than a few percent. However, contrary to earlier studies, we did not observe significant difference in growth rates and feeding behavior of pikeperch between the TRP-fed and control groups.

In conclusion, there is no simple answer for the question posed in the title. In our opinion, future studies on genetic or dietary factors are needed and should obtain much better effect in decreasing cannibalism in larviculture, alongside routine manipulations such as grading, optimal weaning time, and stocking density.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

ALLOMETRIC GROWTH IN THE *NANNACARA ANOMALA* REGAN, 1905 (CICHLIDAE, PISCES)

K. Kupren[1], M. Prusińska[2], D. Żarski[1], S. Krejszeff[1], and D. Kucharczyk[1]

[1] Department of Lake and River Fisheries, University of Warmia and Mazury in Olsztyn, Poland
[2] Department of Ichtiology. The Stanislaw Sakowicz Inland Fisheries Institute in Olsztyn, Poland

Introduction

Nannacara anomala is a small (max. 5.6cm TL) substrate-brooding neotropical cichlid belonging to Subfamily Cichlinae. This species is found in Guianan rivers from the Aruka River in Guyana east to the lower Marowijne River in Suriname (Kullander, 2003). *N. anomala* is frequently kept in aquaria because it is attractive in appearance, undemanding in maintenance, and easily bred.

Literature on their early development is scarce and limited to general information. The present work is the first analysis of growth patterns during early ontogeny not only for this species, but also all substrate-brooding cichlids belonging to Perciformes, the largest order of the Vertebrata. The analysis of inflexion points in growth curves, in combination with observed changes in morphological development, may be used for both comparative studies of the ontogeny in other species as well as assessing phylogenetic relationships. Moreover, morphological features are very useful indicators for determining optimal rearing conditions for young fish.

Materials and methods

Eggs were obtained from 2 pairs of *N. anomala* which spawned simultaneously. Immediately after activation, the eggs were transferred, together with the substratum to which they were attached, to 50-l glass aquaria with the same water condition. After hatching, newly hatched larvae were placed in three 1-L glass rearing tanks (50 individuals per tank). Each tank was supplied separately with filtered water from a sprinkler. Mechanical and biological filtration was provided with an external (Fluval 405) filter. From hatching until day 18 (loss of all larval characteristics), random samples of 30 larvae were taken each day (10 individuals from each small tank) prior to morning feeding . Sampled larvae were placed on Petri dishes and digital photographs of each specimen were taken us-

ing digital image analysis software DP-Soft software (Olympus, Japan). After measuring, the fish were returned to the same tanks from which they had been taken. On each digital photograph, several body proportions were measured to the nearest 0.01mm. These measurements were: head length (HL), trunk length (TRL); tail (post anal) length (TAL), eye diameter (ED); head depth (HD); body depth (BD); muscle height at anus level (MH); tail depth at the caudal peduncle level (TD), and total length (TL). All measurements were taken along lines parallel or perpendicular to the horizontal axis of the body (Gisbert, 1999). Dead and abnormal specimens were excluded from the analysis.

Data analysis

Growth patterns during the developmental stages were modeled by a power function of TL and the patterns in allometry were described by the growth coefficient (i.e., power function exponent) in the equation $Y = aX^b$, where Y is the dependent variable (measured character) and X is the independent variable (TL), *a* is the intercept, and *b* is the growth coefficient. When isometric growth occurred, b=1, a positive allometric growth occurred when b>1, and a negative one when b<1. If *b* is negative, then this relates to negative growth. In addition, linear regressions were performed on log-transformed data (TL as independent variable) and the inflection points were calculated. Inflection points are the X values where the slope of growth changes. The inflection points were calculated according to van Snik et al. (1997).

Results and discussion

Body proportions changed considerably during early development of *N. anomala*. Inflection points for all the body measurements occurred between days 3 and 8 after hatching, corresponding to the TL interval of 4.03-4.84mm, except for the TRL, which showed weak negative allometric growth (*b*=0.86) throughout the studied period (Fig. 1). In the case of TAL, HL, ED, and HD, there was a clear reduction in the growth coefficients after the inflection point from positive (*b*=1.48, 1.76, 1.79, and 3.13, respectively) to near isometric (*b*=1.08, 0.94, 0.84, and 0.91, respectively) growth. Positive allometric growth after inflexion point was still observed for the MH and TD, but value of growth coefficients were clearly lower (*b* changed from 2.67 to 1.98 and 4.12 to 1.39, respectively). The BD showed first negative (*b*=-0.90) and near isometric (*b*=1.13) growth after inflexion point. Such cumulative changes in growth coefficients are also characteristic for many other teleost species which go through the fast change in swimming mode (from anguilliform type of locomotion to subcarangiform swimming) during flexion stage, when caudal fin rays appeared. Most body parts studied of *N. anomala* showed initially (during yolk sac period) high allometric growth. According to Osse and van den Boogaart (2004), the positive allometry of head and tail regions, prior to the abdominal region, during the endogenous nutrition period reflects the early priority to develop those structures and organs

related to vital functions such as feeding and swimming. Such growth sequences could be also interpreted as an adaptation to reduce and optimize costs of larval transport (van Snik et al., 1997). In juvenile and adult stages, all growth coefficients approach 1. This change to isometry has been considered as a natural transition in growth priorities since primary functions have been fulfilled during the early developmental stages (Fuiman, 1983; van Snik et al., 1997). In *N. anomala* larvae, near isometric growth after inflexion points were characteristic for most studied body regions. The positive allometry of the TD and MH throughout all rearing period contributes to increasing the swimming abilities.

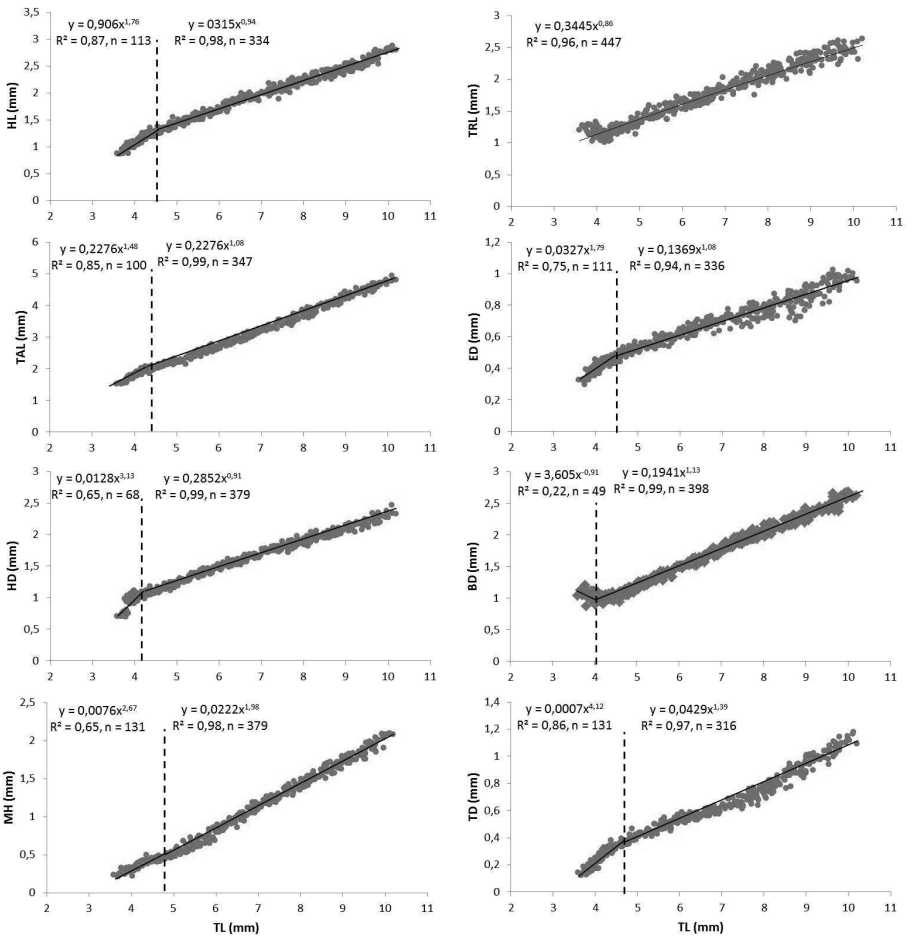


Fig. 1. Allometric growth equations between measured body proportions and total length during *Nannacara anomala* development. The dashed line represents the inflection point of growth.

References

Fuiman L.A.1983. Growth gradients in fish larvae. Journal of Fish Biology 23:117-123

Gisbert E. 1999. Early development and allometric growth patterns in Siberian sturgeon and their ecological significance. Journal of Fish Biology 54:852-862

Kullander S.O. 2003. Cichlidae. Pp. 605-654 In Reis, R.E., S.O. Kullander & C.J. Ferraris, Jr., (eds.), Check list of the freshwater fishes of South and Central America. Edipucrs, Porto Alegre, 729 p.

van Snik G.M.J, J.G.M. van den Boogaart and W.M. Osse .1997. Larval growth patterns in *Cyprinus carpio* and *Clarias gariepinus* with attention to the fanfold. Journal of Fish Biology 50:1339-1352

Osse J.W. and J.G.M. van den Boogaart. 2004. Allometric growth in Fish Larvae: Timing and Function. In: J.J. Govoni (ed.), The Development of Form and Function in Fishes and the Question of Larval Adaptation, American Fisheries Society, Symposium 40, Bethesda, Maryland, 167-194 p.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

MICROENCAPSULATED DIETS FOR ALTRICIAL FRESHWATER FISH LARVAE: PRODUCTION AND EVALUATION

N.J. Leitão[1], J.B. Rodrigues[2], C.R.F. Grosso[2], M. Dal Pai-Silva[1,3], and M.C. Portella[1]

[1] Sao Paulo State University, Aquaculture Center, 14884-900 Jaboticabal, SP, Brazil
[2] Campinas State University, Faculty of Food Engineering, 13083-862 Campinas, SP, Brazil
[3] São Paulo State University, Institute of Biosciences, Department of Morphology, 18618-000 Botucatu, SP, Brazil

Introduction

During the past several decades, enormous efforts have been made to develop microdiets to replace live feed as complete or partial replacements for fish larvae. In recent years, new diet-manufacturing methods have been adopted that potentially have better properties, including microencapsulation. Microencapsulated particles are made using various techniques; these particles are characterized by a capsule wall that separates the dietary materials from the surrounding medium (Yúfera et al., 2009). Microencapsulated diets produced by ionic gelation have been studied in Brazil (Mukai-Correa et al., 2005) and have shown good acceptance by pacu larvae. The ionic gelation technique consists of forming a gel matrix upon which a solution of polysaccharides containing nutrients comes into contact with an ionic solution. However, due to the difficulties of producing particles with high protein content, this method had to be improved to obtain higher incorporation of protein. The combination of ionic gelation and the complex coacervation methods proposed by Rodrigues et al. (2012) resulted in particles with high levels of moisture and protein that were similar to the natural foods of fish larvae. This characteristic can make these particles interesting for use as a microdiet during fish larviculture.

In this study, microencapsulated diets were produced by ionic gelation followed by complex coacervation, and a weaning experiment was conducted with pacu (*Piaractus mesopotamicus*) larvae to investigate their effectiveness. The diets were evaluated by measuring physical (size and shape) and chemical (proximal composition) parameters, attractiveness (intestinal fullness), efficiency of the diet (larval survival and growth), development of the digestive tract (histological analysis), skeletal muscle cellularity (distribution of muscle fiber size), and expression of genes related to muscle growth (MyoD and myogenin).

Materials and methods

Production and characterization of microencapsulated diets
The methods for the preparation of the microencapsulated diets were based on Rodrigues et al. (2012). Briefly, the particles were formed using a pectin emulsion (2% w/w) by the dropwise addition of the suspension into a calcium chloride (2% w/w) solution. The oil and a mineral and vitamin mixture were emulsified with the pectin. Later, the particles were coated with whey protein and fish hydrolysate CPSP-90 (Sopropêche, France). Different oil sources were used to prepare the diets: olive (OO), sunflower (SO), linseed (LO), sacha inchi (SiO), and fish (FO). Samples of each of the diets were analyzed for their crude protein, lipid, ash, and moisture contents. The diet size was determined automatically using the Mastersizer 2000 instrument (Malvern Instruments, UK).

Feeding experiment
The microencapsulated diets were tested in vivo with pacu larvae. Larvae (16 days post hatching, dph; 20.0±6.5mg) were stored (10 larvae.l^{-1}) in 28 50-l polyethylene tanks with continuously flowing water and constant artificial aeration (29.8°C and 5.1mg.l^{-1} dissolved oxygen). They were given seven treatments: OO, SO, LO, SiO, and FO microencapsulated diets, commercial diet (Hatchfry Encapsulon, Argent, USA) and *Artemia* nauplii for 28 days. For statistical analysis, a completely randomized experimental design with four replicates for each treatment condition was used. Larvae (n=25 per tank) were individually weighed and measured weekly; and the mean wet weights and lengths were calculated. At 44dph, all of the remaining larvae in each tank were counted and the survival rates were determined. Larval intestinal fullness was used as a measure of the ingestion rate of the diets. Fifty larvae from each tank were sampled at 44dph and scored (0%, <50%, >50%, 100%) according to the quantity of food ingested.

Histological analyses
Histological samples (10 specimens per treatments) were collected at 16 and 44dph. For morphological analyses of the stomach, intestine, liver, and pancreas, samples of the digestive tract kept in a 4% paraformaldehyde solution were embedded in Historesin® (Leica, Germany), cut longitudinally into sections (4µm thick), and stained with hematoxylin-phloxine B, periodic acid-Schiff (PAS), Alcian blue pH 2.5, eosin-orange G-phloxine, and Sudan black B. For the muscle cellularity analyses, muscle samples were fixed in Karnovsky solution and embedded in Historesin®, cut transversally into sections (4µm thick), and stained with hematoxylin-phloxine B. The muscle cellularity was examined on one section per fish. The muscle fiber diameter was quantified using an image analysis system (Leica Qwin, Germany); 200 muscle fibers per larva were measured in the deep compartment of the epaxial region.

RT-qPCR analysis of MyoD and myogenin gene expression
Muscle samples (n=10 for each treatment) were collected from the dorsal region at 16 and 44dph. Total RNA was extracted using TRIzol® Reagent (Invitrogen, USA) according to the manufacturer's protocol. The total RNA was reverse-transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, USA). The samples were amplified using primers specific for MyoD, myogenin, and the 18S rRNA reference gene (GenBank accession numbers: FJ686692, FJ810421 and GQ337002, respectively). The MyoD and myogenin mRNA expression analyses were performed using an ABI 7300 Real-Time PCR System (Applied Biosystems, USA). The relative expression ratio of the MyoD and myogenin genes was obtained using the comparative Ct method (Livak and Schmittgen, 2001); the value was expressed in arbitrary units and then normalized to the 18S rRNA endogenous reference gene.

Statistical analysis
Analyses of variance and Cramer Von Mises and Brown-Forsythe tests were performed using the SAS version 9.0 software (SAS Institute Inc., USA). Data were statistically analyzed using one-way ANOVA at a probability level of 5%.

Results and discussion

The microencapsulated diets had 550g.kg^{-1} protein, 240g.kg^{-1} lipid, and 11g.kg^{-1} ash on average. The moisture content was greater than 850g.kg^{-1}. The size distribution of the particles was 90-750µm; the particle diameters D(0.5) were approximately 300µm. Our results showed that the microencapsulation technique used in this study was able to produce particles similar to live food for fish larvae in terms of macronutrients and size (Baskerville-Bridges and Kling, 2000).

None of the microencapsulated diets were completely rejected by the fish; however, lower amounts of the diets were ingested than the *Artemia* nauplii ($p<0.05$). The intestinal fullness data indicated that at least 98% of the fish fed any of the microdiets scored lower than 50% full. In contrast, only 36% of the fish fed the *Artemia* nauplii were scored at intestinal fullness values lower than 50%. Chemical stimuli from the *Artemia* nauplii may have favored pacu feeding behavior (Tesser and Portella, 2006).

The survival rates of the pacu larvae to 44dph ranged from 73.1-90.8% with the microencapsulated diets, 61.4% with the commercial diet, and 97.5% with live prey ($p<0.05$). However, the pacu growth performance and development of the larval digestive tract were negatively affected by the use of the microdiets. The fish fed the *Artemia* nauplii and the commercial diet showed high growth rates and significantly higher average final body weights (250.5±4.1mg and 211.3±3.0mg, respectively) compared with fish fed microencapsulated diets (final body weight values ranging from 42.3-32.1mg). Fish fed microencapsulated

diets also showed lower body lengths at 44dph. Histological observations of the digestive system are considered a good indicator of a fish's nutritional condition (Ostaszewska et al., 2005). The poorly developed intestinal folds, few glycogen (PAS-positive) storage areas in hepatocytes, few proenzyme granules in pancreatic cells (eosin-orange G-phloxine weak reaction), absence of intrahepatic pancreas, and retardation in the thickening of the glandular stomach observed in fish fed the microencapsulated diets indicated poor nutritional condition.

The decreased growth rates of the pacu fed the microencapsulated diets changed the dynamics of their white muscle growth and altered the expression of genes involved in myogenesis such as MyoD and myogenin. A high frequency of small fibers (diameter ≤10mm) was observed in the deep layer of the epaxial muscle ($p<0.05$) as well as low MyoD and myogenin expression levels ($p<0.05$).

In conclusion, the microencapsulation technique (ionic gelation followed by complex coacervation) in this study was able to produce particles with similar size and macronutrient profiles to live food; these particles were satisfactorily accepted by pacu larvae. However, the microencapsulated diets produced in this study were not adequate to promote growth in pacu larvae during weaning, suggesting that the poor development of the digestive tract and skeletal muscle observed in these fish could negatively affect growth at later developmental stages.

References

Baskerville-Bridges B. and L.J. Kling. 2000. Development and evaluation of microparticulate diets for early weaning of Atlantic cod *Gadus morhua* larvae. Aquaculture Nutrition 6:171–182.

Livak K.J. and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408.

Mukai-Correa R., A.S. Prata, I.D. Alvim, and C. Grosso. 2005. Caracterização de microcápsulas contendo caseína e gordura vegetal hidrogenada obtidas por gelificação iônica. Brazilian Journal of Food Technology 8:73–80.

Ostaszewska T., K. Dabrowski, K. Czuminska, W. Olech, and M. Olejniczak. 2005. Rearing of pike-perch larvae using formulated diets – first success with starter feeds. Aquaculture Research 36:1167–1176.

Rodrigues J.B, N.J. Leitão, J.T. Kojima, R. Takata, M.C. Portella, and C.R.F. Grosso. 2011. Development of microcapsules containing *Lactobacillus acidophilus* to feed fish larvae. Anais WAS. Natal, RN, Brazil.

Tesser M.B. and M.C. Portella. 2006. Ingestão de ração e comportamento de larvas de pacu em resposta a estímulos químicos e visuais. Revista Brasileira de Zootecnia 35:1887–1892.

Yúfera M., M.C. Portella, S. Drust, and D. Poncelet. 2009. Microencapsulation and aquaculture. Aquaculture Europe 34:18–20.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

GENE REGULATION OF ATLANTIC COD (*GADUS MORHUA*) LARVAE WITH FOCUS ON LIPID DIGESTION AND PHOSPHOLIPID METABOLISM

K. Li*, M.-A. Østensen, K. Attramadal, P. Winge, T. Sparstad, I. Bakke, A.M. Bones, O. Vadstein, E. Kjørsvik, and Y. Olsen

Norwegian University of Science and Technology, Department of Biology, NO-7491 Trondheim, Norway. *E-mail: keshuai.li@ntnu.no

Introduction

Phospholipids (PL) are essential dietary components for normal development and growth in fish larvae (Cahu et al., 2009). The main hypothesis today is that fish larvae need dietary PL because they are not able to biosynthesize PL through the normal pathway from digested triacylglycerides (TAG) precursors (Tocher et al., 2008). The biochemically limiting steps for PL synthesis in fish larvae are unknown. The objective of this study was to investigate the influence of lipid levels of rotifers on gene regulation of PL biosynthesis of Atlantic cod (*Gadus morhua*) larvae.

Materials and methods

Rotifers (*Brachionus* Cayman) were cultivated to contain high (163±3.0mg g^{-1} DW) and low (117±1.2mg g^{-1} DW) lipid levels, termed Cayman-high and Cayman-low, respectively, and were fed to cod larvae from 3 day post-hatching (dph). Atlantic cod eggs were hatched and maintained in darkness the first 3 days and thereafter in continuous light. *Nannochloropsis oculata* algae paste (Reed Mariculture) was added (1mg $C.l^{-1}$) to the fish tanks at feeding from day 2. Rotifers were fed to larval tanks by a robot system 4 to 6 times a day to maintain the concentration of 5000 to 12 000 rotifers l^{-1}. Four replicate tanks (100 l, water exchange rate 2-3×.d^{-1}) were used for each treatment with initial larvae density of 100 individuals l^{-1}.

Total lipids were determined using a modified method of Bligh and Dyer (1959). Gene expression profiling was analyzed with a cDNA microarray covering 22 101 genes on cod larvae.

Results and discussion

Survival at 17dph was around 40% with no significant differences between treatments ($p>0.05$). On 8 and 13dph, the dry weight of CodL (cod larvae fed Cayman-low) was significantly higher than that of CodH (cod larvae fed Cayman-high) ($p<0.05$, Fig. 1), but there were no significant differences in dry weight on 17dph ($p>0.05$). No significant differences were found in the total lipid levels ($p<0.05$, CodL, 134±1.0mg.g^{-1} DW and CodH, 136±0.8mg.g^{-1} DW).

The log_2-transformed gene expression ratios between the two treatments were not significantly ($p>0.05$) different. Therefore, they were combined in Figure 2. The main digestion enzymes bile salt-activated lipase, phospholipase A2, and phospholipase B1 were significantly ($p<0.05$) up-regulated in 17dph larvae, which was in agreement with the quantitative real-time PCR results obtained by Kortner et al. (2011). However, most of the genes encoding PL biosynthesis enzymes were not significantly up-regulated ($p>0.05$, Fig. 2), indicating that the biosynthesis of PL at early larval stage was very limited. It is possible that the biosynthesis pathway shown in Fig. 2B was running, and it is likely that the biosynthesis of PC (phosphatidylcholine) was more up-regulated than that of PE (phosphatidylethanolamine) on 17dph (Fig. 2). A close association of endoplasmic reticulum (ER) and mitochondrial membranes is crucial for PL biosynthesis. The cod larvae have very immature mitochondria in the enterocytes and hepatocytes during the first weeks after hatching (Wold et al., 2008), and an increased amount of ER and MAM-like (mitochondria- associated-membranes) associations was only observed in older larvae (30dph).

Conclusion

The lipid levels of rotifers tested in this experiment did not affect the gene expressions involved in phospholipids biosynthesis. Cod larvae had limited ability to biosynthesize phospholipids until 17dph.

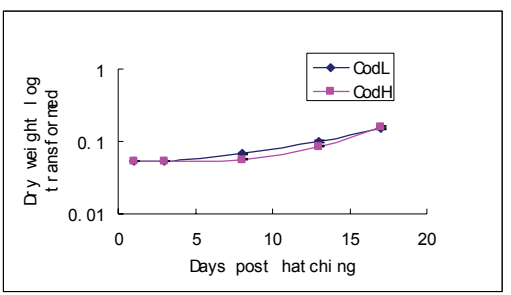


Fig. 1. Dry weight of fish larvae. CodL, cod larvae fed Cayman-low; CodH, cod larvae fed Cayman-high.

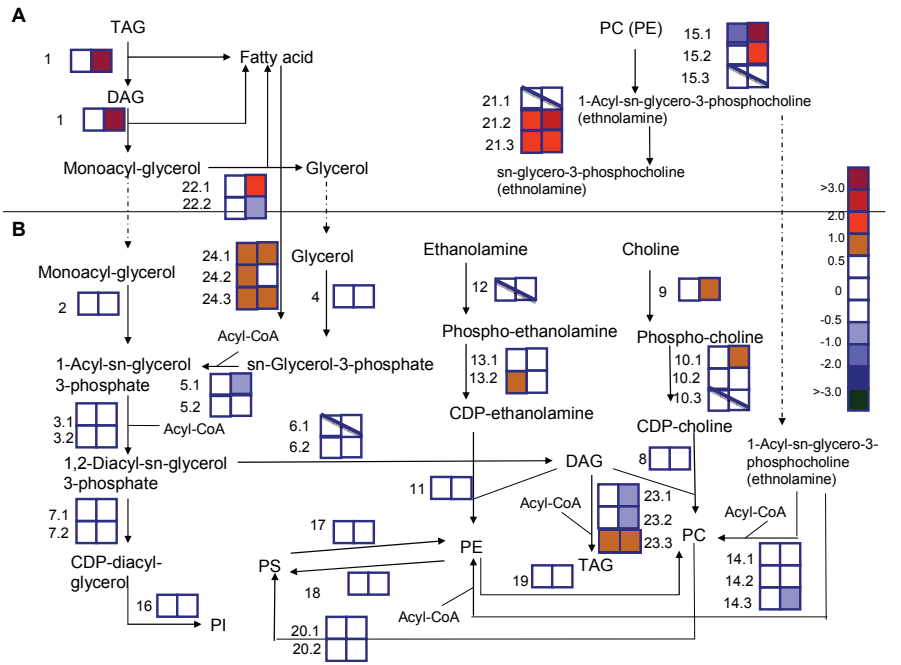


Fig. 2. Regulation of genes encoding triacylglycerides and phospholipids metabolism enzymes in Atlantic cod larvae. A. Digestion pathway. B. Biosynthesis pathway. Colored squares indicate expression levels at 3dph (left) and 17dph (right), and the data are normalized to 1dph. The color scale indicates log_2-transformed gene expression ratios. Squares with a diagonal line inside indicate genes were unlikely turned on. TAG, triacylglycerol; DAG, diacyl- glycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phospha- tidylserine; PI, phosphatidylinositol. 1, Bile salt-activated lipase-like protein; 2, acylglycerol kinase, mitochondrial precursor; 3.1(3.2), 1-acyl-sn-glycerol- 3-phosphate acyltransferase delta (gamma); 4, glycerol kinase; 5.1, glycerol-3- phosphate acyltransferase 1; 5.2, glycerol-3-phosphate acyltransferase 2; 6, phosphatidic acid phosphatase type 2c; 7, phosphatidate cytidylyltransferase 2; 8, cholinephosphotransferase 1-like; 9, choline kinase alpha-like; 10.1, phosphate cytidylyltransferase 1, choline, beta b; 10.2(3) choline-phosphate cytidylyl- transferase A(B); 11, ethanolaminephos- photransferase 1-like; 12, ethanolamine kinase 2; 13, ethanolamine-phosphate cytidylyltransferase; 14.1(14.2), lyso- phosphatidylcholine acyltransferase 2(1); 15, phospholipase A2, major isoenzyme; 16, CDP-diacylglycerol-inositol 3-phosphatidyltransferase; 17, phosphatidylserine decarboxylase proenzyme; 18, phosphatidylserine synthase 2; 19, phosphatidylethanolamine N-methyl-transferase; 20, phosphatidylserine synthase 1; 21, phospholipase B1, membrane-associated-lik; 22.1(22.2), mono- acylglycerol lipase ABHD12(6-A); 23.1(23.3), diacylglycerol O-acyltransferase 1(2); 23.2, diacylglycerol O-acyltransferase ho-molog 1b; 24.1, acyl-CoA synthetase family member 2, mitochondrial precursor; 24.2, acyl-CoA synthetase short-chain family member 3, mitochondrial; 24.3, very long-chain acyl-CoA synthetase isoform 1.

References

Bligh E.G. and W.J. Dyer. 1959. A Rapid Method of Total Lipid Extraction and Purification. Canadian Journal of Biochemistry and Physiology. 37:911-917.

Cahu C.L., E. Gisbert, L.A.N. Villeneuve, S. Morais, N. Hamza, P.A. Wold, J.L.Z. Infante. 2009. Influence of dietary phospholipids on early ontogenesis of fish. Aquaculture Research. 40:989-999.

Kortner T.M., I. Overrein, G. Oie, E. Kjorsvik, T. Bardal, P.A. Wold, and A. Arukwe. 2011. Molecular ontogenesis of digestive capability and associated endocrine control in Atlantic cod (Gadus morhua) larvae. Comp Biochem Physiol A Mol Integr Physiol. 160:190-199.

Wold P.A., K. Hoehne-Reitan, J. Rainuzzo, and E. Kjorsvik. 2008. Allometric growth and functional development of the gut in developing cod Gadus morhua L. larvae. Journal of Fish Biology. 72:1637-1658.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

HOST-INDUCED INCREASE OF SEA BASS MORTALITY IN A GNOTOBIOTIC CHALLENGE TEST WITH *VIBRIO ANGUILLARUM*

X. Li, T. Defoirdt, Q. Yang, P. Bossier, and K. Dierckens

Laboratory of Aquaculture & *Artemia* Reference Center, Ghent University, Rozier 44, Gent 9000, Belgium

Introduction

Vibrio anguillarum is the major cause of a haemorrhagic septicaemia, vibriosis, which is a severe disease affecting marine fish. This is especially significant for European sea bass (*Dicentrarchus labrax*), a species that is very sensitive to stressors and pathogens, where infections are often the major limiting factor in successful rearing. A few virulence-related factors and genes have been identified in *V. anguillarum* (Frans et al., 2011), including genes affecting chemotaxis and motility, an iron uptake system, haemolytic activity, extracellular metalloprotease, lipopolysaccharides, and exopolysaccharides. However, the pathogenicity mechanisms behind this disease caused by the bacterium are not yet completely understood.

As virulence factors produced by vibrios are often costly metabolic products, their production generally is tightly regulated. Important regulatory mechanisms that have been reported to control virulence gene expression in vibrios include quorum sensing and the ToxR regulon. However, neither of these has been found to affect the virulence of *V. anguillarum.* In addition to these regulatory mechanisms, host cues are also known to affect virulence gene expression in pathogenic bacteria. As far as we know, the role of host cues in the pathogenicity of *V. anguillarum* has not yet been investigated. In this study, we aimed at investigating the effect of dead larvae on the virulence of *V. anguillarum* towards sea bass larvae cultured under highly controlled gnotobiotic conditions.

Materials and methods

V. anguillarum strains 43, NB10, and HI610 were used in this study. The challenge tests with sea bass larvae were performed according to Dierckens et al. (2009). Lipase, phospholipase, caseinase, gelatinase, and hemolysin activity were assessed according to Natrah et al. (2011). Motility was assessed as described previously (Wang et al., 2011). Data were analysed using the SPSS software, version 19. All analyses were done at a significance level of 0.05.

Results and discussion

In this work, it was found that the mortality of *V. anguillarum* challenged sea bass larvae increased with increasing numbers of 'dead before challenge' larvae in the vials (Fig. 1). Based on this finding, the effect of dead larvae on the virulence of *V. anguillarum* towards sea bass larvae was investigated. Addition of homogenised sea bass larvae led to increased larvae mortality in *V. anguillarum* strain 43, NB10, and HI610 (Fig. 2). The bacteria number in the larvae as well as in the culture water increased in the presence of fish homogenate (Data not shown). The motility of all three *V. anguillarum* strains was significantly increased by the addition of fish homogenate. However, it was not affected by the addition of double amount of peptone (Table I). Lipase, phospholipase, caseinase, gelatinase and hemolysin activities were not affected by the addition of fish homogenate. These results suggest that fish homogenate increased the larvae mortality caused by *V. anguillarum* strains not only by offering nutrients to increase bacteria number, but also by increasing the motility of these bacteria.

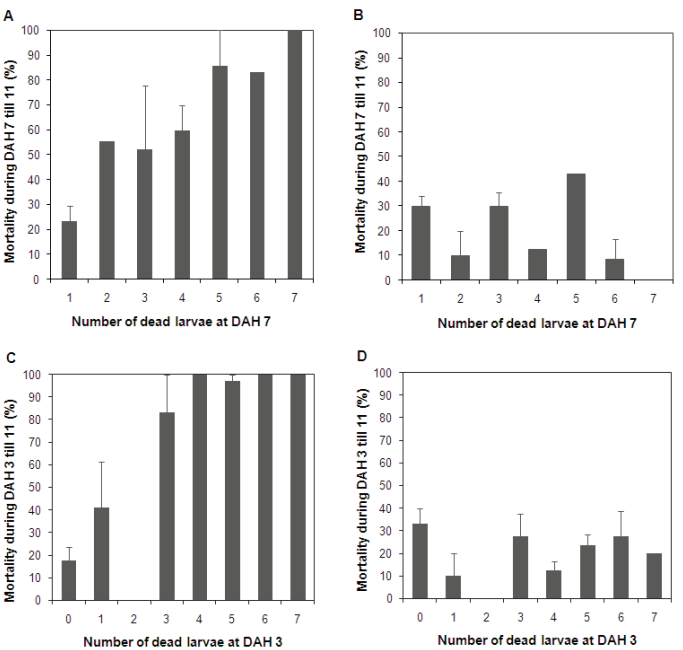


Fig. 1. Mortality between 7 and 11DAH of gnotobiotic sea bass larvae challenged with *V. anguillarum* HI 610 at 7DAH (A) and of axenic larvae (B) as function of the number of dead larvae present in the vial at 7DAH, and mortality between 3DAH and 11DAH of gnotobiotic larvae challenged with *V. anguillarum* HI 610 at 3DAH (C) and of axenic larvae (D) as function of the number of dead larvae present in the vial at 3DAH. Error bars represent the standard error.

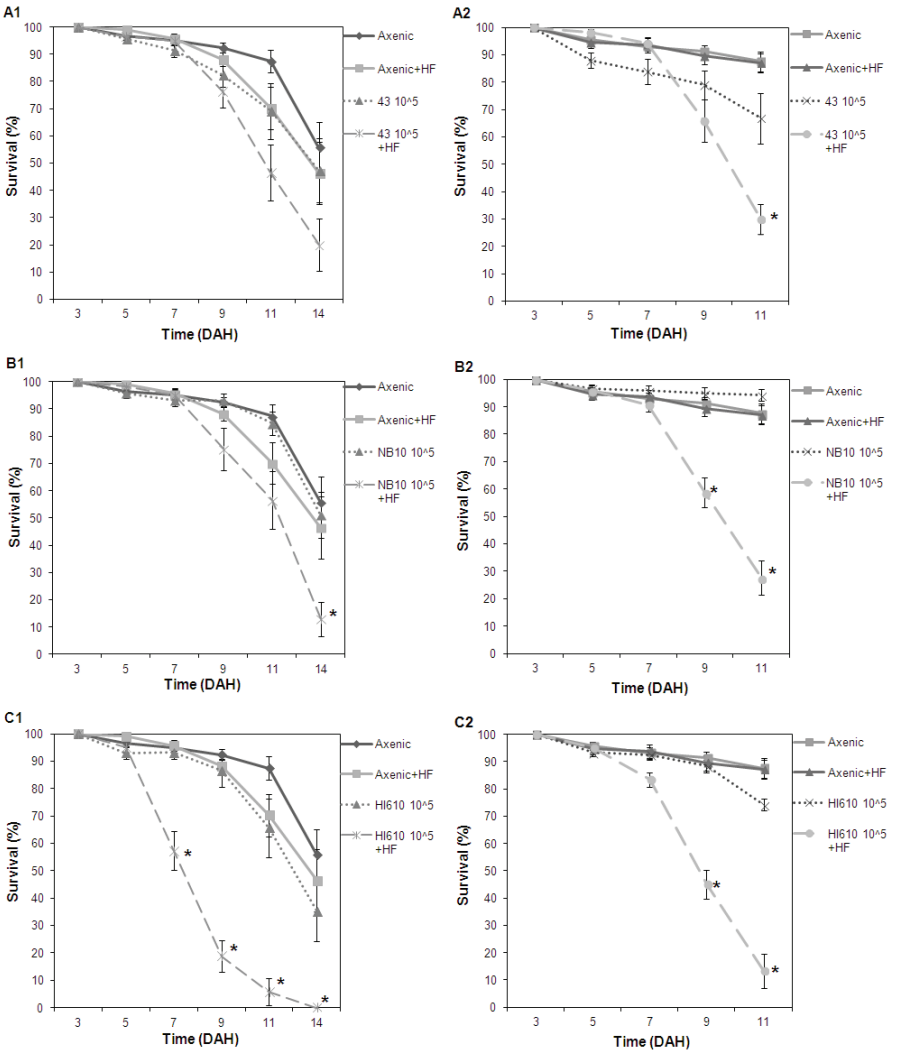


Fig. 2. Survival of gnotobiotic sea bass larvae challenged with *V. anguillarum* strains 43 (panels A1 and A2), NB 10 (panels B1 and B2), and HI 610 (panels C1 and C2) in two independent challenge tests. Error bars represent the standard error of 10 fish cultures. The asterisks denote significant differences when compared to the corresponding axenic control with or without homogenised fish ($p<0.05$). HF: homogenised fish

Table I. Motility of *V. anguillarum* strains 43, NB10, and HI 610 (average ± standard error, n=3 for each sampling point).

Treatment	Motility halo (mm)		
	43	**NB 10**	**HI 610**
Control	29 ± 3^{a}	35 ± 3^{A}	55 ± 0^{x}
Fish Homogenate	53 ± 9^{b}	55 ± 2^{B}	74 ± 1^{y}
Peptone	30 ± 3^{a}	39 ± 2^{A}	46 ± 1^{z}

Values in the same column with different superscript letters are significantly different ($p<0.05$).

References

Dierckens K., A. Rekecki, S. Laureau, P. Sorgeloos, N. Boon, W. Van den Broeck, and P. Bossier. 2009. Development of a bacterial challenge test for gnotobiotic sea bass (*Dicentrarchus labrax*) larvae. Environmental Microbiology 11:526-533.

Frans I., C.W. Michiels, P. Bossier, K.A. Willems, B. Lievens, and H. Rediers. 2011. *Vibrio anguillarum* as a fish pathogen: virulence factors, diagnosis and prevention. Journal of Fish Disease 34:643-661.

Natrah F.M., H.A. Ruwandeepika, S. Pawar, I. Karunasagar, P. Sorgeloos, P. Bossier, and T. Defoirdt. 2011. Regulation of virulence factors by quorum sensing in *Vibrio harveyi*. Veterinary Microbiology 154:124-129.

Wang X., Q.Y. Wang, M.J. Yang, J.F. Xiao, Q. Liu, H.Z. Wu, and Y.X. Zhang. 2011. QseBC controls flagellar motility, fimbrial hemagglutination and intracellular virulence in fish pathogen *Edwardsiella tarda*. Fish & Shellfish Immunology 30:944-953.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

ONTOGENY OF THE DIGESTIVE SYSTEM OF *OCTOPUS BIMACULATUS* PARALARVAE

D.J. López-Peraza*, M. Hernández-Rodríguez**, and B. Barón-Sevilla

Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE). E-mail: *dlopez@cicese.edu.mx; **mhernand@cicese.mx

Introduction

In Mexico, the octopus fishery is the fourth most commercially important and is concentrated mainly on the coasts of the Gulf of Mexico and Caribbean Sea, with *Octopus vulgaris* and *O. maya*, the last endemic species of the Yucatan Peninsula (CONAPESCA, 2011). *O. hubbsorum*, *O. macrocopus*, and *O. bimaculatus* are captured in the Pacific Ocean. The capture of *O. bimaculatus* predominates throughout the year in Bay of Los Angeles, Baja California (SEMARNAT, 2004). With the intention of developing the culture of octopus, different institutions have studied the larval development, and on a recurring basis have registered large mortalities, which are mainly due to nutritional deficiencies of supplied food and lack of standardization of culture techniques (Moxica et al., 2002; Iglesias et al., 2004). Studies of the biological development of the digestive system and the changes associated with the digestion and absorption processes of nutrients of the diet are crucial to understand the nutritional physiology of octopus paralarvae and to identify nutritional factors that limit their growth and survival.

Studies on the embryological development of octopus paralarvae have been made in a few species (Marthy, 1975; Boletzky, 2003; Ignatius and Srinivasan, 2006; Avila-Poveda et al., 2009), but knowledge is insufficient and even null in some species, including the spotted octopus *O. bimaculatus*. As this species holds economic value and potential for culture in Mexico, understanding paralarvae development is of fundamental importance. Therefore, in this work, a histological description of the organs that integrate the digestive system in the different phases of the ontogeny of *O. bimaculatus* paralarvae is provided. This serves as a basis for future studies of the digestive physiology of this species.

Materials and methods

Female of *O. bimaculatus* were collected in Bay of Los Angeles, Mexico, Baja California (B.C.), located between 29° 02' and 28° 57'N and 113° 32' and 113°

26'W. The organisms were transported to the Department of Aquaculture of the Centro de Investigación Científica y de Educación Superior de Ensenada, B.C. (CICESE) and they were individually placed in 500-l plastic tanks with continuous sea water replacement at a 200% daily rate and constant aeration; in addition a clay pot was placed as a refuge. Temperature, salinity, dissolved oxygen, and total ammonia as nitrogen were measured daily and the day in which the female started with the egg laying was registered. In order to study the ontogeny of the organs of digestive system, samples were taken from eggs at the beginning of laying (0 days post-laying, DPL) until the day of hatching (0 days post-hatching, DPH). The samples were fixed in the Davidson's solution, in a relation sample-fixation of 1:5, then they were dehydrated in a histokinette processor Leica model TP1040 and immediately they were embedded in paraffin, by means of a paraffin dispenser Leica model EG1160 to make sections of 5µm thickness of eggs and newly hatched paralarvae with a rotatory microtome American Optical Spencer-820-320. In order to contrast tissues, the technique of Arteta trichromic stain (Valderrama et al., 2004) was used. Images of the histological sections were captured by means of a microscope Olympus model CKX41, equipped with an Olympus camera C-5060 model. Finally, the organs and tissues that integrate the digestive system of paralarvae were identified and described histologically, and their relation to each other, taking as a reference the digestive system from an adult octopus of *O. bimaculatus*.

Results and discussion

The egg incubation lasted 61 days with a thermal variation of 16.5-21.5°C from April to June 2011.The embryonic development of O. bimaculatus could be grouped three stages: (a) from fertilization to the first inversion, in which the germinal layers were developed and the embryo prepared itself for organogenesis; (b) from first inversion to second inversion, where the development of the organs of the anterior region of the digestive system occurred, and the primordiums of most of the posterior region appeared; and (c) from second inversion to hatching, in which organs of the posterior region of the digestive system developed, and ended when the paralarvae hatched.

At 30DAL, more than 50% of embryos had experienced the first inversion towards the anterior part of the egg. In general, the pattern of embryonic development of *O. bimaculatus* was similar to that described by Arnold (1965) for *Loligo paeli*, however, his description did not include formation of internal organs. The formation of the ectodermal stomodeum in the embryo of *O. bimaculatus* (33DAL) agreed with stage 19 on the scale of Arnold (1965) for L. paeli, while the complete formation of the buccal mass (44DAL) agreed with the stage 24.

At 37DAL, the outlines of the stomach, caecum and digestive gland were observed. At 55DAL, more than 50% of embryos experienced the second inver-

sion. The structures that formed the buccal mass, i.e., mandibles, radula and anterior salivary glands, already resembled a newly hatched paralarvae. The stomach increased its size and its lumen was better defined, however, the epithelium of this organ still was undifferentiated. The caecum displayed a simple cylindrical epithelium very folded, with long cilia. The digestive gland had a greater size, greater development of the secretory tubules, which were constituted by a simple epithelium of ciliate cells, and the space between them was reduced. Also a section of the intestine was observed, constituted by a columnar epithelium.

In *O. bimaculatus* paralarvae at 0DAH (Fig. 1), the organs and tissues of the digestive system were differentiated in a similar arrangement to an adult. The buccal mass (Fig. 1A) was located in the anterior region, surrounded by a close sine that facilitates its movement; it had two mandibles and a radula that allow tearing and crushing of food. In *O. bimaculatus* paralarvae we noted that a row of teeth of the radula consists of 30 longitudinal teeth positioned parallel to the main axis. The anterior salivary glands, whose secretions (with secretions of the posterior salivary glands) were probably used to pre-digest the food. The esophagus was very narrow and crossed the cephalic region.

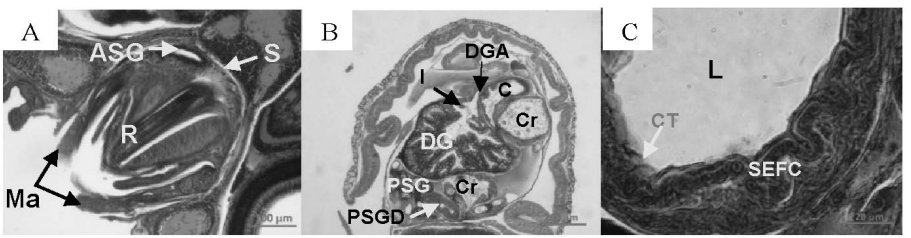


Fig. 1. Longitudinal cuts of the digestive system of *O. bimaculatus* paralarvae at 0DAH. A: buccal mass. ASG, anterior salivary gland; Ma, mandible; R, radula; S, sine. B: location of the digestive system organs in the internal cavity of the mantle. DGA, digestive gland appendage; C, caecum; Cr, crop; DG, digestive gland; PSG; posterior salivary gland; PSGD, posterior salivary glands ducts. C: approach of stomach epithelium. L, lumen; SEFC, simple epithelium of flat cells.

The posterior region of the digestive system of the paralarvae (Fig. 1B) was constituted by the crop, which internally was covered by a pseudo-stratified epithelium of flat cells. In the anterior portion of the crop, adjacent to the brain, were the posterior salivary glands. These glands were connected by a common duct with the buccal cavity. The stomach (Fig. 1C), whose lumen was covered by a simple epithelium of flat cells very folded. The external wall was made up of a thick layer of muscle fibers arranged in circular position. In the *O. bimaculatus* paralarvae, the goblet cells were not observed that are characteristic of the stomach epithelium of adult cephalopods (Budelmann et al., 1997), probably because the stomach was not yet completely developed. The caecum (Fig. 1B) that was connected to the digestive gland through two appendages was spiral-shaped; to-

wards the lumen it had a simple columnar epithelium with long cilia and mucous cells. The digestive gland was the organ of greatest volume of paralarvae and was delimited by a thin membrane and its interior was constituted by secretory tubules with a simple cylindrical epithelium of ciliated cells. In the distal portion of the digestive system was the intestine, which was in contact with the caecum and was folded in the mantle cavity. It ended in the distal opening or rectum located near the distal portion of the ink sac, both adjacent to the siphon.

The present research constitutes the first description at histological level of the ontogenic development of the digestive system of the of *O. bimaculatus* paralarvae. This information is essential because it will serve as a foundation for futures digestive physiology studies of this species.

References

Arnold J. 1965. Normal embryonic stages of the squid, Loligo pealii (Lesueur). Biological Bulletin. 128(1):24-32.

Ávila-Poveda O.H., R.F. Colin-Flores, and C. Rosas. 2009. Gonad development during the early life of Octopus maya (Mollusca: Cephalopoda). Biological Bulletin 216:94-102.

Boletzky S. 2003. Biology of early life stages in cephalopod molluscs. Advances in Marine Biology, 44:143-203.

Budelmann B.U., R. Schipp, and S.V. Boletzky. 1997. Cephalopoda. 119-414p. In: Microscopic Anatomy of Invertebrates. Mollusca II. Vol.6A. s Harrison F.W. and Kohn (Ed.) Wiley-Liss, New York, London. 480p.

CONAPESCA. 2011. Anuario Estadístico de Acuacultura y Pesca. Secretaria de agricultura, ganadería, desarrollo rural, pesca y alimentación. 34p.

Iglesias J., J.J. Otero, C. Moxica, L. Fuentes, and F.J. Sánchez. 2004. The completed life cycle of the octopus (Octopus vulgaris, Cuvier) under culture conditions: paralarval rearing using Artemia and zoeae, and first data on juvenile growth up to 8 months of age. Aquaculture International 12:481-487.

Ignatius B. and M. Srinivasan. 2006. Embryonic development in Octopus aegina Gray, 1849. Current Science 91(8):1089-1092.

Marthy H. 1975. Organogenesis in Cephalopoda: further evidence of blastodisbound developmental information. Journal of embryology and experimental morphology 33:75-83.

Moxica C., F. Linares, J.J. Otero, J. Iglesias, and F.J. Sánchez. 2002. Cultivo intensivo de pararlarvas de pulpo, Octopus vulgaris Cuvier, 1797, en tanques de 9 m3. Boletín Instituto Español de Oceanografía 18(1-4):31–36.

SEMARNAT. 2004. Carta Nacional Pesquera. Comisión Nacional de Acuacultura y Pesca, México. 128p.

Valderrama K., M. Oliva, B. Campos, and D. Brown. 2004. Parasitic castration of Eurhomalea lenticularis (Bivalvia:Veneridae) by a digenetic trematode: quantitative histological analysis. Disease of aquatic organism 59:151-158.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

LOW-COST PRODUCTION OF THE MARINE THRAUSTOCHYTRID ISOLATE, *SCHIZOCHYTRIUM* SP. LEY7 AS LARVAL LIVE FEED ENRICHEMENT FOR THE MANGROVE SNAPPER, *LUTJANUS* SP.

G. Ludevese-Pascual, M. de la Peña, O. Reyes, and J. Tornalejo

Southeast Asian Fisheries Development Center/Aquaculture Department (SEAFDEC/AQD), Brgy. Buyuan, Tigbauan, Iloilo 5021 Philippines

Introduction

Thraustochytrids are eukaryotic organisms ubiquitous in marine and estuarine environments, in both tropical and sub-tropical areas (Leano et al., 2003). They are now established candidates for commercial production of docosahexaenoic acid (DHA) (Raghukumar, 2008). Despite growing evidences on its applicability in aquaculture, its use is primarily hampered due to the high cost of culture media. This study evaluates the use of low-cost production substrates and determines the optimum culture conditions for stable biomass production of *Schizochytrium* sp. LEY7 isolated from mangrove leaves in Baybay, Southern Leyte, Philippines as potential source of lipid and polyunsaturated fatty acid (PUFA) in the larviculture of the mangrove snapper, *Lutjanus* sp.

Materials and methods

Growth of *Schizochytrium* sp. LEY7 in standard and alternative low-cost substrates was assessed with a haemocytometer every 24h for 5 days. Costs were compared among the production substrates tested.

Optimum growth of *Schizochytrium* sp. LEY7 was further determined in different salinity (15, 25, 30ppt) and pH (4, 6, and 8) combinations at different incubation temperatures (20, 25, and 30°C) using the best low-cost production substrates obtained in the previous experiment. Thraustochytrid biomass, expressed as mg freeze-dried weight (FDW) per 50ml culture medium, was analyzed for macronutrients, total lipid content, and fatty acid profile.

Preliminary trials on the application of *Schizochytrium* sp. LEY7 as enrichment for *Artemia* nauplii used in the larviculture of the mangrove snapper, *Lutjanus* sp. was conducted. Feeding trials were carried using the following dietary treatments: thraustochytrid-enriched *Artemia* nauplii; hatchery-prepared emulsion-enriched *Artemia* nauplii; A1 DHA SELCO-enriched *Artemia* nauplii, and non-

enriched *Artemia* nauplii serving as the control diet. Percent survival and increase in total length (cm) was quantified at the end of the feeding trial. Fifteen fry were randomly collected at the end of the feeding trial for salinity stress test (5, 15, 25, 35, and 45ppt) for 24h.

Results and discussion

Growth of *Schizochytrium* sp. LEY7 in standard and alternative low-cost substrates differed after 24h (Table I). Cost was substantially reduced when using the production substrate containing commercial glucose and yeast extract from NaCl-treated baker's yeast as source of carbon and micronutrients, respectively. Growth pattern of *Schizochytrium* sp. LEY7 characterized by a short duration of lag phase with highest increased in biomass at 48h concurred with the study of Arafiles et al. (2011). The short duration of lag phase displayed by *Schizochytrium* sp. LEY7 irrespective of the type of production substrates used in this study indicates a good growth advantage in that cells are readily able to utilize the medium's components and thus possibly reducing the growth of contaminants that can be present during the early phase of the culture.

Table I. Growth of *Schizochytrium* sp. LEY7 in standard and alternative production substrates after 24h at ambient temperature and salinity (means of 3 replicates ± SEM).

Production substrates	Cell density (cells.ml^{-1})	Estimated production cost (€.ml^{-1})
Standard production substrates		
JTBaker glucose + Hi-Media yeast extract	1 042 500±2.47^{b}	2.52
Alternative low-cost production substrates		
JTBaker glucose + NaCl-treated yeast extract	1 160 625±40.12^{ab}	2.30
Commercial glucose + Hi-Media yeast extract	1 151 875±4.54^{ab}	0.50
Commercial glucose + NaCl-treated yeast extract	1 440 000±17.75^{a}	0.28
Cane molasses + Hi-Media yeast extract	383 750±3.50^{d}	0.48
Cane molasses + NaCl-treated yeast extract	513 125±2.27^{cd}	0.26
Commercial honey + Hi-Media yeast extract	695 625±53.35^{c}	0.73
Commercial honey + NaCl-treated yeast extract	715 625±82.99^{c}	0.51

Values in a column with different letter superscripts are significantly different (p<0.01).

Biomass production of *Schizochytrium* sp. LEY7 was optimized using an incubation temperature of 30°C and salinity level of 30ppt (Fig. 1). Biomass production did not however differ at 4, 6, and 8 pH levels. Crude protein and nitrogen free extracts (soluble carbohydrate) were the two most abundant macronutrients found (Table II). Total lipid averaged to 19.4%. Principal fatty acids were palmitic acid (C16:0) with 33.52% and docosahexanoic acid (DHA, C22:6n3) with 39.92% proportion to total fatty acid. Thraustochytrids are therefore considered suitable for the production of DHA (Kobayashi et al., 2011).

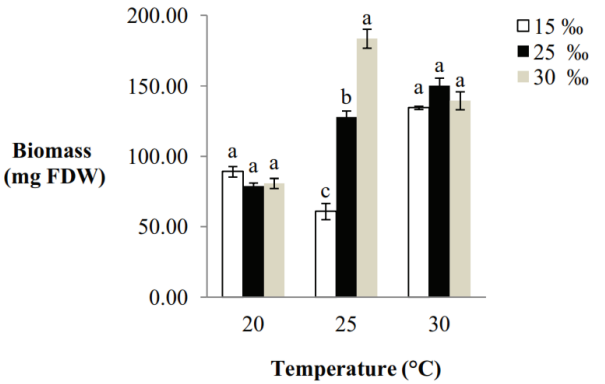


Fig. 1. Biomass production (per 50ml broth medium) of *Schizochytrium* sp. LEY7 at 3 salinity levels incubated at 20, 25, and 30°C. Data are means of 5 replicates±SEM. Letter superscript per salinity level indicates significant difference ($p<0.01$).

Table II. Chemical composition of *Schizochytrium* sp. LEY7 produced using low-cost production substrate at optimum culture conditions.

Macronutrients	Percent content (%)
Moisture content	2.36
Crude protein	34.53
Crude fat	14.97
Crude Fiber	0.08
Nitrogen Free extract (soluble carbohydrates)	40.38
Ash	10.03

Raw data on the average total length (ATL) and percent survival at the end of the feeding trial were highest in snapper fry fed thraustochytrid-enriched *Artemia* nauplii (data not shown). Snapper fry fed SELCO-enriched *Artemia* nauplii were diagnosed for amyloodiniosis towards the end of the feeding trial while snapper fry fed thraustochytrid-enriched *Artemia* nauplii remained unaffected. Raw data on salinity stress test showed lower percent morbidity for snapper fry fed thraustochytrid-enriched *Artemia* nauplii (data not shown). Thraustochytrid enrichment might have increased the resistance of the fry. Confirmatory experiment has to be conducted to verify the results obtained.

Conclusion

This study demonstrates the potential of low-cost production substrates in mass producing the *Schizochytrium* sp. LEY7 for maximum DHA production. High biomass yield was achieved by optimizing the culture conditions particularly the incubation temperature and salinity levels. Preliminary data on feeding trials have shown promising results and therefore confirmatory experiments need to be conducted to verify the results obtained.

References

Arafiles K.H.V., J.C.O. Alcantara, P.R.F. Cordero, J.A.L. Batoon, F.S. Galura, E.M. Leaño, and G.R. Dedeles. 2011. Cultural Optimization of Thraustochytrids for Biomass and Fatty Acid Production. Mycosphere 2(5):521–531.

Kobayashi T., K. Sakaguchi, T. Matsuda, E. Abe, Y. Hama, M. Hayashi, D. Honda, Y. Okita, S. Sugimoto, N. Okino, and M. Ito. 2011. Increase of Eicosapentaenoic Acid in Thraustochytrids through Thraustochytrid Ubiquitin Promoter-Driven Expression of a Fatty Acid Δ5 Desaturase Gene. Applied and Environmental Microbiology 77(11):3870–3876.

Leaño E., R. Gapasin, B. Polohan, and L.Vrijmoed. 2003. Growth and fatty acid production of thraustochytrids from Panay mangroves, Philippines. Fungal Diversity 12:111-122.

Raghukumar S. 2008. Thraustochytrid Marine Protists: production of PUFAs and Other Emerging Technologies. Marine Biotechnology (NY) 6:631-40.

Santhanam A. and S. Sreenivasan. 2010. Microbial production of polyhydroxy alkanotes (PHA) from *Alcaligens* spp. and *Pseudomonas oleovorans* using different carbon sources. African Journal of Biotechnology 9(21):3144-3150.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

IMPORTANCE OF DHA FOR FIRST FEEDING PIKEPERCH LARVAE – INFLUENCE ON BEHAVIOURAL RESPONSES

I. Lund, E. Höglund, and P.V. Skov

Technical University of Denmark, DTU Aqua, Section for Aquaculture, The North Sea Research Centre P.O. Box 101, DK-9850 Hirtshals, Denmark

Pikeperch (*Sander lucioperca*) is a strong candidate for the potential diversification of freshwater aquaculture in Europe. In aquaculture it is crucial to produce robust, stress-resilient fish and the vulnerability of fish to dietary fatty acid composition may affect cognitive function and the ability to cope with stress. DHA is important in terms of brain and neural development and plasticity, as well as in the support of learning and memory. Recent studies have indicated that dietary LC-PUFAs (especially DHA) have immediate and long-term consequences on mortality and salinity stress sensitivity in larval and juvenile pikeperch and affect the size of the brain. In the present study dietary emulsions with various DHA or phospholipids levels were provided to pikeperch larvae until 28dph, when they were subjected to a suite of tests to examine behavioural responses to visual and mechanosensory stressors. Learning ability was quantified by a maze test, where the duration to find a way out was used as a learning index. Four dietary emulsions were made by the substitution of extra refined virgin olive oil (Seatons 79.1% oleic acid) with either DHA oil (Incromega DHA500TG, DHA content ≥51% of total fatty acids) or a fish oil rich in phospholipids. Three emulsions contained either (A) 0g, (B) 50g, or (C) 500g.kg^{-1} DHA oil and one emulsion (D) 500g.kg^{-1} phospholipid rich fish oil (440g phospholipids.kg $^{-1}$). *Artemia* were enriched with one of the four emulsions for 24h and fed to triplicate groups of larvae from 7-28dph for 18h.day^{-1}. From 29-40dph, all groups were weaned onto dry feed made of fish meal and fish oil/rapeseed oil and fed until 140dph.

At 27dph growth of larvae was recorded and larvae sampled for body FA composition. Larvae body fatty acid composition reflected the dietary composition of the four treatments, while growth was not significantly affected. In experiments looking at behavior in the presence and absence of a simulated predator, pikeperch larvae fed *Artemia* deficient or low in DHA (treatment A and B) exhibited a high degree of apprehensiveness, as evidenced by a significantly higher proportion of time (~90%) spent at the edge of the Petri dish. A remarkable shift in this behavior was observed for treatment C and D with a high DHA or phospholipid inclusion, as <10% of the time was spent at the edge. When a visual stressor

was applied in the form of a moving ball, all dietary treatments increased the fraction of time spent at the edge, although fish on diets rich in DHA or phospholipids continued to spend less time at the edge, suggesting that fish on a more balanced FA diet are less prone to seeking safety under unfamiliar conditions. The application of a moving shadow (i.e. simulating the presence of a predator) in the upper half of the dish caused fish to spend an increased amount of time in the lower half. The fact that all fish, regardless of diet, displayed an equal response to a visual stimulus indicates no impaired visual acuity in fish on diets with low DHA content. Fish on dietary treatment A and B with low levels of DHA had significantly higher routine swimming speeds, averaging 2 and 1.3BL.s^{-1} respectively, while fish on treatment C and D had comparable routine swimming speeds of 0.1-0.2BL.s^{-1} under control conditions. Visual observation of the former dietary groups indicates that this is erratic behavior correlated to the continuously swimming around along the edge. Such behavior is likely to incur a high energetic cost, and combined with the observed apprehensiveness, compound effects on growth performance. For the diets low in DHA, swimming speeds were not affected by a simulated predator, however, in C and C diets, swimming speeds increased five- to eightfold. The amount of time holding station decreased significantly with decreasing DHA enrichment, to a minimum of 10% in the groups fed diet A. In the face of a simulated predator, all dietary treatments spent an equal amount of time holding station. In a different series of experiments, we assessed the responsiveness to a mechanosensory stimulus by performing a series of fast escape experiments. Larvae reared on diet C and D performed better than larvae on low DHA diets, in that they had peak accelerations significantly higher than for larvae fed on diet A and B.

Long-term effects on learning ability and stress responsiveness were investigated 121-140 days after termination of larvae treatment by a maze test and cortisol response to confinement. The maze consisted of a 40×40cm square with four potential exits in each corner. One of the exits led juvenile fish out of the maze to a darker area with a cover. Initial freezing time before searching, time spent searching, and number of visits to exits before leaving the maze were recorded. Juveniles were trained in six sessions in the maze during three days and time spent in the maze decreased during training, an effect that was related to a decrease in initial freezing time. Moreover, juveniles fed diet (A) as larvae had longer initial freezing time compared to juveniles fed diet C or D with a high content of DHA and phospholipids, which was independent of training. This indicates a more anxious behaviour profile and suggests consistent effects of LC PUFAs during early larval feeding on brain function. However, dietary DHA content or phospholipids did not affect the juvenile stress-induced cortisol release, suggesting that this difference in brain function is not directly related to the neuroendocrine stress axis. However, an anxious behaviour profile may reflect a general behavioural inhibition in responses to stressful events, such as reduced appetite, and thereby may directly affect production parameters.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

USE OF *PHAEOBACTER* SP. PROBIOTIC BACTERIA FOR THE REARING OF SEA BASS LARVAE (*DICENTRARCHUS LABRAX*)

P. Makridis, N. Papandroulakis, and P. Divanach

Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Center for Marine Research, Herakleion, Crete, Greece

Introduction

Bacteria of the *Phaeobacter* clade have been previously used in the rearing of marine fish larvae (Prol Garcia et al., 2012; D'Alvise et al., 2012). Probiotic bacteria *Phaeobacter* sp. were bioencapsulated in rotifers and *Artemia* and used for the rearing of sea bass larvae (*Dicentrarchus labrax*). The aim of this experiment was to determine if bioencapsulation of *Phaeobacter* bacteria in the live diet could improve the biological performance of sea bass larvae in terms of growth and survival, modify the intestinal microbiota of the larvae and provide protection against fish pathogens in a small scale challenge trial.

Materials and methods

Sea bass yolk-sac larvae were stocked in 500-l cylindroconical fiberglass tanks (about 50×10^3 larvae per tank). *Phaeobacter* isolated from bonito larvae (*Sarda sarda*) were cultured in Marine Broth (Difco Laboratories USA) and bioencapsulated in rotifers or *Artemia* after 30min incubation, where live food organisms grazed bacteria added to seawater at a final cell density of 5×10^7cells ml^{-1}. Four tanks received bioencapsulated bacteria three times per week and four tanks were used as control. Microalgae *Chlorella minutissima* were added to the tanks at a final density of about 25×10^4 cells.ml^{-1}. Samples of fish were taken 4, 11, 18, 25, and 32 days after hatching. Five larvae from each tank were homogenized in glass homogenizers and spread on Marine agar and TCBS (thiosulfate-citrate-bile salts-sucrose) agar after serial dilutions. An aliquot of the homogenate was stored at -20°C and used for DGGE (denaturing gradient gel electrophoresis) analysis, which was run as described by Massana et al. (1997).

A small-challenge trial was run with 30-day-old sea bass larvae. Six batches of 25-30 larvae that had received probiotics were transferred to 1-l beakers and these were incubated at 20°C with careful aeration for 3 days. A pathogenic *V. harveyi* was added to three of the beakers at about 10^5 cells final concentration.

A similar procedure was followed for larvae from the control tanks. Mortality in the beakers was monitored and dead larvae were removed daily.

Results and discussion

Survival 60 days after hatching was significantly higher ($P<0.05$) in tanks with added probiotics in the diet compared with control treatment (Fig. 1), whereas no significant difference was shown in the growth of the larvae between the two treatments (results not shown).

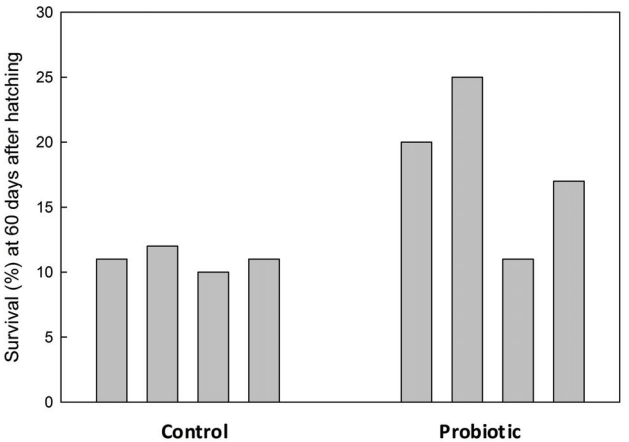


Fig. 1. Survival 60 days after hatching in each tank of the experiment as a percentage of initial number of yolk-sac sea bass larvae

Total numbers of bacteria in terms of colony-forming units in Marine Agar was similar in the two treatments, whereas presumptive *Vibrio*, as determined by counts in TCBS agar were significantly lower ($P<0.05$) in probiotic treatment (results not shown). DGGE analysis showed that microbial diversity in general was highest at day 4 after hatching (yolk-sac stage) and that it gradually decreased at later stages. A comparison between the treatments showed that microbial diversity was lower in the probiotic treatment (Fig. 2). If probiotic bacteria inhibited growth of *Vibrio* bacteria as implied by previous results, this suggests that this is the case here as well, both from classical microbiological results and DGGE analysis.

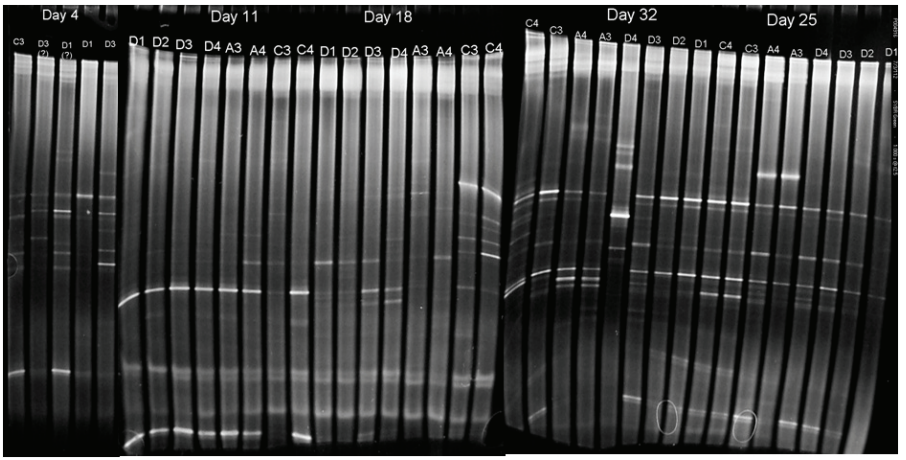


Fig. 2. DGGE analysis in sea bass larvae from control tanks (A3, A4, C3, C4) and tanks added probiotics (D1-D4) on day 4, 11, 18, 25, and 32 after hatching.

In the challenge test it was shown that sea bass larvae from the probiotic treatment showed a survival of 51% and 49% in beakers added pathogen and contol beakers, respectively. In the case of larvae from control tanks, survival was 18% and 51% in beakers added pathogen and control beakers, respectively. These results showed that probiotic-treated larvae showed a higher resistance against the pathogen *V. harveyi*.

References

D'Alvise P.W., S. Lillebø, M.J. Prol-Garcia, H.I. Wergeland, K.F. Nielsen, K.F. Nielsen, Ø. Bergh, and L. Gram. 2013. *Phaeobacter gallaciensis* reduces *Vibrio anguillarum* in cultures of microalgae and rotifers, and prevents vibriosis in cod larvae. PLoS ONE 7(8): art. no. e43996.

Massana R., A.E. Murray, C.M. Preston, and E.F. DeLong. 1997. Vertical distribution and phylogenetic characterization of marine planktonic *Archaea* in the Santa Barbara Channel. Applied and Environmental Microbiology 63:50-56.

Prol-Garcia M.J., M. Gomez, L. Sanchez, and J. Pintado. *Phaeobacter* grown in biofilters: A new strategy for the control of *Vibrionaceae* in aquaculture. Aquaculture Research (in press, DOI: 10.1111/are.12046)

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

DIGESTIVE AND IMMUNE RESPONSES TO PROBIOTICS OR PREBIOTICS IN PERCID LARVAE

S.N.M. Mandiki, C. Mathieu, F. Chaffa, T.M. Rakotomalla, J. Douxfils, and P. Kestemont

University of Namur, Research Unit in Environmental and Evolutionary Biology, Rue de Bruxelles, 61, B-5000 Namur, Belgium.

Introduction

Application of probiotics or prebiotics are nowadays tested as growth and immune defence stimulators in fish, but little information is available on the synergic effects expected from association of some probiotics and prebiotics. Moreover, the physiological pathways by which bio-stimulants act are still not clarified for fish. Specifically, the use of bio-stimulants has received little attention in percid fish, but the few available data indicate beneficial actions on some immune compounds (Siwicki et al., 2009; Mandiki et al., 2011). While a high mortality is still limiting the development of the culture of some percid species, no efficient prophylactic strategy has been established for optimizing fish rearing conditions. The study aimed to determine the potential effects of probiotics or prebiotics on growth-related parameters, digestive enzyme activities, and immune compounds of larvae of Eurasian perch (*Perca fluviatilis*) or pikeperch (*Sander lucioperca*) under intensive culture conditions.

Materials and methods

Experiment 1 tested the effects of increasing doses of either probiotic bacteria or prebiotics on pikeperch larvae. Probiotic bacteria were a commercial mixture of *Bacillus* spp. composed of *B. subtilis*, *B. licheniformis*, and *B. pumilus* (Sanolife MIC-F = B, INVE Aquaculture, Belgium), and prebiotics consisted of phytobiotics (P) composed of Chine herbal extracts (INVE, Belgium). This was conducted in triplicate 40-l tanks containing 200 larvae (8mg, initial body weight, IBW). After training to co-feed on live and dry fed, larvae were fed with enriched metanauplii with increasing doses of *Bacillus* (B: 4, 12, and 24×10^{11}CFU.l^{-1} incubation medium = B1, B3, B6) or phytobiotics (P) coated on a dry feed (3-6g.kg^{-1} feed = P3, P6) for 30 days. Growth, deformity rate, digestive enzyme activities, and immune compounds were measured on D15 and 30. The second experiment tested the influence of increasing doses (100, 200, 400mg.l^{-1} incubation medium) of inulin or a mixture of inulin and the same probiotic bacteria

(Sanolife MIC-F = B) as in experiment 1 using Eurasian perch larvae. Dose treatments were compared in triplicates of 40-l tanks containing 200 larvae (8mg, IBW), and growth parameters as well as digestive enzyme activities were measured on D15 and 30. Experiment 3 emphasized the influence of prebiotics using pikeperch larvae (5mg, IBW). Increasing doses (400, 800, 1200mg.l^{-1} incubation medium) of inulin (I) or oligofructose (OF) were tested in triplicates of 40-l tanks containing 700 larvae (5mg of IBW), and growth-related parameters, digestive enzyme activities, and immune compounds were measured on D14 and 28.

Results

In experiment 1, only high bacteria doses (B6) increased growth rate, while digestive enzyme activities were improved with almost all the tested doses, namely α-amylase and chymotrypsine (Table I). No growth or physiological effects were observed for the phytobiotic treatments. Bacteria treatments also increased some immune compounds (Table I) but final survival did not differ for both probiotic bacteria and phytobiotics, no matter the dose. The mixture of probiotic bacteria (B3) and inulin showed a dose-dependent effect for growth rate, and the increase varied between 13-34% depending on inulin doses. Inulin alone also stimulated growth rate (10-31%), and this was associated with elevated digestive enzyme activities, especially phosphatase alkaline (33%) and trypsine (41%). Experiment 3 is ongoing and results will be presented during the Larvi symposium 2013.

Table I. Growth and physiological responses (%) to probiotic bacteria or phytobiotics (B1, B3, B6: 4, 12, 24×10^{11}CFU.l^{-1}. P3, P6: 3-6g.kg^{-1} feed).

	B1	**B3**	**B6**	**P3**	**P6**
SGR	8	8	23*	6	0
a-amylase	0	128*	88*	14	0
Chymotrypsine	78*	99*	88*	14	16
Lysozyme activity	80*	155*	128*	29*	29*
Total immunoglobulins	12	50*	80*	11	8

Conclusions:

The results showed that probiotic bacteria or inulin can stimulate growth rate by improving digestive enzyme activities and some immune functions, while the targeted phytobiotics did not show any stimulatory effects in percid larvae.

References

Mandiki S.N.M., S. Milla, N. Wang, G. Blanchard, T. Djonkack, S. Tanascaux, and P. Kestemont. 2011. Effects of probiotic bacteria on growth parameters

and immune defence in Eurasian perch *Perca fluviatilis* larvae under intensive culture conditions. Aquaculture Research 39:647–656.
Siwicki A.K., Z. Zakes, E. Terech-Majewska, A. Kowalska, and J. Malczeweska, 2009. Supplementing the feed pikeperch juveniles with macrogard and its influence on non-specific cellular and humoral defense mechanisms. Aquaculture Research 40:405-4011.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

DO IMMUNOSTIMULANTS AFFECT SPERM QUALITY IN SENEGALESE SOLE?

S. Martínez-Páramo[1], F. Arfuso[1,2], S. Engrola[1], J. Dias[1,3], C. Faggio[2], and C. Aragão[1*]

[1] CCMAR-CIMAR L.A., Universidade do Algarve, Campus de Gambelas, edf. 7, 8005-139 Faro, Portugal
[2] Department of Biological and Environmental Sciences, University of Messina, Viale Ferdinando Stagno d'Alcontres 31, 98166 S.Agata-Messina, Italy
[3] Sparos, Centro Regional para a Inovação do Algarve (CRIA), Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal; *caragao@ualg.pt

Introduction

The use of immunostimulants is a preventive approach important in aquaculture to improve the fish's innate defense and resistance against diseases. β-glucans and some plant constituents are immunostimulants, which may directly initiate activation of the innate defence mechanisms that may result in production of anti-microbial molecules. However, in birds, it has been recently shown that immune activation reduces sperm quality (Losdat et al., 2011). Sperm production in Senegalese sole (*Solea senegalensis*) is very low and variable and sperm quality is usually better in wild than in G1 males (Cabrita et al., 2006). Moreover, an impairment of the immune system in Senegalese sole under stressful husbandry conditions has been recently confirmed (Costas et al., 2013). Therefore, in this work, paprika oleoresin and β-glucan-supplemented diets were tested in G1 Senegalese sole subjected to repeated handling stress, and the effect of these additives on sperm quality was evaluated.

Materials and methods

Senegalese sole (G1 specimens; 862±192g, mean±SD) were divided in groups of six fish and placed in six flat-bottomed rectangular tanks (density=4.3kg m^{-2}, open water circulating system at 21.0±0.1ºC and 36.6±0.5g l^{-1}). Three experimental diets were used: Control, Paprika and β-Glucan. All diets had the same basal formulation presented in Table I, which is considered the Control diet. Paprika oleoresin (0.3%; Ingredientes Naturales Seleccionados S.L., Spain) and β-glucans (0.1%; Leucogard®, Fibona Health Products GmbH, Germany) were emulsified in fish oil and applied post-extrusion by vacuum coating to the Control diet, obtaining the Paprika and β-Glucan diets, respectively. Each diet was

assigned to duplicate tanks. Fish were fed six days a week by automatic feeders. After three weeks of feeding, fish were subjected to repeated handling stress for eight days (air exposure for 5min before feeding).

Table I: Diet formulation.

Ingredients (%)	Control diet
Fishmeal LT70	17.3
Fishmeal 60	25.0
CPSP 90	6.7
Squid meal	22.4
Wheat gluten	3.0
Whole wheat	5.0
Whole peas	9.0
Fish oil	10.5
Vit & Min premix	1.0
Choline chloride	0.1

Sperm evaluation

After 8 days of repeated handling stress all fish were sampled. Fish were anaesthetised with 2-phenoxyethanol (150mg l^{-1}) and checked for skin injuries. In male specimens, sperm samples were collected from the urogenital pore using a syringe without a needle by gently pressure on the testes. The urogenital pore was previously clean and dry to avoid urine contamination. Samples were maintained at 4°C for further processing.

Sperm motility and concentration were analysed immediately after extraction using the computer-assisted sperm analysis (CASA) coupled to a microscope (Nikon E200, Tokyo, Japan) with a 10× negative phase contrast objective. For motility analyses, 1µl of sperm was placed on a makler chamber and activated with 10µl of seawater. Motility was recorded at 15, 30, 45, and 60s post-activation using a Basler camera A312f (25 frames.s^{-1}) (Basler Afc, Ahrensburg, Germany) and images were processed with the software ISAS (Proiser, Valencia, Spain). Total motility (TM, %; spermatozoa with a VCL>10µm.s^{-1}), progressiveness (PM, %), curvilinear velocity (VCL, µm.s^{-1}) and straight line velocity (VSL, µm.s^{-1}) were determined in duplicate for each sample.

To quantify cell concentration, sperm was diluted (1:5, v.v^{-1}) in a non-activating solution (300mM sacarose). Ten microlitres of the dilution was placed in a makler chamber and 3 microscope fields were capture per sample and processed with the ISAS software.

Statistical analyses

The SPSS 18.0 package for Windows was used for the statistical analysis. Skin injuries were analysed using the chi-square test. Significant differences between treatments for sperm concentration were determined using a one-way ANOVA

followed by Tukey's test. Sperm motility parameters were compared using a general linear model with the Bonferroni correction. Statistical analysis was performed with the whole curve during the whole motility recorded time, comparing the tendency of the curve for each treatment during the 60s recorded. Statistical significance was tested at a 0.05 probability level.

Results and conclusions

Results showed that Paprika diet significantly decreased skin injuries in fish subjected to repeated handling stress [Chi-square test: asymp. sig. (2-sided) = 0.021].

Paprika diet significantly increased sperm concentration compared to the Control diet, showing values of 1.8×10^{9}spz.ml^{-1} and 0.1×10^{9}spz.ml^{-1} respectively. β-Glucan diet resulted in similar values to the Control (Fig. 1).

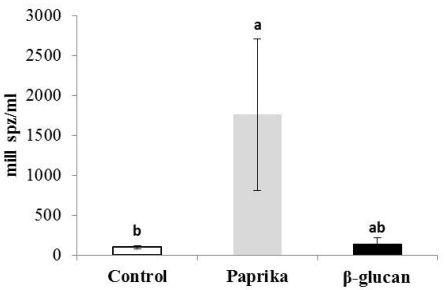


Fig.1. Sperm concentration in *Solea senegalensis* males fed with the experimental diets (means±SEM). Different superscripts indicate significant differences among treatments.

Sperm motility, despite not being significantly different among treatments, showed similar trends for all parameters, with the higher values for the Paprika diet and the lowest values for the β-Glucan diet. Sperm of Control and Paprika treatments showed a normal pathway of movement, with high motility values at 15s post-activation decreasing until 60s, while β-Glucan diet promoted low sperm motility since activation. Fifteen seconds after activation, total sperm motility of Paprika and Control treatments were 61.0±8.7% and 53.5±9.5% respectively, while with the β-Glucan diet the percentage of spermatozoa motile was 19.5±6.5%. Similarly, progressiveness of sperm from males fed with the Control (22.0±6.0%) and Paprika (21.7±6.9%) diets were similar, while less than 10% of the spermatozoa were progressive with the β-Glucan diet (Fig. 2). Curvilinear and straight line velocities were also not significantly different among treatments, although the lowest values were recorded with the β-Glucan diet. The highest curvilinear velocity, 15s post-activation, was obtained in sperm from males fed with the Paprika diet (112.5±35.2μm.s^{-1}), followed by the Control

(96.5±15.5µm.s^{-1}) and β-Glucan diets (34.0±1.0µm.s^{-1}). The straight line velocities, 15s after activation, were 68.3±21.9µm.s^{-1}, 61.5±0.5µm.s^{-1} and 35.0±16.0µm.s^{-1} for the Paprika, Control, and β-Glucan diets, respectively.

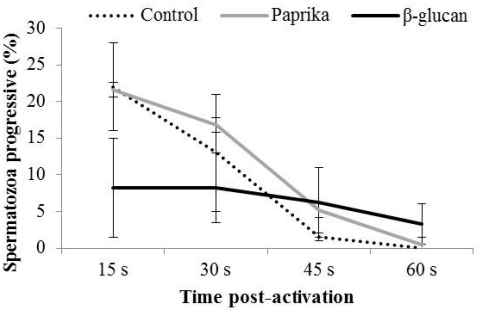


Fig.2 Spermatozoa progressiveness during 1 minute post-activation in *Solea senegalensis* males fed with the experimental diets (means±SEM). No significant differences were found among treatments.

In conclusion, paprika supplemented diet increased sole performance by reducing skin injuries and increasing sperm concentration. β-Glucan supplemented diet tend to reduce sperm motility and did not improve fish resistance to skin injuries compared with Control diet. Positive effects of Paprika diet may be related with the capacity of this compound to counteract oxidative stress, which is one of the causes of sperm damage. Further studies are necessary to explore the immunostimulator effect of the paprika oleoresin.

Acknowledgements

This study benefited from funding by Project BONAQUA (programme POCTEP, co-funded by FEDER, European Commission). SMP, CA and SE were supported by Fundação para a Ciência e Tecnologia postdoctoral fellowships (BPD/48520/2008, BPD/37197/2007, BPD/49051/2008) funded by National Funds (MEC).

References

Cabrita E., F. Soares, and M.T. Dinis. 2006. Characterization of Senegalese sole, *Solea senegalensis*, male broodstock in terms of sperm production and quality. Aquaculture 261:967–975

Costas B., C. Aragão, J. Dias, A. Afonso, and L.E.C. Conceição. 2013. Interactive effects of a high-quality protein diet and high stocking density on the stress response and some innate immune parameters of Senegalese sole *Solea senegalensis*. Fish Physiology and Biochemistry, *in press*

Losdat S., H. Richner, J.D. Blount, and F. Helfenstein. 2011. Immune activation reduces sperm quality in the great tit. PLoS ONE 6(7):e22221

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EVALUATION OF DAILY RHYTHMS IN FEEDING ACTIVITY AND DIGESTIVE FUNCTIONS IN GILTHEAD SEA BREAM (*SPARUS AURATA*) LARVAE

J.A. Mata[1], G. Martínez-Rodríguez[1], F.J. Moyano[2], and M. Yúfera[1]

[1] Instituto de Ciencias Marinas de Andalucía (ICMAN-CSIC). Campus Universitario Río San Pedro. 11510-Puerto Real, Cádiz, Spain
[2] Universidad de Almería. Departamento Biología y Geología. La Cañada de San Urbano s/n. 04120-Almería, Spain

The larval stage is considered the main bottleneck in marine fish culture due to the high mortalities produced during this period. Most of the problems related with feeding and nutrition may be related to the lack of detailed knowledge on the digestive process during these early life stages. The aim of this study was to describe the relation between feeding activity and digestive functions (enzymatic activity and gene expressions) at different moments during development (10, 18, 30, and 60 days post-hatch; dph) of gilthead seabream (*Sparus aurata*) larvae.

Larvae were maintained in 300-l circular tanks under a photoperiod of 12h L:D and fed on live food (rotifers followed by *Artemia* nauplii). Samples were taken in the selected days every 2h for a 24-h period, with active feeding confirmed by evaluation of stomach contents. The relation between food intake and digestive functions was assessed by measuring activities of different digestive enzymes (trypsin, lipase, and α-amylase) as well as their gene expression together with that of chymotrypsin, phospholipase A_2, proton pump, and pepsinogen.

The activities of digestive enzymes were determined using specific fluorescent substrates: Boc-Gln-Ala-Arg-7-amido-4-methylcoumarin hydrochloride (BOC-SIGMA B4153) for trypsin; 4-methylumbelliferyl butyrate (MUB-Fluka 19362) for lipase, and BODIPI FL (EnzChek® Ultra Amylase Assay Kit) for α-amylase. For gene expression, samples were preserved in RNA*later*® (Life Technologies™) and total RNA was subsequently extracted using the NucleoSpin® RNA XS (for 10 and 18dph larvae) or the NucleoSpin® RNA II (for 30 and 60dph larvae) kits (Macherey-Nagel). Total RNA was reverse-transcribed using the qScript™ cDNA synthesis kit (Quanta Biosciences). Specific primers were designed for trypsin, chymotrypsin, phospholipase, α-amylase, BAL-lipase, proton pump, and pepsinogen. qPCR reactions were performed with 1ng of cDNA, sense and antisense primers and PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences). β-actin was used as the internal reference gene.

The feeding activity of larvae was closely related to the photoperiod and showed a clear diurnal pattern. The larvae started to feed when the light was turned on and showed a progressive increase in their gut content until the light was turned off. In relation to the digestive function, the activity of trypsin was closely related to the feeding behaviour in all ages and showed an increase during the diurnal period. The activity of lipase showed a similar pattern in larvae at 10 and 60dph, but an opposite trend in larvae at 18 and 30dph. In the case of amylase, no clear pattern was observed except in larvae at 60dph.

Gene expression for trypsin, chymotrypsin, phospholipase, α-amylase, and BAL-lipase at all ages showed the same pattern, with a gradual decrease during the diurnal hours and a gradual increase during darkness. Nevertheless, the expression of proton pump and pepsinogen showed a peak during the day at 60dph.

In all ages, the activity of digestive enzymes and their gene expression showed an inverse pattern marked by the transition from light to dark. It is concluded that both feeding behavior and digestive function in gilthead seabream larvae are markedly determined by the photoperiod.

Study supported by the Spanish Government MINECO + FEDER/ERDF (projects AGL2011-23722 and Aquagenomics CSD2007-0002). Study benefited from participation in LARVANET COST action FA0801.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

ADVANCES IN LARVAL REARING PROTOCOLS OF SOLE, *SOLEA SENEGALENSIS*

A.C. Mendes[1], S. Castanho[1], M. Gamboa[1], J. Coutinho[2], N. Bandarra[2], L. Conceição[3], S. Morais[4], and P. Pousão-Ferreira[1]

[1] IPMA - Portuguese Institute for the Ocean and Atmosphere Aquaculture Research Station - Av. 5 Outubro s/n 8700-305 Olhão, Portugal
[2] IPMA - Portuguese Institute for the Ocean and Atmosphere, Av. de Brasília, 1499-006 Lisboa, Portugal
[3] Sparos Lda, Área Empresarial de Marim, 8700-221 Olhão, Portugal.
[4] IRTA, Ctra. Poble Nou Km 5.5, 43540 Sant Carles de la Rápita, Spain

Introduction

Senegalese sole, *Solea senegalensis*, is a flatfish species which garners huge interest for its cultivation, mostly in Portugal and Spain. Although larval rearing seems to be easier, less demanding in terms of dietary lipid profile (Conceição et al., 2007; Dâmaso-Rodrigues et al., 2010), and with a high survival rate compared with sea bream or sea bass, weaning is still an important bottleneck for sole cultivation. Compared to sea bass and sea bream, this is a problematic stage, quite reliant on specific good quality feeds and often presents large mortality.

In this work we will present the results and the overall practices followed in the IPMA Aquaculture Research Station for the juvenile production, describing the rearing protocols for larvae and post-larvae with special emphasis on the feeding schedule, dietary lipid profile, and weaning process.

Materials and methods

At IPMA Aquaculture Research Station, sole broodstock has been established for several years with different approaches tested and improvements made. Broodstock tanks have a bottom area of 16m^2 with a water depth of ~1.2m. Photoperiod is natural and the water temperature is controlled. Larvae are reared in 200-1500 l white or light fiberglass tanks. During the larval period we use a photoperiod of 10D:14L. For post-larvae and juvenile rearing we have been using 1.7-m^{2}fiberglass raceways. During spawning and larval rearing water temperature is at 19±1ºC and post-larvae and juvenile rearing is done at 20±2ºC. Oscillations in water temperature must be avoided in order to prevent fish stress and disease outbreaks.

Results and discussion

Continuous and natural spawning has been achieved for more than 10 years from a broodstock of approximately 100 fishes with a sex ratio of 1:1 (Fig. 1). Viable eggs are obtained for around 6 months per year in two seasons (spring and autumn, approximately March-June and October-December, respectively) by rigorously controlling the water temperature, heating or cooling as appropriate (Fig. 2). However, we observed that egg lipid content and quality decreased along the spawning season, which can be related to the extended reproductive period and also to broodstock nutrition. It is known that nutrient reserves of the yolk are dependent on the maternal nutrient reserves and consequently on the daily feeding regime of the broodstock, including nutrient levels and duration. Therefore broodstock feeds must be specific and fortified in HUFAs in order to avoid this decrease.

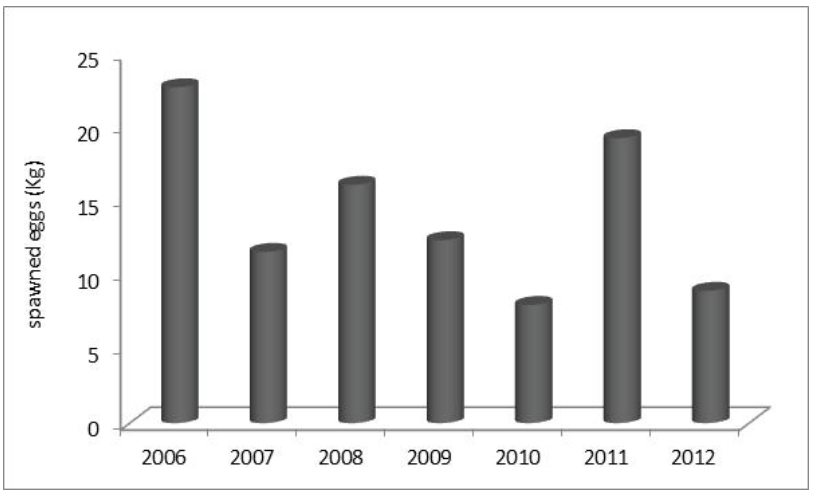


Fig. 1. Total amount of spawned sole eggs at IPMA Aquaculture Research Station.

Larval rearing protocols

For sole larval rearing we usually use a density between 30-50 larvae.l^{-1} depending on the tank volume and bottom surface. The average survival ranges 50-70%. In juvenile rearing we use densities of 3500-4000 individuals.m^{-2}.

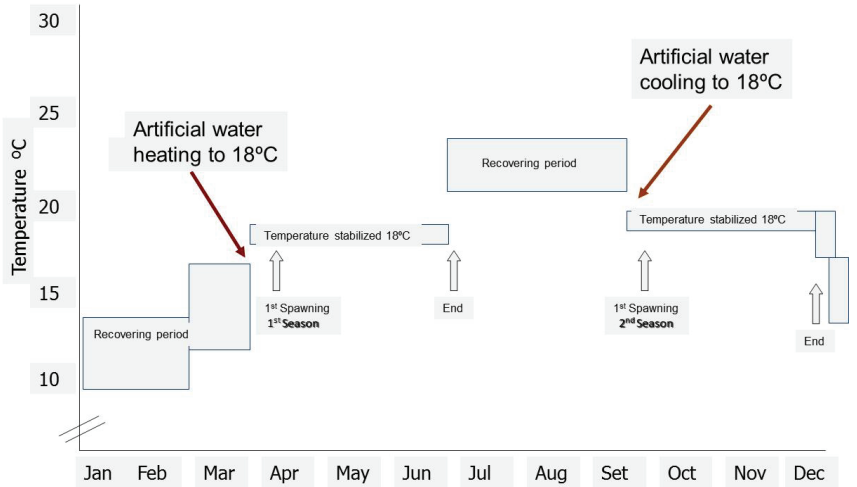


Fig. 2. Schematic control of water temperature for broodstock spawning.

Feeding schedule and food amount

Due to the scarce availability of good quality and high market cost of *Artemia* nauplii to be used as Instar I, we have used rotifers as first feed for some years and with good results. Enriched *Artemia* Instar II are supplied from 6 days after hatching (DAH) and rotifers are used until 10DAH. After metamorphosis and settlement of the majority of fishes, live *Artemia* Instar II is replaced by frozen enriched *Artemia* Instar II/III, easier to be captured by the benthic fish and with a more controlled fatty acid profile. In Table I the feeding protocol is summarized for a density of 40 larvae.l^{-1}. Prey are distributed to the tanks in several meals, during the light period and it is important to control the relation prey perlarva. The metamorphosis begins at 11DAH and may be completed at 16DAH, when the fish become benthic. Also due to the important changes that are occurring, sole considerably reduce food ingestion and in some case even stop eating during the metamorphosis climax. However, afterwards they need to re-establish energy stores, hence the oscillation in the feeding table. In addition, it is important to understand that the food supply may differ in the different stages. During the planktonic stage and during the rotifer-only period 2-3 meals are enough, but with the introduction of *Artemia* its important to ensure a more frequent food supply, with 3-4 meals daily and up to 6 meals later.

Weaning

With regards to weaning, despite considerable recent improvements in diets and protocols, weaning success is still quite variable, and large mortalities occur at times. The interaction diet:water quality:microbiota certainly plays a critical role in defining the weaning success. Therefore, the stabilization of environmental

parameters such as water quality (very dependent of food quality) and water temperature is crucial.

Table I. Feeding protocol established for *S. senegalensis* at IPMA Aquaculture Research Station (preys.ml^{-1}).

DAH	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Rotifers	5	7	10	10	7.5	11	11	10							
Artemia				0.4	0.5	0.7	1.4	2.0	2.0	2.5	2.5	3.5	2.0	3.5	4.2
frozen *Artemia*												0.5	0.8	1.2	1.2

Conclusions

The early acceptance of frozen prey allows a more convenient feeding protocol which is a huge advantage with regards to other marine species that require live food for a longer period of time. In addition, and perhaps a key factor in the easiness of larval rearing, sole seem to perform well with low HUFA diets which, although not accurately assessed, is likely also the case in juvenile stages. We now know that this is due to the unique capability, within commonly cultivated marine fish species, of Senegalese sole to biosynthesise DHA (Morais et al., 2012). Even during metamorphosis, as dramatic as it is in flatfish species, survival is quite high. The main bottleneck which still leads to high mortalities, in spite of large improvements during the last years, is the weaning into formulated diets. At this time, the stabilization of water quality and microbiota, which are very dependent on feed quality and feeding protocol, is crucial to the rearing success.

Acknowledgments

FCT (Portugal), project EFARFish – PTDC/MAR/67017/2006.

References

Conceição L.E.C., L. Ribeiro, S. Engrola, C. Aragão, S. Morais, M. Lacuisse, F. Soares, F., and M.T. Dinis. 2007. Nutritional physiology during development of Senegalese sole (*Solea senegalensis*). Aquaculture 268:64-81.

Dâmaso-Rodrigues M.L.; P. Pousão-Ferreira, L. Ribeiro, J. Coutinho, N.M. Bandarra, P.J. Gavaia, L. Narciso, and S. Morais. 2010. Lack of essential fatty acids in live feed during larval and post-larval rearing: effect on the performance of juvenile Soleasenegalensis. Aquaculture International 18(5):741-757.

Morais S., F. Castanheira, L. Martinez-Rubio, L.E.C. Conceição, and D.R. Tocher. 2012. Long chain polyunsaturated fatty acid synthesis in a marine vertebrate: Ontogenetic and nutritional regulation of a fatty acyl desaturase with Δ4 activity. BBA - Mol Cell Biol Lipids 1821:660-671.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EFFECT OF INCREASING DHA CONTENT IN NEW WEANING DIETS FOR LONGFIN YELLOWTAIL (*SERIOLA RIVOLIANA)*

A. Mesa-Rodriguez[*], C. Hernández-Cruz, M. Izquierdo, H. Fernández-Palacios, and J. Roo

Grupo de Investigación en Acuicultura: Instituto Canario de Ciencias Marinas & Universidad de Las Palmas de Gran Canaria, PO Box 56, E-35200 Telde, Las Palmas, Canary Islands, Spain. ([*]E-mail: a.mesa.rodriguez@gmail.com)

Introduction

With the common name of longfin yellowtail, *Seriola rivoliana* (Valenciennes and Cuvier, 1833) is one of the species proposed for the diversification in marine aquaculture, mostly due to the fast growth rate, high flesh quality, and worldwide distribution (Roo et al., 2012). This species belongs to the family of Carangidae, shared with the widely known species like greater amberjack *S. dumerilii* (*Risso,* 1810), Japanese amberjack *S. quinqueradiata* (Temminck and Schlegel, 1845), yellowtail amberjack *S. lalandi*, (Valenciennes, 1833), and longfin yellowtail *S. rivoliana* (Valenciennes, 1833). In general, polyunsaturated fatty acids (PUFAs) play an important role in larval nutrition and rearing success. Moreover, several studies have shown that DHA has a greater potential as an essential fatty acid for marine fish larvae than EPA (Watanabe et al., 1989; Watanabe, 1993), since its requirement more limiting for growth and survival than those for other n-3 HUFA (Izquierdo, 1996).

Due to the general importance of DHA as a main dietary lipid for larval marine finfish rearing success, the purpose of this study was to evaluate the effect of increasing DHA levels in weaning diets for *S. rivoliana* larvae, in terms of larval growth, survival, and larval quality measured as incidence of skeletal malformations and resistance to stress.

Materials and methods

S. rivoliana eggs were cultured under semi-intensive system conditions (7.5 ind.l^{-1} in 40-m^3 tanks) according to the methodology described by Roo et al., (2012). At 30 days after hatch (dah), larvae were settled in a total of 15, 200-l tanks (90 larvae per tank, in triplicates), under natural water conditions (temperature: 22.5±0.6°C; oxygen levels: 6.5±0.3 ppm). Five isoproteic (54.8%) and isolipidic (24.1%) experimental microdiets with increasing levels of DHA con-

tent from 0.5-5.0% (dw) were manufactured. Diets were named according to its DHA content (D0.5; D1.5; D3.0; D4.0, and D5.0). Larval growth was assessed measuring the dry weight and the standard length of the larvae's using a profile projector (Nikon V-12A, NIKON™) at 30, 42, and 50dah. For deformity characterisation, a total of 15 larvae per tank were fixed in 10% buffered formalin and stained with alizarin red according with the modified methodology of Vandewalle et al. (1998). Activity test was determined by larvae 30 seconds air exposure at 50dah. Proximate analysis and fatty acid composition of the experimental microdiets were performed.

Results

At the end of the feeding period (50dah), larvae weaned with diets with highest DHA levels showed better final survival (Fig. 1) and resistance to stress. Contrarily, the highest DHA content resulted in smaller larvae (Fig. 2).

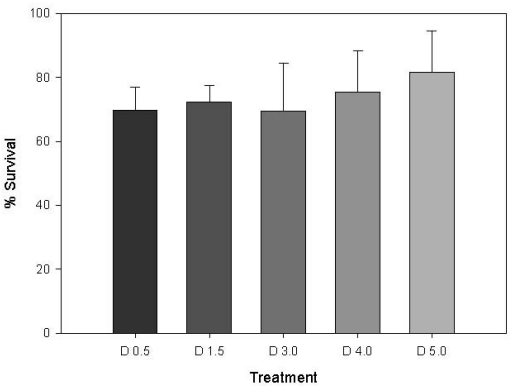


Fig. 1. Final survival rates per diet.

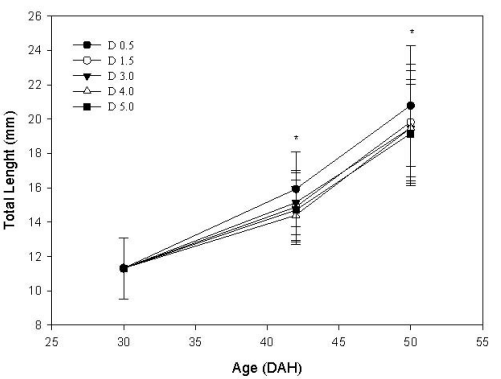


Fig. 2. Total length progress.

Regarding to the deformity characterisation, DHA content in weaning diets clearly influenced appearance of skeletal anomalies, mainly related with spinal column deformities, particularly lordosis and kiphosis. The highest incidence of skeletal deformities was obtained in the Diets 3 and 4, whereas the lower incidence was obtained in diet 0.5 (Fig. 3).

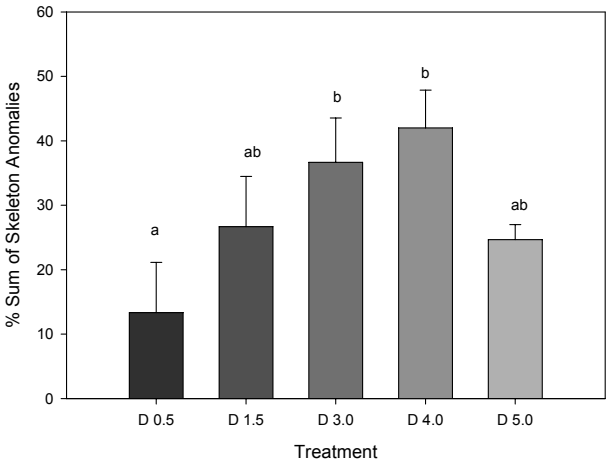


Fig. 3. Incidence of skeletal deformities in *S. rivoliana* larvae fed graded levels of DHA in formulated diets (Sum of total anomalies included lordosis, kyphosis, vertebral fusions and jaw deformities).

Conclusions

Survival rate was not significantly different for all the levels of DHA assayed, but highest survival was obtained in Diet 5. Similarly, the increased in DHA content promotes better resistance to acute stress test. On the contrary, highest growth and the lowest incidence of skeletal deformities were obtained with the lowest DHA content.

Acknowledgements

This study was performed under the project: "Mejora de las técnicas de cría de larvas de (*Seriola rivoliana*): Determinación de requerimientos de ácidos grasos esenciales en su etapa larvaria y optimización de la secuencia alimentaria (METCSER) funded by the Canary Gobermend throught out the program: Proyectos de Investigación Científco-Tecnológicos del Gobierno de Canarias 2011.

References

Izquierdo M.S. 1996. Essential fatty acid requierements of cultured marine fish larvae. Review article. Aquaculture Nutrition 2:183-191.

Roo J., H. Hernández-Palacios, M. Hernández-Cruz, A. Mesa-Rodriguez, D. Schuchardt, and M.S. Izquierdo. 2012. First results of spawning and larval rearing of longfin yellowtail Seriola rivoliana as fast-growing candidate for European marine finfish aquaculture diversification. Aquaculture Research.1-12. doi:10.1111/are.12007.

Vandewalle P., I. Gluckmann, and F. Wagemans.1998. A critical assessment of the alcian blue/alizarin double staining in fish larvae and fry. Belgian Journal of Zoology 128(1):93-95.

Watanabe T., M.S. Izquierdo, T. Takeuchi, S. Satoh, and C. Kitajima. 1989. Comparison between eicosapentaenoic and docosahexaenoic acids in terms of essential fatty acid efficacy in larval red seabream. Nippon Suisan Gakkai-shi 55:1635-1640.

Watanabe T. 1993. Importance of docosahexaenoic acid in marine larval fish. Ournal of the World Aquaculture Society 24:152-161.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EVALUATION OF COLOUR DEVELOPMENT OF ROSY BARB, *PUNTIUS CONCHONIUS* (HAMILTON) DURING ONTOGENY

G. Mitra[1*], J.K. Jena[2], P. Jayasankar[1], P.C. Das[1], and S.K. Swain[1]

[1] Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar-751002, Odisha, India ([*]E-mail:gopamitra@yahoo.com)
[2] National Bureau of Fish Genetic Resources, Canal Ring Road, Dilkhusa, Lucknow-226002, India

Introduction

Wide variety of colour and colour patterns are the characteristics of ornamental fish species. The commercial value of these fish depends on the vibrant colour along with the size and shape of body and fins. Rosy barb (*Puntius conchonius*) is one of the prospective indigenous ornamental fish species of India having considerable high commercial importance (Felix et al., 2012; Kurup et al., 2012). The bright orange and yellow colour of the fish is the consequences of ingestion and metabolism of the pigment carotenoids, which the fish cannot synthesize de novo but acquires from the food. However, a few studies have tracked carotenoid status and colour development in rosy barb during ontogeny. Therefore, the present study was carried out to evaluate the developmental pattern of colour and their relation with tissue carotenoid content in rosy barb during ontogeny, from hatching to 39 days post-hatch (dph).

Materials and methods

Yolk-sac larvae were obtained from the Aquarium fish farm of Central Institute of Freshwater Aquaculture. A random sample of 30 larvae was collected every third day for 1-39dph from six larval rearing tanks (water temperature 28-31°C, dissolved oxygen 5.03mg.l^{-1}) and brought to the laboratory for further analysis of carotenoid and changes in colour intensity. Larvae were reared with mixed pond-collected plankton (both phytoplankton and zooplankton). Extraction of larval carotenoid and estimation of astaxanthin was done following standard methods (Britton, 1995; Hongxia et al., 2005). The isolation of total carotenoid from fish larvae was done using acetone and methanol solvent (1:1) (Guillou et al., 1993) and concentration of astaxanthin was estimated by spectrophotometer with the extinction coefficient of astaxanthin in n-hexane (E1%/1cm, 2100) at 470nm (Britton, 1995; Rodriguez-Amaya, 2001).

Larval skin colour was assessed by capturing its colour image with a high-resolution USB CCD colour camera (Model No. DFK51AU02), the major components of the Colour Machine Vision System (CMVS), and the procedures followed in capturing colour bitmap images of the live anesthetized fish larvae (with MS222, tricaine methanesulphonate) was as described by Wallat et al. (2002). The camera was set in Video format, Software of IC capture unit was programmed to capture 24-bit red, green, blue (RGB) values per pixel, frame rate was 12 images per second, and resolution was 1600×1200 pixels. The captured bitmap images were used for colour analysis by Colour.exe software to get RGB values and converted to HSI (Hue, Saturation, and Intensity) colour scheme (Wyszecki and Stiles, 2000).

Results and discussion

The ontogeny of colouration of rosy barb is condition-dependent involving a trade-off between ingested and metabolized colour pigments and physiological condition of the body. The relation between astaxanthin content and hue during ontogeny of rosy barb, has been depicted in Figure 1, showing a polynomial relationship ($Y=-317.62X^2+77.845X+34.22$, $R^2=0.92$) and revealing the role of astaxanthin in the development of bright hue in the species. The goodness of fit of nonlinear model for both pigments and hue ($R^2=0.92$) during larval development implied that the maximum bright hue due to carotenoid deposition could be achieved at tissue astaxanthin level of 0.12µg.g^{-1}wet weight in which point the plateau in the curve was observed.

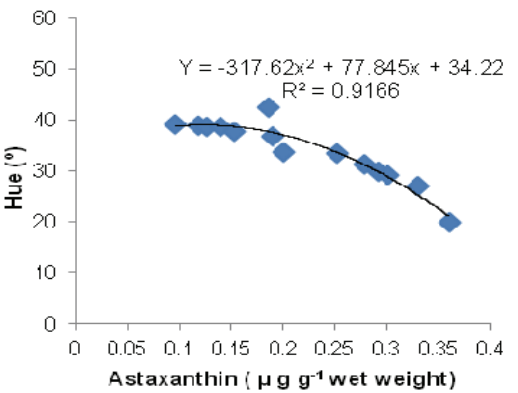


Fig.1. Relation between astaxanthin content and hue during ontogeny of rosy barb, *Puntius conchonius*.

The orange-red colouration was more vivid towards 21-39dph and a decreasing trend in hue angle was observed along with increasing age and weight (Fig. 2).

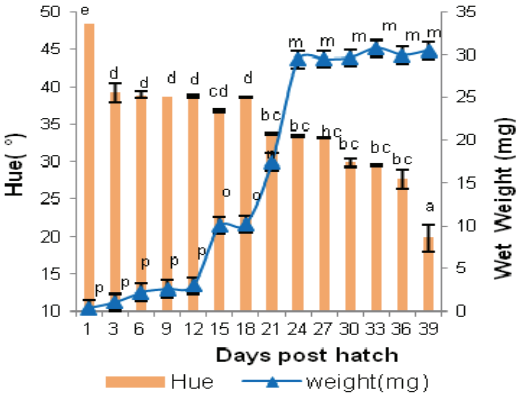


Fig. 2. Development of hue during ontogeny of rosy barb, *Puntius conchonius*.

From the inflection point of response curve with hue and saturation ($Y=8E\text{-}05x^3-0.0085x^2+0.2534x-1.7116$, $R^2=0.96$) (Fig. 3) the most advantageous value of hue has been estimated as 35.42. Further, the optimum saturation or chroma was 0.64 ($Y=-139.59x^3+265.8x^2-196.69x+78.948$, $R^2=0.84$). More often, the measurement of colour variables such as hue and chroma or saturation gives information about the development of skin colouration due to deposition of carotenoids in the skin (Kalinowski et al., 2011). Negative correlation (r=-0.91) between the tissue astaxanthin content and hue also substantiated the finding that hue angle decreased due to increase in astaxanthin content in tissue giving bright orange red colour to the fish. This finding accentuated that colour pattern in rosy barb is most likely pigment-based. As the pigment could not be synthesized de novo by the fish, it must have been obtained from the live food as mixed plankton. Besides, it might have metabolized and allocated between colour traits and other body functions consequently as per their physiological efficiency.

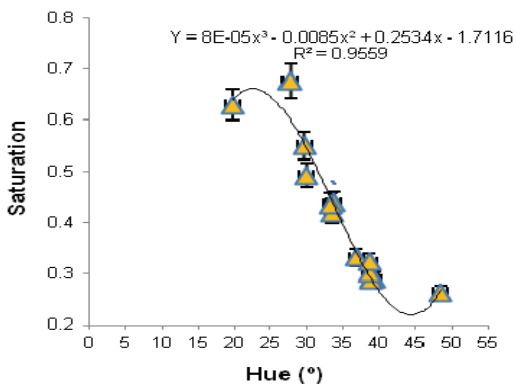


Fig. 3. Relation between hue and saturation during rosy barb, *Puntius conchonius* colour development.

These results with special reference to the assessment of the colour development and their relation with astaxanthin during ontogeny could be extrapolated during feed development for persistent colour improvement of the species to overcome the major problem of colour losses during production process.

Acknowledgements

Research grant from Department of Science and Technology, New Delhi for the research work and Women Scientist Fellowship to the first author is thankfully acknowledged. The authors would like to thank the Director of this Institute for providing necessary facilities to conduct the research work.

References

Britton G. 1995. UV/visible spectroscopy. p 13-63 In:Carotenoids: spectroscopy, vol 1B., Britton, G., S. Liaaen-Jensen, and H. Pfander, (Eds), Birkhäuser Verlag, Basel.

Felix N., J. D. Jameson, and R.A. Brindo. 2012.Ornamantal fish farming-a successful cottage industry in rural and urban India. Aquaculture Asia XVII (3):14-18.

Guillou A., G. Choubert , and J. de la Noue 1993. Separation and determination of carotenoids, retinol, retinal, and their dehydro forms by isocratic reversed phase HPLC. Food Chemistry, Analytical Methods Section 47:93-99.

Hongxia L, S.T. Tyndale, D.D. Heath, and R.J. Letcher. 2005. Determination of carotenoids and all trans-retinol in fish eggs by liquid chromatography-electrospay ionization-tandem mass spectrometry. Journal of Chromatography B.816:49-56.

Kalinowski C.T., L.E. Robaina, and M.S. Izquirdo. 2011. Effect of dietary astaxanhtin on the growth performance, lipid composition and post mortem skin colouration of red porgy *Pagrus pagrus*. Aquaculture International 19:811-823.

Kurup M.B., M. Harikrishnan, .and K. Renjith. 2012.Breeding farming and trade of ornamental fishes in India-prospects and challenges. Ornamentals Kerala, Department of Fisheries, Govt of Kerala,Cochin,India.

Rodriguez-Amaya D.B. 2001. A guide to carotenoid analysis in food. International Life Sciences Institute, Washington, D. C. 20005-5802, 64 p.

Wallat G.K., D. A. Luzuriaga, M. O. Balaban, and F.A. Chapman. 2002. Analysis of skin color development in live goldfish using a Color Machine Vision System. North American Journal of Aquaculture 64:79-84.

Wyszecki G. and W.S. Stiles. 2000. Colour Science: Concepts and methods, Quantitative data and formulae. Second Edition, John Wiley and Sons Ltd., Chichester, UK.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

DHA REQUIREMENT OF LARVAL JAPANESE FLOUNDER *PARALICHTHYS OLIVACEUS* IN THE ROTIFER FEEDING PERIOD

A. Miyashima, T. Kamiya, H. Fushimi[1], N. Sato[2], T. Kotani[3], and T. Kusano[4]

[1] Institute of Marine Bio-resources, Fukuyama University, Innoshima, Onomichi, Hiroshima 722101, Japan
[2] Nagase Sanbio Co. Ltd., Nishi-ku, Osaka 550-8668, Japan
[3] Kagoshima University, Korimoto, Kagoshima 890-8580, Japan
[4] Maruha Nichiro Sea foods, Inc., Toyosu, Tokyo 100-8608, Japan

Introduction

The nutritional composition of euryhaline rotifers used as initial food for finfish larviculture is important for improvement of larval health. It is well known that DHA is one of the essential fatty acids of larval marine finfish (Watanabe et al., 1989; Takeuchi et al. 1990; Watanabe, 1993). Usually, many marine finfish hatcheries use rotifers and commercial diets for DHA enrichment. However, we have little knowledge on the DHA requirement of larval marine finfish in the rotifer feeding period. In order to examine this, we conducted experimental larval rearing of Japanese flounder *Paralichtys olivaceus* using rotifers containing differing DHA levels.

Materials and methods

The experimental period was from 0 days post hatching (dph) to metamorphosis to juveniles. 1-m^3 tanks were used and larvae were initially stocked at 20 larvae.l^{-1}. In order to produce rotifers with increasing quantity of DHA, we made enrichment diets while controlling the quantity of DHA, EPA, and the DHA:EPA ratio. The quantity of EPA was fixed as the amount of rotifers enriched with *Nannochloropsis oculata*, and the levels of DHA ranged from 2-5× that of EPA. Larvae were fed the rotifers at four DHA levels and enriched with commercial diet (DHA PROTEIN SELCO, INVE Technologies NV, Dendermonde, Belgium) as well as a control from 3dph. *Artemia* nauplii enriched with Selco-S.presso (INVE Technologies NV, Dendermonde, Belgium) were used from 13dph. The feeding of formulated diets (LOVE.LARVA, Maruha Nichiro Sea foods Inc, Hayashikane Sangyo Co. Ltd.) began once larvae attained an average of 11mm total length. In order to examine the DHA requirement of larval Japanese flounder, we analyzed the fatty acid composition of rotifers and larval fish at each developmental stage.

Results and discussion

The DHA content of rotifers after enrichment has an upper limit of 9mg.g^{-1} dry basis (D.B.; Fig. 1). The best larval rearing performance was shown using rotifers with 4:1 of DHA:EPA ratio, followed by 1:3. The relationships between the DHA content of rotifers and larval Japanese flounder changed with larval developmental stage. From the relationship between DHA content in rotifers and larvae of each developmental stage, DHA content (mg.g^{-1}DW) of larvae reached 15mg, reflecting the 7.2mg.g^{-1}DW DHA content of rotifers. Larval DHA contents were stable after getting rotifers containing more DHA (Fig. 2). We determined that the DHA requirement of larval Japanese flounder in the rotifer feeding period is 7mg.g^{-1}DW.

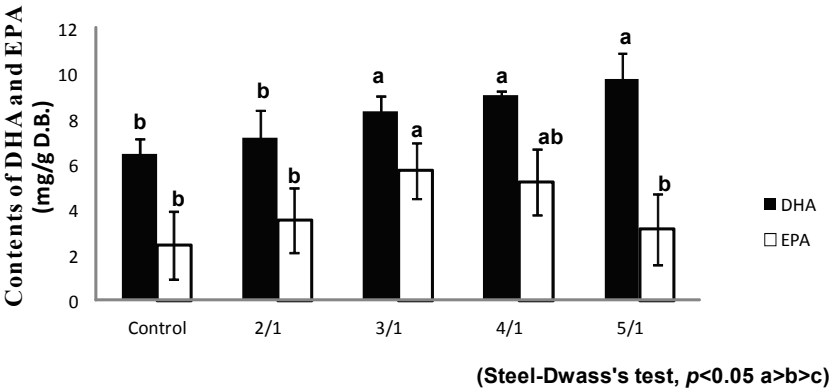


Fig.1. Contents of DHA and EPA quantities (mg.g^{-1} D.B.) of rotifers. Black and white bar indicates average content of DHA (mg.g^{-1} D.B.) and EPA (mg.g^{-1} D.B.), respectively. Each error bar shows standard deviation. Each alphabetical superscript indicates significance of result of Steel-Dwass's test (P<0.05, a>b).

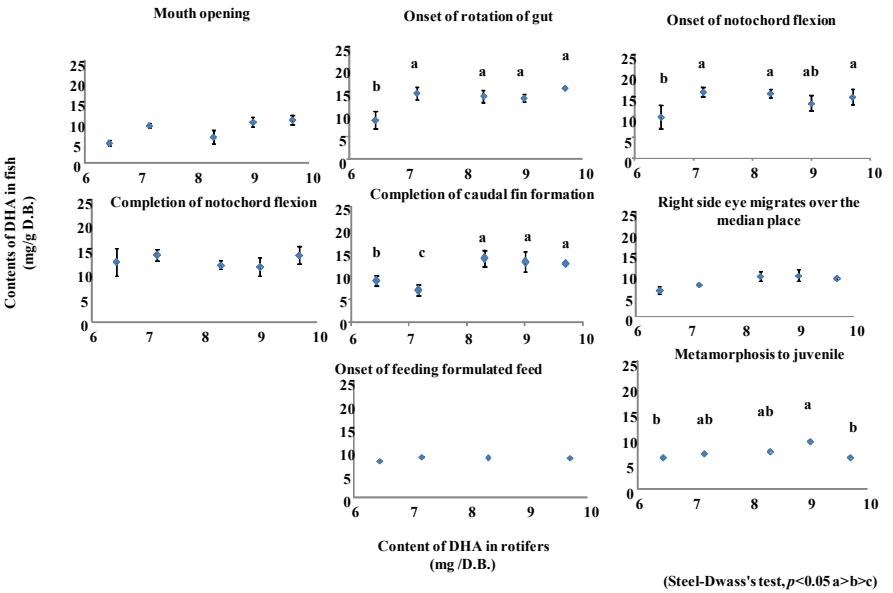


Fig.2. Relationship between DHA content in rotifers and larvae of each developmental stage. Each alphabetical superscript indicates significance of result of Steel-Dwass's test (P<0.05, a>b).

Acknowledgements

We thank Marua Suisan Co., Ltd for providing fertilized eggs. We express our gratitude to Maruha Nichiro Sea foods, Inc. for their financial support.

References

Watanabe T., M.S. Izquierdo, T. Takeuchi, S. Satoh, and C. Kitajima. 1989. Comparison between eicosapentaenoic and docosahexaenoic acids in terms of essential fatty acid in larval red sea bream. Nippon Suisan Gakkaishi 55:1635-1640.

Takeuchi T., M. Toyota, S. Satoh, and T. Watanabe. 1990. Requirement of juvenile red sea bream *Pagrus major* for eicosapentaenoic and docosahexaenoic acids. Nippon Suisan Gakkaishi 56:1263-1269.

Watanabe T. 1993. Importance of docosahexaenoic acid in marine larval fish. Journal of the World Aquaculture Society 24:152-161.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

INVESTIGATING THE ESSENTIAL FATTY ACID REQUIREMENTS OF THE CEPHALOPOD *SEPIA OFFICINALIS*: A MOLECULAR APPROACH

Ó. Monroig[1], F. Hontoria[1], I. Varó[1], D.R. Tocher[2], and J.C. Navarro[1]

[1] Instituto de Acuicultura Torre de la Sal (IATS-CSIC), Ribera de Cabanes 12595, Spain
[2] Institute of Aquaculture, School of Natural Sciences, University of Stirling, Stirling FK9 4LA, Scotland, UK

Introduction

Polyunsaturated fatty acids (PUFA) are critical compounds for early life-cycle stages of organisms and this has been established for cephalopods including the common cuttlefish (*Sepia officinalis*) (Navarro and Villanueva, 2000). Our overarching aim is to identify the dietary essential fatty acids (FA) for cuttlefish early life-cycle stages (hatchlings). Due to the difficulties in conducting feeding trials with hatchlings, our approach is to characterise the enzymes involved in the PUFA biosynthesis as they dictate the ability of a certain species to endogenously produce PUFA (Bell and Tocher, 2009). On one hand, fatty acyl desaturases (Fad) are key enzymes that mediate the introduction of a double bond (unsaturation) at the Δx carbon counting from the carboxyl group of the fatty acid (FA). On the other, elongases of very long-chain fatty acids (Elovl) catalyse the addition of two carbons to pre-existing fatty acyl chains.

Materials and methods

Total RNA from adult male cuttlefish tissues (hepatopancreas and gonad) was extracted by homogenisation in Tri Reagent (Sigma-Aldrich) and cDNAs were subsequently synthesised. In order to isolate the first fragment of cuttlefish Fad and Elovl, degenerate primers were designed in conserved regions deduced from the alignment of Fad and Elovl proteins from a variety of organisms. The PCR fragments were sequenced and specific primers were designed to produce the full-length cDNA by 5' and 3' rapid amplification of cDNA ends (RACE) PCR.

In order to functionally characterise the cuttlefish Fad and Elovl, their coding sequences, cloned into pYES2 (Invitrogen), were expressed in *Saccharomyces cerevisiae* (INVSc1 strain, Invitrogen) and grown in presence of potential FA substrates for Fad (18:3n-3, 18:2n-6, 20:3n-3, 20:2n-6, 20:4n-3, 20:3n-6, 22:5n-3, and 22:4n-6) or Elovl (18:3n-3, 18:2n-6, 18:4n-3, 18:3n-6, 20:5n-3, 20:4n-6,

22:5n-3, and 22:4n-6). After two days, transgenic yeast samples were collected for further FA analysis. Conversion rates were expressed as a percentage of total FA substrate converted to desaturated or elongated products.

Results and discussion

The cuttlefish Fad cDNA encodes a putative protein of 445 aa, being 80.7% identical to the *Octopus vulgaris* Fad (Monroig et al., 2012a) and 53.6-65.8% identical to gastropod (*Lottia gigantea* and *Haliotis discus hannai*) homologues. Moreover, the cuttlefish Fad possesses all the typical features of these enzymes including three histidine boxes (HXXXH, HXXHH, and QXXHH), the putative cytochrome b5-like domain and the heme-binding motif, HPGG. The cuttlefish Elovl cDNA encodes a protein of 295 aa that shows 77.7% identity to the *O. vulgaris* Elovl-like protein (Monroig et al., 2012b) and lower identity scores to other molluscan (*L. gigantea* and *Lymnaea stagnalis*) Elovl-like proteins. The deduced aa sequence of the cuttlefish elongase contains the diagnostic histidine box (HXXHH) conserved in all members of the Elovl protein family, as well as two lysine (K) residues at the carboxyl terminus (KKXX), regarded as putative ER retrieval signals.

The functional characterisation assays in yeast revealed that the cuttlefish Fad and Elovl cDNAs have highly similar functions to those of the octopus homologues that we recently investigated (Monroig et al., 2012ab). Thus, the gene product of the cuttlefish Fad exhibited Δ5-desaturase specificity, enabling this species to endogenously biosynthesise the physiologically essential FA eicosapentaenoic (20:5n-3, EPA) and arachidonic (20:4n-3, ARA) acids from 20:4n-3 and 20:3n-6, respectively (Table I). Additionally, the Δ5 Fad enables the cuttlefish to convert the FA substrates 20:3n-3 and 20:2n-6, respectively, to $^{\Delta5,11,14,17}$20:4 and $^{\Delta5,11,14}$20:3, two non-methylene-interrupted (NMI) FA reported to occur in marine invertebrates and with unknown functions (Barnathan, 2009). Regarding the cuttlefish elongase, transgenic yeast expressing the cuttlefish Elovl were able to elongate C18 and C20 PUFA substrates, but showed no activity towards C22 PUFA (Table II). These results are consistent with the substrate specificities of the common octopus Elovl cDNA and also those of Elovl5-like proteins from vertebrates (Monroig et al., 2012b).

Conclusions

The present study demonstrates that the common cuttlefish expresses Fad- and Elovl-like genes that participate in the PUFA biosynthesis pathways. The functional characterisation assays of the cuttlefish Fad and Elovl cDNAs enable us to hypothesise the PUFA biosynthetic pathways in this species (Fig. 1). Thus, we can speculate that EPA and ARA are dietary essential FA for *S. officinalis* because their biosynthesis from 20:4n-3 and 20:3n-6, respectively, it is likely to be

limited. First, the availability of 20:4n-3 and 20:3n-6 in diets for cuttlefish is extremely low. Second, the endogenous production of 20:4n-3 and 20:3n-6 via biosynthesis from C18 PUFA (18:2n-6 and 18:3n-3) appears to be restricted by the absence of critical desaturase (Δ6 or Δ8) activities. Similarly, lack of Δ4- or Δ6-desaturases strongly suggests that docosahexaenoic acid (22:6n-3, DHA) cannot be endogenously biosynthesised by cuttlefish and therefore it needs to be supplied in the diet to ensure normal growth.

Table I. Substrate conversions of yeast *Saccharomyces cerevisiae* transformed with pYES2 containing the coding sequence of the cuttlefish desaturase. Transgenic yeast were grown in presence of exogenously added polyunsaturated fatty acid (FA) substrates. FA are designated using the 'n-' nomenclature, except for non-methylene interrupted FA where the 'Δ' nomenclature was used.

FA substrates	Product	Conversion rate (%)
18:3n-3	$^{\Delta5,9,12,15}$18:4	0
18:2n-6	$^{\Delta5,9,12}$18:3	0
20:3n-3	$^{\Delta5,11,14,17}$20:4	24
20:2n-6	$^{\Delta5,11,14}$20:3	14
20:4n-3	20:5n-3	48
20:3n-6	20:4n-6	39
22:5n-3	22:6n-3	0
22:4n-6	22:5n-6	0

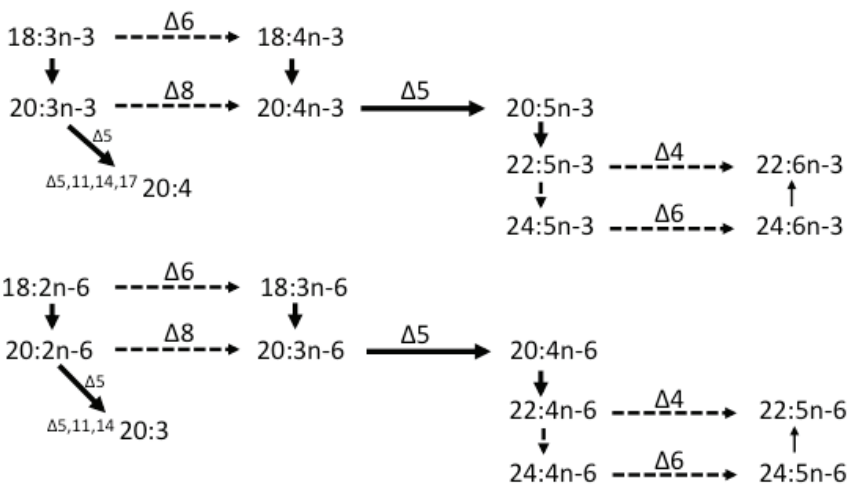


Fig. 1. Biosynthetic pathways of PUFA. Solid arrows indicate pathways demonstrated in cuttlefish, whereas dashed arrows indicated pathways shown in vertebrates but unconfirmed in cephalopods.

Table II. Substrate conversions of yeast *Saccharomyces cerevisiae* transformed with pYES2 containing the coding sequence of the cuttlefish elongase.

FA Substrate	Product	% Conversion	Activity
18:3n-3	20:3n-3	32	C18→20
18:2n-6	20:2n-6	50	C18→20
18:4n-3	20:4n-3	51	C18→20
18:3n-6	20:3n-6	51	C18→20
20:5n-3	22:5n-3	8	C20→22
20:4n-6	22:4n-6	37	C20→22
22:5n-3	24:5n-3	0	C22→24
22:4n-6	24:4n-6	0	C22→24

Acknowledgements

This research and OM were supported by a Marie Curie Reintegration Grant within the 7th European Community Framework Programme (PERG08-GA-2010-276916, LONGFA), with additional support from "Ministerio de Ciencia e Innovación" through the OCTOPHYS Project (AGL-2010-22120-C03-02) and a Juan de la Cierva postdoctoral contract for OM, and from the Generalitat Valenciana through a PROMETEO Project (2010/006).

References

Barnathan G. 2009. Non-methylene-interrupted fatty acids from marine invertebrates: Ocurrence, characterization and biological properties. Biochimie 91:671-678.

Bell M.V. and D.R. Tocher. 2009. Biosynthesis of polyunsaturated fatty acids in aquatic ecosystems: general pathways and new directions. p.211-236. In: Lipids in Aquatic Ecosystems. Arts, M.T., M. Brett, and M. Kainz (Eds.). Springer-Verlag, New York. 380p.

Monroig Ó., J.C. Navarro, J.R. Dick, F. Alemany, and D.R. Tocher. 2012a. Identification of a Δ5-like fatty acyl desaturase from the cephalopod *Octopus vulgaris* (Cuvier 1797) involved in the biosynthesis of essential fatty acids. Mar. Biotechnol. 14: 411–422.

Monroig Ó., D. Guinot, F. Hontoria, D.R. Tocher, and J.C. Navarro. 2012b. Biosynthesis of essential fatty acids in *Octopus vulgaris* (Cuvier, 1797): Molecular cloning, functional characterisation and tissue distribution of a fatty acyl elongase. Aquaculture 360–361: 45–53.

Navarro J.C. and R. Villanueva. 2000. Lipid and fatty acid composition of early stages of cephalopods: an approach to their lipid requirements. Aquaculture 183:161–177.

'13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EFFECT OF SENEGALESE SOLE BROODSTOCK NUTRITION ON EARLY LARVAL PERFORMANCE AND METABOLISM OF LONG-CHAIN POLYUNSATURATED FATTY ACIDS (DHA AND EPA)

S. Morais[1], A. Candeias Mendes[2], F. Castanheira[3], J. Coutinho[2], N. Bandarra[2], J. Dias[3,4], L.E.C. Conceição[3,4], and P. Pousão-Ferreira[2]

[1] IRTA, Ctra. Poble Nou Km 5.5, 43540 Sant Carles de la Rápita, Spain
[2] IPIMAR-INRB, Av. Brasilia, Lisboa and IPIMAR/CRIP Sul, Av. 5 de Outubro, Olhão, Portugal
[3] CCMAR/CIMAR LA, Universidade do Algarve, Campus de Gambelas, Faro, Portugal
[4] Sparos Lda, CRIA - Universidade do Algarve, Faro, Portugal

Introduction

Fish larvae rely on their yolk reserves for the supply of energy and essential fatty acids (EFA), such as EPA and DHA, for growth and development during the endogenous feeding stage. As the yolk biochemical composition reflects the maternal nutrient reserves, which in turn depends on the broodstock diet, in this study we tested two new specifically formulated semi-moist diets for *Solea senegalensis* broodstock; a control diet (CTR) and a diet containing higher lipid, vitamin, and DHA levels (DHA). The objective was to analyse effects of broodstock nutrition on early (during endogenous feeding, until 3 days post-hatching: dph) and later (at 7dph and17 dph) stages. Effects were assessed by egg hatching rate, body fatty acid (FA) composition, survival, growth, EFA requirements assessed by metabolism of EPA and DHA, and by quantification of the expression of genes involved in the LC-PUFA biosynthesis pathway, of sole larvae.

Materials and methods

Two semi-moist diets were formulated and manufactured by Sparos Lda.: a CTRL diet with high levels of marine-derived ingredients and a DHA diet with higher lipid level (23 vs. 16%) and supplemented with vitamins and long-chain polyunsaturated fatty acids (LC-PUFA), mainly DHA. The experimental diets were manufactured using a temperature-controlled low shear extruder (die size: 8mm) and were kept frozen for the duration of the trial. Sole broodstock were divided into two groups and fed the experimental diets during two years (except during the summer resting season, when only the CTR diet was fed). The larval rearing trial was initiated when simultaneous natural spawns were obtained from both groups, which were reared at the IPMA Aquaculture Research Station in

the same standard conditions (Conceição et al., 2007) until the end of the pelagic stage (17dph). Eggs were incubated at a density of 0.3g.l^{-1} in filtered and U.V disinfected seawater at 19ºC. After evaluating the hatching rate, larvae were transferred and reared, in triplicate 200-l tanks per treatment, in semi-recirculation, at an initial density of 40 larvae.l^{-1}, a temperature of 19±1.0ºC and salinity of 36±1.0‰. Larvae were fed ad libitum several times a day with rotifers until 10dph and live *Artemia* metanauplii from 6dph onwards, with both preys being enriched in Red Pepper® (Bernaqua™).

Triplicate samples per treatment (one per tank) were collected for FA and molecular analysis (RT-qPCR quantification of *elovl5* and *Δ4fad*, according to Morais et al., 2012). To examine the absorption and metabolism of ^{14}C-EPA and ^{14}C-DHA, a tube feeding trial was conducted at 7 and 17dph, according to the methodology described by Rønnestad et al. (2001).

Results and discussion

Hatching rate was 93.3 and 79.6% for the eggs spawn from broodstock fed the CTR and DHA diets, respectively. The larvae hatched from these eggs did not show significant differences in mortality (around 55%) until 17dph. However, larvae originating from the CTR broodstock were significantly larger and heavier, with differences in total length (TL) being significant already at 3dph.

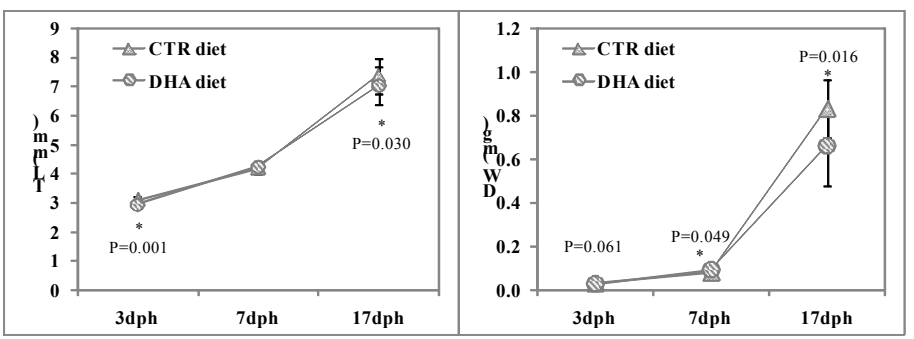


Fig. 1. Total length (TL) and dry weight (DW) of larvae originating from broodstock fed the CTR or the DHA diet. Asterisks indicate significant differences between treatments (P values are indicated).

The two experimental diets presented similar levels of total saturated and monounsaturated FA's (MUFA), as well as of n-6 PUFA. Levels of n-3 LC PUFA were higher in the DHA diet, compared to the CTR diet (as % dry feed; EPA: 2.3 vs. 1.5 and DHA: 2.7 vs. 0.9). The broodstock diet affected the FA composition of eggs and larvae (Fig. 2), but with a stronger impact on EPA levels, which were significantly higher in the DHA treatment during the whole endogenous feeding period and at 7dph. DHA levels were also significantly higher, but only

in eggs and newly hatched larvae from broodstock fed the DHA-supplemented diet, and again at 3dph. On the other hand, eggs and 0dph larvae from the CTR treatment presented significantly higher levels of MUFA and linoleic acid.

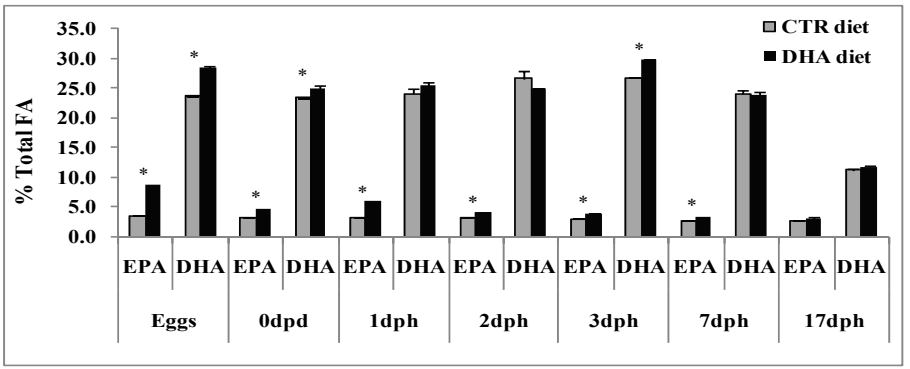


Fig. 2. Levels of EPA and DHA (% total FA) in eggs and larvae from broodstock fed the CTR or DHA diet (average of n=3 with SD). Asterisks represent significant differences ($P<0.05$) between the two treatments.

The expression of genes involved in LC-PUFA biosynthesis showed a similar early developmental pattern as that previously described in Morais et al. (2012) but the novel result shown here is that the levels of transcripts in eggs (only for *elovl5*, since *Δ4fad* is not expressed at this stage) and newly hatched larvae (0dph) can be affected by the broodstock diet, with significantly higher levels found in spawns from fish fed the CTR diet (Fig. 3). This difference is however later reversed, with significantly higher levels of *elovl5* and *Δ4fad* transcripts being found in the DHA treatment at 2dph (only for *Δ4fad*) and 7dph. Given that these enzymes are capable of elongating and desaturating EPA into DHA, the early up-regulation in the CTR treatment might explain the lack of significant differences in DHA content from 1dph onwards, with the exception of a significant rise in start-feeding larvae (at 3dph) of the DHA treatment. It is tempting to correlate this result with the significant up-regulation of *Δ4fad* expression in the DHA treatment at 2dph, which was previously shown to be the ontogenetic peak of *Δ4fad* expression (measured up to 6dph; Morais et al., 2012), but this cannot be ascertained with certainty. On the other hand, the up-regulation of both enzymes at 7dph in larvae originating from the DHA treatment is unexpected, given that at this point larvae have totally consumed their endogenous yolk reserves and were being fed the same standard diet.

Finally, the tube feeding trial performed at 7dph and 17dph did not show any significant differences between larvae originating from both treatments, indicating that broodstock nutrition did not have an effect on later larval LC-PUFA metabolism. Both ^{14}C-EPA and ^{14}C-DHA were equally well absorbed (60-70%), of which 75-93% was retained in body tissues and 7-25% oxidized.

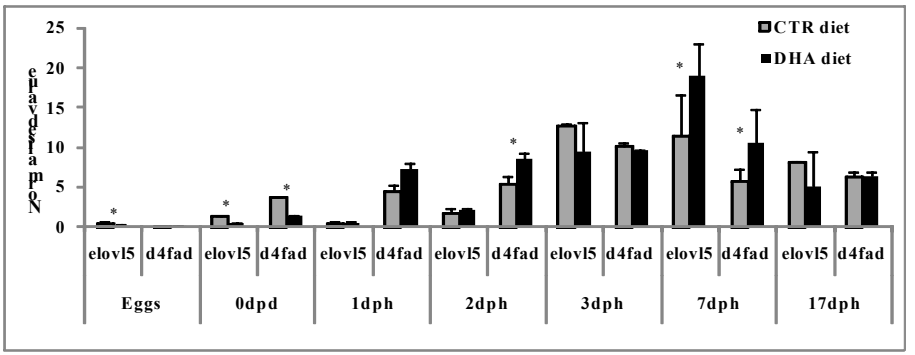


Fig. 3. Expression of *elovl5* and *Δ4fad* in whole eggs and larvae, determined by RT-qPCR. Results are normalised values (ref. genes: *ef1a+rpsa+ubq*) with SD. Asterisks represent significant differences ($P<0.05$) between the two treatments.

Conclusions

Nutrition of Senegalese sole broodstock significantly affected the FA composition of the eggs but later effects (until 7dph) were only visible in EPA levels, which were significantly higher in the DHA treatment. Levels of DHA were also significantly higher in eggs and 0dph larvae from the DHA treatment but by 1dph there were no longer significant differences. This could be explained by the DHA biosynthesis capacity of Senegalese sole larvae, which may have been up-regulated at hatching in the CTR treatment. Nevertheless, larvae spawned from broodstock fed the CTR diet were significantly larger and heavier. This might be explained by either genetic differences between the two broodstocks or by the higher level of MUFA, which are better energy substrates fueling growth, in eggs from the CTR treatment.

Acknowledgments

FCT (Portugal), project EFARFish – PTDC/MAR/67017/2006.

References

Conceição L.E.C., L. Ribeiro, S. Engrola, C. Aragão, S. Morais, M. Lacuisse, F. Soares, and M.T. Dinis. 2007. Nutritional physiology during development of Senegalese sole (*Solea senegalensis*). Aquaculture 268:64-81.

Morais S., F. Castanheira, L. Martinez-Rubio, L.E.C. Conceição, and D.R. Tocher. 2012. Long chain polyunsaturated fatty acid synthesis in a marine vertebrate: Ontogenetic and nutritional regulation of a fatty acyl desaturase with Δ4 activity. BBA - Mol Cell Biol Lipids 1821:660-671.

Rønnestad I., C.R. Rojas-García, S.K. Tonheim, and L.E.C. Conceição. 2001. In vivo studies of digestion and nutrient assimilation in marine fish larvae. Aquaculture 201:161-175.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

GENE EXPRESSION IS STRONGLY REGULATED BY NUTRIENTS IN FIRST FEEDING ATLANTIC COD LARVAE (*GADUS MORHUA*)

M. Moren[1], K. Kvalheim[1*], A. Nordgreen[1**], T. Harboe[2], and K.K. Lie[1]

[1] NIFES, P.O.Box 2029 Nordnes, N-5817 Bergen, Norway
[2] IMR, Austevoll Research Station, N-5392 Storebø, Norway
[*] Present address: Marine Harvest Labrus, Marine Harves Norway AS, Sandviksboder, 78a, N- 5035 Bergen, Norway
[**] Present address: Norsildmel AS, Kjerrediviken 16, N-5141 Fyllingsdalen, Norway

The aim of the study was to evaluate how both vitamin A (VA) and arachidonic acid (ARA), alone or in combination, affected the growth and development of the skeleton in Atlantic cod larvae (*Gadus morhua*). Both nutrients are known to influence the skeletal development and can vary in diets to fish larvae in aquaculture. VA is added to some commercial live prey enrichments while in nature, the carotenoids are the main source. ARA levels are low in most diets, commercial and natural, but a higher n-6/n-3 ratio is commonly found in commercial live prey (rotifers and *Artemia*). Since both components can vary in commercial diets we performed a two-factorial feeding experiment. Four different diets of enriched rotifers were randomly given to (four tanks per diet of) A. cod larvae from start-feeding until day 29 dph (days post hatch).

The enrichments consisted of our experimental oil mixtures (60%) and fish meal (40%), where the oil was mixed to make two very different contents of ARA and EPA without changing the level of DHA. Retinyl palmitate was then added to half of the two different oil mixtures. The rotifers were sampled for analysis three times during the feeding period. Analysis of these rotifers showed that diet 1 (high EPA/ARA ratio and no extra VA) contained 17 % EPA (of total lipids), 1.7% ARA, 12.5% DHA and 0.14 µg VA g^{-1} dry wt. Diet 1+ had identical levels of EPA and ARA and 1.1 µg VA g^{-1} dry wt. Diet 2 contained 8% EPA, 8.6% ARA, 12.9% DHA and 0.2µg VA g^{-1} dry wt. Diet 2+ contained the same levels of fatty acids as diet 2 and 1.8 µg VA g^{-1} dry wt. and results.

There were no differences in survival and only the larvae fed diet 2+ had a lower growth rate than the three other larval groups. Pooled samples of larvae from each tank were taken at 17 and 29 dph. Content of fatty acids and total VA were determined in samples taken at 29 dph, while samples from both 17 and 29 dph were used in microarray analysis as well as in RT-qPCR analysis. The larval content of EPA, ARA, DHA and VA reflected the diets. More than 1000 genes

were differently regulated in larvae fed diet 2+ (high VA and high ARA/EPA ratio) compared to larvae fed diet 1 (low VA and low ARA/EPA ratio), with larvae fed diets 1+ and 2 as intermediates. The diets with high ARA/EPA ratio (diet 2 and 2+) had the strongest effect on the skeletal markers, while increase in VA (diet 1+ and 2+) seemed to have less influence, perhaps due to smaller differences between low and high VA compared to low and high ARA/EPA ratio. Calcification seemed to be less developed in larvae fed diet 2+, but these larvae were also smaller at 29 dph. Combining these in a two-dimensional factorial design revealed that the high ARA/EPA ratio had an effect on VA metabolism regardless of the VA level in the diet. Larvae fed diet 2 or 2+ had three times as much 13-*cis*-retinoic acid compared to larvae fed diet 1 or 1+. Results from microarray and RT-qPCR will be presented and discussed together with semi-quantitative measurements of skeletal calcification and concentration of the different retinoids.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

IDENTIFICATION AND MIGRATION OF PRIMORDIAL GERM CELLS IN ATLANTIC SALMON (*SALMO SALAR*) AND ATLANTIC COD (*GADUS MORHUA*)

K. Nagasawa[1], C. Presslauer[1], G. Yoshizaki[2], M. Miwa[2], J.M.O. Fernandes[1], and I. Babiak[1]

[1] Faculty of Biosciences and Aquaculture, University of Nordland, 8049 Bodø, Norway
[2] Department of Marine Biosciences, Tokyo University of Marine Science and Technology, Minato-ku, 108-8477 Tokyo, Japan

Introduction

Development of primordial germ cells (progenitor cells of eggs and sperm; PGCs) is fundamental to further gonad formation in teleosts (Molyneaux and Wylie, 2004). The developmental pattern of PGC during embryogenesis has been described in several fish species so far: Cyprinidae, including *Danio rerio* (Yoon et al., 1997), *Carassius auratus* (Otani et al., 2002), and *Gobiocypris rarus* (Cao et al., 2012); Cobitidae, such as *Misgurnus anguillicaudatus* (Fujimoto et al., 2006); Adrianichthyidae, namely *Oryzias latipes* (Herpin et al., 2007); and Gobiidae, such as *Gymnogobius urotaenia* (Saito et al., 2004) and *Leucopsarion petersii* (Miyake et al., 2006). However, there is no information available for PGC specification during early development in superorders Protacanthopterygii and Paracanthopterygii even though a series of studies using *vasa-gfp* transgenic technique and chimeric RNA injection have identified PGCs in larval stage in salmonids such as *Oncorhynchus mykiss*, *O. masou*, *Salvelinus fontinalis*, and *Salmo trutta.* (Sakao et al., 2009; Yoshizaki et al., 2005). Therefore, it would be noteworthy to describe PGC development in both taxa for understanding its basic mechanism among teleosts. In this study, we chose *S. salar* and *Gadus morhua* as representatives of Salmonidae and Gadidae, respectively. Marker genes are powerful and useful probes for specifying target cell types and their stages of differentiation. In some teleost species, there are cell marker genes for PGC identification, such as *vasa* and *nanos* (Xu et al., 2010). We therefore aimed to identify appropriate PGC marker genes in *S. salar* and *G. morhua* and to characterize PGC localization during embryogenesis by whole-mount in situ hybridization and in vivo PGC labeling using chimeric *gfp* RNA microinjection.

Materials and methods

Two-year-old *S. salar* and *G. morhua* were provided from Morkvedbukta Research Station (University of Nordland, Bodø, Norway) and CodJuveniles ASA (Bodø, Norway), respectively. Embryos of *S. salar and G. morhua* were obtained from the broodstocks in AquaGen AS (Trondheim, Norway) and the F1 generation of a MarinBreed AS (Bodø, Norway) selective breeding program, respectively. Total RNA was extracted from specimens and used for cDNA synthesis. Full-length *vasa*, *dnd*, and *ly75* cDNAs from salmon and *vasa* and *nanos*3 cDNAs from cod were amplified by RACE-PCR and sequenced. Deduced amino acid sequences were used for domain structure analysis and phylogenetic tree analysis. Tissue distribution of candidate genes for germ cell marker was determined by RT-PCR. Whole-mount in situ hybridization with probes of germ cell marker gene (*vasa* for salmon, *vasa/nanos*3 for cod) was performed with PFA-fixed embryos. The microinjection of *gfp-rt-vasa* or *gfp-zf-nos*1 3'-UTR RNAs was performed with salmon egg.

Results and discussion

Transcripts of candidate genes displayed gonads specific distribution in adult tissues, namely *vasa*/*dnd* in salmon and *vasa*/*nanos*3 in cod. *In situ* analysis with selected germ cell markers (*vasa* for salmon, *vasa*/*nanos*3 for cod) described PGC migration pattern in both species during embryogenesis. Interestingly the typical *vasa* localization pattern of four clusters during blastulation found in zebrafish (Yoon et al. 1997) was not present in *S. salar* but was observed in *G. morhua* (Fig. 1).

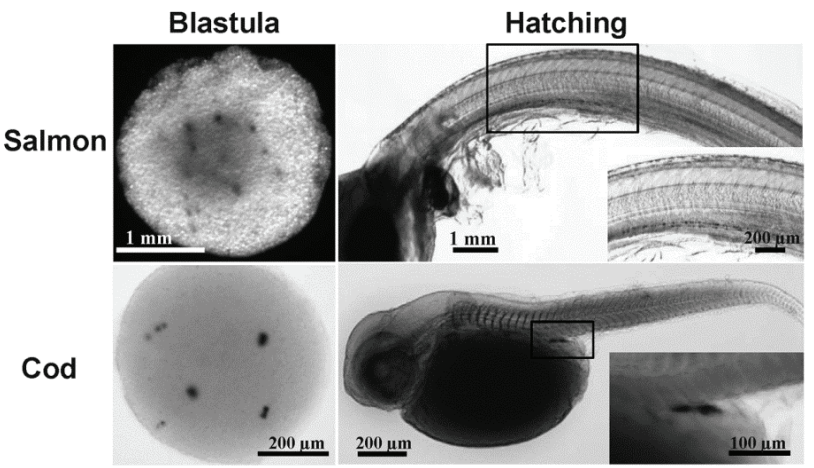


Fig. 1. Localization of *vasa* mRNA and PGCs in *S. salar* (upper panels) and *G. morhua* embryos (lower panels) at different developmental stages (blastula and hatching stages).

In addition, salmon PGCs could be specifically labeled with a green fluorescence protein using *gfp-rt-vasa* 3'-UTR RNA microinjection, which enabled us to track these cells during embryogenesis and early larval stages (Fig. 2). These findings of PGC development in both Salmonidae and Gadidae contribute to our understanding the basic mechanisms of teleost PGC development. Importantly, this knowledge may assist in the development of reproductive biotechnology techniques (Okutsu, et al. 2006), such as germ cell purification, transplantation, cell culture, cryopreservation, gene knockdown, and further transcriptome analysis by next-generation sequencing.

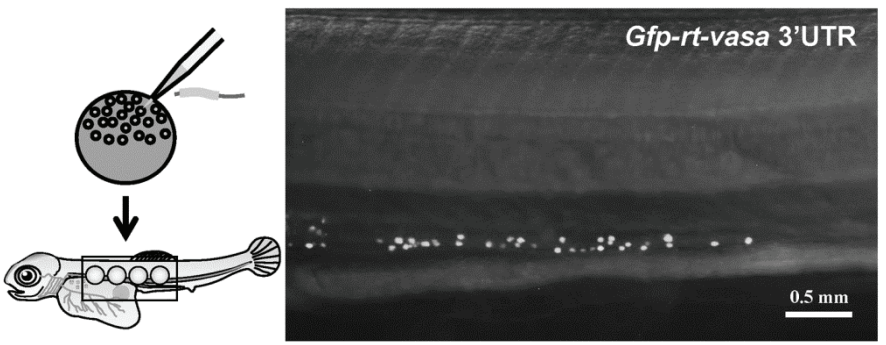


Fig. 2. GFP labeling of PGCs by microinjection of *gfp-rt-vasa* 3'-UTR RNA in *S. salar* embryo (60 days post-injection).

References

Cao M., Y. Yang, H. Xu, J. Duan, N. Cheng, J. Wang, W. Hu, and H. Zhao. 2012. Germ cell specific expression of Vasa in rare minnow, Gobiocypris rarus. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 162: 163-170.

Fujimoto T., T. Kataoka, S. Sakao, T. Saito, E. Yamaha, and K. Arai. 2006. Developmental stages and germ cell lineage of the loach (Misgurnus anguillicaudatus). Zoological Science 23: 977-989.

Herpin A., S. Rohr, D. Riedel, N. Kluever, E. Raz, and M. Schartl. 2007. Specification of primordial germ cells in medaka (Oryzias latipes). BMC Developmental Biology 7: 3.

Miyake A., T. Saito, N. Kashiwagi, D. Ando, A. Yamamoto, T. Suzuki, N. Nakatsuji, and T. Nakatsuji. 2006. Cloning and pattern of expression of the shiro-uo vasa gene during embryogenesis and its roles in PGC development. The International Journal of Developmental Biology 50: 619-625.

Molyneaux K., and C. Wylie. 2004. Primordial germ cell migration. The International Journal of Developmental Biology 48: 537-544.

Okutsu T., A. Yano, K. Nagasawa, S. Shikina, T. Kobayashi, Y. Takeuchi, and G. Yoshizaki. 2006. Manipulation of fish germ cell: visualization, cryopreservation and transplantation. Journal of Reproduction and Development 52: 685-693.

Otani S., S. Maegawa, K. Inoue, K. Arai, and E. Yamaha. 2002. The germ cell lineage identified by vas-mRNA during the embryogenesis in goldfish. Zoological Science 19: 519-526.

Saito T., S. Otani, T. Fujimoto, T. Suzuki, T. Nakatsuji, K. Arai, and E. Yamaha. 2004. The germ line lineage in ukigori, Gymnogobius species (Teleostei: Gobiidae) during embryonic development. The International Journal of Developmental Biology 48: 1079-1085.

Sakao S., T. Fujimoto, T. Kobayashi, G. Yoshizaki, E. Yamaha, and K. Arai. 2009. Artificially induced tetraploid masu salmon have the ability to form primordial germ cells. Fisheries Science 75: 993-1000.

.Xu H.Y., M.Y. Li, J.F. Gui, and Y.H. Hong. 2010. Fish germ cells. Science China-Life Sciences 53: 435-446.

Yoon C., K. Kawakami, and N. Hopkins. 1997. Zebrafish vasa homologue RNA is localized to the cleavage planes of 2- and 4-cell-stage embryos and is expressed in the primordial germ cells. Development 124: 3157-3165.

Yoshizaki G., Y. Tago, Y. Takeuchi, E. Sawatari, T. Kobayashi, and T. Takeuchi. 2005. Green fluorescent protein labeling of primordial germ cells using a nontransgenic method and its application for germ cell transplantation in Salmonidae. Biology of Reproduction 73: 88-93.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

INTERACTION BETWEEN DIETARY LEVELS OF LC-PUFA AND VEGETABLE OIL SOURCES IN SENEGALESE SOLE (*SOLEA SENEGALENSIS)* POST-LARVAE: PUZZLING RESULTS SUGGESTING COMPLETE BIOSYNTHESIS PATHWAY FROM C18 PUFA TO DHA

C. Navarro-Guillén[1], S. Engrola[2], F. Castanheira[2], N. Bandarra[3], I. Hachero-Cruzado[4], L.E.C. Conceição[5], and S. Morais[6]

[1] ICMAN-CSIC, Campus Río San Pedro, 11519 Puerto Real, Cádiz, Spain
[2] CCMAR/CIMAR LA, Universidade do Algarve, Campus de Gambelas, Faro, Portugal
[3] IPMA, Av. Brasilia, Lisboa, Portugal
[4] IFAPA "El Toruño", Ctra. N. IV Km. 654ª, 11500 El Puerto de Santa María, Cádiz, Spain
[5] Sparos Lda-CRIA, Universidade do Algarve, Faro, Portugal
[6] IRTA, Ctra. PobleNou Km 5.5, 43540 Sant Carles de la Rápita, Spain

Introduction

The major emphasis so far in lipid nutrition of marine fish larvae has been to study essential fatty acid (EFA) requirements. However, more attention needs to be given to the balance between EFA and other fatty acids which are a main source of metabolic energy and to determine suitable ratios leading to an optimized utilization (absorption and retention) of dietary EFA. In this study we addressed this question by testing *Artemia* enrichment emulsions containing different DHA levels in combination with two vegetable oil sources – olive oil, rich in the monounsaturated fatty acid (FA) oleic acid (18:1n-9), and soybean oil, rich in the polyunsaturated FA (PUFA) linoleic acid (18:2n-6) – on lipid (triolein) absorption and oxidation and DHA metabolism in Senegalese sole post-larvae.

Materials and methods

Solea senegalensis larvae were obtained from IPMA Aquaculture Research Centre (Olhão, Portugal) at 19 days post hatching (dph), with an average dry weight of 0.56±0.25mg. These larvae were transferred to a recirculation system of eighteen 3-l flat-bottom trays with 240 larvae each at CCMAR facilities and fed one of six experimental treatments (Table I) in triplicate trays in excess to satiation four times daily. Triplicate samples of enriched *Artemia* and 31dph post-larvae (20 per tray) were collected for FA analysis.

The expression of genes involved in the pathway of long chain (LC)-PUFA biosynthesis – a fatty acyl elongase (*elovl5*) and desaturase (*Δ4fad*) – was analysed at 31dph, by RT-qPCR, in a pool of 10 post-larvae per tray (according to Morais et al., 2012).To examine the absorption and metabolism of ^{14}C-DHA and ^{14}C-triolein, a tube feeding trial was conducted at 30 and 31dph, according to the methodology described by Rønnestad et al. (2001). Results were analyzed by two-way ANOVA looking at the effect of "lipid source" and "DHA level".

Results and discussion

The FA composition of the enriched *Artemia* (Table I) generally followed that of the experimental emulsions, with high levels of oleic acid in the olive oil treatments and increasing percentages of LC-PUFA, particularly DHA, and high levels of linoleic acid in the soybean oil treatments and equally increasing levels of DHA in the Low to High DHA treatments. High levels of alpha-linolenic acid were also characteristic, independently of the oil source used, with decreasing amounts from Low DHA to High DHA treatments.

Table I. Fatty acid composition (% TFA) of the six experimental treatments. Different letters within a row represent significant differences between treatments (Tukey test; P<0.05)

	Olive oil			Soybean oil		
	Low DHA	**Medium DHA**	**High DHA**	**Low DHA**	**Medium DHA**	**High DHA**
Total SFA	20.0±0.4^{ab}	20.0±0.6^{ab}	16.5±0.2^{d}	21.0±0.5^{a}	19.4±0.3^{bc}	18.5±0.2^{c}
18:1	31.2±0.3^{c}	31.9±0.3^{b}	45.7±0.2^{a}	26.8±0.1^{e}	26.9±0.2^{e}	29.5±0.1^{d}
Total MUFA	35.6±0.3^{b}	36.1±0.3^{b}	49.0±0.2^{a}	31.4±0.2^{d}	31.1±0.4^{d}	32.2±0.1^{c}
18:2n6	5.9±0.1^{de}	5.7±0.1^{e}	6.3±0.1^{d}	8.0±0.1^{c}	13.4±0.2^{b}	24.4±0.2^{a}
18:3n3	26.1±0.7^{ab}	25.4±0.7^{b}	15.2±0.2^{c}	27.5±0.5^{a}	24.7±0.5^{b}	14.3±0.2^{c}
20:4n6	0.2±0.0^{b}	0.2±0.0^{b}	0.3±0.0^{a}	0.2±0.0^{b}	0.2±0.0	0.2±0.0^{b}
20:5n3	0.7±0.1^{c}	0.8±0.1^{c}	1.8±0.1^{a}	0.7±0.1^{c}	0.8±0.0^{c}	1.3±0.0^{b}
22:5n3	0.0±0.0^{d}	0.1±0.0^{c}	0.5±0.0^{a}	0.0±0.0^{d}	0.0±0.0^{d}	0.3±0.0^{b}
22:5n6	0.0±0.0^{c}	0.0±0.0^{c}	0.2±0.0^{a}	0.0±0.0^{c}	0.0±0.0^{c}	0.1±0.0^{b}
22:6n3	0.3±0.0^{d}	0.8±0.0^{c}	4.0±0.2^{a}	0.1±0.1^{d}	0.6±0.0^{c}	2.6±0.1^{b}
Total PUFA	39.9±0.6^{c}	39.6±0.8^{c}	31.9±0.5^{d}	43.1±0.6^{b}	45.8±0.7^{a}	46.5±0.3^{a}
n-3 HUFA	2.5±0.1^{de}	3.2±0.2^{c}	7.2±0.3^{a}	2.4±0.1^{e}	2.9±0.1^{cd}	5.1±0.1^{b}

In general, the FA composition of the post-larvae reflected their diet, but there were some interesting deviations (Table II). DHA levels showed significant differences between treatments but, overall, were higher than would be expected based on diet composition. In the olive oil treatments DHA level was indeed higher in the high DHA treatment but, surprisingly, the highest level was obtained in the soybean oil-Low DHA treatment. What was also remarkable was that this treatment was the one that least reflected the diet composition, showing much lower levels than expected of 18:3n-3 and 18:4n-3 and higher levels of EPA (22:5n-3), DHA, as well as of ARA and 22:5n-6.

Table II. Fatty acid composition (% TFA) of post-larvae. Different letters within a row represent significant differences between treatments (Tukey test; P<0.05)

	Olive oil			Soybean oil		
	Low DHA	**Medium DHA**	**High DHA**	**Low DHA**	**Medium DHA**	**High DHA**
Total SFA	24.7±2.2^{b}	25.0±1.5^{b}	23.4±1.4^{b}	31.3±1.8^{a}	25.9±1.7^{b}	25.4±1.7^{b}
18:1	28.4±1.1^{b}	26.0±1.3^{c}	34.8±0.8^{a}	22.6±1.0^{d}	23.1±1.5^{d}	24.2±2.1^{cd}
Total MUFA	32.6±1.3^{b}	30.5±1.5^{b}	38.3±1.0^{a}	26.3±1.1^{c}	26.9±1.5^{c}	27.2±0.7^{c}
18:2n6	7.3±0.4^{b}	5.9±0.2^{b}	6.4±0.3^{b}	6.7±0.9^{b}	11.1±0.7^{ab}	14.1±6.8^{a}
18:3n3	15.5±1.8^{ab}	17.4±0.8^{a}	12.7±0.5^{bc}	11.5±1.6^{c}	15.0±1.0^{abc}	13.4±4.0^{bc}
20:4n6	1.8±0.3^{bc}	1.9±0.2^{bc}	1.5±0.1^{c}	2.8±0.3^{a}	2.0±0.1^{b}	1.9±0.3^{bc}
20:5n3	1.5±0.2^{c}	1.7±0.2^{abc}	2.1±0.2^{a}	2.0±0.3^{ab}	1.6±0.2^{bc}	1.8±0.2^{abc}
22:5n3	1.0±0.1^{d}	1.0±0.1^{d}	1.5±0.1^{a}	1.3±0.1^{b}	1.1±0.1^{cd}	1.2±0.1^{bc}
22:5n6	1.1±0.2^{bc}	1.1±0.1^{bc}	0.9±0.0^{c}	1.7±0.2^{a}	1.2±0.1^{bc}	1.3±0.4^{b}
22:6n3	5.6±0.9^{c}	5.6±0.5^{c}	7.0±0.3^{ab}	7.5±0.7^{a}	6.0±0.4^{bc}	6.4±0.5^{bc}
Total PUFA	40.4±2.0^{b}	41.4±1.5^{b}	36.3±1.1^{c}	39.1±0.9^{b}	44.6±1.3^{a}	45.3±1.5^{a}
n-3 HUFA	10.5±1.1^{c}	10.6±0.7^{c}	12.3±0.7^{ab}	12.8±0.9^{a}	10.9±0.5^{bc}	11.3±0.8^{bc}

The tube-feeding trial revealed no significant differences in absorption, retention and catabolism of DHA and triolein radiotracers between individual treatments. However, two-way ANOVA evidenced a significant effect of oil source in DHA retention, which was globally higher in larvae fed the soybean oil treatments (P=0.029). Nonetheless, this does not entirely explain the differences in post-larval FA composition, given that this effect was not particularly accentuated in the soybean oil-Low DHA treatment.

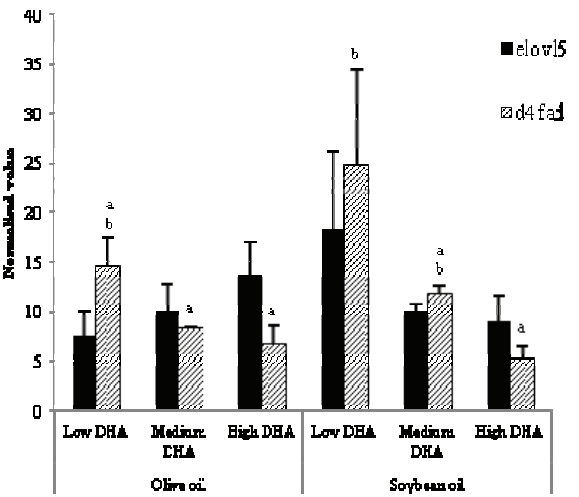


Fig. 1. Analysis of *elovl5* and *Δ4fad* expression by qPCR. Different letters represent significant differences in *Δ4fad* expression between treatments (Tukey test; P<0.05).

The molecular analysis (Fig. 1) revealed non-significant differences in the expression of *elovl5* between treatments, in spite of a significant interaction between the two factors (P=0.040). However, a significant effect of DHA level was

observed in the expression of*Δ4fad* (P=0.000), which was up-regulated by decreasing levels of DHA in the diet, irrespective of the oil base. This has been described previously in Morais et al. (2012) and explains the low requirements for dietary DHA in Senegalese sole, as this enzyme was shown to be capable of converting 22:5n-3 into DHA, and 22:4n-6 into 22:5n-6. However, this study further suggests that Senegalese sole is also capable of biosynthesising DHA from lower chain (C18) PUFA precursors, under conditions of high dietary availability of these precursors. In addition, the results suggest that this pathway appears to be tightly regulated given that biochemical signs of desaturation and elongation from C18 PUFA (that is, lower levels of precursors and intermediate substrates and higher levels of end-products) were only observed in the soybean oil-low DHA treatment, combining both high 18:2n-6 and the highest 18:3n-3 level. It remains to be determined whether this activity is performed by a different enzyme or if by the same *Δ4fad*, having alsoΔ5/Δ6 activity.

In conclusion, we suggest that even though the previously characterized Δ4fad showed in vitro mainly a Δ4 activity, in vivo, and under particular nutritional conditions of low DHA levels combined with high levels of C18 PUFA from both the n-3 and n-6 series, the expression of this enzyme is highly up-regulated and possibly capable of performing all of the desaturation steps, Δ6-Δ5-Δ4, from C18 to DHA. This hypothesis needs to be further examined but, if proven correct, would be remarkable and would establish Senegalese sole as a model in which to study this important pathway and its regulation in lower vertebrates.

Acknowledgments

The authors wish to acknowledge Dr Pedro Pousão-Ferreira and co-workers at IPMA Aquaculture station (Portugal) for supplying the sole larvae for this experiment. This study was supported by FCT (Portugal), project EFARFish – PTDC/MAR/67017/2006, and grant SFRH/BPD/49051/2008.The study also benefited from LARVANET COST Action FA0801.

References

Morais S., F. Castanheira, L. Martinez-Rubio, L.E.C. Conceição, and D.R. Tocher. 2012. Long chain polyunsaturated fatty acid synthesis in a marine vertebrate: Ontogenetic and nutritional regulation of a fatty acyl desaturase with Δ4 activity. BBA - Mol Cell Biol Lipids 1821:660-671.

Rønnestad I., C.R. Rojas-García, S.K. Tonheim, and L.E.C. Conceição. 2001. In vivo studies of digestion and nutrient assimilation in marine fish larvae. Aquaculture 201:161-175.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EFFECTS OF GLUCOSE AND PROBIOTIC SUPPLEMENTATION IN NURSING JUVENILE CLAM, *MERETRIX LYRATA*

Ngo Thi Thu Thao and Le Quang Nha

College of Aquaculture and Fisheries, Cantho University, Vietnam

Abstract

This study was conducted to evaluate the effects of different glucose concentration on the survival and growth rate of juvenile hard clam, *Meretrix lyrata*. Juvenile clams *M. lyrata* were cultured with algae from a tilapia-greenwater system and different concentrations of glucose (0, 35, and 70µg.l^{-1}). After 70 days of culture, highest survival rate was obtained with 35µg.l^{-1} glucose supplementation (99.5-100%). However, in highest concentration of glucose, weight gain and shell length of clams reached highest values ($p<0.05$). Our findings showed that supplementation of probiotics and suitable glucose concentration in the nursery period could increase survival and growth rate of clams.

Introduction

White clam (*M. lyrata*) is the most important bivalve mollusc species and has been cultured for domestic and export markets. In 2009, there were 7 artificial seed clam hatcheries in the Mekong Delta. On average, their capacity is 1750m^3 per hatchery, productivity is 52 800 stage II spat per cycle, and the hatcheries were operated 8-10 cycles annually; however the realized capacity was only about 26.1%. The most important obstacle was the lack of broodstock clams and quantity and quality of algal biomass (Le Tan Thoi, 2010). From 2006-2009, the hard clam seed were mainly wild seed, in which 10% was sold locally and 90% was sold to Northern provinces at around 91 000 ind.kg^{-1}. The major problem was the poor quality of the seed because of the selection and preservation from the harvester. Uchida et al. (2010) discovered that the growth of *Ruditapes philippinarum* in aquaculture was accelerated by about 30% by supplementing particulate diet with glucose. Glucose is given not as a main dish for the clams but as a nutritional supplement and improves their nutritional state. The objective of this study was to evaluate effects of glucose supplementation on survival and the growth rate of juvenile clams during nursing period.

Materials and methods

This experiment was set up with 4 treatments and 3 replicates were run per each treatment with the following supplements: (1). greenwater (Control); (2). greenwater and probiotic (Pro); (3). greenwater, probiotic, and glucose @35µg.l^{-1} (Pro+GC35); (4). greenwater, probiotic, and glucose @70µg.l^{-1} (Pro+GC70).

One hundred clams with shell length 7-8mm were cultured in 250-l plastic tanks with sandy bottoms. Water temperature was recorded at 7:00 AM and 14:00 PM, and environmental parameters such as pH, NH_4^+, and NO_2 were determined weekly by SERA test (Germany). Total weight and shell length of clams were measured biweekly. Water samples from all treatments were also collected biweekly to determine total bacteria, *Vibrio*, and *Bacillus* densities.

Data were analyzed by SPSS 16.0 with ANOVA and Turkey test to find the significant difference of average values among treatments at level of $p<0.05$.

Results

Variation of environmental parameters

Mean concentrations of NH_4^+ and NO_2^- in control treatment were significantly higher than those from probiotics (based on *Bacillus)* or glucose-supplemented treatments (Table I).

Table I: Mean concentrations of NH_4^+ and NO_2^- among treatments

Parameters	Control	Pro	Pro+GC35	Pro+GC70
NH_4^+ (mg.l^{-1})	0,39± 0,00^a	0,29 ± 0,01^b	0,26 ± 0,01^b	0,27 ± 0,00^b
NO_2 (mg.l^{-1})	3,10 ± 0,19^a	2,44 ± 0,31^b	2,39 ± 0,10^b	2,02 ± 0,06^b

Data in the same row with the same letters indicated non significant different ($p>0.05$)

Total bacteria density (CFU.ml^{-1})

Total bacterial counts in all treatments varied from 1.9-3.7×10^{4}CFU.ml^{-1}. From day 15-70 of the cultured period, total bacterial counts increased rapidly from 0.6-4.21×10^{4}CFU.ml^{-1} in probiotic and glucose-supplemented treatments.

Density of Vibrio bacteria (CFU.ml^{-1})

Vibrio bacterial density was highest in the control treatment (4.6×10^{3}CFU.ml^{-1}) and lowest in probiotic together with 70-µg.l^{-1} glucose treatment (1.3×10^{3}CFU.ml^{-1}). The densities of *Vibrio* in probiotic or probiotic together with glucose supplementation were always lower and less variable than in the control (Fig.1).

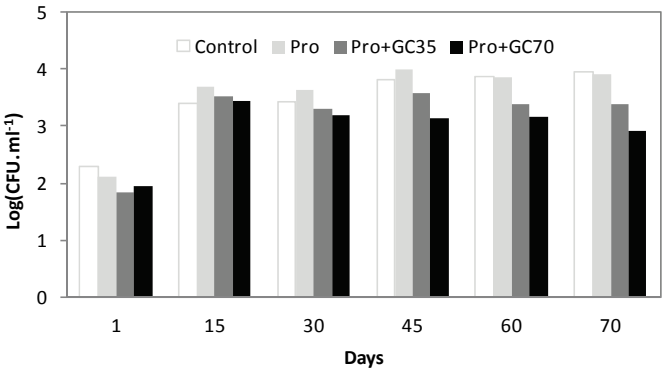


Fig. 1. Variation of *Vibrio* density (CFU.ml^{-1}) during the culture period,

Density of Bacillus bacteria (CFU.ml^{-1})

Density of *Bacillus* was highest in probiotic together with 70-µg.l^{-1} glucose treatment (2.5×10^{4}CFU.ml^{-1}) and lowest in control (0.84×10^{4}CFU.ml^{-1}). Supplementation probiotics or probiotics together with glucose resulted in higher numbers of *Bacillus* and lower numbers of *Vibrio*, indicating the better competition of *Bacillus* (Fig. 2) and better environmental conditions.

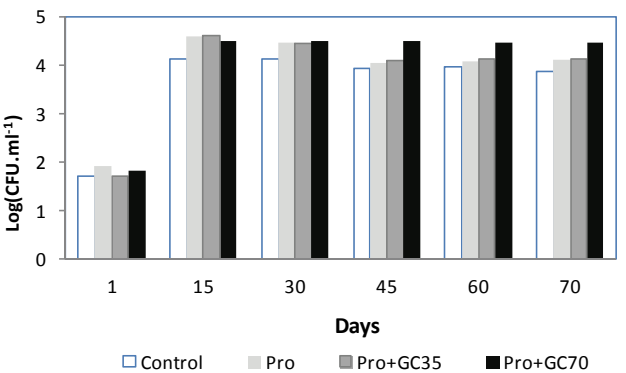


Fig. 2. Variation of *Bacillus* density (CFU.ml^{-1}) during the culture period.

Survival rate of juvenile clams (%)

Survival rates of clams were high in probiotics together with glucose-supplemented treatments (100%) compared to control (98.85%), however the significant different ($p>0.05$) was not seen after 70 days of cultured period.

Growth of clams

Shell length of clams in probiotic-supplemented treatments was 1.7 times higher than those from the control (Fig. 3).

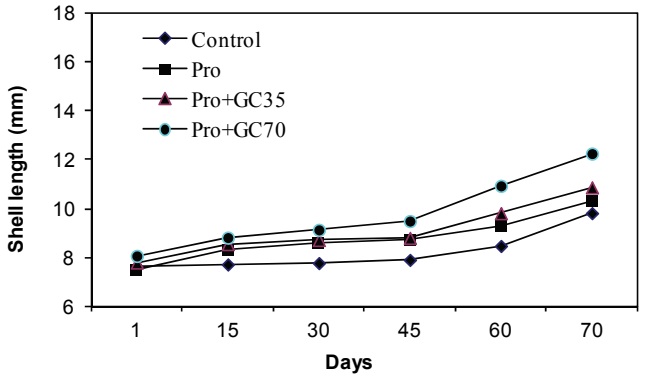


Fig. 3. Shell length of clams during the culture period

After 70 days of culture, clams in probiotics and 70-µg.l^{-1} glucose treatment reached highest total weight (0.5g) compared to the lowest value from the control (0.27g). Supplementation probiotics together with glucose in cultured medium resulted in faster growth rate of juvenile clams (Fig. 4).

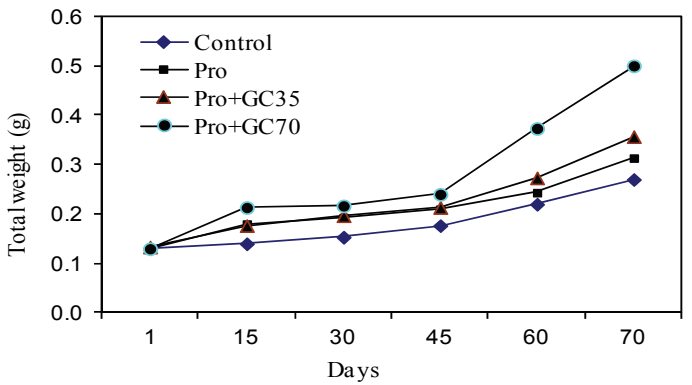


Fig.4. Total weight of clams during cultured period

References

Le Tan Thoi. 2010. An analysis of production and trade of hard clam (*Meretrix lyrata*) industry in the Mekong Delta. Thesis of Master of Aquaculture. College of Aquaculture & Fisheries, Cantho University, Vietnam. 89 pages.

Uchida M., M. Kanematsu, and T. Miyoshi. 2010. Growth promotion of the juvenile clam, *Ruditapes philippinarum*, on sugars supplemented to the rearing water. Aquaculture 302(3):243-247.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EFFECT OF ENVIRONMENTAL FACTORS ON HERITABILITY AND ITS BIOMETRICS OF *ARTEMIA FRANCISCANA* VINH CHAU BY MASS SELECTION OF SMALL SIZED CYSTS

Nguyen Thi Hong Van[1,2], Nguyen Van Hoa[2], P. Bossier[1], P. Sorgeloos[1], and G. Van Stappen[1]

[1] Laboratory of Aquaculture & Artemia Reference Center, Faculty of Bioscience Engineering - Department of Animal Production, Ghent University, Ghent, Belgium.
[2] College of Aquaculture and Fisheries, Cantho University, Cantho, Vietnam

Introduction

Artemia and rotifers are the most widely used live prey in aquaculture, and a lot of efforts have been made to replace rotifers by *Artemia* nauplii at first-feeding of shrimp, marine fish, and crab, but prey size limitations resulting in reduced larval survival is still the main challenge (Baylon et al., 2004; Nhu et al., 2009; Watkins and Le Vay (2011)). Small-sized *Artemia* cysts by truncated selection is probably both a cost and nutritionally effective option to meet this demand. However, environmental effects and food availability were reported as playing an important role in *Artemia* growth and reproduction (Baert et al., 1997; Anh et al., 2009); and consequently might influence gametes. Browne et al. (2002), studying *Artemia* reproductive and life span traits confirmed that the environmental component contributes more to total phenotypic variation than the genetic one. This has been demonstrated for *Artemia* cyst size as well, which has been documented as negatively correlated with salinity and temperature of salt ponds during the reproduction phase (Guermazi et al., 2009). To assess the interference of environmental factors – i.e., food quantity, culture temperature, and salinity of the parental generation in the selection process, quantified as selection response R and heritability h^2 – a factorial experiment was set up to select for small-sized cysts over two successive generations in laboratory conditions..

Materials and methods

Vinh Chau strain, Vietnam, was utilized as parental material in this study. Based on the frequency distribution of the Vinh Chau cyst diameter, 210-212µm, corresponding to about 5% of the population, was set as the targeted selection truncation point. The selection procedure followed the method described by Nguyen Thi Hong Van et al. (2012)

A factorial experiment was set up as described in Figure 1. P cysts were hatched and cultured in three replicate 20-l glass tanks per treatment (F1) and 1-l plastic cones (F2) according to Figure 1 with moderate aeration, using a stocking density of 400 nauplii.l^{-1}. *Artemia* were fed twice a day with spray-dried algae (Spirulina pacifica, Cyanotech-HAWAII, USA) combined with Lansy® PZ shrimp feed (INVE, Belgium) according to the feeding rate used by Hoa (2002). Temperature was maintained among treatments by putting culture tanks in a water baths that were heat-controlled. The culture lasted for 45 days for each trial.

Cyst diameter, naupliar length, and shell-thickness was determined manually by analyzing at least 300 cysts per sample under a microscope equipped with a graduated ocular.

The realized heritability (h^2; Luzt, 2001) is expressed as the result of R (response; the difference in mean phenotype between the progeny generation and the previous generation) and S (selection differential; the difference in mean phenotype between the selected parents and the entire parental population)

Results

From the parental population, cyst diameter was 227±10.7µm and after sieving, that of the selected population (Ps) was 215 ±13.3µm. After one generation under experimental conditions, the cyst size in the selected lines decreased about 7.8-11.4µm (3.4-5%) compared to 1.9-8.1µm (1-3.5%) of the non-selected lines. In the second selection, the cyst sizes in selected lines were reduced less than in the first selection (only 1.0-1.5µm) and there were a little increase in size in non-selected lines (0.2-3.8 µm) compared to their parent.

Similarly to the cyst size, the nauplii length in the selected lines was 409-421 µm and 398-409 µm correspondingly to first and second selection, which was also smaller than none-selected treatments. Although the chorion thickness of F1 and F2 cysts was thicker than the parental (P) but there were not significant differences between them (Table I).

ANOVA analysis demonstrated that selection and food quantities were significant effects ($p<0.001$) to the cyst size trait, naupliar length, but not shell thickness. The only significant interaction between factors was "selection and food quantity" during first selection. However, in the second selection, cyst size trait was found not only independently affected by four experimental factors but also by the interaction between them.

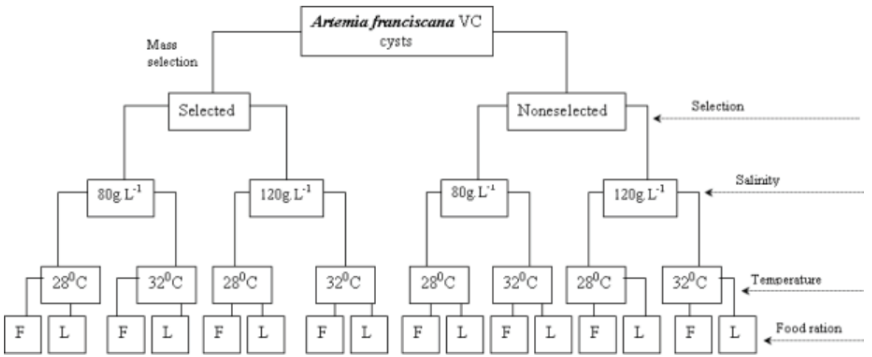


Fig. 1: Schematic outline of designed experiment in which F refers to full feeding (100% requirement) and L (lacking) is only half.

Table I: Mean ±SD of cyst diameter, chorion thickness, and nauplii length of *Artemia franciscana* after first and second selection (s: selected and ns as nonselected

Treatment	First selection (P to F1)			Second selection (F1 to F2)		
	Cyst diameter (µm)	Shell thickness (µm)	Nauplii length (µm)	Cyst diameter (µm)	Shell thickness (µm)	Nauplii length (µm)
Parent	227±10.7 ^{e}	7.7±0.01 ^{a}	432±22.7 ^{d}		-	-
s_100%_28ºC_80‰	219±14.1^{abcd}	9.1±0.97^{a}	421±36.8 ^{bcd}	218±16.5^{cde}	8.8±0.19 ^{a}	409±34.0 ^{d}
s_100%_28 ºC_120‰	218±14.7 ^{abc}	8.5±1.15 ^{a}	420±31.6 ^{bcd}	217±16.5 ^{cd}	8.3±0.50 ^{a}	407±31.5^{cd}
s_100%_32ºC_80‰	218±14.5 ^{abc}	8.8±1.28 ^{a}	419±32.8 ^{abcd}	217±17.1^{cd}	8.3±0.33 ^{a}	408±36.9^{cd}
s_100%_32ºC_120‰	218±15.2 ^{abc}	8.2±0.90 ^{a}	419±31.1^{abcd}	216±17.6 ^{bc}	8.0±0.92 ^{a}	404.±32.6^{bc}
s_50%_28ºC_80‰	218±13.5 ^{abc}	9.4±1.10 ^{a}	413±26.9 ^{abc}	215±12.9 ^{ab}	8.4±0.57 ^{a}	401±31.6^{ab}
s_50%_28ºC_120‰	217±14.9 ^{a}	8.7±0.81 ^{a}	411±36.5 ^{ab}	215±14.4 ^{ab}	8.7±0.73 ^{a}	401±36.9^{ab}
s_50%_32ºC_80‰	218±14.4 ^{ab}	9.2±1.28 ^{a}	411±35.2 ^{ab}	214±14.4 ^{a}	8.4±0.36 ^{a}	401±32.8^{a}
s_50%_32ºC_120‰	215±13.1 ^{a}	8.3±0.88 ^{a}	409±26.6 ^{a}	214±13.7 ^{a}	8.7±0.42 ^{a}	398±33.5^{a}
ns_100% _28ºC_80‰	225±12.5 ^{e}	9.3±0.92 ^{a}	427±37.4 ^{d}	223±12.7 ^{f}	8.8±0.72 ^{a}	424±35.7^{i}
ns_100%_28ºC_120‰	224±12.7 ^{de}	9.0±1.21 ^{a}	422±27.0 ^{bcd}	223±13.3 ^{f}	9.4±0.19 ^{a}	421±27.2^{hi}
ns_100%_32ºC_80‰	222±13.5 ^{cde}	9.1±0.65 ^{a}	422±27.6 ^{cd}	222±14.2 ^{f}	9.2±0.68 ^{a}	419±29.2^{gh}
ns_100%_32ºC_120‰	222±14.1^{bcde}	8.8±0.47 ^{a}	421±29.5 ^{bcd}	220±13.3 ^{e}	8.5±0.47 ^{a}	419±28.3^{fgh}
ns_50%_28ºC_80‰	219±14.3 ^{abc}	8.9±1.27 ^{a}	418±28.0 ^{abcd}	219±15.3 ^{de}	8.9±0.36 ^{a}	416±30.1^{efg}
ns_50%_28ºC_120‰	219±14.8 ^{abc}	8.4±0.74 ^{a}	419±38.1^{abcd}	217±15.0 ^{cd}	8.3±0.28 ^{a}	415 ±37.4^{ef}
ns_50%_32ºC_80‰	219±13.0^{abcd}	8.1±0.68 ^{a}	418±41.7 ^{abcd}	218±15.5 ^{cd}	8.3±0.48 ^{a}	413±36.3^{e}
ns_50%_32ºC_120‰	219±15.2 ^{abc}	8.3±0.55 ^{a}	417±32.0 ^{abcd}	218±16.6 ^{cd}	8.8±0.98 ^{a}	414±28.2^{e}

The heritability of cyst size varied from 0.60 to 0.95 after first selection and there were no significant differences found among treatments. Surprisingly, h^2 in the second selection was severely reduced for those treatments fed with 100% but not those fed only 50 % feeding rate (Table II).

Table II. The heritability value of cyst diameter after first and second selection

Treatment	Heritability value (h^2)	
	First selection	Second selection
100%_28°C_80‰	0.60±0.16 [a]	0.25±0.14 [a]
100%_28°C_120‰	0.68±0.20 [a]	0.34±0.07 [ab]
100%_32°C_80‰	0.67± 0.18 [a]	0.34±0.03 [ab]
100%_32°C_120‰	0.72±0.16 [a]	0.30±0.12 [ab]
50%_28°C_80‰	0.74±0.13 [a]	0.73±0.19 [bc]
50%_28°C_120‰	0.83±0.08 [a]	0.56±0.29 [bc]
50%_32°C_80‰	0.75±0.11 [a]	0.82±0.09 [c]
50%_32°C_120‰	0.95± 0.22 [a]	0.47±0.14 [abc]

Discussion

The results of the experiment showed that unidirectional mass truncation selection for small-sized cysts could be obtained but was influenced by numerous culture conditions as mentioned by previous studies (Vanhaecke and Sorgeloos, 1980; Camargo et al., 2005, Nguyen Thi Hong Van et al., 2012). In this study, the statistical results indicate that in the first selection only food quantities and selection affected cyst biometrics, but there were more independently affected factors and their combination were found in the second selection (F2), especially for selected lines of low feeding level (1). Beside that, heritability (h^2) was also high in the first selection and reduced to a lower level at second selection; lowest values were found with sufficient feeding (2). From these issues (1 and 2), it is possible that when selected animals were subjected to a new environment, they may have exposed the trait that they inherited from the selection process due to strong selection pressure, but after that they became more adapted with living conditions, the traits were more or less affected, and showed a tendency towards their original characters. This explains why in some none-selected treatments, the cyst sizes are increasing in the second selection.

References

Conceiçao L.E.C., M. Yufera, P. Makridis, S. Morais, and M.T. Dinis. 2010. Live feeds for early stages of fish rearing. *Aquaculture Research* 41:613-640.

Guermazi W., A. Habib, and A. Lotfi. 2009. Correspondence of the seasonal patterns of the Brine Shrimp, *Artemia salina* (Leach, 1819) (Anostraca) with several environmental factors in an arid solar saltern (Sfax, Southern Tunisia). *Crustaceana* 82:327-348.

Nguyen Thi Hong Van, Nguyen Van Hoa, P. Bossier, P. Sorgeloos, and G. Van Stappen. 2012. Mass selection for small-sized cysts in *Artemia franciscana* produced in Vinh Chau salt ponds,Vietnam. *Aquaculture Research*:1-9.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

POTENTIAL USES OF GUT WEED *ENTEROMORPHA* SPP. AS A FEED FOR HERBIVOROUS FISH

Nguyen Thi Ngoc Anh, Tran Thi Thanh Hien, and Tran Ngoc Hai

College of Aquaculture and Fisheries, Can Tho University, Vietnam

Abstract

Three separate experiments were performed to assess the potential use of gut weeds *Enteromorpha* spp. as a food source for herbivorous fish. The fresh or dried gut weeds were used as a direct feed to replace commercial feed in an alternative approach for feeding spotted scat (*Scatophagus argus*), red tilapia (*Oreochromis* sp.), and giant gourami (*Osphronemus goramy*) juveniles for 60 days, 45 days, and 56 days, respectively. Four feeding regimes were applied to triplicate tanks and fish was fed daily either commercial feed or gut weed: (1) single commercial feed everyday as a control treatment, (2) single gut weed daily and 2 alternative feeding regimes where (3) 1 day commercial feed and 1 consecutive day gut weed or and (4) 2 consecutive days gut weed. The results indicated that survival of experimental fish was not affected by the feeding treatments. Growth performance of the *S. argus* fed single gut weed was not significantly different from the control group ($P>0.05$). Growth rates of *Oreochromis* sp. and *O. goramy* in the alternative feeding treatments were comparable to the control treatment. Application of the combined feeding regimes, feed conversion ratio could be reduced from 26.1 to 57.8%. These results indicated that fresh and dried gut weed can be used as a feed to substitute commercial feed for herbivorous fish. Moreover, using gut weeds as a feed could improve water quality in the rearing tanks.

Introduction

Gut weed (*Enteromorpha* spp.) belonging to green macroalgae have high nutritional values, containing 9-14% protein; 2.0-3.6% lipid; 32-36% ash, and n-3 and n-6 fatty acids 10.4 and 10.9g.100g^{-1} of total fatty acid, respectively, and are rich in essential amino acids, pigments, and minerals (Aguilera-Morales et al., 2005). They can be used as an ingredient in diets or as direct feed for fish/shrimp and for integrated aquaculture system (FAO, 2003). In Vietnam, gut weed was found abundantly in the extensive shrimp farms and other brackish water bodies of the Mekong delta (SUDA, 2009). Growing well in brackish water areas and about 600 000ha of in shrimp farming areas in the region, there is a

large quantity of gut weed is available for aquaculture feeds. The aim of this study was to assess the effect of commercial feed replacement with fresh or dried gut weed as direct feed on water quality, growth, and feed efficiency of the herbivorous fish. This work could encourage farmers to use locally available gut weed as food source for fish in the Mekong Delta and it could contribute to reduce feed costs.

Materials and methods

Experimental design

Three separate experiments were conducted in Can Tho University, Vietnam. The fresh or dried gut weeds were used as a direct feed to replace commercial feed in an alternative regimes for feeding spotted scat (*Scatophagus argus*), red tilapia (*Oreochromis* sp.), and giant gourami (*Osphronemus goramy*) juveniles for 60d, 45d, and 56d, respectively. Feeding regimes were assigned to triplicate tanks and each day fish were fed either commercial feed or gut weed. Four treatments comprised (1) single commercial feed daily as a control treatment (CF), (2) single gut weed everyday (GW), and 2 alternative feeding regimes where (3) 1 day commercial feed and 1 consecutive day gut weed (1CF_1GW) and (4) 2 consecutive days gut weed (1CF_2GW). The commercial feed (GROBEST-GB640) used in this studies had ≥30% crude protein, ≥6% lipid, and the gut weed had 12.7±2.6% protein content and 2.4±0.2% lipid.

Culture system

100-l plastic tanks were filled with 80 l water with salinity of 5g.l^{-1} for spotted scat and with freshwater for red tilapia and giant gourami. 30 fish were stocked in each tank and provided with continuous aeration and fish were fed to satiation twice a day. Uneaten feed was collected after 1.5h of feeding. Water exchange was done every 3 days and about 30% of the tank volume.

Data collection

Daily water temperature and pH were recorded at 0700h and 1400h and the concentrations of NO_2, and NH_4/NH_3 (TAN) were determined weekly using test kits (Sera, Germany). Initial and final weights were measured at the beginning and at the end of experiment. Specific growth rate (SGR), feed conversion ratio (FCR), and survival were calculated using the following equations:

$$SGR\,(\%.day^{-1}) = \frac{(\ln final\ weight - \ln initial\ weight)}{Days\ of\ culture} \times 100$$

$$Survival\,(\%) = \frac{Final\ number\ of\ fish}{Initial\ number\ of\ fish} \times 100$$

$$FCR = \frac{Feed\ consumed(dry\ weight)}{Weight\ gain(wet\ weight)}$$

Statistical analysis

Data for all measured parameters were analyzed by one-way ANOVA using SPSS version 14.0. The Tukey HSD post-hoc analysis was used to detect differences between means. Significant differences were considered at $P<0.05$.

Results and discussion

Water quality parameters

Daily water temperature and pH over three experiments fluctuated in the ranges of 26.5-30.2°C and 7.1-8.3, respectively. The mean levels of NO_2 and TAN were in the ranges of 0.33-1.50ppm and 0.28-0.90ppm, respectively, in which the lowest and highest values were observed in the single gut weed and solely commercial feed treatments. These factors were generally within the acceptable range for fish growth (Boyd, 1998).

Survival

Results showed that the survival of experimental fish was not significantly different among treatments ($P>0.05$), varying in the ranges of 88.3-93.3%, 82.7-85.3%, and 93.3-100% for spotted scat, red tilapia, and giant gouramy, respectively (Table I).

Table I. Growth, survival, and feed conversion ratio (dry weight) by experimental fish reared on 4 different feeding regimes (mean values ± SD).

Treatment	Final weight (g)	SGR (% day^{-1})	Survival (%)	FCR		CF reduced vs. control (%)
				CF	GW	
Spotted scat, *Scatophagus argus* reared for 60 days						
CF (control)	9.66±2.27^{c}	2.66±0.42^{b}	93.3±6.7^{a}	2.07±0.17	-	
1CF-1FGW	8.13±2.06^{a}	2.36±0.46^{a}	86.7±3.3^{a}	1.53±0.04	0.97±0.06	-26.1
1CF-2FGW	8.26±1.89^{ab}	2.40±0.38^{a}	88.9±1.9^{a}	1.18±0.14	1.46±0.04	-43.0
FGW	9.03±1.35^{bc}	2.58±0.26^{b}	88.3±7.1^{a}	-	2.82±0.03	-100
Red Tilapia *Oreochromis* sp. reared for 45 days						
CF (control)	10.76±2.33^{b}	2.31±0.51^{b}	84.0±0.0^{a}	1.56±0.12	-	
1CF-1DGW	10.63±2.59^{b}	2.28±0.51^{b}	82.7±2.3^{a}	1.05±0.17	0.27±0.28	-32.7
1CF-2DGW	10.25±2.13^{b}	2.20±0.48^{b}	85.3±2.3^{a}	0.87±0.09	0.41±0.05	-44.2
DGW	7.10±1.29^{a}	1.40±0.40^{a}	84.0±4.0^{a}	-	2.47±0.35	-100
Giant gouramy, *Osphronemus goramy* reared for 56 days						
CF (control)	6.10±0.88^{c}	1.74±0.26^{c}	98.3±2.9^{a}	1.99±0.27	-	
1CF-1DGW	5.97±0.83^{c}	1.71±0.26^{c}	100±0.0^{a}	1.03±0.07	0.95±0.06	-48.2
1CF-2DGW	5.28±0.86^{b}	1.48±0.28^{b}	93.3±2.9^{a}	0.84±0.06	1.41±0.04	-57.8
DGW	4.60±0.70^{a}	1.24±0.27^{a}	95.0±5.0^{a}	-	3.29±0.12	-100

Values are mean ± standard deviation. Mean values in each column bearing different superscripts are significantly different ($P<0.05$). CF: commercial feed; FGW: fresh gut weed; DGW: dried gut weed. Mean initial weights of fish were 1.90g, 3.72g, and 2.27g for the spotted scat, red tilapia, and giant gourami, respectively.

Growth performance

There was high variability in the final weight and specific growth rate of experimental fish in the different dietary treatments for 3 experiments (Table I).

For the spotted scat, alternative feeding regimes between commercial feed and fresh gut weed (1CF_1FGW and 1CF-2FGW) resulted in significantly poorer growth ($P<0.05$) compared to other treatments. However, fish fed single GW had similar growth to the control group ($P>0.05$). For the red tilapia, growth of fish received alternative feeding regimes of commercial feed and dried gut weed were comparable to the control group ($P>0.05$) while the lowest growth rates were observed in the group fed single dried gut (DGW) and significantly different from other treatments ($P<0.05$). For the giant gouramy, growth rate followed similar pattern as observed for the red tilapia.

Feed conversion ratio

The highest FCR of commercial feed in the fish fed single commercial feed were found in three experimental fish species. When combination of feeding CF with fresh or dried gut weed, FCR of CF was considerably reduced: 26.1-43.0%, 32.7-44.2%, and 48.2-57.8% for *S. argus*, red tilapia, and *O. gouramy*, respectively. Using gut weed as single feed, FCR of GW was much higher (2.47-3.29) than using solely CF (1.56-2.07).

It can be concluded that using *Enteromorpha* spp. to replace commercial feed can reduce feed costs and maintain better water quality. In this way, farmers maximize the use of on-farm resources without additional cost for feeds.

References

Aguilera-Morales M., M. Casas-Valdez, S. Carrillo-Dominguez, B. Gonzalez-Acosta, and F. Perez-Gil. 2005. Chemical composition and microbiological assays of marine algae Enteromorpha spp. as a potential food source. Journal of food composition and Analysis 18, 79-88.Boyd C.E. 1998. Water quanlity in ponds aquaculture. Auburn University, Alabana 36849 USA.

FAO. 2003. A guide to the seaweed industry, Fisheries Technical paper 441.

SUDA. 2009. Selecting aquatic biofilters with commercial value in the Mekong Delta. Final Report. Sustainable Development of Aquaculture (SUDA) Programme, 108 pp.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

INFLUENCE OF VIRULENT AND AVIRULENT BACTERIAL STRAINS ON HSP70 CONTENT OF *ARTEMIA* INSTAR II LARVAE

Nguyen Thi Xuan Hong[1], D. Vanrompay[2], and P. Bossier[1]

[1] Laboratory of Aquaculture and Artemia Reference Center. 44 Rozier - 9000 Gent, Belgium
[2] Department Molecular Biotechnology. Coupure Link 653, B-9000 Gent, Belgium

Introduction

Bacteria play an important role as a direct feed source for herbivorous zooplankton and can contribute to the nutritional value of *Artemia* feeds by being a major source of protein, vitamins essential amino acids, fatty acids, and enzymes (Marques et al, 2004).

Heat shock proteins (HSP) are known as molecular chaperones, which are produced by prokaryotes and eukaryotes. Previous studies showed that HSP70 and DnaK protein (the bacterial HSP70 homologue) were associated with protection against virulent vibrios and improve the survival of *Artemia* challenged with *Vibrio campbellii* (VC) in axenic conditions (Sung, et al., 2008; Baruah, et al., 2010; Wang, et al., 2010).

The general aim of this experiment is to investigate whether some of these bacterial strains influence the HSP70 content of instar II *Artemia* nauplii.

Materials and methods

Bacterial strains and growth conditions
Ten (virulent and non-virulent) bacterial strains were examined (Table I). All bacterial strains were inoculated in Marine Broth for 24h at 28°C. Bacteria were harvested by centrifugation at 2200xg for 15min, and then added to gnotobiotic *Artemia* at 10^7cells.ml^{-1}. *Artemia* samples were collected at 3; 6; 9, and 24h after exposure, immediately frozen in liquid nitrogen, and stored at -80°C for HSP70 assay by Western blot. The *Artemia* samples were used to extract protein as described in the next section.

Table I. Bacterial strains used in this experiment (The quorum sensing (QS) mutant strains are isogenic strains from BB120, named BB152, MM30, and JAF483). (+): Challenge with VC; (-): Non-challenge with VC

Strain	Gram	Virulence	Identity	Challenge
LVS2	+	-	*Bacillus* sp.	+/-
LVS3	-	-	*Aeromonas hydrophila*	+/-
LT3	+	-	*Bacillus* sp.	+/-
HT6		-	New isolation from ARC	+/-
LMG21363	-	+	*Vibrio campbellii*	-
BB120	-	+	*Vibrio harveyi*	-/+
BB152	-	+	HAI-1 negative	-/+
MM30	-	-	AI-2 negative	-/+
JAF483	-	+	Maximally active QS	-/+
LVS8		-	*Vibrio* sp.	

Protein extraction, SDS polyacrylamide gel electrophoresis, and immunoprobing of Western blots

One hundred milligrams (wet weight) of tissue were homogenized in cold buffer K and supplemented with a protease inhibitor cocktail (Catalogue # P8340, Sigma-Aldrich, Inc. USA) as recommended by the manufacturer. The samples were then centrifuged at 4000×g for 1 minute. The supernatant (protein extract) was combined with equal volumes of sodium dodecyl sulfate (SDS) sample buffer in new Eppendorf tubes, vortexed, heated at 95°C for 5 minutes, cooled, and centrifuged at 4000×g for 1 minute. The equal volumes of samples (10µl) were applied to each lane of a 15% SDS polyacrylamide gel. Two gels were run simultaneously at 130V for 15min and subsequently 150V for 1h. One gel was stained with Coomassie Biosafe. Another gel was transferred to a polyvinylidene fluoride transfer membrane for antibody probing. After incubation in blocking buffer for 1h the membrane was incubated with Mouse monoclonal anti-Hsp70 antibody clone 3A3 (Affinity BioReagetns Inc., Golden, CO) as primary antibody and secondary antibody (Donkey anti-mouse IgG coupled with horseradish peroxidase conjugate) at the recommended dilution. Detection was implemented with 0.7mM diaminobenzidine tetrahydrochloride dehydrate as substrate in association with 0.01% (v/v) H202 in 0.1 M Tris-HCl (pH7.6).

Results

Influence of avirulent bacterial exposure on Artemia HSP70 content

A difference in HSP70 content was observed between the control (non-feeding *Artemia*) and the treatments (Fig. 1). Just as in cysts, HSP70 content is high in instar II nauplii. The HSP70 was detectable until 24h in the control in the absence of bacterial exposure. In contrast, in the treatments, HSP70 content decreased more rapidly becoming undetectable after 9-h exposure.

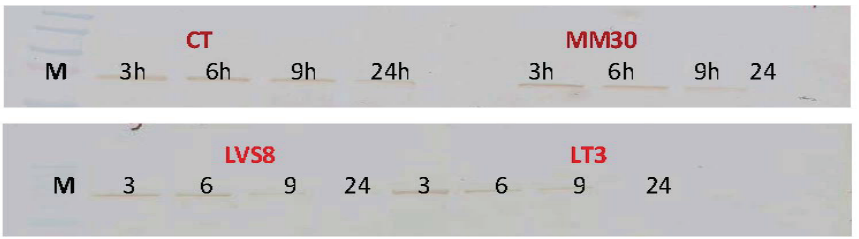


Fig. 1. Evolution of HSP70 content upon exposure to avirulent bacteria

Influence of virulent bacterial exposure on Artemia HSP70 content
The HSP70 content decreases with time and tend to become undetectable at 9h after treatment (Fig. 2). The nauplii exposed to the VC showed the fastest decrease in HSP70 content in comparison to the other treatments.

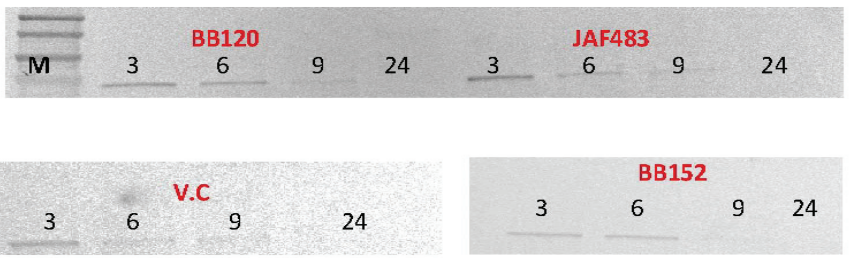


Fig. 2. Evolution of HSP70 content upon exposure to virulent bacteria

Influence of multibacterial exposure on Artemia HSP70 content
There was no significant difference in HSP70 content in all treatments (Fig. 3). The HSP70 content in *Artemia* nauplii was stably maintained from 3-9h after exposure. The exposure to more than one bacterial species might postpone the HSP70 content decrease in nauplii.

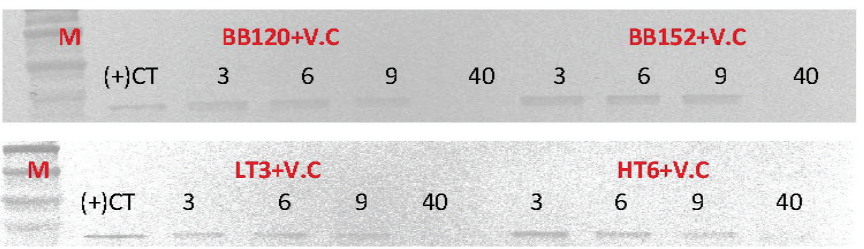


Fig. 3. Evolution of HSP70 content upon exposure to multiple bacteria

Discussion

HSPs are molecular chaperones. They play an important role in folding, stabilization, and translocation of proteins under both normal and stress conditions. In addition, HSP molecules have a critical role in the generation of immune responses against bacterial pathogens.

The results of this study revealed that there was no obvious relationship between the virulence of the bacterial strain to which *Artemia* was exposed and *Artemia* HSP70 content decrease. The multibacterial exposure tends to delay HSP70 content decrease in *Artemia* nauplii, which could be an indication of HSP70 production. These results did not correspond with previous studies which reported that *V. anguillarum* injection resulted in HSP70 induction in shrimp *Fenneropeneus chinensis* (Zhenyu, 2004). In addition, research done in white shrimp and tiger shrimp showed that the level of HSP70, HSP90, and HSP70 mRNA expression in the haemocytes was significantly up-regulated after injection with either *Staphylococcus aureus*, *V. alginolyticus*, or *V. harveyi*. Besides, it was also verified that HSP70 mRNA expression was dependent on the tested organs of shrimp (Rungrassamee, et al., 2010; Zhou, et al., 2010).

We assume that the mode of challenge (in this study, immersion rather than injection) might influence the outcome with relation to pathogen induced HSP70 production in crustacean. By injection, major barriers are overcome, and the host response to bacterial infection might be faster probably by the considerable cellular damage inflicted by the bacterium. On the other hand, it is also possible that the response to a pathogen is host-dependent.

References

Baruah K., J. Ranjan, P. Sorgeloos, and P. Bossier. 2010. Efficacy of heterologous and homologous heat shock protein 70s as protective agents to Artemia franciscana challenged with Vibrio campbellii. *Fish & shellfish immunology* 29(5):733–9. doi:10.1016/j.fsi.2010.07.011

Marques A., J. Dhont, P. Sorgeloos, and P. Bossier. 2004. Evaluation of different yeast cell wall mutants and microalgae strains as feed for gnotobiotically grown brine shrimp Artemia franciscana. *Journal of Experimental Marine Biology and Ecology* 312(1):115–136. doi:10.1016/j.jembe.2004.06.008

Rungrassamee W., R. Leelatanawit, P. Jiravanichpaisal, S. Klinbunga, and N. Karoonuthaisiri. 2010. Expression and distribution of three heat shock protein genes under heat shock stress and under exposure to Vibrio harveyi in Penaeus monodon. *Developmental and comparative immunology* 34(10):1082–9. doi:10.1016/j.dci.2010.05.012

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

NEW APPROACHES FOR *ARTEMIA* POND CULTURE

Nguyen Van Hoa[1*], Tran Huu Le[1], Nguyen Thi Hong Van[1], P. Sorgeloos[2], and G. Van Stappen[2]

[1] College of Aquaculture and Fisheries, Cantho University, 3/2 street, Cantho City, Vietnam; [*]E-mail: nvhoa@ctu.edu.vn
[2] Laboratory of Aquaculture & *Artemia* Reference Center, Faculty of Bioscience Engineering, Department of Animal Production, Ghent University, Rozier 44, B-9000 Gent, Belgium

Abstract

A project for intensive culture of *Artemia* in Vinhchau solar saltwork was funded by Soctrang Authority. The aim of this project is to increase the average cyst yield of 50kg.ha^{-1}.crop, and to build up a stable culture technique with a better yield for local farmers. Multiple laboratory experiments were set up with inert food including fermented rice bran, tiger shrimp feed (PL_{15}), as well as their combination with live algae (*Chaetoceros*). Results showed that, under laboratory conditions, fermented rice bran and tiger shrimp feed can be used as supplemental food sources. The shrimp feed alone or in combination with algae always gave better cyst production compared to the others, but should not account for more than 50% of the diet. In the field trials, aeration of *Artemia* ponds also increased cyst yields (from 195.8±44.2 to 207±46.1kg.ha^{-1}.crop with 6 and 12h aeration a day, respectively) compared to ponds with no aeration (88.2± 27.5kg.ha^{-1}.crop), however the returns on investment (ROI=2.73-2.71 with aeration vs. 2.24 without) are not significantly different. Utilization of fermented rice bran (20kg.ha^{-1}.day) and shrimp feed (6kg.ha^{-1}.day) as a supplementary feed during pond production in combination with greenwater supplies (10% of pond volume daily) resulted in higher yields (96.0±15.9 and 157.2±15.0kg.ha^{-1}.crop, respectively) than traditional culture; Shrimp feed as a supplemental feed supported the cyst yield but their negative effect was at a high cost vs. traditional culture and use of fermented rice bran. Based on the cyst yield and ROI, fermented rice bran should be a promising item for poor farmers.

Materials and methods

The influence of feeds on survival and reproduction of *Artemia* under laboratory conditions was studied using the following treatments: treatment 1 (I) 100% fermented rice-bran (RB); treatment 2 (II) 100% Chaetoceros (Chae); treatment

3 (III) 100% tiger shrimp feed (SF, 42% protein); treatment 4 (IV) 75% RB:25% Chae; treatment 5 (V) 50%RB:50% Chae; treatment 6 (VI) 25% RB:75% Chae; treatment 7 (VII) 25%SF:75% Chae; treatment 8 (VIII) 50%SF:50% Chae; and treatment 9 (IX) 75%SF:25% Chae. *Artemia* were reared in 20-22°C, salinity 80ppt. Feeding rate per day was according to Nguyen Van Hoa (1993). SF is a commercial tiger shrimp feed (No 0) with approximately 42%protein content.

For the effect of aeration on *Artemia* pond culture, there were three treatments: treatment 1 (NT1) as a control (without aeration); treatment 2 (NT2) aeration in the pond bottom in 6h.day^{-1}; and treatment 3 (NT3) aeration in the pond bottom in 12h.day^{-1}. The pond of was 500m^3 and treatment was triplicated, aeration performed during 18h-0h (A2) and 18h-6h (A3); salinity upped to 80 ppt and water level varied around 50cm; ponds were fed with greenwater (GW) from a fertilizer pond and SF was used at a rate of 200-300g.pond^{-1}.day (4-6 kg.ha^{-1}.day).

Results and discussion

Survival and growth

During day 1-3, treatment I and IV showed lowest the survival rate (~78%), however in the next 4-10 days, there were no significant differences in survival (>94%). After day 10 *Artemia* fed 100% Chae (II) displayed highest survival (91.3±2.67%). Treatment VI (25% rice bran:75% Chae) and treatment IX (75% SF:25% Chae) benefitted growth of *Artemia* as both could reach 9.14-9.33mm after 10 days, similar to Coutteau et al. (1992).

Reproductive characteristics

Replacing up to 75% Chae by SF resulted in the same survival in treatments II and IX. The main significant differences ($p<0.05$) were B (reproduction period); C (pre-reproduction period); and D (post reproduction period) (see Table I); other reproductive characteristics included treatment I with less reproductive cycles (6) but the number of embryos (cysts or nauplii) were higher than the others. Interestingly, all the combination of food led females to release total numbers of embryos averaging 1110±196 to 1357±145 embryos per female (cysts and nauplii). Mono fed with only RB, SF, or Chae enhanced the total fecundity of females involved (1466±371, 1707±671, and 1531±223 embryos per female (cysts and nauplii), respectively ($p <0.05$)). Moreover, RB-fed females tended to release nauplii (67.9%), while females fed with SF (III) or replaced 25% Chae by SF (VII) were stimulated to release more cysts (77.8%).

Table I: Influence of feed on survival and reproduction characteristics of *Artemia franciscana*. A: life span; B: reproduction period; C: pre-reproduction period; D: post reproduction period. Values with the same letter in a column are not significantly different ($p<0.05$)

Treatment	A ($p<0.05$)	B ($p<0.05$)	C ($p<0.05$)	D ($p<0.05$)
I	59.2 ± 6.31^{a}	45.8 ± 6.30^{a}	12.93 ± 1.68^{b}	0.00^{a}
II	68.0 ± 5.21^{cd}	48.0 ± 4.40^{a}	11.0 ± 1.13^{a}	8.90 ± 2.35^{d}
III	61.9 ± 4.73^{ab}	50.9 ± 4.93^{a}	9.00 ± 1.02^{c}	2.10 ± 2.43^{bc}
IV	59.0 ± 5.52^{a}	47.0 ± 5.70^{a}	12.0 ± 1.43^{ab}	0.00^{a}
V	60.0 ± 6.57^{ab}	47.0 ± 6.81^{a}	12.1 ± 1.30^{ab}	0.97 ± 1.40^{ab}
VI	63.0 ± 7.02^{ab}	48.0 ± 8.74^{a}	12.1 ± 1.41^{ab}	2.90 ± 2.95^{bc}
VII	61.4 ± 6.93^{ab}		11.0 ± 1.89^{a}	5.00 ± 3.72^{c}
VIII	64.0 ± 6.48^{bc}		11.0 ± 1.25^{a}	2.93 ± 3.30^{e}
IX	69.3 ± 4.87^{d}		19.3 ± 3.58^{d}	10.3 ± 3.06^{d}

Tiger shrimp feed was chosen as supplemental feed for *Artemia* pond production in farmer ponds. The yield recorded averaged 145.3±52.7 kg.ha^{-1}.crop (the best farmer got 204kg.ha^{-1}.crop) which earned ~US$3500-9000 net income, with an average of US$6000 per ha in season 2012.

Effect of aeration on Artemia pond culture

Variation of oxygen at 7h was 4.19±1.42 to 4.93±1.27mg.l^{-1} and 14h was 9.62±2.0mg.l^{-1} to 10.45±1.46mg.l^{-1}, almost double the oxygen content in the morning.

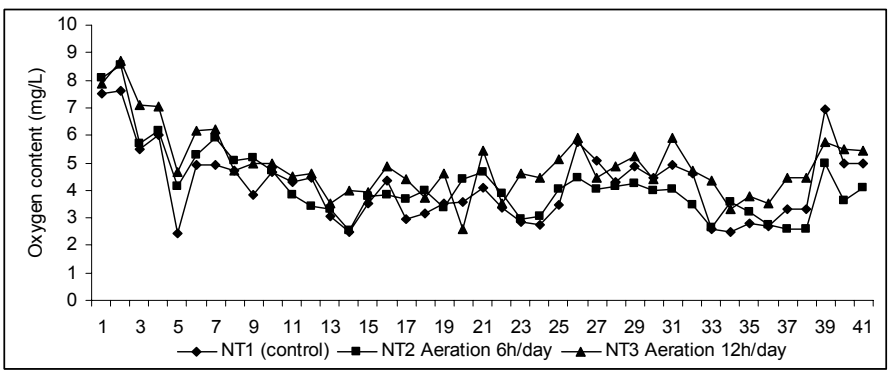


Fig. 1: Variation of oxygen throughout the culture

In the control, oxygen was higher than 2mg.l^{-1} and did not cause any negative effect to *Artemia* population in terms of survival or reproduction (Sorgeloos et al., 1986; Nguyen Van Hoa et al., 2007).

Cyst yield (kg.ha^{-1}.crop)

Aeration affected cyst yields; at NT3 and NT2 ponds could produce in average 207±46.1kg.ha^{-1}.crop vs. 195.8±44.2kg.ha^{-1}.crop at aeration 12h and 6h, respec-

tively. Both were double that of and significantly different to the control (NT1) (Fig. 2) but no significant difference between NT2 and NT3.

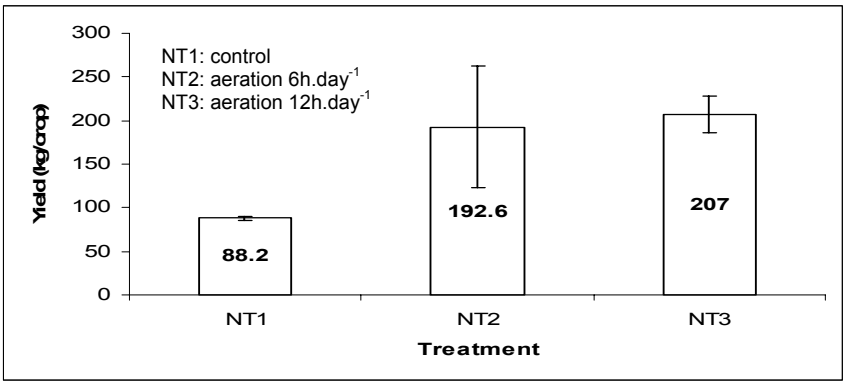


Fig. 2: Cyst yield as a function of aeration.

Conclusions

Replacing SF with 25% Chae during first 3 days in *Artemia* culture will support their survival, then adult *Artemia* fed with 100% RB or SF (~100% survival); SF showed its stimulation of *Artemia* to release cysts while RB promoted nauplii reproduction. Aeration in *Artemia* ponds showed significant support for cyst production as cyst yield was almost double (195.8±44.2 to 207±46.1kg. ha^{-1}.crop) compared to traditional culture pond (88.2±27.5kg.ha^{-1}.crop)

References

Coutteau P., L. Brendonck, P. Lavens, and P. Sorgeloos, 1992. The use of manipulated baker's yeast as an algal substitute for the laboratory culture of Anostraca. Hydrobiologia.Vol. 234. 25- 32.

Nguyen Van Hoa. 1993. Effect of environmental conditions on the quantitative feed requirements of the brine-shrimp *Artemia* franciscana (Kellogg). Msc. thesis, Ghent University, Ghent, Belgium

Nguyen Van Hoa (Ed.). 2007. *Artemia* – Research and application in aquaculture (Vietnamese). Agiculture publishing house. 135 pp.

Sorgeloos P., P. Lavens, P. Léger, W. Tackaert, and D. Versichele. 1986. Manual for the culture and use of brine shrimp *Artemia* in aquaculture, Ghent University, Ghent, Belgium, 319 p.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

USE OF POLY–B-HYDROXYBUTYRATE IN BIVALVE LARVICULTURE

Nguyen Van Hung, P. De Schryver, P. Bossier, and N. Nevejan

Laboratory of Aquaculture & ARC, Ghent University, Rozier 44 - 9000 Gent, Belgium

Introduction

Bivalve aquaculture is significantly affected worldwide by diseases that cause high losses in hatcheries. The main factors responsible for these mortalities are a number of *Vibrio* species that are considered important pathogens in aquaculture. A sustainable strategy using poly-β-hydroxybutyrate (PHB) has been proven effective to combat diseases in fish and crustacean larviculture. PHB is a natural bacterial polymer that is thought to be intestinally depolymerized into water-soluble 3-hydroxybutyrate. This monomer can act as a microbial control agent (Defoirdt et al., 2007). The efficiency of PHB as an antimicrobial agent for larvae of different bivalve species is investigated. This paper describes the effect of PHB delivery forms and concentrations on the performance of blue mussel (*Mytilus edulis*) larvae.

Materials and methods

Adult blue mussels from Zeeland, The Netherlands, were induced to spawn in the lab using temperature shock. Two days after fertilization, D-larvae were concentrated on a sieve (30µm), rinsed with 0.2-µm filtered seawater, and counted. The larvae were stocked at a density of 5 larvae.ml^{-1} in 8-l Züger bottles and fed an optimal algae diet consisting of *Isochrysis galbana, Chaetoceros calcitrans,* and *Tetraselmis suecica* (1:1:1, based on cell numbers) at increasing concentrations from 30 cells.µl^{-1} on day 1 to 100 cells.µl^{-1} on day 18. The control treatment consisted of the algal diet, and the larvae of the PHB treatments received an additional 0.1mg l^{-1} and 1mg.l^{-1} sieved (30µm) and autoclaved PHB particles, while the larvae of the *Alcaligenes* treatments received an addition 0.1mg.l^{-1} and 1mg.l^{-1} lyophilized *Alcaligenes eutropha* bacteria that contained 75% of PHB by dry weight. Each treatment was performed in triplicate and each bottle was aerated and incubated at ambient temperature (16.7±0.5°C). Water was changed completely every two days, and feed was administered and samples were taken for counting and height measurements.

When the larvae showed eye spots (day 18), the larvae from each bottle that remained on a 150-µm sieve were stocked in a downwelling PVC cylinder (Ø 12cm, H 14cm) at a concentration of 1000 larvae.cylinder^{-1}. The cylinders were placed in rectangular tanks containing 60 l of 0.2-µm filtered natural seawater at 16.7±0.5°C. The young spat were fed the same mixed algae diet as the larvae at a concentration of 100cells.µl^{-1} without any addition of PHB or *Alcaligenes*. After 15 days, settlers were counted and measured.

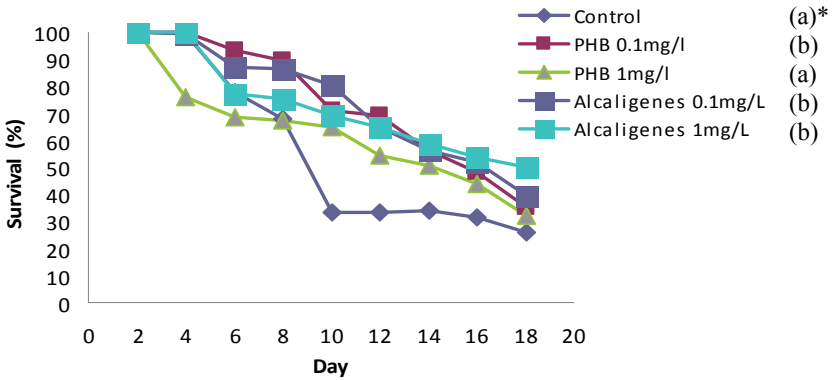
All remaining mussel larvae were sampled for denaturing gradient gel electrophoresis (DGGE) analysis and for isolation of PHB-degrading bacteria (currently ongoing). Based on molecular analysis, it will be possible to determine if PHB treatment of mussel larvae selectively suppresses or promotes intestinal bacteria and if these are pathogenic or have anti-pathogenic properties. The isolation of bacteria able to degrade PHB will offer opportunities to develop a probiotic strategy to increase the effects of PHB in mussel larvae.

Mean survival of mussel larvae was compared with a factorial ANOVA analysis, using SPSS (v16.0). Height measurements and settlement success were compared with a one-way ANOVA. Differences were considered significant at $P<0.05$. A Duncan test was used for the post-hoc analysis.

Results and discussion

Larvae of the PHB (1mg.l^{-1}) treatment did not show significantly different survival compared to the larvae from the control treatment, while the addition of the PHB (0.1mg.l^{-1}) and the PHB containing *Alcaligenes* (both concentrations) significantly increased the survival up to 50±11% (Fig. 1). The larvae of the latter treatments are likely to benefit from the energy that is released from the PHB in the form of 3-hydroxybutyric acid during gastrointestinal depolymerization. Similar observations have already been made for *Macrobrachium rosenbergii* larvae (Nhan et al., 2010) and European sea bass juveniles (De Schryver et al., 2010). The control over opportunistic pathogens in the PHB and *Alcaligenes* treatments may also have contributed to the increased survival in these treatments. Molecular analysis will give more information in this respect. Since the PHB particles have an irregular edgy shape, the higher concentration of 1mg l^{-1} may have resulted in physical damage to the larvae. Alternatively, excessive bacterial growth in the water due to the availability of this carbon source may also have been an additional reason for this reduced survival.

There were no significant differences in height between the treatments: the larvae measured on average between 181±8µm (*Alcaligenes* 1mg.l^{-1}) and 197±3µm (*Alcaligenes* 0.1mg.l^{-1}) on day 18, just before settlement.



.

Fig.1. Survival of blue mussel larvae fed PHB particles and *Alcaligenes eutropha* (75% PHB) compared to the control diet. *Treatments with a different letter in the legend are significantly different $P < 0.05$.

There were no significant positive effects of PHB on the settlement success of mussel spat, although spat of treatment *Alcaligenes* (1mg.l^{-1}) tended to settle more successfully while the opposite is true for spat of treatment PHB (1mg.l^{-1}) (Table I). This confirms the pattern observed during the larval stage of the mussels, although differences in spat settlement were not significant. There was no significant difference in spat size between the treatments, although the spat of the control treatment had a tendency to be bigger. It remains to be determined if a difference in energy delivery as the result of PHB supplementation can be the cause of these observations.

Table I. Metamorphosis performance of mussel spat after 15 days of settlement

Treatment	**Control**	**PHB 0.1mg.l^{-1}**	**PHB 1mg.l^{-1}**	***Alcaligenes* 0.1mg.l^{-1}**	***Alcaligenes* 1mg.l^{-1}**
Settled larvae (%)	41 ± 19^{a}	43 ± 5^{a}	28 ±7^{a}	43 ± 17^{a}	51 ± 9^{a}
Height (µm)	388 ± 10^{a}	347 ± 31^{a}	352 ± 27^{a}	369 ± 6^{a}	352 ± 24^{a}

Acknowledgements

Nguyen Van Hung receives a PhD-scholarship from the Vietnamese Ministry of Education and Training (VIED). Peter DS is a post-doctoral research fellow of the Fund for Scientific Research (FWO) in Flanders.

References

Defoirdt T., D. Halet, H. Vervaeren, N. Boon, T. Van de Wiele, and P. Sorgeloos. 2007. The bacterial storage compound poly-beta-hydroxybutyrate protects *Artemia franciscana* from pathogenic *Vibrio campbellii*. Environmental Microbiology 9:445-452.

De Schryver P., A. Sinha, P. Kunwar, K. Baruah, W. Verstraete, N. Boon, G. De Boeck, and P. Bossier. 2010. Poly-β-hydroxybutyrate (PHB) increases growth performance and intestinal bacterial range-weighted richness in juvenile European sea bass, *Dicentrarchus labrax*. Applied Microbiology and Biotechnology 86:1535-1541.

Liu Y., P. De Schryver, B. Van Delsen, L. Maignien, N. Boon, P. Sorgeloos, W. Verstraete, P. Bossier, and T. Defoirdt. 2010. PHB – degrading bacteria isolated from the gastrointestinal tract of aquatic animals as protective actors against luminescent *Vibriosis*. FEMS Microbiology Ecology 74:1-9.

Nhan D., M. Wille, P. De Schryver, T. Defoirdt, P. Bossier, and P. Sorgeloos. 2010. The effect of poly-β-hydroxybutyrate on larviculture of the giant freshwater prawn (*Macrobrachium rosenbergii)*. Aquaculture 302:76-81.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

IMPROVING VISUAL ENVIRONMENT IN COD LARVAL REARING BY FACTORIAL DESIGNS

O. Nicolaisen[1], M. Cuny[2], and S. Bolla[1]

[1] Faculty of Biosciences and Aquaculture, University of Nordland, N-8049 Bodø, Norway
[2] Science Insight, Frognerveien 1B, 0257 Oslo, Norway

Hatchery production is a bottleneck in marine fish production and the tank environment needs further optimization to improve growth and larval quality. Traditionally, tank environment effects are studied using a one-variable-at-a-time (OVAT) approach, though real-world problems are often multivariate and more effectively addressed by factorial designs. Marine fish larvae exhibit species-specific sensitivity to light, which affects behaviour, foraging, and (ultimately) growth and survival. Larval vision develops during the larval period, which indicates that larval demands for visual environment will change over time. We applied factorial short-term experiments to study how factors altering the visual environment jointly affect foraging success, attraction towards light, and spatial distribution in cod (*Gadus morhua*) larvae at different larval ages/sizes.

The three experiments all followed the same general design scheme. At a series of predefined larval ages, a desired number of larvae, reared at standard conditions in terms of light, temperature, water exchange, and live feed density, were collected from black 100-l holding tanks for use in the respective experiments.

In experiment 1, a 2^4 factorial screening design, replicated in the centre point (n=4), was applied at each of the ages 5, 10, 15, and 20 days post-hatch (DPH). Experimental factors and factor levels were light intensity (100, 650, or 1200 lx), tank bottom colour (black, grey, or white), added microalgae paste (Instant Algae Nanno 3600®, Reed Mariculture Inc.; 0.5, 1.25, or 2 million cells.ml^{-1}), and prey density (5, 12.5, or 20 rotifers.ml^{-1}. The response variable was average number of rotifers eaten by larvae starved beforehand, and experimental units were 10-l cylindrical black tanks. At each age, the experiment duration was 4h. Effect from experimental factors on foraging changed with increased larval age/size. Grey tank bottom generally enhanced foraging at all ages, while microalgae improved foraging only the first week after hatching. At 15DPH a stronger positive effect from increased feed density was seen for black than for light tank bottoms, while increased light intensity caused foraging to decrease in black and

increase in white bottomed tanks. At 20DPH, high algal density enhanced foraging only at high light intensity.

In experiment 2, a duplicate 2^3 factorial screening design was applied at ages 5, 10, 15, 20, 27, and 35DPH using a glass aquarium (length: 1m; width: 0.1m; depth: 0.15m) with black inner walls and lid. One end was illuminated (100 or 1200lux) while at the other, the wall colour was manipulated (white or black). Microalgae paste was added at densities of none or 2 million cells.ml^{-1}, respectively. At each individual experimental run, 40 larvae were transferred to the tank midpoint, and after 20 minutes their position was registered visually using a marked scale (1-10) at the tank bottom. Larvae moved on average horizontally towards the light source at all ages. The strength of this effect changed with age ($p<0.001$), increasing from 5 to 27DPH and then decreasing, indicating a curved response with a peak around 27DPH. At 5DPH, added algae increased orientation towards light ($P<0.001$) while at 10 and 15DPH only light intensity affected orientation. At 20DPH, all three factors interacted, while at 27DPH increased light and white wall colour additively affected larval orientation. At 35DPH the only significant factor was wall colour ($P=0.005$).

In experiment 3, duplicated 2^2 factorial designs were applied at ages 6, 11, 16, 21, and 28DPH. Radius of the circular units was 8cm, depth 9cm, and experimental factors were tank wall (black or white) and light intensity (100 or 1200lx). At each experimental run, 0.5 l seawater and 50 dark-adapted larvae were transferred to the unit, and the tank was then photographed from above after 20 minutes. The distance of individual larvae from the tank center was measured from pictures using Cell P software, and expressed as a fraction of the full tank radius. Experiment 3 also revealed a strong effect from tank wall color ($P<0.0001$) on spatial positioning in tanks at all ages. In white walled tanks, larvae were attracted towards the tank wall, while no added effect was seen from light intensity in the range 100-1200lx.

Our results demonstrate the potential of factorial designs in studies of larval rearing conditions. Within the chosen experimental domains, the most influential factors at different larval ages/sizes were identified, which guide further studies to optimize settings of environmental factors. The presence of interactions between factors shows the strength of factorial designs as compared to OVAT designs. When interactions are present, the latter designs are less able to identify optimal conditions of multiple factors. Specifically, grey tank bottoms improved larval foraging at all ages. Larvae oriented towards the light source and this effect changed during the larval period. Also, the strong effect from tank wall colour on spatial distribution of larvae suggests that care should be taken when choosing tank wall colour. Due to the simplicity of execution and relatively low costs, the applied experimental designs are suited for extensive multi-factorial experiments, useful for both screening and optimization of rearing protocols.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

THE PROTECTIVE AND INTESTINAL MICROBIOTA STEERING EFFECT OF A NOVEL HEAT SHOCK INDUCER ON BRINE SHRIMP LARVAE

Y. Niu, T. Van de Wiele, and P. Bossier

Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Rozier 44, 9000 Ghent, Belgium

Introduction

A novel heat shock protein inducing compound (Hspi) Tex-OE® has been demonstrated to provide protection on *Artemia fransiscana* against abiotic stressors in the gnotobiotic system by a priming procedure which induces the overproduction of Hsp70 (Baruah et. al., 2012). However, how this compound works in a conventional rearing system and its putative interaction with the microorganisms in the aquatic system is unknown. Thus, in our study, we investigated the effect of Tex-OE® on *Artemia* in a conventional rearing system. The impact of this compound on the intestinal microbiota was also investigated.

Materials and methods

Axenic hatching of Artemia and rearing water
Axenic *A. franciscana* were obtained by decapsulation and hatching (Sung et al., 2008). The incubation water used as the model of the conventional rearing system was obtained from the wastewater in a shrimp rearing recirculation system.

In vivo pre-treatment of Artemia with Tex-OE®
The product Pro-Tex® (containing the active compound Tex-OE®), supported in food grade ethanol, was kindly provided by Bradan Ltd., Campbeltown. The detailed information of this product is referred to in the study of Buruah et al. (2012). Five different concentrations of this product were applied in this study: 20, 40, 80, 160, and 320µl.l^{-1}. In total, three separate studies were performed. In the first study, a dose-response relationship of Tex-OE® was determined. For that, the *Artemia* were pretreated within a fixed time (1h) in filtered autoclaved seawater (FASW) with increasing concentrations of Tex-OE® (20, 40, 80, 160, and 320µl.l^{-1}) or with ethanol alone (ethanol control). The final ethanol concentration in the ethanol control or Tex-OE® treatments corresponds to the amount added in the treatment with highest Tex-OE® concentration. A negative control was also maintained without the addition of Tex-OE® and ethanol. The second

study involved testing the effect of pretreating *Artemia* with the highest and lowest effective doses in the first study (40 and 160µl.l^{-1}) for different time intervals (1, 2, and 4h). In the third study, the optimal combination of dose and pre-treatment time was applied (160µl.l^{-1} and 1h) to investigate the steering effect of Tex-OE® on intestinal microbiota.

Survival assay

The pretreated *Artemia* in study one and two were then incubated in shrimp wastewater and the survival was scored after 36 hours.

Study on intestinal microbiota

In study three, after pretreatment, the *Artemia* were incubated in shrimp wastewater. After 36 hours incubation, *Artemia* were collected for the study of intestinal microbiota by using PCR-DGGE, following the procedure described by (Niu et al., 2012).

Results

Dose response of Tex-OE®

In this initial study, a dose-response relationship of Tex-OE® was determined. As shown in Figure1, *Artemia* pretreated with 40, 80, or 160µl.l^{-1} Tex-OE® were significantly protected in the shrimp water compared to the control and ethanol control. However, Tex-OE® pretreatment with 20 or 320µl.l^{-1} provided no protection.

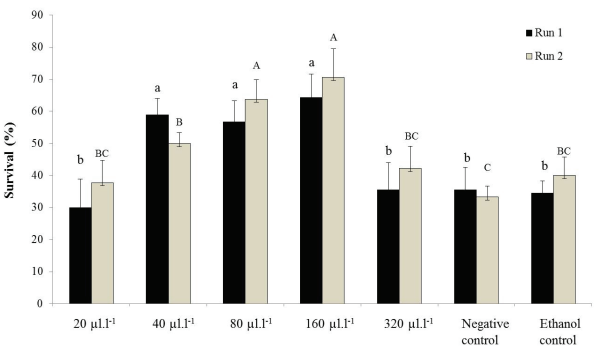


Fig. 1. Survival of Tex-OE® pretreated *Artemia* nauplii after incubation in shrimp water. Experiments were repeated once indicated as Run 1 and Run 2. Error bars with different alphabet letters (small and capital letters for Run 1 and Run 2, respectively) indicate significant difference ($P < 0.05$).

Time-response of Tex-OE® pretreatment

In this study, *Artemia* nauplii were pretreated with 40 and 160µl.l^{-1} Tex-OE® as described in the previous dose-response experiment; however, the exposure time was prolonged to 1, 2, and 4 hours. As shown by Figure 2, pre-treatment of Tex-

OE® with both 40µl.l^{-1} and 160µl.l^{-1} significantly improved the survival of *Artemia* compared to the controls. Pre-treatment of 40µl.l^{-1} Tex-OE® with different time indicated no significant difference. However, pre-treatment of 160µl.l^{-1} Tex-OE® with one hour showed best protection compared to the other treatments.

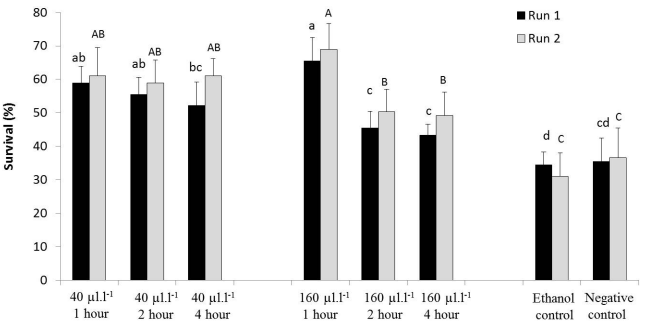


Fig. 2. Survival of Tex-OE® pre-treated *Artemia* nauplii after 36 hours incubation in shrimp water. Experiments were repeated once indicated as Run 1 and Run 2. Error bars with different alphabet letters (small and capital letters for Run 1 and Run 2, respectively) indicate significant difference (P<0.05).

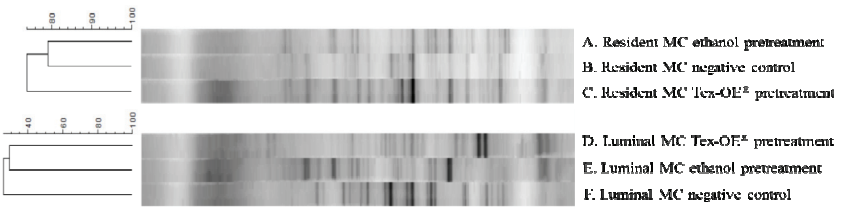


Fig. 3 DGGE fingerprints of intesintne resident and luminal microbiota 36 hour after incubation in shrimp water. Lane.A, B, C, corresponds to the resident microbiota obtained from ethonal pretreatment, negative control, Tex-OE® pretreatment respectvely. Lane.D, E, F corresponds to the luminal microbiota obtained from ethonal pretreatment, negative control, Tex-OE® pretreatment respectvely.

Effect of Tex-OE® intestinal microbiota

In this study, we investigated whether Tex-OE® pretreatment influence the structure of intestinal microbiota of *Artemia*. The resident microbiota and luminal microbiota were distinguished by cellulose purging (Niu et. al., 2012). As indicated by Figure 3B, 36h post-incubation, the luminal microbiota was very distinguished among each treatment, with a similarity less than 40%. However, the intestinal resident microbiota (Fig. 3A) shared a much similar pattern, interestingly, the microbiota with Tex-OE® pre-treatment was distinguished from the negative and ethanol control. Moreover, the evenness of microbiota in each treatment and control was studied by forming the Pareto-Lorenz curve. As

indicated by Figure 4A, the luminal microbiota in pretreatmet and control groups shared a similar eveness, however, the resident microbiota in Tex-OE® pre-treatment group was more unevened than the ethanol and negative control..

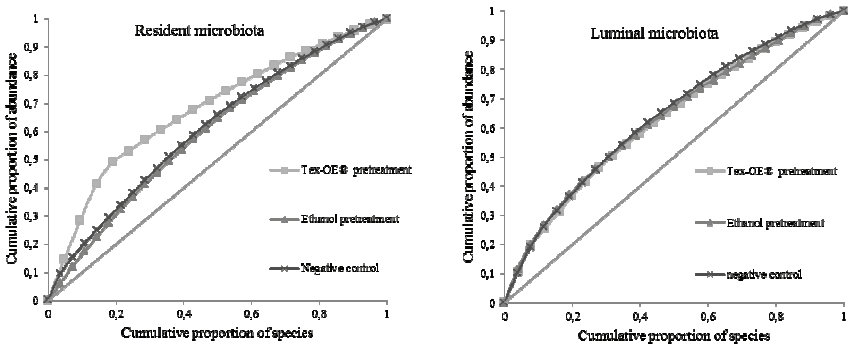


Fig. 4. Pareto-Lorenz curves derived from *Artemia* intestinal microbiota.

Conclusion and discussion

In summary, we have demonstrated that the novel Hspi Tex-OE® can provide protection to *Artemia* nauplii in a conventional rearing system. Meanwhile, a steering effect of Tex-OE® pre-treatment on the structure of the resident intestinal microbiota was observed, suggesting that the Hspi pretreatment may influence the microbial colonization in the gut of *Artemia*. The modulation on resident microbiota may also associate with the protection on *Artemia* against the stressors in a conventional rearing system.

Reference

Baruah K., P. Norouzitallab, R.J. Roberts, P. Sorgeloos, and P. Bossier. 2012. A Novel Heat-Shock Protein Inducer Triggers Heat Shock Protein 70 to protect Artemia franciscana against Abiotic Stressors. Aquaculture 334-337:152-158.

Niu Y.F., T. Defoirdt, A. Rekecki, P.D. Schryver, W. Van den Broeck, S.L. Dong, P. Sorgeloos, N. Boon, and P. Bossier. 2012. A method for the specific detection of resident bacteria in brine shrimp larvae. Journal of microbiological methods 89(1):33-7

Sung Y.Y., C. Pineda, T.H. Macrae, P. Sorgeloos, and P. Bossier. 2008. Exposure of gnotobiotic Artemia franciscana larvae to abiotic stress promotes heat shock protein 70 synthesis and enhances resistance to pathogenic Vibrio campbellii. Cell Stress and Chaperones 13:59-66.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

ONTOGENY OF *KISS2* AND *KISS1R* GENE EXPRESSION IN GILTHEAD SEA BREAM (*SPARUS AURATA*) LARVAE

C.C.V. Oliveira[1], A. Davie[2], E. Cabrita[1,3], M.F. Castanheira[1], M.T. Dinis[1], and H. Migaud[2]

[1] CCMAR, University of Algarve, Campus de Gambelas, ed 7, 8005-139 Faro, Portugal
[2] Institute of Aquaculture of the University of Stirling, Stirlingshire, FK9 4LA, UK
[3] ICMAN/CSIC, Campus Universitario Rio San Pedro, s/n. Puerto Real, 11519 Cadiz, Spain

Introduction

Animals synchronize their biological rhythms with cyclic environmental cues, mostly relying on the pineal organ and its hormone melatonin as photo-temperature transducers. However, the exact mechanism of action in terms of controlling the reproduction rhythms is yet unknown. The recent discovery of the so-called kisspeptin might help understanding such a complex photo-neuroendocrine system. Kisspeptins form a family of structurally related peptides encoded by the *kiss1* gene in mammals and activated through the G-protein coupled receptor 54 (*kiss1r*, previously called GPR54). They have been shown to play a major role in the regulation of the gonadotropic axis, especially in the timing of puberty onset and the control of gonadotropin secretion in mammals (Zohar et al., 2009), as well as in fish (Filby et al., 2008). In fish, two kisspeptin genes have been identified with *kiss2* acting on the GnRH neurons and initiating reproduction (Felip et al., 2009). This system has also been suggested to integrate both environmental cues and metabolic signals and to transduce this information onto the reproductive axis (Zohar et al., 2009), possibly having an involvement in the seasonal control of reproduction (Migaud et al., 2012). In Nile tilapia (*Oreochromis niloticus*), it was actually hypothesised that melatonin could have a direct or indirect role in regulating *kiss1r* expression levels, as shown in mammals (Martínez-Chávez et al., 2008). However, little is known about the role of this system in early life stages of fish development. The ontogeny of the KiSS receptor has been studied in cobia (*Rachycentron canadum*) (Mohamed et al., 2007), with expression detected very early in larvae, in parallel with GnRH expression. In gilthead sea bream, a species with a high commercial interest, the ontogeny of the GnRH system and reproductive axis was investigated, with expression of related genes being also detected very early in development (Wong et al., 2004). Knowing also when KiSS genes start to express in this species ontogeny will allow better understanding of the early development

of the reproductive axis as well as the role of this system in such a complex process.

Thus, the objective of this research was to investigate the ontogeny of *kiss2* and *kiss1r* genes, previously cloned in gilthead sea bream (*Sparus aurata*), because these genes have been suggested to be functionally more important in fish. Gene expression was measured in larvae reared under standard aquaculture conditions (Moretti et al., 1999), to determine when they start to express during the early development of this species.

Materials and methods

Gilthead sea bream larvae were reared in CCMAR at the "Ramalhete" experimental station, using standard rearing protocols for this species (Moretti et al., 1999). Eggs were incubated in a 100-l fibreglass conical cylindrical tank kept at 18±1ºC during approximately 48 hours. Newly hatched larvae were transferred to 3 fibreglass conical cylindrical tanks (100 l) in a closed water recirculation system. Larvae were fed with rotifers (*Brachionus plicatilis*) from the onset of exogenous feeding (3 days after hatching; DAH) until 11DAH. From 12-21DAH they were co-fed with rotifers and *Artemia* (Branchiopoda, Anostraca) nauplii and finally from 22-30DAH just with *Artemia* nauplii. Photoperiod was set as 16h light, 8h darkness, and water temperature was 18±1ºC.

Egg samples were collected in the morning after spawning (gastrula stage) and also prior to hatching (embryo stage). Larvae samples were collected at 0, 5, 10, 20, and 30DAH from each tank at 11:00am. All samples were washed with Mili-Q water and immediately frozen in liquid nitrogen. Gene expression analyses were performed at the Institute of Aquaculture of the University of Stirling, Scotland. The genes *kiss2* and *kiss1r* were previously cloned using gonad and brain samples obtained from broodstock from ICMAN.CSIC in Cádiz, Spain.

Sample total RNA was first extracted using commercial Trizol, according to manufacturer protocol. All samples were treated with DNase RNase-Free and reverse transcribed using a cDNA synthesis kit (Applied Biosystems®). Quantitative PCR (qPCR) was performed in a iCycler iQ real-time PCR detection system (Bio-Rad) with SYBR Green I master mix. Specific primers for each gene were used, and controls were performed using water instead of target cDNA. Quantification was achieved by a parallel set of reactions containing standards consisting of serial dilution of linearised plasmid for each gene. In order no normalise the results, a series of previously validated housekeeping genes was tested, and ßactin was selected as the most stable throughout the range of samples analysed. The normalised gene expression was compared between the several sampling points using a one-way ANOVA, following Duncan post-hoc tests. Statistical significance was taken at $p<0.05$.

Results and discussion

The results of the ontogeny of the KiSS system in gilthead sea bream larvae showed a similar profile for both genes tested, *kiss2* and *kiss1r* (Fig. 1). *Kiss2* and *kiss1r* mRNA gene expression was very low in eggs sampled prior to hatching and then increased significantly at 5 days post hatch (DAH), doubling at 10DAH in the case of *kiss2*. *Kiss2* gene expression then decreased at 20 and 30DAH while *kiss1r* expression levels decreased at 30DAH.

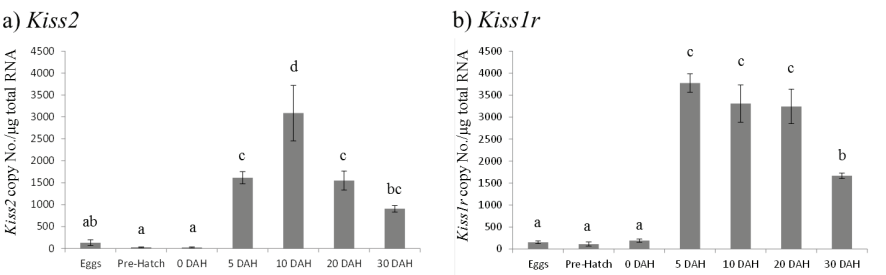


Fig. 1. Ontogeny of *kiss2* (a) and *kiss1r* (b) expression in gilthead sea bream eggs at gastrula and embryo stage (pre-hatch), and larvae during early development (values expressed as mean ± S.E.M.). Letters a, b, c, d, indicate groups with statistical significant differences within each gene (ANOVA, Duncan's test).

The elevated peak of expression observed at 10DAH for *kiss2*, and between 5 and 20DAH for *kiss1r* are very prompt to be related with the early ontogenetic events occurring at this stage. During the first five days several structures are developing: mouth opens at 3-5DAH starting exogenous feeding; and the eyes are totally black and stomach developed at 5DAH (Szisch et al., 2005). As to the brain, there are evidences that it is in a developing state from the embryo until the 15-18DAH (Wong et al., 2004; Ortiz-Delgado et al., 2009), however, there is still scarce information about this organ development. Wong and co-authors (2004) suggested that the ontogeny and organization of the reproductive axis may start as early as 5 days post fertilization (DPF), become more activated at 14DPF, and maintain a stable development after 28DPF, what is within the time range both *kiss2* and *kiss1r* were highly expressed in the present work. Knowing that in another fish species, the cobia, the early expression of *kiss1r* gene observed during the first days of larval development, was parallel to the expression of three GnRH genes, suggesting a close association between *kiss1r* and multiple GnRHs during this stage (Mohamed et al., 2007), reinforces the idea of a possible role of the KiSS system in the ontogeny of the reproductive axis in gilthead sea bream.

Briefly, our study showed for the first time the expression levels of *kiss2* and *kiss1r* during the ontogeny of gilthead sea bream larvae, presenting high levels

of expression between days 5 and 20 after hatching, suggesting an involvement of the KiSS system in the early development of the reproductive axis.

Acknowledgements

CO is supported by a Post Doctoral grant from the Portuguese Ministry of Science and Technology (SFRH/BPD/63933/2009) co-founded by POPH - QREN - Tipologia 4.1 (FEDER and MCTES). Thanks also to AquaExcel project for the funding, and Dr. Luis Conceição from Sparos Lda. for the larvae.

References

Felip A., S. Zanuy, R. Pineda, L. Pinilla, M. Carrillo, M. Tena-Sempere, and A. Gómez. 2009. Evidence for two distinct KiSS genes in non-placental vertebrates that encode kisspeptins with different gonadotropin-releasing activities in fish and mammals. Mol. Cell. Endocrinol. 312:61-71.

Filby A.L., R. van Aerle, J.W. Duitman, and C.R. Tyler. 2008. The Kisspeptin/gonadotropin-releasing hormone pathway and molecular signalling of puberty in fish. Biol. Reprod. 78:278–89.

Martínez-Chávez C.C., M. Minghetti, and H. Migaud. 2008. GPR54 and rGnRH I gene expression during the onset of puberty in Nile tilapia. Gen. Comp. Endocrinol. 156:24-233.

Migaud H., R. Ismail, M. Cowan, and A. Davie. 2012. Kisspeptin and seasonal control of reproduction in male European sea bass (*Dicentrarchus labrax*). Gen. Comp. Endocrinol. 179:384–399.

Mohamed J.S., A.D. Benninghoff, G.J. Holt, and I.A. Khan. 2007. Developmental expression of the G protein-coupled receptor 54 and three GnRH mRNAs in the teleost fish cobia. J. Molecular Endocrinol. 38:235–244

Moretti A., M.P.F, Criado, G. Cittolin, and R. Guidastri. 2009. Manual on hatchery production of seabass and gilthead seabream. Volume 1. FAO, Rome. 194p.

Ortiz-Delgado J.B., H. Segner, and C. Sarasquete. 2009. Brain CYP1A in seabream, *Sparus aurata* exposed to Benzo(a)pyrene. Histol. Histopathol. 24: 1263-1273.

Szisch V., N. Papandroulakis, E. Fanouraki, and M. Pavlidis. 2005. Ontogeny of the thyroid hormones and cortisol in the gilthead sea bream, *Sparus aurata*. Gen. Comp. Endocrinol. 142:186–192.

Wong T.T., Y. Gothilf, N. Zmora, K.E. Kight, I. Meiri, A. Elizur, and Y. Zohar. 2004. Developmental expression of three forms of gonadotropin-releasing hormone and ontogeny of the hypothalamic–pituitary–gonadal axis in gilthead seabream (*Sparus aurata*). Biol. Reprod. 71:1026–1035.

Zohar Y., J.A. Muñoz-Cueto, A. Elizur, and O. Kah. 2009. Neuroendocrinology of reproduction in teleost fish. Gen. Comp. Endocrinol. 165:438-455.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

TANK WALL COLOR AFFECTS SWIMBLADDER INFLATION IN EURASIAN PERCH, *PERCA FLUVIATILIS* L., UNDER CONTROLLED CONDITIONS

K. Palińska-Żarska, D. Żarski*, S. Krejszeff, J. Nowosad, M. Biłas, and D. Kucharczyk

Department of Lake and River Fisheries, University of Warmia and Mazury in Olsztyn, Poland; *E-mail: danielzarski@interia.pl

Introduction

The Eurasian perch, *Perca fluviatilis* L., is one of the very promising candidates for diversification of European intensive freshwater aquaculture. Despite the number of studies on this species (Fontaine, 2004), very limited data is still available on the first days of life of perch larvae under controlled conditions. Swimbladder inflation effectiveness (SBIE) is, among others, a limiting factor in freshwater percids larviculture (Bein and Ribi, 1994; Kestemont and Melard, 2000). Additionally, the variable success of early rearing creates the need for standardization of rearing protocols during this important period.

Eurasian perch is a physoclistous fish species which inflates its swimbladder up to ten days post-hatch (DPH), usually coinciding with the onset of exogenous feeding (Żarski et al. 2011). To date, scarce and contradictory SBIE data have been reported, ranging from 11-76% (Bein and Ribi, 1994, Żarski et al., 2011).

In finfish larviculture, the tank wall colour (TWC) has already been reported to have significant effect on rearing effectiveness, related with the behavioral response of particular species to different TWC (Martin-Robichaud and Peterson, 1998; Cobcroft and Battaglene, 2009) and the visibility (contrast) of the food in the tank with different TWC (Martin-Robichaud and Peterson, 1998). Martin-Robichaud and Peterson (1998) also proved that TWC affected SBIE in striped bass, *Morone saxatilis* (Walbaum). In Eurasian perch, it was reported that black TWC had a positive effect on the growth rate during the first five weeks of rearing (Jentoft et al. 2006). On the other hand, Tamazouzt et al. (2000) reported that black TWC significantly affected lower survival (3%) as compared to the white TWC (13% survival rate) up to 15DPH. However, none of those studies investigated the effect of TWC on the SBIE. The aim of the present study was to determine the effect of white and black TWC on the SBIE in Eurasian perch.

Materials and methods

Eurasian perch larvae were obtained after induced spawning (single injection of hCG, 500IU.kg^{-1}) of wild spawners caught during the spawning season (from Sasek Wielki lake). Freshly hatched larvae (0DPH, 0.78±0.10mg, 5.22±0.55mm) were stocked into six square 50-l tanks (25 000 ind. per tank) – three with white TWC (wTWC) and three with black TWC (bTWC) – supplied with water from a top inlet. Constant light (1500lux) and temperature (15°C) were provided, but no surface skimmer used. Larvae were fed with *Artemia* sp. (SF origin) from 5DPH onward. On day 20 of the experiment, SBIE (%) and total length (TL, ±0.01mm) was recorded with the use of a stereomicrocope (Zeiss steREO Discovery V.20, Germany). Wet body weight (WBW) was determined with a precise balance (±0.1mg). Survival rate was verified on the base of the number of live larvae at the end of the experiment.

Data were analyzed with the t-test at a significance level of ($p<0.05$). Before the analysis, the data expressed as percentages (survival, SBIE) were subjected to arcsine transformation.

Results

Survival rate was significantly affected by the TWC ($P<0.05$). Use of wTWC caused over 95% mortality, whereas bTWC caused less than 76%. Final WBW, TL, and SBIE were also negatively affected by the application of wTWC as compared to bWTC ($P<0.05$) (Table I).

Table I. Final parameters (TL – total length, WBW – wet body weight, SBIE – swim-bladder inflation effectiveness) of Eurasian perch larvae reared for 15 days (from hatching) in the tanks with different tank wall colours: white (wTWC) and black (bTWC). Data (mean ±SD) in columns marked with different letters were statistically different (t-test, $P<0.05$).

	Final TL (mm)	**Final WBW (mg)**	**Survival (%)**	**SBIE (%)**
wTWC	10.36±1.15^{b}	7.35±3.41^{b}	4.9±3.2^{a}	7.7±2.7^{b}
bTWC	12.07±1.00^{a}	14.35±3.25^{a}	24.6±6.4^{b}	32.9±3.6^{a}

Discussion

These results clearly show for the first time that the TWC significantly affects the SBIE, where bTWC was found to be more effective as compared to wTWC.

The survival and SBIE of Eurasian perch larvae was reported to be very variable. Survival rate obtained in the present study in bTWC treatment was close to that reported by Jentoft et al. (2006) (approx. 25%) and higher than obtained by Tamazouzt et al. (2000) (max. 17%). The SBIE was also very similar to that reported by Bein and Ribi (1994), who recorded SBIE between 20.1-42.8%.

Therefore, the obtained results, as well as the rearing conditions provided, may be considered satisfactory.

The positive effect of the black TWC on the swimbladder inflation was already reported for striped bass by Martin-Robichaud and Peterson (1998). Those authors suggested that it was probably linked to the light reflection from the white TWC and thus attract the phototactic larvae better than the light above the water surface. The TWC were found to affect so-called 'walling behaviour' in striped trumpeter, *Latris lineata* (Forster), larvae which resulted in high jaw malformations rate (Cobcroft and Battaglene, 2009). In walleye, *Sander vitreus* (Mitchill), Bristow et al. (1996) recorded reduced 'walling behavior' in turbid water which positively affected SBIE. Therefore, it may be concluded, that application of bWTC in the present study affected higher SBIE due to the prevention of 'walling behaviour' in Eurasian perch larvae. In effect, phototactic perch larvae swam to the source of light (located above the tank) rather than to the tank walls. This caused a direct contact with the water surface and allowed more effective passage through the water surface and air gulping.

References

Bein R. and G. Ribi. 1994. Effect of larval density on the development of perch larvae (*Perca fluviatilis* L.). Aquatic Sciences 56:97-105.

Bristow B.T., R.C. Summerfelt, and R.D. Clayton. 1996. Comparative performance of intensively cultured larval walleye in clear, turbid, and colored water. The Progressive Fish-Culturist 58:1-10.

Cobcroft J.M. and S.C. Battaglene. 2009. Jaw malformation in striped trumpeter *Latris lineata* larvae linked to walling behaviour and tank colour. Aquaculture 289:274-282.

Fontaine P. 2004. L'élevage de la perche commune, une voie de diversification pour l'aquaculture continentale. INRA Prouctions Animales 17:189–193.

Jentoft S., S. Øxnevad, A.H. Aastveit, and Ø. Andersen. 2006. Effects of Tank Wall Color and Up-welling Water Flow on Growth and Survival of Eurasian Perch Larvae (*Perca fluviatilis*). Journal of the World Aquaculture Society 37:313-317.

Kestemont P. and C. Melard. 2000. Aquaculture. P.191–224. In: Percid fishes. Systematics, ecology and exploitation. J.F. Craig (Ed.). Blackwell Science, Oxford, UK.

Martin-Robichaud D.J. and R.H. Peterson. 1998. Effects of light intensity, tank colour and photoperiod on swimbladder inflation success in larval striped bass, *Morone saxatilis* (Walbaum). Aquaculture Research 29:539-547.

Tamazouzt L., B. Chatain, and P. Fontaine. 2000. Tank wall colour and light level affect growth and survival of Eurasian perch larvae (*Perca fluviatilis* L.). Aquaculture 182:85-90.

Żarski D., K. Palińska, K. Targońska, Z. Bokor, L. Kotnik, S Kreszeff, K. Kupren, A. Horvath, B. Urbanyi and D. Kucharczyk. 2011. Oocyte quality indicators in Eurasian perch, Perca fluviatilis L., during reproduction under controlled conditions, Aquaculture 311: 84–91.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

BURBOT, *LOTA LOTA*, L., LARVAE REQUIREMENTS FOR *ARTEMIA* SP. NAUPLII DURING EXPERIMENTAL REARING IN LABORATORY CONDITIONS

K. Palińska-Żarska*, D. Żarski, I. Trąbska, S. Krejszeff, B. Łączyńska, R. Deptuła, and D. Kucharczyk

Department of Lake and River Fisheries, University of Warmia and Mazury in Olsztyn, Poland. *E-mail: katarzyna.palinska@uwm.edu.pl

Introduction

Burbot, *Lota lota* L., is the only member of the Gadidae family which occurs in almost entirely holarctic freshwaters (McPhail and Paragamian, 2000). Disappearance of the populations, low thermal requirements (e.g., Edsall et al., 1993; Tiitu and Vornanen, 2002), fast growth, and high market prices led to increased interest to develop efficient procedures of burbot aquaculture production, both for commercial and enhancement purposes (e.g., Harzevili et al., 2004; Żarski et al., 2010; Jensen et al., 2011).

Larvae are very sensitive to deficiency of food and even a short-term starvation could induce changes in their digestive tracts, which impair digestion (Dąbrowski, 1984). That is why the knowledge of appropriately selected feed rations on different developmental stages is so important (e.g., Wooley et al., 2012). Ad libitum feeding of larvae is beneficial for fast growth, but knowledge of the minimal feeding ratio, which still guarantees high growth rate, is very significant from an economic point of view. Knowledge about the minimal feeding rations is more important, since some fish species (like burbot) need to be fed with live food for a long time (over 50 days) in order to obtain good quality juveniles after weaning (Palińska-Żarska et al., 2013). In the literature, there is a lack of data on the minimal live food ratio for early stages of burbot.

The aim of the study was to define the optimal requirement for freshly hatched *Artemia* nauplii for 22-day-old burbot larvae reared in laboratory conditions.

Materials and methods

Burbot larvae were obtained from artificial spawning of wild fish caught in the Szczecin Lagoon (northeast Poland). Spawners were stimulated only by thermal manipulations as described by Żarski et al. (2010). The initial pre-experimental

rearing of larvae was carried out in a 150-l tank in a semi-closed recirculating aquaculture system. During the first 15 days post-hatching (DPH), the temperature was set at 12°C and from 16DPH, it was increased to 17°C. Before and during the experiment, the photoperiod was 24h light, the concentration of oxygen maintained above 80%, and the concentration of ammonia below 0.01mg l^{-1}. From 5DPH, the larvae were fed three times a day ad libitum with freshly hatched *Artemia* sp. nauplii (GSL origin, INVE Aquaculture, Belgium).

Experimental rearing lasted 12 days and it began when burbot larvae were 22DPH, average wet body weight (WBW) 3.90±0.60mg, and total length (TL) 8.05±0.84mm. Burbot larvae were kept in 15 1-l tanks (100 ind.l^{-1}, constant light 1500lux; temperature 17°C). The larvae were divided into 5 experimental groups: G2, G4, G6, G8, and G10 (in triplicates) and were fed daily with freshly hatched *Artemia* nauplii in doses of 2, 4, 6, 8, and 10% of biomass, respectively. Each dose was counted based on dry weight of *Artemia* as per Leger et al. (1983). The biomass of larvae was verified everyday based on 10 random in vivo weighted specimens (as described by Krejszeff et al., 2013) and on the last day of the experiment, the TL and WBW of 30 randomly chosen larvae were determined. Based on the TL and WBW, the specific growth rate (SGR%.d^{-1}) and the condition factor (K) were also calculated for each group. Before any handling, larvae were anaesthetized in MS-222 solution at a dose of 150mg.l^{-1}.

Statistical differences were analyzed with one-way analysis of variance (ANOVA) and Tukey's post hoc test at a significance level of 5% ($p<0.05$). Before analysis, data expressed in percentages (survival) was subjected to arcsine transformation.

Results

On 34DPH (end of the experiment) the lowest TL, WBW, and SGR ($p<0.05$) were recorded in group G2. In the remaining groups similar TL, WBW, and SGR ($p>0.05$) were noted. At the end of experiment, there were no statistically significant differences ($p>0.05$) between condition factor and survival rate between all of the experimental groups (Table I).

Table I. The results (mean±SD) obtained during burbot rearing, fed at different feeding levels (2, 4, 6, 8, and 10% of the biomass per day on the base of the dry weight of the food). Data in rows marked with different letters were statistically different ($p<0.05$). WBW – wet body weight, TL – total length, S – survival, SGR – specific growth rate, K – condition factor.

Parameter	Feeding level (% of biomass)				
	2	4	6	8	10
	The end of experiment (34 DPH)				
WBW (mg)	19.02±2.82b	23.95±1.53a	24.98±3.28a	25.73±0.79a	25.47±0.48a
TL (mm)	11.86±0.79b	13.09±0.22a	13.19±0.93a	13.56±0.07a	13.62±0.31a
S (%)	93.0±2.0a	94.7±0.6a	92.3±1.5a	91.3±2.1a	96.3±2.5a
SGR (% d^{-1})	13.14±1.23b	15.11±0.54a	15.43±1.14a	15.72±0.26a	15.64±0.15a
K	1.14±0.06a	1.07±0.09a	1.09±0.09a	1.03±0.05a	1.01±0.05a

Discussion

Experimental rearing of burbot larvae allowed, for the first time, to determine the minimal feeding ratio of freshly hatched *Artemia* nauplii (based on the dry weight of the nauplii and with maintaining high survival rate and development of the larvae), which should be given to the fish after 22DPH.

The high survival of the larvae is an important indicator which provides information about well-chosen rearing procedures. In the experiment, the survival was very high (above 90%) in all the groups. It may be concluded that feeding the larvae with 2% of their biomass per day (like in G2 group) provided a sufficient level of nutrition for maintaining high survival rate, but it was not enough to obtain high WBW and TL of the larvae.

It was shown that after 22DPH (with the range of WBW from 3.30-4.50mg and TL from 7.76-9.3mm) the burbot larvae should be given live food at a rate of 4% of their biomass per day. The use of the dry weight of food as a basis gives the possibility to exploit the presented data for using, for example, another "origin" of the *Artemia* in feeding the burbot larvae or even another type of live food, as suggested Jensen et al. (2011). The presented data could be also helpful when calculating the costs of production connected with live food. Moreover, the knowledge of properly determined doses of nauplii is important in the practice of *Artemia* enrichment with vitamins and fatty acids during the specific culture protocols (e.g., Akbary et al., 2011). Regardless of priority of burbot rearing (commercial or enhancement), the results presented may allow to minimize the production costs due to the optimized feeding protocol with the live food.

References

Akbary P., S.A. Hosseini, and M.R Imanpoor. 2011. Enrichment of Artemia nauplii with essential fatty acids and vitamin C: Effect on rainbow trout (Oncorhynchus mykiss) larvae performance. Iranian Journal of Fisheries Sciences 10:557-569.

Dąbrowski K. 1984. The feeding of fish larvae: present "state of the art" and perspectives. Reproduction Nutrition and Development 24:807-833.

Edsall T.A., G.W Kennedy, and W.H. Horns. 1993. Distribution, abundance, and resting microhabitat of burbot on Julian's Reef, southwestern Lake Michigan. Transactions of the American Fisheries Society 122:560-575.

Harzevili S.A., I. Dooremont, I. Vught, J. Auwerx, P. Quataert, and D. De Charleroy. 2004. First feeding of burbot, Lota lota (Gadidae, Teleostei) larvae under different temperature and light conditions. Aquaculture Research 35:49-55.

Jensen N.R., P.J. Anders, C.A. Hoffman, L.S Porter, S.C. Island, and K.D. Cain. 2011. Performance and Macronutrient Composition of Age-0 Burbot Fed Four Diet Treatments. North American Journal of Aquaculture 73:360-368.

Krejszeff S., D. Żarski, K. Palińska, I. Trąbska, K. Kupren, K. Targońska, M. Bowszys, and D. Kucharczyk. 2013. Procedure of harmless estimation of fish larvae weight. Italian Journal of Animal Science 12:e44.

Léger P., P. Vanhaecke, and P. Sorgeloos. 1983. International study on Artemia XXIV: Cold storage of live Artemia nauplii from various geographical sources: potentials and limits in aquaculture. Aquacultural Engineering 2:69-78.

McPhail J.D., and V.L. Paragamian. 2000. Burbot biology and life history. p. 11–23. In: Burbot Biology, Ecology and Management. Washington USA.

Palińska-Żarska K., D. Żarski, S. Krejszeff, J. Nowosad, M. Biłas, K. Trejchel, A. Brylewski, K. Targońska, and D. Kucharczyk. 2013. The effect of age, size and digestive tract development on burbot, *Lota lota* (L.), larvae weaning effectiveness. Aquaculture Nutrition, in press.

Tiitu V., and M. Vornanen. 2002. Morphology and fine structure of the heart of the burbot, a cold stenothermal fish. Journal of Fish Biology 61:106-121.

Trabelsi A., J-N. Gardeur, F. Teletchea, and P. Fontaine. 2011. Effects of 12 factors on burbot *Lota lota* (L., 1758) weaning performances using fractional factorial design experiment. Aquaculture 316:104-110.

Woolley L.D., G.J Partridge, and J.G. Qin. 2012. Mortality reduction in yellowtail kingfish (*Seriola lalandi*) larval rearing by optimizing Artemia feeding regimes. Aquaculture 344–349:161-167.

Żarski D., D. Kucharczyk, W. Sasinowski, K. Targońska, and A. Mamcarz. 2010. The Influence of Temperature on Successful Reproductions of Burbot, *Lota lota* (L.) under hatchery conditions. Polish Journal of Natural Science 25:93-105.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

THE IMPACT OF QUORUM SENSING-DISRUPTING COMPOUNDS ON SURVIVAL AND GROWTH OF GIANT FRESHWATER PRAWN (*MACROBRACHIUM ROSENBERGII*) LARVAE

G.S.J. Pande[1,2], P. Bossier[1], and T. Defoirdt[1]

[1] Laboratory of Aquaculture & *Artemia* Reference Center, Ghent University, Rozier 44, 9000 Gent, Belgium
[2] Department of Aquatic Resources Management, Faculty of Marine Science and Fisheries, Udayana University, Bukit Jimbaran Campus, 80361 Bali, Indonesia

Introduction

Due to the high economic value of giant freshwater prawn (*Macrobrachium rosenbergii*) and the ability to produce the postlarvae in hatcheries, the number of prawn farming has significantly increased. However, disease outbreaks are amongst the major obstacles to produce healthy and high quality seed for further expansion of giant freshwater prawn culture (Nhan et al., 2010). One of the pathogen species that has been identified from affected giant freshwater prawn larvae is the opportunistic luminous bacterium *Vibrio harveyi* (Tonguthai, 1997).

The virulence of *V. harveyi* has been known under control of quorum sensing, a regulatory mechanism based on secreting and sensing small signal molecules called autoinducers (Henke and Basler, 2004; Defoirdt et al., 2008; Natrah et al., 2011). We previously reported that *V. harveyi* quorum sensing regulates its virulence towards giant freshwater prawn larvae (Pande et al., 2013). Consequently, the application of quorum sensing-disrupting agents might be a valid strategy to control vibriosis in this species. In this study, we aimed at investigating the impact quorum sensing-disrupting compounds such as cinnamaldehyde, brominated furanones and brominated thiophenones on the survival and growth of giant freshwater prawn larvae when challenged to pathogenic *V. harveyi*.

Materials and methods

In this study we used *V. harveyi* BB120 (=ATCC BAA-1116) while the quorum sensing-disrupting compounds used in this study are cinnamaldehyde (Sigma), the brominated furanone (Z-)-4-bromo-5-(bromomethylene)-2(5H)-furanone (Sigma), and the brominated thiophenone (Z)-4-((5-(bromomethylene)-2-oxo-2,5-dihydrothiopen-3-yl)methoxy)-4-oxobutanoic acid (synthesized as described in Defoirdt et al. (2012)).

Giant freshwater prawn experiments were performed as described in Pande at al. (2013). The freshly hatched prawn larvae were distributed in groups of 25 larvae in glass cones containing 100ml fresh autoclaved brackish water ($12g.l^{-1}$ synthetic sea salts) with aeration line. The larvae were fed daily with 5 axenic *Artemia* nauplii.larvae^{-1}. Prawn larvae were challenged with 10^6CFU.ml^{-1} *V. harveyi*. Either bacteria or quorum sensing-disrupting compounds were adding to the culture water on the day after first feeding. Survival was counted daily in the treatment challenged to *V. harveyi* without addition of the quorum sensing-disrupting compounds as negative control. The challenge test was stopped when more than 50% mortality was achieved in the negative control. At this time, the larval stage index and larval survival was determined in all treatments by considering that only those larvae presenting movement of appendages were alive. The data was analyzed statistically using SPSS 20.

Results and discussion

In vivo challenge experiments revealed that the survival of challenged larvae significantly increased when these compounds were added to the culture water at 1µM, offering a complete protection (no significant difference in survival when compared to non-challenged larvae) (Table I).

Table 1. Percentage survival of giant freshwater prawn larvae (average ± standard deviation of five replicates) after 5 and 8 days of challenge with *Vibrio harveyi* BB120, with and without quorum sensing-disrupting compounds.

Treatments	Survival (%)[1]	
	Day 5	Day 8
Control	86.4 ± 3.58^{c}	84.0 ± 2.83^{D}
BB120	64.8 ± 6.57^{b}	42.4 ± 6.07^{B}
BB120 + Cinnamaldehyde 1µM	78.4 ± 4.56^{bc}	76.8 ± 5.22^{CD}
BB120 + Cinnamaldehyde 10µM	79.2 ± 8.67^{bc}	76.0 ± 6.32^{CD}
BB120 + Furanone 1µM	79.2 ± 9.12^{bc}	76.0 ± 9.38^{CD}
BB120 + Furanone 10µM	44.8 ± 3.35^{a}	0^{A}
BB120 + Thiophenone 1µM	77.6 ± 7.80^{bc}	75.2 ± 5.22^{CD}
BB120 + Thiophenone 10µM	71.2 ± 7.69^{b}	68.8 ± 7.69^{C}

[1]Values in the same column with different superscript letters are significantly different ($p<0.05$)

Increasing the concentration of the compounds to 10µM did not further increase the survival of the larvae and 10µM of furanone even resulted in complete mortality of the larvae. Importantly, the quorum sensing-disrupting compounds showed no negative impact on the growth of surviving prawn larvae since no difference in larval stage index (LSI) was observed between treated and untreated larvae (Table II).

Table II. Larval stage Index (LSI) of giant freshwater prawn larvae (average ± standard deviation of five replicates) after 5 and 8 days of challenge with *Vibrio harveyi* BB120, with and without quorum sensing-disrupting compounds.

Treatments	LSI[1]	
	Day 5	Day 8
Control	3.6 ± 0.52^{a}	5.2 ± 0.79^{A}
BB120	3.4 ± 0.52^{a}	5.2 ± 0.63^{A}
BB120 + Cinnamaldehyde 1µM	3.5 ± 0.53^{a}	5.2 ± 0.79^{A}
BB120 + Cinnamaldehyde 10µM	3.6 ± 0.52^{a}	5.1 ± 0.88^{A}
BB120 + Furanone 1µM	3.5 ± 0.53^{a}	5.1 ± 0.74^{A}
BB120 + Furanone 10µM	3.2 ± 0.42^{a}	ND
BB120 + Thiophenone 1µM	3.5 ± 0.53^{a}	5.1 ± 0.74^{A}
BB120 + Thiophenone 10µM	3.4 ± 0.52^{a}	5.0 ± 0.82^{A}

[1]Values in the same column with different superscript letters are significantly different ($p<0.05$); ND: not determined

Our results are consistent with previous reports showing that cinnamaldehyde is able to protect brine shrimp larvae against *V. harveyi* (Brackman et al., 2008) and burbot (*Lota lota* L.) larvae from *Aeromonas hydrophila* and *Aeromonas salmonicida* (Natrah et al., 2012). Brominated furanones have been reported before to protect brine shrimp larvae from pathogenic *V. harveyi* (Defoirdt et al., 2006) and rainbow trout (*Oncorhyncus mykiss*) from *Vibrio anguillarum* (Rasch et al., 2004), and the thiophenone compound has been reported before to protect brine shrimp larvae from *V. harveyi* (Defoirdt et al., 2012). As far as we know, this study provides the first evidence of a protective effect of this kind of compounds in a commercial crustacean species. The observation that higher levels of furanone resulted in high mortality of the prawn larvae is also consistent with the literature, in that this kind of compounds is known to have considerable toxicity towards higher organisms (Hentzer and Givskov, 2003; Defoirdt et al., 2006). In conclusion, in this study, we found that the use of quorum sensing-disrupting compounds is a valid strategy to protect giant freshwater parwn larvae from *V. harveyi* without negative effect on growth of the prawn larvae.

References

Brackman G., T. Defoirdt, C. Miyamoto, P. Bossier, S.V. Calenbergh, H. Nelis, and T. Coenye. 2008. Cinnamaldehyde and cinnamaldehyde derivatives reduce virulence in *Vibrio spp*. by decreasing the DNA-binding activity of the quorum sensing response regulator luxR. BMC Microbiology, 8(149):1-14.

Defoirdt T., T. Benneche, G. Brackman, T. Coenye, P. Sorgeloos, and A.A. Scheie. 2012. A quorum sensing-disrupting brominated thiophenone with a promising therapeutic potential to treat luminescent vibriosis. Plos ONE 7(7):e41788.

Defoirdt T., N. Boon, P. Sorgeloos, W. Verstraete, and P. Bossier. 2008. Quorum sensing and quorum quenching in *Vibrio harveyi*: lessons learned from in vivo work. ISME. J. 2:19-26.

Defoirdt T., R. Crab, T.K. Wood, P. Sorgeloos, W. Verstraete, and P. Bossier. 2006. Quorum sensing-disrupting brominated furanones protect the gnotobiotic brine shrimp *Artemia franciscana* from pathogenic *Vibrio harveyi*, *Vibrio campbelli*, and *Vibrio parahaemolyticus* isolates. Applied and Environmental Microbiology 72(9):6419-6423.

Henke J.M. and B.L. Bassler. 2004. Three parallel quorum sensing systems regulate gene expression in *Vibrio harveyi.* Journal in Bacteriology 186: 6902–6914.

Hentzer M. and M. Givskov. 2003. Pharmacological inhibition of quorum sensing for treatment of chronic bacterial infections. J. Clin. Invest. 112: 1300-1307.

Natrah F.M.I., M.I. Alam, S. Pawar, S. Harzevili, N. Nevejan, N. Boon, P. Sorgeloos, P. Bossier, and T. Defoirdt. 2012. The impact of quorum sensing on the virulence of *Aeromonas hydrophila* and *Aeromonas salmonicida* towards burbot (*Lota lota L.*) larvae. Veterinary Microbiology 159:77-82.

Natrah F.M.I., H.A.D. Ruwandeepika, S. Pawar, I. Karunasagar, P. Sorgeloos, P. Bossier, and T. Defoirdt. 2011. Regulation of virulence factors by quorum sensing in *Vibrio harveyi*. Veterinary Microbiology 154:124-129.

Nhan D.T., D.T.V. Cam, M. Wille, T. Defoirdt, P. Bossier, and P. Sorgeloos. 2010. Quorum quenching bacteria protect *Macrobrachium rosenbergii* larvae from *Vibrio harveyi* infection. Journal of Applied Microbiology 109:1007-1016

Pande G.S.J., F.M.I. Natrah, P. Sorgeloos, P. Bossier, and T. Defoirdt. 2013. The three *Vibrio harveyi* quorum sensing signals have a different impact on virulence towards different hosts. Submitted.

Rasch M., C. Buch, B. Austin, J. Slierendrecht, K.S. Ekmann, J.L. Larsen, C. Johansen, K. Riedel, L. Eberl, M. Givskov, and L. Gram. 2004. An inhibitor of bacterial quorum sensing reduces motalities caused by vibriosis in rainbow trout (*Oncorhynchus mykiss* Walbaum). Syst. Appl. Microbiol. 27:350-359.

Tonguthai K. 1997. Diseases of the freshwater prawn, *Macrobrachium rosenbergii*. The Aquat. Anim. Health Res. Inst. Newsletter 4(2):1-9.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EFFECT OF DHA ON THE EXPRESSION OF THE Δ6-DESATURASE DURING LARVAL DEVELOPMENT OF YELLOW SNAPPER, *LUTJANUS ARGENTIVENTRIS*

B. Paz-Raymundo*, D. Tovar-Ramírez, E. Palacios-Mechetnov, and J.C. Pérez-Urbiola

Centro de Investigaciones Biológicas del Noroeste S.C. (CIBNOR). Mar Bermejo 195 Col. Playa Palo Sta. Rita C.P.23090 La Paz, B.C.S. México

Introduction

The importance of the n-3 series highly unsaturated fatty acids (HUFA) – docosahexaenoic acid (DHA, 22: 6n-3) and eicosapentaenoic (EPA, 20: 5n-3) – in the diet has been well studied in marine fish and there has been successful larval production by providing sufficient amounts of these fatty acids since the aquatic environment is characterized by large amounts of long-chain polyunsaturated fatty acids (PUFA) or HUFA. Biosynthesis of HUFAs in fish involves the sequential desaturation and elongation of precursor C18 PUFAs, and Δ6-desaturase (Δ6D) and Elovl5 elongase are the critical enzymes in the biosynthetic pathway of HUFAs. The inclusion of high HUFAs levels in the diets can result in limited energy for larvae, since these substrates are relatively poor systems for biosynthesis of fatty acids. Therefore, an appropriate balance between polyunsaturated, monounsaturated, and unsaturated fatty acids is necessary, and this can be obtained through the knowledge of the metabolic pathways of each species (Hastings et al., 2005)

The yellow snapper *Lutjanus argentiventris* (Peters) is one of the target species for commercial fish farming in the Southern California Gulf. Generally, many estuarine species of snapper are amenable to handling and captivity and adapt to stress of cultivation (Guerrero-Tortolero et al., 2008). From 1996, *L. argentiventris* has been held in captivity at CIBNOR near La Paz, B.C.S., Mexico. Juveniles inhabiting the coastal area are captured throughout the year with common fishing techniques and transported to aquaculture facilities for grow-out to maturation.

The objective of this study was to determine the effect of the concentration of docosahexaenoic acid (DHA) on growth and expression of the Δ6-desaturase enzyme in larvae of yellow snapper *L. argentiventris*.

Materials and methods

CIBNOR *L. argentiventris* broodstock were spawned naturally. The viable floating eggs were collected and reared in 35-l fiberglass tanks (80% water exchange daily) with constant aeration. Water temperature was maintained in 24°C. Photoperiods of 12:12h day:night were maintained. There were 5 treatments: unfed larvae (negative control) and larvae fed rotifer (*Brachiounus plicatilis*) cultivated with microalgae *Nannochloropsis oculata*, DHA administered through enriched live food using two emulsions ICES (50%, 0% HUFAs), and a dilution of both with a ratio (1:4). The enrichment was carried out according to Nghia et al. (2008).

Samples were taken after spawning and newly hatched larvae at 1, 3, and 5 days after the first feeding (~50-80mg), settled in 500µl of RNA LATER in 1.5-ml Eppendorf tubes, labelled, and stored at -80°C). They were also sampled at 24, 48, 72, 96, and 120h to hatch to measure growth and the decrease in lipid reserves. At the end of the experiment, survival of each treatment will be determined.

For the cloning of the partial *L. argentiventris* desaturase cDNA, mRNA FADS2 of *Dicentrarchus labrax* and *Sparus aurata* were compared using the Blast alignment algorithm. Primers were chosen corresponding to the most conserved coding regions of D6-desaturase. TRIzol® Reagent (Invitrogen, Breda, Netherlands) was used for total RNA extraction. Then, 1µg total RNA was reverse-transcribed to cDNA with the Improm II Transcription kit (Promega). cDNA pooled from the yellow snapper larvae fed DHA and control diet were used to construct standard curves for each analyzed gene and conditions according to Tovar et al. (2010). Standard curve allowed us to check efficiency for each cDNA amplification. The gene Ef1α was chosen as reference gene. The relative gene expressions were determined according to the ΔΔCt method using CFX Manager Software (Bio-Rad)

Results and discussion

For teleosts, the nutritional modulation of Δ6-desturase has been extensively studied to test whether desaturase activity could compensate for the reduction of tissue HUFA levels due to the inclusion of dietary vegetal oils. Recent studies have attempted to discover whether the nutritional modulation of Δ6D gene expression is a persistent adaptation at the molecular level or a transient acclimation to the nutritional environment. It was possible to modulate Δ6D gene expression using nutritional conditioning during the larval phase. In this experiment, it was only possible to quantify the expression in the treatment of the dilution 1:4 of ICES as shown in Figure 1; the expression is significantly higher in the first 24 hours post-hatching and then decreases at minimum levels. Morais et

al. (2011) suggested an alternative role for these enzymes in marine fish, based on a requirement to maintain membrane DHA levels, particularly in neural tissues at times of high demand such as embryonic and larval development.

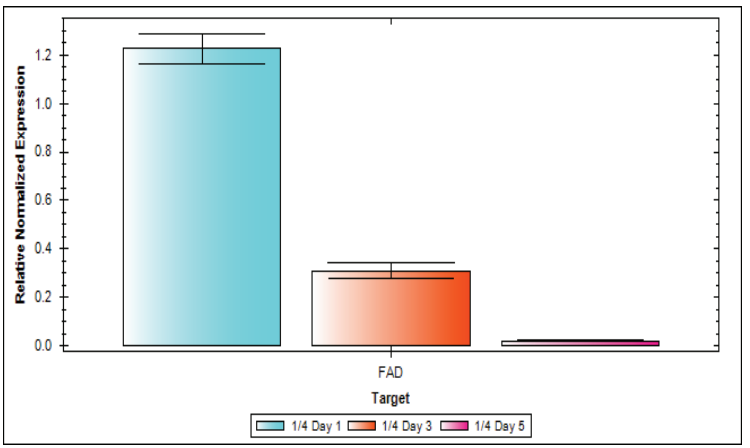


Fig. 1. Expression Relative of Δ6-Desaturase in treatment of the dilution1:4 ICEs.

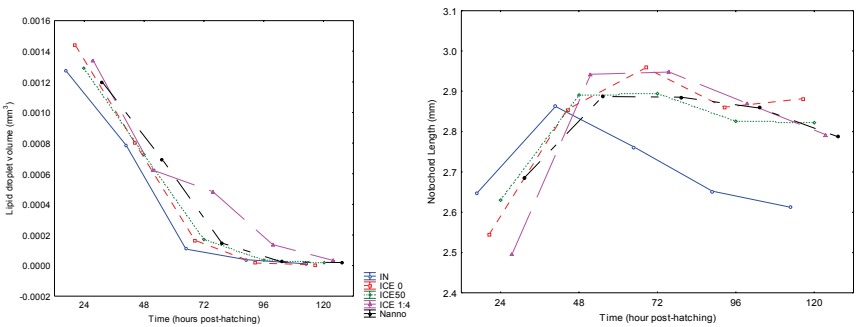


Fig. 2. Evolution of Lipid droplet (left) and notochord length (right) post hatching.

.

There were no significant differences in growth as a result of dietary DHA levels found at 5 days post-hatching ($p<0.05$). However, larvae fed the highest DHA level showed highest notochordal length and the lipid drop volume decreased significantly more slowly ($p>0.005$) in the treatment of ICE 1:4, which could suggest that this proportion of DHA favors a less immediate demand for the lipid reserves.

Acknowledgments

This work was made possible thanks to the CIBNOR and funding was provided by Grant 157763 from CONACYT. The first author received the grant no. 412859/260429 from CONACYT.

References

Guerrero-Tortolero D.A., R. Campos-Ramos, J.C. Pérez-Urbiola, and A. Muhlia-Melo. 2008. Photoperiod manipulation of yellow snapper (*Lutjanus argentiventris*) broodstock induced out-of-season maturation, spawning, and differences in steroid profiles.Cybium Supplement 32:327-328.

Hastings N., M.K. Agaba, D.R. Tocher, X. Zheng, C.A. Dickson, J.R. Dick, and A.J. Teale. 2005. Molecular cloning and functional characterization of fatty acyl desaturase and elongase cDNAs involved in the production of eicosapentaenoic and docosahexaenoic acids from α-linolenic acid in Atlantic salmon (*Salmo salar*) Mar Biotechnol. 6(5):463-474.

Seiliez I., Panserat S., Corraze G., Kaushik S., Bergot P.2003. Cloning and nutritional regulation of a D6-desaturase-like enzyme in the marine teleost gilthead seabream (*Sparus aurata*). Comparative Biochemistry and Physiology Part B 135:449-460.

Tovar-Ramírez D., Mazurais, D., Gatesoupe, J. F., Quazuguel, P., Cahu, C. L. and Zambonino-Infante, J.L. 2010. Dietary probiotic live yeast modulates antioxidant enzyme activities and gene expression of sea bass (*Dicentrarchus labrax*) larvae. Aquaculture 300:142-147.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

SELECTION AND IDENTIFICATION OF PROBIOTIC BACTERIA FOR USE IN THE MARINE SHRIMP CULTURE

S. Peixoto*, J. Vogeley, R. Nery, C. Barros, J.V. Lima, and R. Soares

Av. Dom Manoel Medeiros, S/N, Dois Irmãos, Recife, PE, Brazil (*silvio.peixoto@gmail.com,)

Introduction

Farming of white shrimp *Litopenaeus vannamei* has become a significant economic activity in several developing countries, such as Brazil. However, this industry has been suffering with an increased incidence of infectious pathogens. Bacterial diseases are often associated with low survival rates during larvae production, and the genus *Vibrio* has been closely related with this problem (Saulnier et al., 2000; Decamp et al., 2008).

The use of probiotics has been increasingly reported as an alternative to antibiotic treatment (Balcázar et al., 2006). The genus *Bacillus* is known to antagonize potential pathogens in the aquatic environment (Irianto and Austin, 2002). These microorganisms compete with other bacteria for nutrients and space, as well as exclude them through the production of different antibiotic compounds (Moriarty, 1998). Therefore, the genus *Bacillus* contains most of the bacteria species used as probiotics by the aquaculture industry (Ninawe and Selvin, 2009).

Some authors suggest the isolation of probiotics candidates from the host or culture system could increase their chance to multiply and survive, while exercising their beneficial effects (Verschuere et al., 2000; Vine et al., 2006; Defoirdt et al., 2007). In this context, the aim of this study was to select *Bacillus* from the wild shrimp for use in Brazilian marine aquaculture.

Materials and methods

For bacterial isolation, *Farfantepenaues subtilis* adults were caught on the southern coast of the Pernambuco state, Brazil (08º 38'S; 35º 03'W). A sample of the gut was collected and inoculated into MYP Agar (Mannitol Egg Yolk Polymyxin Agar). Nine bacteria were presumptively identified as *Bacillus* by Gram stain, catalase test, and spore formation using malachite green staining.

Antagonism assays were performed by the agar well diffusion plate method, adapted from Vaseeharan and Ramasamy (2003). All assays were carried out in triplicate. Strains of *Vibrio alginolyticus*, *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* and the nine *Bacillus* spp. isolated were grown in TSB supplemented with 2.0% NaCl at 30°C. After 24h of incubation, samples of culture *Vibrio* species were plated in TSA enriched with 2.0% NaCl and incubated overnight at 30°C. Colonies were suspended in sterile saline solution (2.5% NaCl) and centrifuged (4000rpm for 15min). The bacterial suspension was adjusted to an optical density of 0.5 MacFarland Standard, corresponding to 10^8 $CFU.ml^{-1}$. *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* were inoculated evenly into the separate TSA plates enriched with 2.0% NaCl containing four punched wells of 3mm diameter, using swabs soaked in the bacterial suspension. The *Bacillus* spp. culture at two days old was centrifuged (4000rpm for 15min) and supernatant was discarded. The bacterial cells were suspended in sterile TSB enriched with 2.0% NaCl. Thereafter, 20µl of *Bacillus* spp. suspension was introduced into the three wells of the agar medium and incubated. An additional well served as a control and was inoculated only with sterile TSB with 2.0% NaCl. After incubation at 30°C for 24h, the diameter of the clear zone around each well was measured using a dial caliper.

Results and discussion

Among the nine *Bacillus* spp tested, two bacteria showed inhibitory activity: *Bacillus circulans* against *V. cholerae, V. alginolyticus*, and *V. vulnificus* with diameters of the inhibitory zones around the wells of 14.7±0.6, 12.0±0.0, and 16.3±3.8mm, respectively; and *Paenibacillus thiaminolyticus* against *V. vulnificus*, with 13.3±0.1mm of inhibitory zone. No inhibitory zone was found around the control wells.

Balcázar and Luna-Rojas (2007) tested in vitro *B. subtilis* UTM 126 and other bacterial species against *V. parahaemolyticus* and encountered clear zones of 8-12mm. In another study, the same authors observed clear zones of 10-15 mm against different *V. harveyi* strains and *V. alginolyticus* (Balcázar et al., 2007). The inhibitory mechanism between *Bacillus* spp. and *Vibrio* spp. was not characterized in our study. Nevertheless, Verschuere et al. (2000) reported that the antibacterial mechanism of bacteria could be related to several factors, such as production of antibiotics, bacteriocins, lysozymes, proteases, hydrogen peroxide, and the alteration of pH values by the production of organic acids.

Ma et al. (2009) observed antimicrobial activity of the *Lactobacillus* spp. against *V. parahaemolyticus* and *V. harveyi*. These authors suggested that the antimicrobial activity was caused by bacteriocin-like substance. Nakayama and Nomura (2009) investigated in vitro the effect of *Bacillus* species against *V. harveyi* and observed that *B. subtilis* inhibited *Vibrio* growth while *B.*

licheniformis and *B. megaterium* suppressed the haemolytic activity in *Vibrio*, and the haemolysins are considered virulence factors in *V. harveyi*.

In the present study, the results demonstrated an effective inhibitory action of two *Bacillus* spp. tested against *Vibrio* bacteria under in vitro conditions. Thus, these bacteria can be considered as potential probiotic for use in marine shrimp culture. However, in vivo studies should be performed to assess the efficiency of these bacteria in culture conditions.

References

Balcázar J.L., de Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., Múzquiz, J.L. 2006. The role of probiotics in aquaculture. Veterinary Microbiology 114: 173-186.

Balcázar J.L. and T. Luna-Rojas. 2007. Inhibitory activity of probiotic *Bacillus subtilis* UTM 126 against *Vibrio* species confers protection against vibriosis in juvenile shrimp (*Litopenaeus vannamei*). Current Microbiology 55:409–412.

Balcázar J.L., T. Rojas-Luna, and P.D. Cunningham. 2007. Effect of the addition of four potential probiotic strains on the survival of pacific white shrimp (*Litopenaeus vannamei*) following immersion challenge with *Vibrio parahaemolyticus*. Journal of Invertebrate Pathology 96:147–150.

Decamp O., D.J.W. Moriarty, and P. Lavens. 2008. Probiotics for shrimp larviculture: review of field data from Asia and Latin America. Aquaculture Research 39:334-338.

Defoirdt T., N. Boon, P. Sorgeloos, W. Verstraete, and P. Bossier. 2007. Alternatives to antibiotics to control bacterial infections: luminescent vibriosis in aquaculture as an example. Trends in Biotechnology 25:472-479.

Irianto A. and B. Austin. 2002. Probiotics in aquaculture. Journal of Fish Disease 25:633-642.

Ma C.W., Y.S. Cho, and K.W. Oh. 2009. Removal of pathogenic bacteria and nitrogens by *Lactobacillus* spp. JK-8 and JK-11. Aquaculture 287:266-270.

Nakayama T., H. Lu, and N. Nomura. 2009. Inhibitory effects of *Bacillus* probionts on growth and toxin production of *Vibrio harveyi* pathogens of shrimp. Letters in Applied Microbiology 49:679-684.

Ninawe A.S. and J. Selvin. 2009. Probiotics in shrimp aquaculture: Avenues and challenges. Critical Reviews in Microbiology 35:43-66.

Saulnier D., P. Haffner, C. Goarant, L. Peva, and D. Ansquer. 2000. Experimental infection models for shrimp vibriosis studies: a review. Aquaculture 191:133-144.

Vaseeharan B. and P. Ramasamy. 2003. Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. Letters in Applied Microbiology 36:83-87.

Verschuere L., G. Rombaut, P. Sorgeloos, and W. Verstraete. 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiol. Microbiology and Molecular Biology Reviews 64:655-671.
Vine N.G., W.D. Leukes, and H. Kaiser, H. 2006. Probiotics in marine larviculture. FEMS Microbiology Reviews 30:404-427.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

AMINO ACID PROFILE AND CONSUMPTION DURING THE EMBRYONIC DEVELOPMENT AND YOLK-SAC LARVAE OF PACIFIC RED SNAPPER *LUTJANUS PERU*

R. Peña[1], S. Dumas[1], I. Moguel-Hernández[1], and N. García-Aguilar[2]

[1] Unidad Piloto de Maricultivos. CICIMAR-IPN. Av. IPN s/n La Paz, B.C.S. 23090. Mexico
[2] Centro de Investigación en Alimentación y Desarrollo, Mazatlán, Sinaloa, México

Introduction

The Pacific red snapper (*Lutjanus peru*) is an important species in the local fisheries of the Pacific coast of Mexico. Recent interest in developing a culture strategy for this species has led to induced spawning of broodstock in culture conditions. However, intensive larviculture of this species has been characterized by variable survival during hatching and first-feeding partially due to variable egg quality. The nutritional content of the yolk plays a vital role in the development and differentiation of the embryo and the larvae. The free amino acids (FAA) in the yolk contribute to protein synthesis as well as providing a source of energy for developing embryos and larvae (Ronnestad et al., 1998). Additionally, the profile of free amino acids has been proposed as a good indicator of the amino acids requirements of fish larvae and to egg quality.

The objective of the present study was to describe the profile of amino acids in developing embryo and yolk-sac larvae of the Pacific red snapper. This will provide more information in order to evaluate the biochemical determination of egg quality in this species.

Materials and methods

Eight Pacific red snapper spawns were obtained from wild-captured broodstock, final maturation and spawning was induced by hormonal injection according to Pelcastre-Campos (2006). Gametes were manually stripped and after artificial fertilization, floating (viable) eggs were separated from non-floating (dead eggs) and placed in one 120-l incubation tank at 26ºC, salinity of 35ups, natural photoperiod, and continuous aeration and water flow of 15 $l.min^{-1}$. In every spawn, samples were taken at several developmental stages: starting at fertilized eggs and during early cleavage (8-32 cells stage), blastula, gastrula, newly hatch, and 24h and 48h after hatching. Dead eggs were also sampled. Every sample was

taken directly from the incubation tank using a 100-µm sieve. Each sample was dried and weighted to the nearest 0.0001 g using a digital balance and stored at -80ºC until analysis. Prior to the amino acids analysis all samples were lyophilized and total proteins in each sample was evaluated by the Bradford method (1976) adjusted to the sample type and quantity. A total of 15 free amino acids, 9 essential, and 6 non-essential were analyzed according to the methodology reported by Vázquez-Ortíz et al. (1995).

Results

All the amino acids tested were found throughout the embryonic development and yolk-sac larvae of the Pacific red snapper (Table I). The mean total FAA content increased from the egg stage and during the embryo development and decreased after hatching. The total content of the essential FAA was higher than the non-essential FAA content throughout development. At the egg stage, glutamate, glycine, and alanine were the most abundant non-essential FAA, while leucine, valine, isoleucine, and lysine were the most abundant essential FAA (Table I).

Table I. Mean free amino acid content (nmol.ind^{-1}) during the embryonic development and yolk-sac larvae of the Pacific red snapper, *Lutjanus peru*.

	Developmental Stage							
	Live Eggs	**1st div**	**Blastula**	**Gastrula**	**Hatching**	**24h**	**48h**	**Dead Eggs**
Asp	0.25	0.28	0.31	0.30	0.33	0.34	0.38	0.31
Glu	0.56	0.65	0.72	0.68	0.52	0.55	0.58	0.61
Ser	0.21	0.21	0.27	0.27	0.21	0.21	0.20	0.19
Gly	0.35	0.39	0.44	0.41	0.55	0.60	0.64	0.37
Ala	0.55	0.63	0.74	0.68	0.60	0.43	0.46	0.59
Tyr	0.13	0.14	0.16	0.16	0.15	0.16	0.14	0.14
NEFAA	2.05	2.30	2.64	2.50	2.35	2.29	2.40	2.21
His	0.17	0.39	0.43	0.41	0.45	0.37	0.39	0.39
Thr	0.25	0.52	0.55	0.54	0.58	0.48	0.50	0.51
Arg	0.21	0.35	0.38	0.37	0.40	0.33	0.34	0.35
Met	0.25	0.41	0.44	0.43	0.47	0.39	0.40	0.41
Val	0.38	0.52	0.56	0.54	0.59	0.49	0.51	0.52
Phe	0.27	0.37	0.40	0.39	0.42	0.35	0.36	0.37
Ile	0.33	0.34	0.37	0.35	0.34	0.21	0.23	0.29
Leu	0.50	0.55	0.60	0.57	0.55	0.38	0.38	0.48
Lys	0.34	0.42	0.45	0.44	0.48	0.39	0.41	0.41
EFAA	2.71	3.86	4.19	4.02	4.28	3.39	3.53	3.72
Total FAA	4.76	6.16	6.83	6.52	6.63	5.68	5.93	5.93

Glutamic acid and alanine increased their content during the embryonic development and significantly decreased at hatching, while glycine significantly increases at hatching. Serine, aspartic acid, and tyrosine remained relatively constant during the studied period. The content of all the essential FAA showed the same pattern during the studied period, an increment after the egg stage and only significant in the concentration of histidine, threonine, arginine, methionine,

valine, and phenylalanine and a drastic decrease at hatching in the content of isoleucine, lysine, histidine, threonine, and valine. None of the FAA were totally depleted at 48h after hatching; in fact, there was an increase in the content of aspartic acid, glycine, threonine, histidine, arginine, methionine, valine, and phenylalanine compared with their content at the egg stage (Table I).

Live (floating) and dead (non-floating) eggs differ in their composition of FAA. With the exception of serine, leucine, and isoleucine, the content of the other FAA was higher in the live eggs when compared with the dead eggs (Table I). However, no significant difference was detected in the content of non-essential FAA between dead and live eggs. On the other hand, significantly higher content of histidine, threonine, arginine, methionine, valine, and phenylalanine were detected in the live eggs when compared to the dead eggs (Table I).

Discussion

The changes in the total content of FAA during the early development of the Pacific red snapper are similar to the profiles reported for other species, an increase after fertilization and a constant decrease after hatching and during the yolk-sac stage. The high content of alanine and glutamic acid, in the egg stage of the Pacific red snapper is similar to the reported in the larvae of the Japanese eel *Anguilla japonica* and have been suggested as a potential source of gluconeogenesis during embryonic development (Ohkubo et al., 2008). In a previous study, Moguel-Hernández et al. (in press) showed the importance of some metabolic enzymes involved gluconeogenesis as quality criteria in *L. peru*.

The drastic depletion of alanine, leucine, isoleucine, threonine, histidine, and valine from hatching to 24hph suggest that they play a big role during the first 24h. A similar pattern was reported in *L. campechanus*, *Dicentrarchus labrax*, *Scophthalmus maximus*, *Pleuronectes platessa*, *Sparus aurata*, *Brevoortia tyrannus*, and *Leiostomus xanthurus* (Fyhn and Govoni, 1995; Ronnestad et al., 1998; Hastey et al., 2010). Conceicao et al. (1998) suggested that the early fluctuations of FAA profile during endogenous feeding of *Clarias gariepinus* may be related to the allometric growth as different organs and tissues develop at different rates and the various organs have different AA profiles. The profile of FAA of the live eggs was similar of the profiles reported for other species like *Dentex dentex*, *Lates calcarifer*, and *L. campechanus* with alanine and glutamic acid as predominant FAA (Roonestad et al., 1998; Hastey et al., 2010). The FAA profile of the dead eggs differs from the live eggs particularly in the essential FAA fraction. More studies are required in order to establish the role of these FAA in the egg survival after fertilization.

Conclusions

In general, the changes in the FAA content observed during the embryonic development of the Pacific red snapper resembled the pattern reported for other marine species. Based on the magnitude of depletion during the first 24 h after hatching, alanine, leucine, isoleucine, and valine are important as metabolic fuels in the development of this species. Alanine, glutamic acid, valine, threonine, and leucine were the most abundant FAA in the newly-fertilized eggs.

References

Conceicao L.E.C., R.O.A. Ozorio, E.A. Suurd, J.A.J. Verreth. 1998. Amino acid profiles and amino acid utilization in larval African catfish (*Clarias gariepinus*): effects of ontogeny and temperature. Fish Physiology and Biochemistry 19:43-58.

Fyhn H.J., Govoni, J. 1995. Endogenous nutrient mobilization during egg and larval development in two marine fishes – Atlantic menhaden and spot. ICES Marine Science Symposia. 201:64-69.

Hastey R.P., Phelps, R.P., Davis, D.A., Cummins, K.A. 2010. Changes in amino acid profile of red snapper *Lutjanus campechanus*, eggs, and developing larvae. Fish Physiology and Biochemistry 36:473-481.

Moguel-Hernández I., Peña, R., Nolasco-Soria, H., Dumas, S., Hinojosa-Baltazar, P. 2013. Egg quality criteria in Pacific red snapper (*Lutjanus peru*). Aquaculture Research In press.

Ohkubo N., Sawaguchi, S., Nomura, K., Tanaka, H., Matsubara, T. 2008. Utilization of free amino acids, yolk protein and lipids in developing eggs and yolk-sac larvae of Japanese eel *Anguilla japonica*. Aquaculture 282:130-137.

Pelcastre-Campos V., 2006. Inducción a la ovulación y espermiogénesis en el huachinango del Pacífico *Lutjanus peru* (Nichols y Murphy, 1922) y almacenamiento de su semen. Master Thesis. CICIMAR-IPN. Mexico. 96 pp.

Ronnestad I., Koven, W., Tandler, A., Harel, M., Fyhn, H.J. 1998. Utilisation of yolk fuels in developing eggs and larvae of European sea bass (*Dicentrarchus labrax*). Aquaculture 162:157-170.

Vázquez-Ortíz A., Caire, G., Higuera-Ciapara, I., Hernández, G. 1995. High performance liquid chromatographic determination of free amino acids in shrimp. Journal of Liquid Chromatography 18(10):2059-2068.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

SELECTION STUDY OF POTENTIAL PROBIOTIC BACTERIA FOR SHRIMP HATCHERIES IN NEW CALEDONIA

D. Pham*, D. Ansquer, A. Chevalier, A. Peyramale, C. Dauga, N. Wabete, and Y. Labreuche

Laboratoire Environnement et Aquaculture Durable, BP 2059, Nouméa - New Caledonia

In New Caledonia, shrimp production has been decreasing over the past few years, in part because of mortality outbreaks occurring during larval rearing. With the implication of bacteria being frequently suspected, alternative approaches to antibiotics are currently investigated to prevent these mortality events. However, the use of antibiotics can induce the emergence of multidrug-resistant bacteria. Probiotics could thus be an alternative to antibiotics in larval rearing.

The present work was aimed at 1) selecting (among a collection of 493 autochthonous bacterial isolates sampled from diverse marine environments in New Caledonia) strains showing in vitro antagonistic activities on the growth of *Vibrio* sp. and 2) evaluating their effects on the growth and survival of *L. stylirostris* larvae.

Well-diffusion agar assays were first used to evaluate the antagonistic abilities of bacterial isolates on two *Vibrio* species known to be pathogenic to shrimp: *Vibrio penaeicida* and *V. nigripulchritudo*. After this initial screening, eight candidate strains were selected and further tested, either individually or in combination, to quantify their inhibitory activities on the growth of a GFP-labeled *V. harveyi* strain. These isolates were also characterized both phenotypically and genotypically using 16S rDNA sequencing.

Seven out of these eight strains were found to belong to the genus *Pseudoalteromonas*, the last one being characterized as a member of the genus *Vibrio* and shown to be related to the Harveyi clade. In vitro co-culture experiments clearly established the inhibitory effects of seven out of these eight isolates on the growth of the GFP-indicator strain, one *Pseudoalteromonas* strain being devoid of inhibitory capacities. Combinations of strains were more effective at inducing an inhibition.

Following in vivo challenge tests with shrimp larvae establishing that the selected strains were not pathogenic to the host, we finally investigated under pi-

lot-scale culture conditions the effectiveness of these candidates, used either individually or in combination, to improve shrimp survival. The expression level of some genes (Pen3, lysozyme, etc.) related to immune response was also investigated.

Repeated trials showed that postlarval survival could be significantly improved by the use of some strains, such as strain 201. In addition, significant differences in some lysozyme transcript abundance could be evidenced between control, non-treated shrimp larvae and animals receiving a combination of probiotic candidates.

Together, the results of this work open new insights into the use of these strains as potential substitutes to antibiotherapy in shrimp larval rearing in New Caledonia.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

IDENTIFICATION OF NITRIFYING BACTERIA IN INTENSIVE SHRIMP PONDS IN SOC TRANG PROVINCE, VIETNAM BY BIO-CHEMICAL TEST AND MOLECULAR TECHNIQUE

Pham Thi Tuyet Ngan[1*], Trương Quoc Phu[1], Tran Suong Ngoc[1], and Nguyen Huu Hiep[2]

[1] Department of Applied Hydrobiology, College of Aquaculture and Fisheries, Can Tho University, Vietnam ([*]E-mail: pttngan@ctu.edu.vn)
[2] Biotechnology Research and Development Institute, Can Tho University, Vietnam

Introduction

Black tiger shrimp (*Penaeus monodon*) is reared around the world and is the fastest growing commercially grown shrimp. The rapid development of tiger shrimp farming has created jobs and generated income in many countries. However, the growth of the shrimp farming industry has led to environmental pollution and animal diseases, and thus the shrimp farming industry has encountered major obstacles.

Shrimp yields have declined in many countries, greatly affecting the economic livelihoods of the farmers. To solve this problem, chemicals and antibiotics have been used, however the use of antibiotics has led to drug resistance. Additionally, exports of these fishery products often fail to meet international standards due to antibiotics, pesticides, and pathogenic microorganisms left on the animals. One potential solution has been use of beneficial bacteria to improve rearing environment and increase production. This solution has great potential for micro-management in intensive ponds to minimize antibiotics for food safety, significantly limits the amount of organic waste in the environment and is a sustainable contribution to aquaculture. The study of local, beneficial bacterial strains as a baseline for the mass production of probiotics is necessary and practical step in the current search to improve aquaculture, limit the environmental pollution and enhance the sustainability of farming.

Materials and methods

Isolation and identification of Bacillus *sp.*
Selected media for *Bacillus* isolates was based on Harwood and Archibald (1990). Biochemical tests to identify isolated bacterial strains were based on Andretta et al. (2004), Sharmin and Rahman (2007). These strains were

identified with primers 16F8 and 16R1391 produced by Nam Khoa Company and Can Tho University, Vietnam. Sequencing results were compared with GenBank data (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Isolation and identification of Nitrosomonas *sp.*
Media used to isolate *Nitrosomonas* sp. was based on Ehrlich (1975). Mass culture *Nitrosomonas* sp. was done according to Lewis and Pramer (1958). We checked the shape of bacterial cells under microscope after Gram staining. We amplified the amoA gene of the 16S rRNA region using PCR.

Isolation and identification of Nitrobacter sp.
Selected media used for isolating *Nitrobacte*r sp. were based on the methods of Ehrlich (1975). To grow *Nitrobacter* we used the medium from Aleem and Alexander (1960). We used PCR with the primers PNGT 1F and PNTG 2R to identify specific groups of *Nitrobacter* (Degrange and Bardin, 1995).

Results and discussion

Isolation and identification of Bacillus *sp*.
Bacterial colonies were round, slightly edged, or flat in shape, with a smooth or slightly wrinkled surface, and opaque white to yellowish in color. Cells were Gram-positive, short or long rod-shaped (depending on the species). When *Bacillus* sp. were stained under the microscope, spores had the green color of Malachite Green dye and the bacterial cell had the red color of Safranin. Nine bacterial strains were purified with the aim of selecting strains capable of decomposing organic mater. These strains were stored on agar at 4ºC and in glycerol at -80°C for further study. The molecular technique showed that the isolated strain B9 was homologous with *B. cereus* BRL02-43 (97%); B37 and B38 strains were homologous with *B. cereus* G9842 (100 and 99%); the B41 strain was homologous with *B. amyloliquefaciens* SB3297 (100%); and the B67 strain was homologous with *B. subtilis* BL10 (99%) (Table I).

Table I. Comparing the sequence of the isolated *Bacillus* strains

Code	Description of species	**Max score**	**Total score**	**Max ident**	**Accession number**
B9	*B. cereus* BRL02-43	881	904	97%	DQ339674
B38	*B. cereus* CMST-AP-MSU	894	1024	100%	CP003187.1
B37	*B. cereus* F837176	996	998	100%	CP001186.1
B67	*B. subtilis* BL10	887	887	99%	GU826165
B41	*B. amyloliquefaciens* SB 3297	990	990	100%	GU191913.1
B8	*B. cereus* G9842	894	894	99%	NC_011772.1

Isolation and Identification of Nitrosomonas sp.
Eight isolated strains of *Nitrosomonas* were denoted S5-S12. Samples were stored at -80°C for further study. These strains were Gram-negative and short

rods. The results also showed all these strains were capable of strongly reducing ammonium in 10-day breeding period. Two strains, S8 and S12, had the strongest ability to remove ammonium and were used for identification by PCR. PCR products of all two strains S8 and S12 proved that these two strains were *N. nitrosa* (Table II).

Table II: Comparing the sequence of the isolated *Nitrosomonas* strains

Code	Description of species	Max score	Total score	Max ident	Accession number
S8	*Nitrosomonas nitrosa*	409	409	99%	AF272404.1
S12	*Nitrosomonas nitrosa*	499	499	99%	AF272404.1

Isolation and Identification of Nitrobacter sp.

Eight strains of *Nitrobacter* capable of oxidizing nitrite were stored in the selective media on shaker (Aleem and Alexander (1960). Samples were stored at -80°C for further research. This Gram-negative group is short rod-shaped and sticks to the substrate particles in the medium. Most *Nitrobacter* species isolated completely reduced nitrite in 10-day period. PCR products of 2 strains N10 and N12 were at about 400bp in size based on the standard scale. Identification by molecular technique showed that N10 and N12 were homologous with *N. winogradskyi* ATCC 25381 (99 and 100%, respectively) (Table III).

Table III: Comparing the sequence of the isolated *Nitrobacter* strains

Code	Description of species	Max score	Total score	Max ident	Accession number
N10	*N. winogradskyi* ATCC 25381	432	414	99%	L35506.1
N12	*N. winogradskyi* ATCC 25381	409	409	100%	L35506.1

Conclusions

We have collected 67 strains of the *Bacillus* group, 8 strains of the *Nitrosomonas* group, and 8 strains of the *Nitrobacter* group. The collected bacteria are currently stored at -80ºC in the College of Aquaculture and Fisheries, Can Tho University. Based on the biochemical tests and PCR sequencing, B8, B9, B37, and B38 were identified as *B. cereus*; and B41 and B67 were *B. amyloliquefaciens* and *B. subtilis,* respectively. Among them, *B. cereus* was the dominant species. All strains were originally from the local ponds and not from the commercial Probiotic products. Two strains – S8 and S12 – were *N. nitrosa* and two strains – N10 and N12 – were *N. winogradskyi.*

References

Aleem M.I.H. and M. Alexander, 1960. Nutrition and physiology of *Nitrobacter agilis*. Appl. Microbiol. 8:80-84.

Andretta C.W.S., R. M. Rosa, E.C. Tondo, C.C. Gaylarde and J.A.P. Henriques, 2004. Identification and molecular characterization of a *Bacillus subtilis* IS 13 strain involved in the biodegradation of 4, 5, 6-trichloroguaiacol, Chemosphere, 55:631-639.

Degramge V. and R. Bardin, 1995. Detection and counting of *Nitrobacter* population in soil by PCR, Environ. Microbiol, 61:2093-2098.

Ehrlich G. G., 1975. Water quality: Analytical Methods - "Nitrifying bacteria (most probable number, MPN, method)" In: R. J. Pickering (Ed.) Quality of water branch technical memorandum. No. 75:13p.

Harwood C. and A. Archibald, 1990. Growth, maintenance and general techniques. In: C.R., Harwood, S.M. Cutting, (Eds), Molecular Biologycal Methods for *Bacillus*. Wiley, Chichester, England: pp.1-26.

Lewis R.F and D. Pramer. 1958. Isolation of *Nitrosomonas* in pure culture. Journal of Bacteriology. 76:524-528.

Sharmin F. and M. Rahman, 2007. Isolation and characterization of protease producing *Bacillus* strain FS-1. Agricultural Engineering International. The CIGR Journal 11:1-10.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

CAN DIETARY PHOSPHOLIPID AND TRACE MINERAL SUPPLEMENTATION INFLUENCE ZEBRAFISH REPRODUCTIVE PERFORMANCE?

W. Pinto[1], P. Diogo[2], G. Martins[2], S. Martínez-Páramo[2], P. Gavaia[2], L. Conceição[1], and J. Dias[1]

[1] Sparos, Lda, CRIA - Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal
[2] CCMAR, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

Introduction

Zebrafish (*Danio rerio*) is widely used as a biological model to study vertebrate embryogenesis and organogenesis, as well as several human diseases. Among its advantages as a biological model are important features such as ease of breeding, short generational intervals, fast growth, and abundant offspring production (Lawrence, 2007). These characteristics also allow zebrafish to be used as an informative model for nutritional studies that can ultimately be applied in fish species relevant for aquaculture production. It has been suggested that optimal levels of certain nutrients in broodstock diets may be vital for gonad maturation, gamete quality, and larval development. Among these nutrients, phospholipids and trace minerals are suspected to influence embryo development, egg fertilization, hatching rates, and sperm quality (Izquierdo et al., 2001).

This study aimed at determining the influence of specific dietary supplements on fish reproduction, using zebrafish as a model. Among dietary factors, the effect of phosphatidylcholine and trace minerals (selenium, zinc, manganese, and iodine) supplementation was assessed on zebrafish growth and reproductive performance, using several egg and sperm characteristics as quality indicators.

Materials and methods

Experimental diets
Seven dietary treatments were tested in this experiment. Among these treatments, a high quality commercial marine fish larval diet (CD) commonly used by some of the zebrafish community was used as a positive control, containing 60% crude protein and 14% fat. The remaining diets were formulated with purified or semi-purified ingredients to simultaneously allow a detailed control over its composition and fulfil zebrafish requirements. A purified diet (PURE) was

used as experimental control, containing 60% crude protein and 12% crude fat. On the remaining treatments, the PURE diet was supplemented with phosphatidylcholine (PC; 5g.kg^{-1}), sodium selenite (SE; 1mg.kg^{-1}), zinc oxide (ZN; 750mg.kg^{-1}), manganese oxide (MN; 1000mg.kg^{-1}), and potassium iodate (I; 20mg.kg^{-1}). For diet preparation, powder ingredients were initially mixed and ground. Fish oil was subsequently added and the resulting mash was humidified and agglomerated through low-shear extrusion. The resulting pellets were dried, crumbled and sieved to desired size ranges.

Fish rearing and sampling

Young adult zebrafish (3 months) were selected according to size and separated into 9 experimental groups composed of 3 males and 5 females isolated in 3-l tanks in a water recirculating circuit. The rearing system water was partially (10%) replaced daily and temperature was kept at 28ºC. After 2 days of acclimation, each experimental group was fed ad libitum twice daily with one experimental diet. After 3 weeks of experimental feeding, fish from each group were mated and spawned eggs were collected and cleaned. Egg number, hatching rate, diameter, size of perivitelline space, and embryo diameter were subsequently determined.

At the end of the experiment, zebrafish males from each treatment were sampled for sperm motility analysis. Sperm was extracted from anesthetized fish by applying gentle pressure to the genital region with forceps. Sperm was collected from the previously cleaned and dried genital pore. Immediately after extraction, sperm was activated by mixing with rearing system water and immediately placed on a Makler chamber. Motility parameters were recorded at 15, 30, 45, and 60s after activation, using computer-assisted sperm analysis (CASA) coupled to a microscope. The images were captured with a Basler camera A312f (Basler Afc, Germany) and processed with the software ISAS (Proiser, Spain). Curvilinear velocity (VCL), straight line velocity and total motility (%; spermatozoa with a VCL>10µm.s^{-1}) were determined to assess sperm quality in each treatment.

Statistical analyses

Results were expressed as means±standard deviation (SD). Data were initially tested by Levene's test for homogeneity of variances. When homogeneity of variances was observed, detection of group mean differences was tested by one-way ANOVA followed by Tukey's multiple comparison tests. Data were analysed through Kruskall-Wallis and Mann-Whitney U nonparametric tests when mean variances were significantly different across groups. Sperm motility parameters were compared using a general linear model with Bonferroni correction. The significance level was $P \leq 0.05$. All results expressed as a percentage were based on arcsine-transformed data.

Results

Results showed that at the end of the trial, zebrafish from the CD treatment had a significantly higher wet weight than zebrafish from the remaining treatments, with exception for PC (Fig. 1). Zebrafish from the PURE treatment also had a significantly higher wet weight than zebrafish from the MN and ZN treatment.

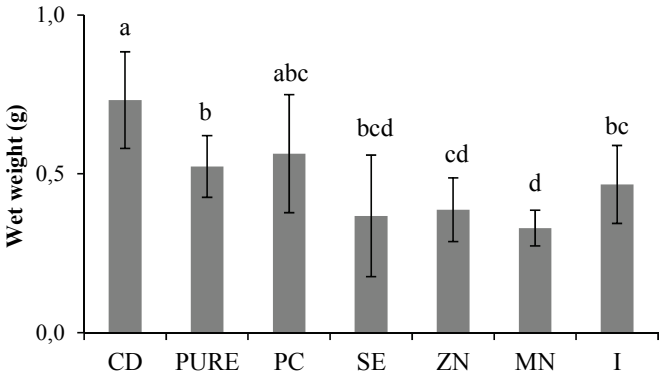


Fig. 1. Final wet weight of zebrafish adults reared under different feeding regimes. Different letters represent significant differences among treatments.

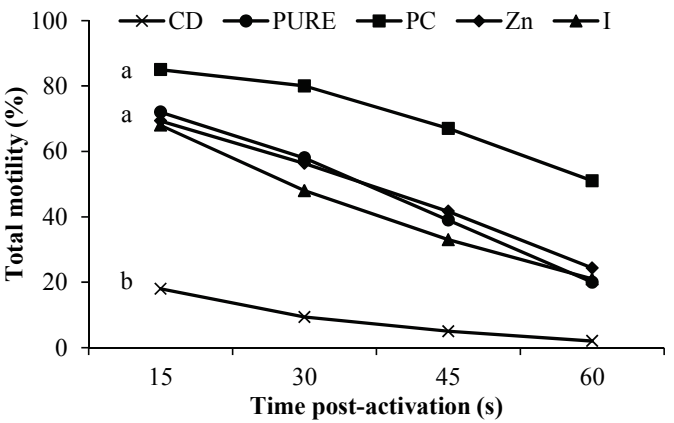


Fig. 2. Total sperm motility of zebrafish reared under different feeding regimes. Different letters represent significant differences among treatments.

Regarding zebrafish reproductive performance, no spawn was obtained for the CD treatment, one spawn was obtained for the PURE and I treatments, while two spawns were obtained for ZN and MN treatments. Results from these spawns showed no significant differences between treatments for the number of eggs produced and hatching rates. However, egg diameter was significantly

higher in the PURE treatment than in the remaining treatments. Embryo diameter was also significantly higher in the PURE and ZN treatments than in the I treatment, whereas no significant differences were obtained between treatments for the perivitelline space size.

Due to technical reasons, no sperm samples were collected from males of the MN and SE treatments. However, results from the remaining treatments showed that total sperm motility was significantly lower in the CD treatment than in the remaining treatments (Fig. 2). Similar findings were observed for sperm curvilinear and straight line velocities.

Discussion

This study showed that dietary phosphatidylcholine supplementation did not affect growth and reproductive performance of zebrafish. Conversely, dietary supplementation of trace minerals (zinc, manganese, selenium and iodine) significantly decreased growth or reproductive performance of zebrafish. Taken together, these results indicate that the purified diet used in the current study may already fulfill zebrafish phospholipid and mineral needs for an adequate growth and reproduction, and further supplementation of these nutrients may cause detrimental effects. In addition, this work also indicated that growth potential and reproductive performance in fish may not be directly proportional, since zebrafish fed the commercial diet had a higher growth than the remaining diets, but a dramatic impairment of sperm motility and reproductive performance was concomitantly observed. These findings may be of significant relevance not only for the zebrafish community, but may also be extrapolated for important fish species targeted for production by the aquaculture industry.

Acknowledgments

S. Martínez-Páramo was supported by FCT postdoctoral fellowship (SFRH/BPD/48520/2008) co-funded by POPH-QREN, Typology 4.1 (FEDER and MCTES). This work is part of project 23000-ZEBRAFEEDS co-financed by FEDER through PO Algarve 21 in the framework of QREN 2007-2013 and was also co-funded by the Portuguese Foundation for Science and Technology Foundation (FCT) through PTDC/MAR/105152/2008 (SpecialK) project.

References

Izquierdo M.S., H. Fernández-Palacios, and A.G.J. Tacon. 2001. Effect of broodstock nutrition on reproductive performance of fish. Aquaculture 197:25-42. awrence, C. 2007. The husbandry of zebrafish (*Danio rerio*): A review. Aquaculture 269:1-20.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

THE DEVELOPMENT OF AN AXENIC BLUE MUSSEL (*MYTILUS EDULIS*) LARVAE TEST SYSTEM

A. Plovie*, F. Gonzaga, N. Nevejan, and P. Bossier

Laboratory of Aquaculture and Artemia Reference Center, Rozier 44, 9000 Gent, Belgium. *E-mail: aaron.plovie@ugent.be

Introduction

Today, farmed products represent over 50 percent of the seafood consumed by humans. In 2005, the blue mussel (*Mytilus edulis*) was the primary cultured bivalve in Europe (FAO, 2008). Mussels are cultured by capturing larvae or fishing juveniles (seed or spat) from the wild and on-growing these to marketable size. However, the dependence on natural seed resources means the industry risks a limited production yield after low annual spat falls. Also, ecosystem destruction during mussel seed fishery necessitates alternatives. One of the proposed solutions is the production of hatchery seed (Galley et al., 2010). Although it is not expected that hatchery-produced spat will totally replace spat caught from wild populations in countries where blue mussels are endemic, the production of mussels in other countries is exclusively dependent on hatchery-produced spat.

Large-scale hatchery production of aquatic organisms, such as the blue mussel, still suffers from major losses due to diseases, often caused by pathogenic or opportunistic bacteria, mainly from the genus *Vibrio* (Prado et al., 2010). Traditionally, bacterial diseases in aquaculture hatcheries were suppressed by using considerable amounts of antibiotics, whether or not in a prophylactic way. However, more sustainable methods of preventing and treating infectious diseases have to be developed since (over)use of antibiotics may lead to resistant pathogens and uncontrollable disease outbreaks. One of the proposed alternatives is the use of probiotics, beneficial bacterial strains that have one or multiple modes of action to protect the animals against pathogens.

Despite numerous studies on probiotics in aquaculture, little emphasis was placed on their actual mechanisms. Thus, the final objective of this project is to gain insight into the different modes of action of probiotics during *M. edulis* larvae development. Therefore, a biotic challenge test using selected pathogens needs to be developed during which the performance, survival, and the immune status of gnotobiotic mussel larvae, and the bacterial communities in the medium

and associated with the larvae, should be followed. Knowledge on the mechanisms of probiotics and its impact on pathogenic bacteria and mussel larvae will provide interesting tools to reduce mortality caused by bacterial pathogens.

Although gnotobiotic larvae of *Mercenaria mercenaria* (hard clam), *Crassostrea gigas* (Pacific oyster), and *C. angulata* (Portuguese oyster) were developed to study host-microbe interactions, no such studies were performed on *Mytilus edulis*. It is evident that the development of a gnotobiotic rearing system for blue mussel larvae is indispensable for studies on mussel-microbe interactions and for the development, commercialization, application, and evaluation of effective and safe bacterial preparations, applicable as biocontrol agents during bivalve culture.

Gordon and Pesti (1971) stated that gnotobiotic experiments offer considerable potential as a tool in the study of host-microbe interactions (i) because it portrays the host either when free from germs and left to its own resources or when modified by known microbial or other associates, (ii) because it permits the study of interflora relationships within the host organism, and (iii) because it may be used in the study of any external or endogenous factor (e.g., nutrition, immune reactions, responses to various forms of injury) where the pure actions of such factors, affected or unaffected by associates in the host, are of interest. The use of gnotobiotes allows increased control of variables, enhanced reproducibility of results, and a more accurate experimental design by separating an animal into host and microbiota in host-microbe studies.

Materials and methods

So far, bacteria-free aquatic animals have been obtained mainly by three ways: (i) removing gametes, fertilized eggs, larvae or embryos not yet contaminated with microbes from parent animals by sterile surgery. In case of gametes, fertilization should happen under aseptic conditions; (ii) using eggs, larvae, or embryos with reduced levels of contamination that are treated with antibiotics or strong antimicrobial solutions to eliminate microbes; or (iii) using conventional eggs, larvae or embryos that initially undergo a series of washings to dilute the unwanted accompanying microbes followed by antibiotic of chemical treatments to completely eliminate microbes. The use of antibiotics and antimicrobial agents poses several disadvantages. They should be completely removed from the medium and the test animals before the actual experiment starts, and they can affect the test animals during the disinfection. Therefore, it is desirable to develop a technique to obtain gnotobiotic mussel larvae without the use of antibiotics or antimicrobial agents, or to develop protocols to eliminate the added antibiotics after disinfection, such as enzymatic degradation.

The second critical step to culture gnotobiotic aquatic animals is their maintenance. Special isolators or recipients are needed. Most studies with gnotobiotic animals use glassware containers easily sterilized by autoclaving, or sterile plastic recipients. Axenic live feeds, for which starter cultures are commercially available, should be used. All handlings of the larvae should be performed using aseptic techniques in a laminar flow hood (Marques et al., 2006). Monitoring periodically several samples from the animal, the feed and the medium is indispensable to assure the gnotobiology of the animals and the reproducibility of the obtained experimental results. Culturing experiments in liquid marine broth or on marine agar plates, microscopic examination after total DNA staining, for example with acridine orange or DAPI, or staining with specific fluorescently labeled probes, and the use of molecular techniques such as PCR, DGGE, and Q-PCR can be applied to monitor samples. Since all verification techniques have their own intrinsic deficiencies, multiple techniques should be applied in parallel to assure gnotobiology. The use of DNA-stained preparations seems to be more accurate and less time-consuming than the use of bacteriological culture media to detect contamination, as these dyes detect all bacteria, even non-culturable organisms, and the results are available in a shorter period. The use of specific fluorescently labeled probes and molecular techniques is even more time-consuming and expensive. However, those techniques are indispensable to monitor bacterial communities in gnotobiotic systems with added bacterial populations since bacteria can only be identified using molecular techniques (Marques et al., 2006).

In order to develop a standardized challenge test for the gnotobiotic mussel system, a set of mussel larvae pathogens has to be selected. According to Paillard et al. (2004), various *Vibrio*, *Aeromonas*, and *Pseudomonas* strains are reported as bivalve pathogens and can be selected after proof of virulence towards *M. edulis* larvae. Using the selected pathogens, a protocol for a proper gnotobiotic challenge test can be developed, as done for sea bass and *Artemia* (Dierckens et al., 2009; Marques et al., 2005).

Conclusions

Obtained knowledge and experience during the development of the mussel gnotobiotic system can be applied in future research to develop gnotobiotic systems for other organisms. Since microbiology becomes more and more important in aquaculture, and gnotobiotic systems are indispensable to study host-microbe interactions (Marques et al., 2006), the importance of this kind of research cannot be overestimated. The knowledge on the role of probiotics and their effects on the mussel host, pathogens, and the culture environment can also be used for investigating hatchery production of spat of bivalves that are more difficult to produce and from which the reproduction season is short, such as the scallop *Pecten maximus*. Due to the difficult production on laboratory scale of

Pecten species, easy growers like mussels are extremely useful as model organisms for those species. Also selective breeding programs for bivalves are dependent on hatchery production of spat. Breeders can select disease-resistant, fast-growing, and other varieties with beneficial properties. Genetic breeding programs were performed especially for oyster. However, it is expected that when hatchery rearing techniques will be optimized, also selective breeding programs for other bivalves will become possible.

References

Dierckens K., A. Rekecki, S. Laureau, P. Sorgeloos, N. Boon, W. Van den Broeck, and P. Bossier. 2009. Development of a bacterial challenge test for gnotobiotic sea bass (*Dicentrarchus labrax*) larvae. Environmental microbiology 11:526-533

FAO. 2008. The state of world fisheries and aquaculture. Food and agriculture organization of the united nations, Rome

Galley T.H., F.M. Batista, R. Braithwaite, J. King, and A.R. Beaumont. 2010. Optimization of larval culture of the mussel *Mytilus edulis* (L.). Aquaculture international 18:315-325

Gordon H.A. and L. Pesti. 1971. The gnotobiotic animal as a tool in the study of host microbial relationships. Bacteriological reviews 35:390-429

Marques A., T. Dinh, C. Ioakeimidis, G. Huys, J. Swings, W. Verstraete, J. Dhont, P. Sorgeloos, and P. Bossier. 2005. Effects of bacteria on *Artemia franciscana* cultured in different gnotobiotic environments. Applied and environmental microbiology 71:4307-4317

Marques A., F. Ollevier, W. Verstraete, P. Sorgeloos, and P. Bossier. 2006. Gnotobiotically grown aquatic animals: opportunities to investigate host-microbe interactions. Journal of applied microbiology 100:903-918

Paillard C., F. Le Roux, and J.J. Borrego. 2004. Bacterial disease in marine bivalves, a review of recent studies: trends and evolution. Aquatic living resources 17:477-498

Prado S., J.L. Romalde, and J.L. Barja. 2010. Review of probiotics for use in bivalve hatcheries. Veterinary microbiology 145:187-197

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

REVIEW ON THE ONTOGENY OF LARVAE FROM NEOTROPICAL FRESHWATER FISHES: THE PACU MODEL

M.C. Portella[1], N.J. Leitão[2], O.C.C. Menossi[1], T.M. Freitas[1], J.T. Kojima[2], T.S. Lopes[2], R.K. Jomori[3], J.A. Clavijo-Ayala[1], and D.J. Carneiro[1]

[1] São Paulo State University, Aquaculture Center, 14884-900, Jaboticabal, SP, Brazil
[2] São Paulo State University, 14884-900, Jaboticabal, SP, Brazil
[3] Faculty Dr Francisco Maeda, Aquaculture Laboratory, 14500-000, Ituverava, SP, Brazil

Fish production by aquaculture in Brazil (394.340 tons in 2012) relies predominantly on freshwater fish. In addition to the exotic tilapia, carp, and trout, approximately 140 000 tons of fish are native South American species. The larviculture techniques usually employed for the production of these juveniles is the outdoor semi-intensive system. In this system the larvae are directly stocked in fertilized ponds just after mouth opening and maintained there to the juvenile phase (30-50 days). However, the survival rates obtained with this system are usually low. As a result, large-scale fish production is unpredictable and highly dependent on environmental conditions. An alternative technique is the intensive system, which provides better survival conditions because the larvae are initially reared in the laboratory for a few days (or weeks) before being transferred to the ponds. Our previous studies demonstrated the economic feasibility of intensive larviculture in the initial rearing of pacu. As fish farming grows into a more important economic activity in Brazil, the demand for larvae and juveniles has been increasing. Therefore, it is necessary to develop and implement better technologies to increase the availability of high-quality juveniles in the market.

The larvae of the native rheophilic species are typically altricial and hatch with little yolk reserve. These species then initiate exogenous feeding while most of their organ systems are still in development and depend on live feed during ontogeny. For the last 15 years, we have investigated the early life history of freshwater Neotropical species to optimize larval feeding in intensive rearing conditions. As a result, we have proposed weaning protocols and diets that meet their abilities and limitations. Our objective is to enhance the production of high quality fish.

The pacu (*Piaractus mesopotamicus*) is one of the most cultivated and studied Neotropical fish in South America and is used as a model in our laboratory. The embryonic development of pacu is fast (approximately 18-19h at 26.5±0.5°C) and the notochordal length and yolk-sac volume at hatching are 3.19±0.04mm

and 0.477±0.061mm^3, respectively. At hatching, pacu larvae have a closed mouth and anus. The digestive tract is a single and undifferentiated tube consisting of a narrow lumen lined by simple epithelium. During the endotrophic phase, a rapid differentiation and development of the liver and pancreas is observed, as well as the differentiation of the segments. Ciliated and microvillous receptor cells are already differentiated in the olfactory epithelium at hatching, which provides early chemical sensitivity to the larvae. The optic vesicles are observed before hatching, the lens is differentiated and separated from the cornea, and the retina is non-stratified, but the eyes have limited pigment present. Only superficial and deep muscle compartments are present in pacu at hatching. The muscle thickening occurs from the beginning of pacu development by hyperplasia and hypertrophy of the fibers, controlled by the myogenic regulatory factors.

The bucco-pharyngeal cavity generally opens before yolk exhaustion, which permits a short mixotrophic phase. At first-feeding, the eyes are pigmented and the olfactory epithelium is covered by a dense layer of non-sensory cilia. The presence of taste buds on the lips and oropharynx cavity suggests that the larva has the ability to select foods. There are solitary chemosensory cells observed on the pacu body surface at the early exotrophic phase. At first feeding, the pacu larva responds to stimuli caused by extracts of live feed (*Artemia* nauplii) and food intake increases as the larvae grow. The mechanoreceptors, as free cranial neuromasts and lateral line neuromasts, develop very fast and at 2dph the hair cells of the neuromast are observed into the cupula. Bone ossification occurs simultaneously in structures of the head and the vertebral column. Additional ossification occurs later in the fin rays.

As the larvae develop, the olfactory epithelium deepens as the nostrils differentiate and the epithelium covers only the internal nasal cavities. The esophagus consists of stratified squamous epithelium and changes into a simple cylindrical epithelium at its distal end; numerous mucous cells are found along the entire length of the esophagus. The transition from the esophagus to the intestine is a dynamic region where the stomach will later develop. The stomach is initially a dilated pouch-shaped intestinal epithelium lined by cylindrical simple epithelium near the junction with the esophagus, decreasing in thickness and becoming simple cuboidal epithelium in the more dilated portion. The intestine consists of cylindrical epithelial cells (with distinguished brush border and basal nuclei) and goblet cells. As the digestive tract matures, the intestinal folds increase. The differentiation of the stomach and the appearance of gastric glands occur later when the larvae reaches approximately 10mg. Taken together, the knowledge of the morphology, physiology, and behaviour of the larvae underlie the identification of strategies that are resulting in high survival rates, the production of high quality fish, and increased economic return in pacu larviculture. This information is also important for the development of microdiets, which is another subject under investigation.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

LARVAL REARING PROTOCOLS FOR MEAGRE *ARGYROSOMUS REGIUS*

P. Pousão-Ferreira[1], S. Castanho[1], L. Ribeiro[1], J. Coutinho[2], N.M. Bandarra[2], and A.C. Mendes[1]

[1] IPMA - Portuguese Institute for the Ocean and Atmosphere Aquaculture Research Center - Av. 5 Outubro s/n 8700-305 Olhão, Portugal
[2] IPMA - Portuguese Institute for the Ocean and Atmosphere, Av. de Brasília, 1499-006 Lisboa, Portugal

Introduction

Meagre culture is very recent and constitutes a promising diversification of aquaculture in Southern Europe, which is very dependent on sea bass and sea bream cultivation. This is a species that presents fast growth, and, unlike sea bass and sea bream, is more appropriate to posterior processing such as filleting.

Its fast growth allows the earlier introduction of prey and inert diet relative to sea bream feeding protocols, with clear advantages in hatchery costs. This study aims to give a contribution to the development of standardized rearing protocol for meagre larvae in order to enhance the larval rearing strategy.

Materials and methods

In the Trial 1 (Fig. 1A) the effect of *Artemia* nauplii Instar I on the larvae development was studied and in the Trial 2 (Fig. 1B) the introduction of early inert food in co-feeding was tested. Eggs from *A. regius* were obtained from wild broodstock kept at IPMA and reared in 200-l white fibreglass conical tanks at a density of 45 larvae.l^{-1} in a semi-recirculation system. Experimental design was 3×3: three different feeding sequences protocols in triplicate. Fish larvae were fed ad libitum accordingly to the feeding sequence described.

The effect of different feeding protocols in the rearing success was assessed by larval growth performance, survival, fatty acids profile, and digestive enzymes activity. Trypsin, pepsin, and alkaline phosphatase activities were determined on 20DAH fish larvae according to the protocols described by Guerreiro et al. (2010). The fatty acid composition of larvae and feeds – rotifers and *Artemia* enriched with Red Pepper® and inert food CAVIAR, from Bernaqua™ –were

determined according to Bandarra et al. (2011). Data were normalized and analyzed using one-way ANOVA, followed by Tukey's multiple comparisons test.

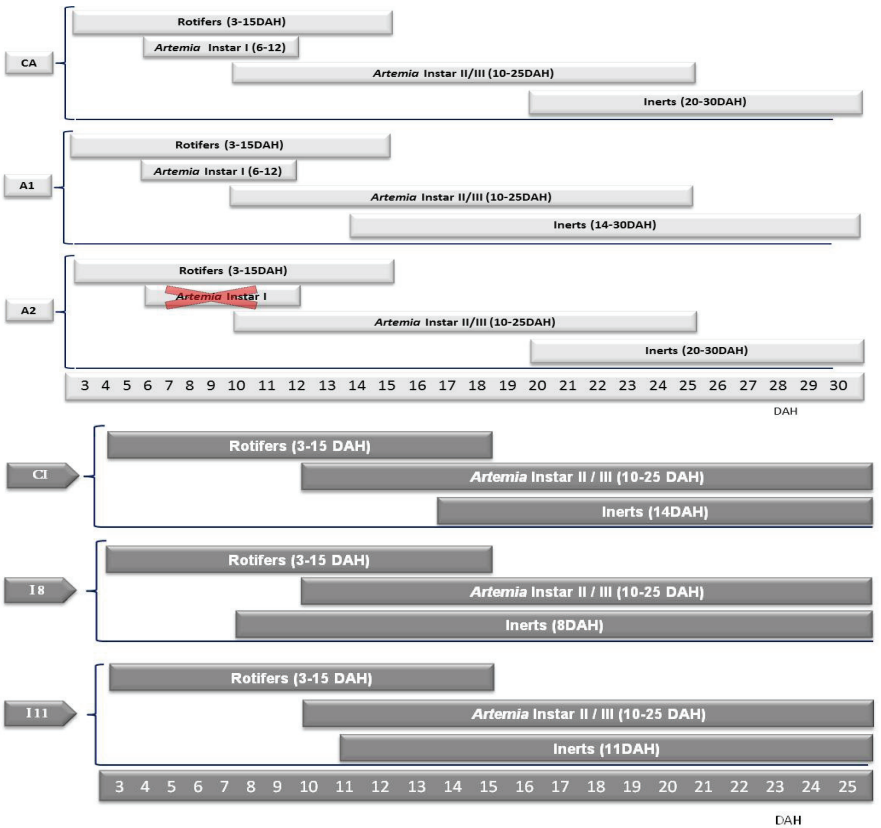


Fig. 1 (Top) Trial 1: CA- control feeding protocol based on sea bream, adapted to meagre growth; A1- inert diet was introduced early at 14DAH; A2 - the main difference is the absence of Instar I *Artemia* nauplii of the "marine strain"; (Bottom) Trial 2: CI - control protocol was based on the better result obtained in Trial 1(A2); I8 and I11 - introduction of inert diet at 8 and 11DAH (co-feeding).

Results and discussion

Trial 1

Larvae fed A2 (without Instar I *Artemia* nauplii) had a signicantly lower (p<0.05) length (TL) at 9 and 13DAH, indicating that the absence of Instar I *Artemia* nauplii constrained larval growth at that age, yet no differences were found either in length or weight at 20DAH (at inert introduction in CA and A2) or at 30DAH (trial end), which apparently seems to indicate that *A. regius* growth performance was not affected by the lack of *Artemia* Instar I.

A1 diet produced fish more consistent in weight (lower SD), which is important for production. Nevertheless, a higher observed mortality 27.4±4.90% occurred in tanks fed with treatment A1 (Instar I *Artemia* nauplii), 20.4±7.7% in A2, and 20.3±11.39% in CA.

Meagre fatty acid (FA) profile of the 3 treatments presented a similar tendency. At the end of the trial there were no significant differences. In the 3 treatments it was observed a significant decrease in ARA (20:4n-6 - Arachidonic acid) and a significant increase in EPA (20:5n-3 – Eicosapentaenoic acid), in DHA (22:6n-3 – Docosahexaenoic acid) also was a decrease in 3 treatments, markedly at 10-14DAH, yet A2 (absence of Instar I *Artemia*) had a less-pronounced decrease, although without statistical significance. Larvae reflected the dietary fatty acid composition, once that some of these changes were clearly associated with the introduction of a new diet item on the feeding protocol.

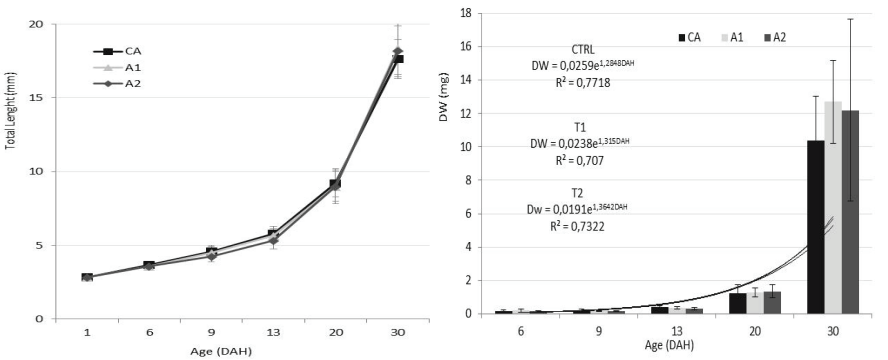


Fig. 2. Total length (TL) and Dry Weight (DW) of *A. regius* larvae in Trial 1.

Trial 2

The earlier introduction of inert food clearly promoted a better growth. Meagre larvae from I8 and I11 treatment was significantly ($p<0.05$) bigger. Larvae from I11 treatment presented higher size variability. The early introduction of inert diet at 8 and 11DAH did not affected digestive enzymes activities when compared to control, 14DAH ($P>0.05$) (Table I).

Table I. Digestive enzyme specific activities of meagre larvae where inert diet was introduced at different stages of development. Results presented as mean±sd (n=3).

	Control (CI)	**I8**	**I11**
Trypsin (mU.mg^{-1} prot)	0.1±0.05	0.1±0.02	0.1±0.03
Pepsin(U.mg^{-1} prot)	0.2±0.04	0.4±0.25	0.2±0.10
Alk.Ph. (mU.mg^{-1} prot)	15.7±6.28	13.3±1.88	14.7±0.65

Also, in the FA profile of meagre larvae no significant differences were observed. In terms of mortality, no significant differences are found during or at the end of the trial, mainly due to the variability between replicates, yet I11

treatment showed less mortality (7.2±0.47%) and more importantly less variability than I8 (8.1±1.62%) and control group CI (8.5±3.23%). Although in co-feeding these results suggest that early weaning is possible and even desirable with meagre.

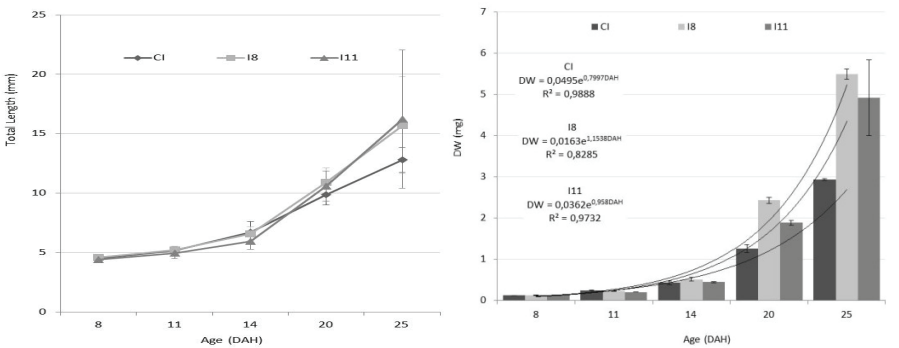


Fig. 3. Total length (TL) and dry Weight (DW) of *A. regius* larvae in Trial 2.

Conclusions

It's possible to successfully produce *A. regius* without the use of *Artemia* Instar I nauplii since no significant differences were found on growth, survival, or larval FA composition.

Moreover, the ability of meagre larva to ingest inert diet from a very early age, without negative impacts on digestive physiology and fatty acid compositions, decreases the use of live feeds and enhance growth.

Acknowledgements

Project AQUACOR - 31-03-05-FEP-03; PROMAR Program (Portugal) & FEP funds.

References

Bandarra N.M, P. Rema, I. Batista, A.C. Figueiredo-Silva, P. Pousão-Ferreira, S.M.G. Batista, and R. Ozório. 2011. Effects of dietary n-3/n-6 ratio on lipid metabolism of gilthead seabream (Sparus aurata). Eur. J. Lipid Sci. Technol. 113:1332-1341.

Guerreiro I., M. Vareilles, P. Pousão-Ferreira, V. Rodrigues, M.T. Dinis, and L. Ribeiro. 2010. Effect of age-at-weaning on digestive capacity of white seabream (Diplodus sargus). Aquaculture 300:194–205.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EFFECTS ON THE SKELETON DEVELOPMENT IN REARED GILTHEAD SEA BREAM (*SPARUS AURATA*)

L. Prestinicola, C. Boglione, and S. Cataudella

Laboratory of Experimental Ecology and Aquaculture, Department of Biology, University of Rome "Tor Vergata", Via Cracovia 1, 00133 Rome, Italy

Although skeletal anomalies in reared fish have been investigated for over 40 years, relatively little progress has been made toward the identification and removal of the causes. The reasons are many, including the fact that while the same factor can provoke a diverse response depending on the species, cohort, body region, tissue, and type of bone, different factors can induce the same anomaly in different species and different factors often act synergistically.

In this scenario, this communication presents a series of case studies aimed at providing a quantitative and qualitative analysis on if and what differences in skeletal anomalies arise in gilthead sea bream (*Sparus aurata*) juveniles when (i) juveniles of different origins are stocked in a similar environment during preongrowing phase(case studies 1 and 2); (ii) newly hatched larvae from the same eggs batch are reared under different rearing conditions (case study 3); and (iii) fish are influenced differently by presence or absence of an inflated swim bladder (case study 4). The aims were to (a) determine if environmentally-induced skeletal anomalies exist, (b) investigate if a relationship between those anomalies and skeletal bone tissues or types of ossification can be found, and (c) identify the best rearing methodology for gilthead sea bream larvae for lowering the deformity rates.

A total of 2079 juveniles, belonging to 19 lots, were monitored. 288 were wild specimens, 52 reared and wild juveniles, while the remaining 1739 were obtained from commercial hatcheries (intensive and semi-intensive rearing). All fish were double-stained in toto for cartilage and bone or X-rayed and data were analyzed through the use of derivate variables, non-parametric tests, and Correspondence Analysis.

The results obtained by the case studies 1 and 2 showed that when fish reared through different methodologies are stocked in a similar (semi-natural) environment, the frequencies of anomalies of vertebral arches, dorsal fins pterygiophores, and dorsal and anal rays decreased independently from the origin (case study 1: wild vs. semi-intensively reared juveniles; case study 2: wild vs. intensively

reared juveniles; in both cases, juveniles were stocked in a coastal lagoon). These case studies, however, did not enhance any peculiar effect on some bone typology or ossification process.

On the contrary, the results obtained in the case study 3 (larvae from the same eggs batch reared under different conditions) highlighted that (i) in all the semi-intensively reared lots, the bones undergoing direct ossification showed consistent lower incidences of anomalies, conversely to sister lots reared in intensive conditions; (ii) the same pattern was not detected in the skeletal elements having a cartilaginous precursor; and (iii) a higher capacity of Large Volumes than Mesocosm (the two tested semi-intensive rearing technologies) of ameliorating the morphological quality with respect to each sister lot reared in intensive condition. Further, all severe anomalies were almost totally absent in semi-intensively reared juveniles, a beneficial effect of larger tanks and lower stocking density.

The higher driving capacity of the Large Volumes to lower the incidence of severe skeletal anomalies with respect to Mesocosm could be ascribed to some nutritional factor: the input of wild plankton in the tank, routinely practiced in the Large Volumes, in fact, results in a larger availability of different sized preys for the larvae, with diverse behaviour and nutraceutical properties. So it seems possible to ameliorate the morphology of reared sea bream juveniles by lowering the stocking densities (maximum 16 larvae.l^{-1}), increasing the volume of the hatchery rearing tanks (minimum 40m^3), and feeding larvae with a wide variety of live (wild) preys.

Eventually, the results from the case study 4 showed that variation of the inner environment (absence of a normally inflated swim bladder) may drive the onset of anomalies in the pectoral fin and pre-haemal vertebrae, at first, and in the haemal ones later, due to the mechanical overload caused by hyperactivity of pectoral fin muscles. This effect was detected only in directly ossifying bones.

In conclusion, this research hypothesizes that directly ossifying bones seem to be influenced by environmental conditions, while all bones which demand for a cartilage template on which mineralization process occur seem to be mainly driven by nutraceutical properties of the administered diet.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

BACTERIAL COMMUNITY COMPOSITIONS OF EJECTED INTESTINE OF JUVENILE SEA CUCUMBER AND THE EFFECT OF SEA SEDIMENT BACILLUS ON IN VIVO ANTAGONISM AGAINST PATHOGENIC *VIBRIO SPLENDIDUS*

Z.-Z. Qi[1], S.-Q. Chen[2], A.-G. Zou[3], L. Tang[1], M.-M. Sun[1], H.-Y. Zhang[1], C.-L. Liu[2], J.-P. Wang[4], and Y. Han[1]

[1] Laboratory of Marine Microbiology, Key Laboratory of Marine Genetics and Breeding (Ocean University of China), Ministry of Education, Qingdao 266003, China
[2] Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, China
[3] Shandong Anyuan Fishery Limited Corporation, Yantai 265600, China
[4] Ningbo Ocean & Fishery Institute, Ningbo 315000, Zhejiang, China

Introduction

Along with the rapid expansion of sea cucumber culture of China, the occurrence of bacterial diseases has been the bottleneck for sustainable development. Pathogens *Vibrio splendidus*, *V. harveyi*, and *V. cyclitrophicus* have been isolated from skin ulceration disease and viscera ejection syndrome of sea cucumber *A. japonicas* (Deng et al., 2009). On the basis of 16S rRNA gene pyrosequencing, we analyzed bacterial community compositions of ejected intestine of juvenile sea cucumber and coastal sea sediment. We formulated a mixture of *Bacillus subtilis*, *B. amyloliquefaciens*, and *B. megaterium* from coastal sea sediment as probiotic candidates. Their probiotic effects on in vivo antagonism against pathogenic *V. splendidus* was evaluated.

Materials and methods

Five freshly ejected intestines were collected from diseased juvenile sea cucumbers (2.2-3.5g) at Hongdao farm in Qingdao. The intestines were pooled together, rinsed with sterilized seawater, and homogenized for bacterial isolation and DNA extraction. An average of 1.5g of coastal sea sediment from 5 sites of the same culture area were collected and mixed for DNA extraction. The sediment samples were dried at outdoor temperature for 3 days for the isolation of *Bacillus*.

The 16S rRNA gene-based sequence was employed for the identification of bacterial isolates by following Deng et al. (2009). The DNA extractions for intes-

tines and sediments were performed via FastDNA® SPIN Kit for Soil (MP Biomedicals, LLC, 29525 Fountain Pkwy. Solon, OH44139). PCR amplification, 16S rRNA V1-V3 based pyrosequencing and sequence information analysis were performed by following Wu et al. (2011).

Infection and probiotic experiment were performed in 2-l beakers at 25°C without feeding. Two hundred and forty juvenile sea cucumbers (0.9-2.8g) were distributed equally into 12 beakers and were divided in 4 groups: Group 1 is blank control; Group 2 was treated with *V. splendidaus* at a level of 10^6cfu.ml^{-1}; Group 3 was treated with a mixture of *B. subtilis*, *B. amyloliquefaciens*, and *B. megaterium* cultures in 1:1:1 ratio at a final level of 10^6cfu.ml^{-1}; Group 4 was treated both *V. splendidaus* and *Bacillus* mixture at 10^6cfu.ml^{-1}. After 7d, the cumulative number of ejected intestine was recorded and analyzed using analysis of variance (SPSS v12, Chicago IL).

Results and discussion

A total of 73 272 valid reads and 4817 OTUs were obtained from intestine and sediment samples via 454 pryrosequencing analysis. Intestinal bacterial communities contained 24 661 reads with 452 OTUs and sediment samples contained 48 611 reads with 4435 OTUs. Good's coverage estimations revealed that 99% and 96% of the bacterial species were obtained in the intestine and sediment samples, respectively. By agar plating method, we obtained 70 and 26 isolates from intestine and sediment samples, respectively.

All sequences were classified from phylum to species. The samples showed different taxonomic composition on the basis 16S rRNA profiles (Fig.1). The sample EI-P (Ejected intestine) was dominated by Proteobacteria, Fusobacteria, and Bacterioidetes, in which more than 19.42% reads relate to *V. tapetis* and 3.89% reads relate to *V. splendidus*. In sample EI-C (ejected intestine for agar plating), 16S rRNA sequence analysis showed that the several isolates related to *V. splendidus*, but none of sequences is similar to *V. tapetis*. The bacterial community composition of CSS-P (fresh coastal sea sediment) sample was dominated by Bacteroidetes (8.72%), Firmicutes-Clostridiales (6.90%), proteobacteria (25.13%), Spirochaetes (3.58%), and Synergistetes (1.61%). A large portion of OTUs presented in low number indicated the high level of bacterial diversity in the sediment sample. We obtained 16 *Bacillus* out of 26 isolates from dried sediment sample (CSS-C), including *B. subtilis*, *B. licheniformis*, *B. aquimaris, B. amyloliquefaciens*, and *B. megaterium*. However we did not find similar *Bacillus* reads from CSS-P pyrosequencing library. It suggested that those *Bacillus* cells/spores might present in low number and/or the DNA extraction from those cells/spores was not efficient.

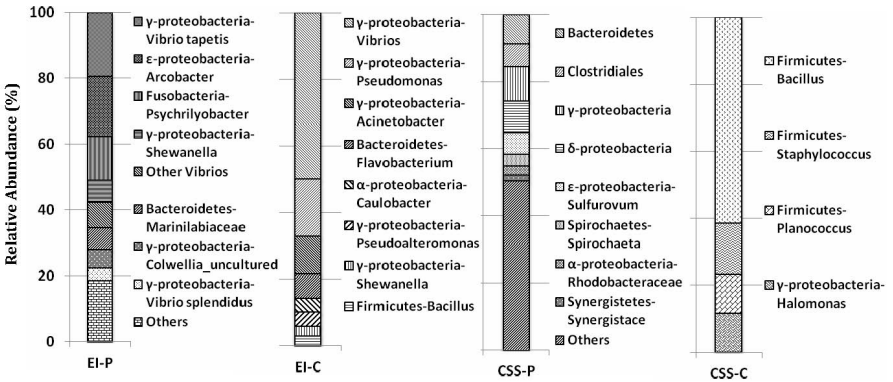


Fig. 1. Bacterial community compositions of ejected *Apostichopus japonica* intestines and costal sea sediment (EI-P: Intestinal microbiota revealed by pyrosequencing; EI-C: Intestinal microbiota revealed by culture-dependent method; CSS-P: Sediment microbiota revealed by pyrosequencing; CSS-C: Sediment microbiota revealed by culture-dependent method).

Pathogens such as *V. splendidus* and *Shewanella marisflavi* have been reported for the cause of *A. japonicas* visceral ejection syndrome (Deng et al., 2009). In our study, strain a17 isolated from ejected intestine of juvenile sea cucumber was identified as *V. splendidus* by 16S rRNA gene analysis. Immersion challenge at a density of 10^6cfu.ml^{-1} of strain a17 induced more than 50% juvenile sea cucumber evisceration in 5 days, following by 100% mortality in 15 days under experimental culture conditions. As fine-grained dried sea sediments are used commonly as part of sea cucumber feed, we isolated *B. subtilis*, *B. amyloliquefaciens*, and *B. megaterium* strains from dried sediment as probiotics candidates.

The *Bacillus* mixture (*B. subtilis, B. amyloliquefaciens,* and *B. megaterium* in 1:1:1 ratio) treatment significantly reduced the cumulative evisceration of sea cucumber infected by immersion of *V. splendidus* ($p<0.05$) (Fig.2). The cumulative evisceration of infected *A. japonicas* reached 70% on the 7th day. At present, various *Bacillus* spp. used as probiotics for aquaculture are of soil origin. Our study provides a mixture of sea sediment-originated *Bacillus* as a probiotic candidate. This mixture showed an in vivo protective effect against *V. splendidus* infection of sea cucumber. Further study is needed to elucidate the exact activities of the Bacillus mixture in the intestine of sea cucumber.

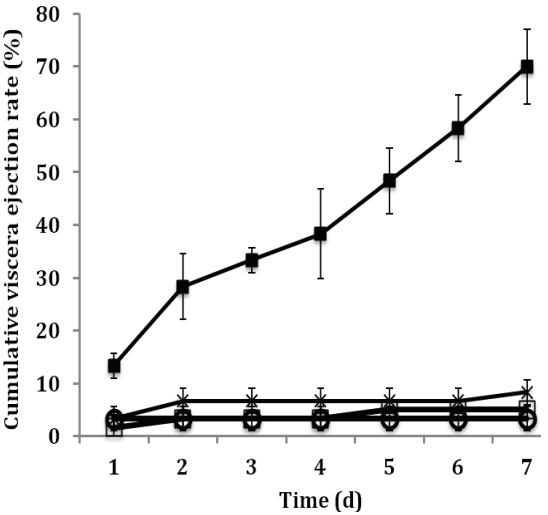


Fig. 2. Cumulative viscera ejection rates of *Apostichopus japonicas* juveniles infected with *V. splendidus* with and without a mixture of *B. subtilis*, *B. amyloliquefaciens*, and *B. megaterium*. ×: Control; Δ: *V. splendidus* immersion treatment; ◊ *Bacillus* mixture treatment; o: *Bacillus* mixture with *V. splendidus* immersion.

Conclusions

The present study showed that the bacterial community compositions of ejected intestine of diseased *A. japonicas* juveniles. The high throughputs sequencing technology revealed that Vibrios appeared to be the core intestinal microbiota of diseased juvenile sea cucumber. *V. splendidus* strains were isolated from ejected intestine and immersion of *V. splendidus* caused high visceral ejection rate at 70%. A costal sea sediment originated Bacillus mixture reduced evisceration rate significantly after immersion *V. splendidus*. The results offer a potential probiotic treatment for *A. japonicas* visceral ejection syndrome.

References

Deng H., C. He, Z. Zhou, C. Liu, K. Tan, N. Wang, B. Jiang, X. Gao, and W. Liu. 2009. Isolation and pathogenicity of pathogens from skin ulceration disease and viscera ejection syndrome of the sea cucumber *Apostichopus japonicas*. Aquaculture 287(1):18-27.

Hamouda T., A. Shih, and J. Baker. 2002. A rapid staining technique for the detection of the initiation of germination of bacterial spores. Letters in applied microbiology 34:86-90.

Wu S., G. Wang, E.R. Angert, W. Wang, W. Li, and H. Zou. 2012. Composition, diversity, and origin of the bacterial community in grass carp intestine. PloS one 7:e30440.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

PAVLOVA – A "NEW" MICROALGAE CANDIDATE SPECIES FOR LIVE FEED CULTIVATION AND FISH LARVAE NUTRITION

S. Rehberg-Haas[*1], S. Meyer[1], S. Lippemeier[2], and C. Schulz[1,3]

[1] GMA – Gesellschaft für Marine Aquakultur, Hafentörn 3, 25761 Büsum, Germany
[2] BlueBioTech, Hafentörn 3, 25761 Büsum, Germany
[3] Institute of Animal Breeding and Husbandry, Christian-Albrechts-Universität, Kiel, Germany

Introduction

The high growth rates in marine aquaculture production lead to an increasing demand of suitable diets for marine fish larvae. Up to this point this demand cannot be sufficiently covered by conventional dry feed diets, especially for the very sensitive first feeding live stages of marine fish larvae. Marine microalgae in combination with live feed organisms (e.g., *Brachionus plicatilis*, *Artemia salina*) are considered optimal diets for marine fish larvae. In addition to providing high quality protein (e.g., essential amino acids) and energy, they provide other key nutrients such as vitamins and essential fatty acids (e.g. eicosapentaenoic acid, EPA, and docosahexaenoic acid, DHA). High growth rates of live feed organisms have been related to diets containing microalgae rich in proteins and essential fatty acids (Renaud et al., 2002). Hirayama et al. (1979) and Okauchi and Fukusho (1984) discovered positive nutritional effects of microalgae used in rotifer cultivation. Further important factors describing the overall rotifer culture performance like growth rate (Yúfera and Pascual, 1980) and filtration and ingestion rates (Savas and Guclu, 2006) are also affected by the diet.

Larval growth, survival, and activity have been reported to be affected by dietary levels of essential fatty acids (Izquierdo 1996). Most of the microalgae frequently used in marine finfish hatcheries (e.g., *Nannochloropsis* sp., *Isochrysis* sp., *Chlorella* sp.) are deficient of at least one of these essential fatty acids. In contrast to this, it is known that other marine microalgae, like *Pavlova* sp., are able to synthesize significant amounts of both EPA and DHA (Volkmann et al. 1991), but it is currently not possible to produce them in a larger scale of commercial relevance. In a previous study, new production techniques for cultivation, harvest and preservation of chosen *Pavlova* sp. microalgae strains have been developed and the large-scale production of *Pavlova* has been successfully implemented (Lippemeier et al., in prep). It was the aim of the present study to evaluate the effects of different commercially cultivated *Pavlova* strains and

Pavlova products (fresh culture, concentrate, frozen concentrate, freeze-dried powder)on the culture performance of rotifers (*B. plicatilis*). These products have commercial potential as "off-the-shelf products" as alternative to expensive hatchery on-site production of microalgae.

Material and methods

In a first small-scale (800ml culture volume per replicate) experiment, three different *Pavlova* strains (*Pavlova* sp. fresh culture; *P. viridis* fresh culture; *P. viridis* concentrate) were used as cultivation products for rotifers (*B. plicatilis*, L-strain) and their potential was evaluated by a comparison to baker's yeast and to *Nannochloropsis* sp. concentrate. Triplicate culture flasks were fed at a daily ratio of 1g DW.million^{-1} rotifers of the respective product. The results of this pilot trial showed positive effects on the culture performance (number of individuals, egg production rate) of all *Pavlova* treatments in comparison to the *Nannochloropsis* sp. concentrate and baker´s yeast. The highest rotifer density was found in the *P. viridis* fresh culture fed group (161 rotifers.ml^{-1}).

The application of different *P. viridis* products for live feed cultivation (*B. plicatilis*) was investigated in a larger-scale trial. An initial rotifer population was cultured in 30-l circular tanks on a combination of baker's yeast and *Nannochloropsis* sp. concentrate. At the start of the cultivation experiment, 10-l plankton tubes were stocked from this population at a density of about 100ind.ml^{-1}. The cultivation products tested were: (1) N=*Nannochloropsis* sp. concentrate; (2) P2=*P. viridis* fresh culture; (3) P1=*P. viridis* concentrate; (4) P3=*P. viridis* frozen concentrate; (5) P4=*P. viridis* freeze-dried powder. Each product was tested in a five-time replication and was applied at a daily ration of 0.75g DW.million^{-1} rotifers. The salinity (20PSU) and the temperature (21.4±0.2°C) were maintained at a constant level during the experimental period of 14 days. Light regime was set on a 24h photoperiod.

A volume of 1.5 l was removed daily from each plankton tube. Samples for counting of individual rotifers and eggs were taken. Furthermore the microalgae concentration was recorded by measuring the optical density (OD at 750nm). After sampling the culture volume was filled up to 10 l including the daily microalgae ration.

Additional experiments concerning the filtration and ingestion rates were conducted at day 6 and day 12. Thus the microalgae concentration in the plankton tubes was measured every hour for seven hours and 24 hours after feeding.

Results

The analyses of the second trial showed positive effects of fresh *Pavlova* culture (P2), *Pavlova* concentrate (P1), and frozen *Pavlova* concentrate (P3) on the culture performance in comparison to freeze-dried *Pavlova* powder (P4) fed rotifers (Fig. 1). The P4 diet showed disadvantages concerning the applicability, as it sank quickly and accumulated at the bottom of the plankton tubes. Furthermore the P2 diet showed a shorter durability than the other products limiting its quality as cultivation product. The *Nannochloropsis*-fed culture showed the highest rotifer density (283 rotifers.ml^{-1}) after the experimental period of 14 days.

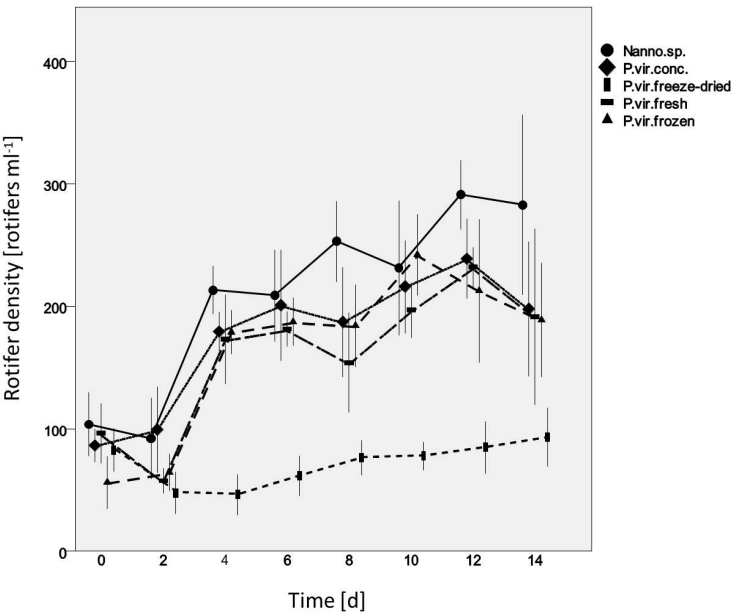


Fig. 1. Culture performance of rotifers fed different cultivation products.

The highest filtration and ingestion rates were found for the *P. viridis* fresh culture treatment.

In an additional experiment newly hatched Atlantic cod (*Gadus morhua*) yolk-sac larvae were fed with rotifers and *Artemia* enriched with different products (*P. viridis*, *Nannochloropsis* sp., commercial enrichment product). First results showed positive effects of the utilization of *Pavlova* in marine fish larvae nutrition. Additional analyses of ingestion rates, standard length, dry weight, protein content, and RNA:DNA ratio of the larvae will give information about the potential of *Pavlova* in comparison to other commonly used enrichment products.

Conclusion

Pavlova represents a readily available microalgae species with a high content of DHA and EPA and other micronutrients of significant importance for both fish larval and human nutrition. The present study demonstrated the high potential of *Pavlova* sp. as cultivation and enrichment product for live feed organisms. Successful implementation of *Pavlova*-based enrichment formulations will be of high economic relevance for both hatcheries and microalgae-producers to replace and accomplish other products.

References

Hirayama K., K. Takagi, and H. Kimura. 1979. Nutritional effect of eight species of marine phytoplankton on population growth of the rotifer, Brachionus plicatilis. Bulletin of the Japanese Society of Scientific Fisheries 45:11-16.

Izquierdo M.S. 1996. Essential fatty acid requirements of cultured marine fish larvae. Aquaculture Nutrition 2:183-191.

Okauchi M. and K. Fukusho. 1984. Food value of a minute alga, Tetraselmis tetrahele, for the rotifer Brachionus plicatilis culture-1. Population growth with batch culture. Bulletin National Research Institute of Aquaculture 5:13-18.

Renaud S.M., L. Thinh, G. Lambrinidis, and D.L. Parry. 2002. Effect of temperature on growth, chemical composition and fatty acid composition of tropical Australian microalgae grown in batch cultures. Aquaculture 211:195-214.

Savas S. and Z. Guclu. 2006. Filtration and ingestion rates of the rotifer Brachionus plicatilis fed five species of microalgae at different cell densities. Israeli Journal of Aquaculture-Bamidgeh 58:39-45.

Volkmann J.K., G.A. Dunstan, S.W. Jeffrey, and P.S. Kearney. 1991. Fatty acids from microalgae of the genus Pavlova. Phyrochemistry 30:1855-1859.

Yúfera M. and E. Pascual. 1980. Estudio del rendimiento de cultivos del rotifero Brachionus plicatilis O.F. Muller alimentados con levadura de panificacion. Investigacion Pesquera 44:361-368.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

ENDOGENOUS METABOLISM OF UNSATURATED FATTY ACIDS IN *ARTEMIA* NAUPLII AS DETERMINED THROUGH INCUBATION WITH ^{14}C-LABELLED FATTY ACID SUBSTRATES

D.B. Reis[1,2,3], N.G. Acosta[2], E. Almansa[3], J.C. Navarro[4], D.R. Tocher[5], Ó. Monroig[4], J.P. Andrade[1], A.V. Sykes[1], and C. Rodríguez[2]

[1] CCMAR-CIMAR L.A., Centro de Ciências do Mar do Algarve, Universidade do Algarve, Campus de Gambelas, 8005-139, Faro, Portugal
[2] Departamento de Biología Animal, Universidad de La Laguna, Avenida Astrofísico Francisco Sánchez, 38206 La Laguna, Santa Cruz de Tenerife, Canary Islands, Spain
[3] Instituto Español de Oceanografía. Centro Oceanográfico de Canarias, Vía Espaldón nº1, Dársena pesquera, Parcela nº8, CP: 38180, Santa Cruz de Tenerife, Canary Islands, Spain
[4] Instituto de Acuicultura Torre de la Sal (IATS-CSIC), 12595 Ribera de Cabanes, Castellón, Spain
[5] Institute of Aquaculture, School of Natural Sciences, University of Stirling, Stirling FK9 4LA Scotland UK

Introduction

Artemia nauplii are widely used as live prey in marine aquaculture but do not naturally provide essential fatty acids (EFA) for marine organisms, such as n-3 long-chain highly unsaturated fatty acids (HUFA). However, the high acceptance of the nauplii as prey by early stages of several marine species and the possibility of manipulating their nutritional content through lipid enrichment makes them a suitable choice in aquaculture (Sorgeloos et al., 2001).

It is well known that the presence of n-3 HUFA into specific phospholipids and total or individual n-3 HUFA ratios are essential factors for marine larvae performance. In this sense, it is interesting to unveil the fate of the fatty acids (FA) during enrichment, including its incorporation into tissular lipids. This study aimed to determine the "*in vivo*" capability of *Artemia* nauplii to incorporate and esterify [1-^{14}C]-labelled fatty acid substrates [18:1n-9 (OL), 18:2n-6 (LO), 18:3n-3 (LNA), 20:4n-6 (ARA), 20:5n-3 (EPA), and 22:6n-3 (DHA)] into the different lipid classes (LC).

Materials and methods

Artemia sp. (Artemia Cyst EG - INVE AQUACULTURE, Belgium) nauplii, obtained according to the methods described by Sorgeloos (1977), were incu-

bated in Sarstedt six flat-bottom-welled tissue culture plates at a density of 10 000 nauplii in 10ml of seawater (35‰). Nauplii were incubated under gentle stirring for 5h, at 24ºC and with 0.2µCi (0.3µM) of either [1-^{14}C]18:1n-9, [1-^{14}C]18:2n-6, [1-^{14}C]18:3n-3, [1-^{14}C]20:4n-6, [1-^{14}C]20:5n-3, or [1-^{14}C]22:6n-3, which were added to the water as their potassium salts bound to BSA. A control group of nauplii was also maintained under similar conditions, but without the addition of labelled FA. After incubation total lipid (TL) extraction was performed as described by Christie (1982). Incorporation of radioactivity into TL (pmol.mg pp^{-1}.h) was performed following Rodríguez et al. (2002). LC were separated by single-dimensional double-development HPTLC as previously described by Tocher and Harvie (1988). The developed HPTLC plates were placed in closed exposure cassettes (Exposure Cassete-K, BioRad, Madrid, Spain) in contact with a radioactive-sensitive phosphorus screen (Imagen Screen-K, Bio-rad, Madrid, Spain). The screens were scanned with an image acquisition system (Molecular Imager FX, BioRad) and quantified in percentage by "Quantity One" image software.

Statistical analysis was assessed by one-way ANOVA followed by Tukey's post hoc test. When normal distribution and/or homogeneity of the variances were not achieved, data were subjected to the Kruskall–Wallis non-parametric test, followed by a Games-Howell non-parametric multiple comparison test. Statistical differences were considered for $p<0.05$. The statistical analysis was performed using the IBM SPSS statistics 20.0 (IBM Corp., Armonk, NY).

Results and discussion

The lipids of *Artemia* nauplii were particularly rich in neutral lipids (NL; 80.55±1.74% of TL), triacylglycerol (TAG) being the main LC (51.86±2.26%), followed by cholesterol (CHO; 14.36±0.51%). Within polar lipids (PL), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were the main LC with 7.80±1.10% and 6.80±0.79% of TL, respectively.

Table I presents the incorporation rate of the radiolabelled substrates into *Artemia* TL. The [1-^{14}C]C18 FA presented similar incorporation. However, within HUFA, some differences were detected with [1-^{14}C]ARA being the most incorporated and [1-^{14}C]DHA presenting approximately half of the incorporation of all other radiolabelled substrates ($p<0.05$). The lower incorporation of [1-^{14}C]DHA could be related to the capability of *Artemia* nauplii to retroconvert DHA into EPA (Navarro et al., 1999). Since the first carbon was the labelled one, a chain shortening would remove this carbon, unlabelling this FA, and turning its products undetectable in the TLC plates.

Table I. Incorporation into total lipid and distribution of radioactivity into lipid classes of *Artemia* nauplii incubated with [1-^{14}C]18:1n-9, [1-^{14}C]18:2n-6, [1-^{14}C]18:3n-3, [1-^{14}C]20:4n-6, [1-^{14}C]20:5n-3, [1-^{14}C]22:6n-3

	18:1n-9	**18:2n-6**	**18:3n-3**	**20:4n-6**	**20:5n-3**	**22:6n-3**
Incorporation	15.6±2.8^{a}	18.3±2.5^{a}	13.6±3.9^{a}	19.4±2.6$^{\Delta a}$	13.4±2.7$^{\circ a}$	6.8±0.8$^{\square b}$
Lipid classes						
SE	8.8±0.9$^{\blacktriangle a}$	8.1±1.2$^{\blacktriangle a}$	3.8±1.6$^{\bullet b}$	2.9±0.9^{b}	4.2±2.0^{b}	5.7±0.9^{ab}
TAG	21.7±2.2$^{\bullet ab}$	20.7±1.9$^{\bullet b}$	25.6±0.8$^{\blacktriangle a}$	9.1±0.5$^{\circ d}$	9.2±1.5$^{\circ d}$	14.4±0.6$^{\Delta c}$
FFA	7.4±0.9$^{\blacktriangle a}$	6.7±1.0$^{\blacktriangle ab}$	4.7±0.3$^{\bullet b}$	4.8±1.4^{ab}	5.9±2.6^{ab}	8.6±0.6^{a}
PAG	5.2±3.9	7.4±2.2	4.6±1.1	5.7±0.9	6.0±2.8	7.3±0.8
PE	29.8±4.8	29.0±5.5	28.0±4.9	20.3±1.3	23.6±5.2	24.0±2.0
PI	4.1±0.9$^{\bullet c}$	5.5±0.5$^{\blacktriangle c}$	4.9±0.5$^{\blacktriangle\bullet c}$	26.3±2.6$^{\Delta a}$	11.2±1.0$^{\circ b}$	9.3±0.7$^{\circ b}$
PS	3.8±1.0^{bc}	3.5±0.5^{c}	2.5±1.00^{c}	6.7±0.4$^{\circ ab}$	8.4±0.2$^{\Delta a}$	6.1±0.6$^{\circ ab}$
PC	19.2±0.8$^{\bullet bc}$	19.4±1.2$^{\bullet bc}$	26.1±0.8$^{\blacktriangle a}$	24.1±0.4^{a}	31.5±3.6^{abc}	24.7±1.6^{ac}
TNL	43.2±2.5^{a}	42.8±4.7^{a}	38.6±3.3^{a}	22.5±3.7^{b}	25.3±8.8^{ab}	35.9±2.5^{ab}
TPL	56.9±2.5^{b}	57.8±4.7^{b}	61.5±3.3^{b}	77.5±3.7^{a}	74.7±8.8^{ab}	64.1±2.5^{ab}

Means ± SD; [1-^{14}C]C18 FA (n=4) and [1-^{14}C]HUFA (n=3). Incorporation: LT = pmoles per mg protein per hour; esterification = % of total radioactivity. ^{abc} in the same row = significant differences within FA ($p<0.05$); $^{\Delta\circ\square}$ and $^{\blacktriangle\bullet\blacksquare}$ within the same row = significant differences within C18 FA and HUFA, respectively ($p<0.05$); SE, sterol esters; TAG, triacylglycerols; FFA, free fatty acids; PAG, partial acylglycerols; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; TNL, total neutral lipids; TPL, total polar lipids.

All radiolabelled substrates were extensively esterified by the *Artemia* nauplii, with less than 10% of the incorporated radioactivity being recovered as FFA (Table I). The majority of the radiolabelled substrates were esterified as PL, mainly into PE and PC, except for [1-^{14}C]ARA that displayed the highest recovery as PI ($p<0.05$). These results differ from those reported by Navarro et al. (1999), where the radiolabelled substrates were mainly recovered as NL, especially as TAG and not total PL. A 24-h enrichment and the addition of the [^{14}C]FA (ethyl esters) together with commercial lipid emulsions performed in that study could explain these differences since, in the present study, [^{14}C]FA where added for only 5h and as the unique lipid source. An increased enrichment time would allow *Artemia* nauplii to metabolize and store the FA as lipid reserves.

Some differences were detected among the [1-^{14}C]C18 FAs and the [1-^{14}C]HUFA substrates esterification patterns ($p<0.05$), namely the higher esterification rates (almost twice) of [1-^{14}C]C18 FAs into TAG, and of [1-^{14}C]HUFA including DHA into PI ($p<0.05$). The esterification of [1-^{14}C]OL and [1-^{14}C]LO was similar. In contrast, the esterification pattern of [1-^{14}C]LNA presented some differences towards the other two [1-^{14}C]C18 radiolabelled FAs. Regarding [1-^{14}C]HUFA, the major difference was the clearly higher esterification of [1-^{14}C]EPA into PS, of [1-^{14}C]ARA into PI and [1-^{14}C]DHA into TAG ($p<0.05$).

In summary, the incorporation of [1-^{14}C]DHA into *Artemia* nauplii TL, was almost half of the incorporation of all other FA. More than 90% of all the radio-

labelled substrates were esterified into the different LC and mainly into PL. Differences between the esterification patterns of [1-^{14}C]C18 FAs and [1-^{14}C]HUFA substrates were determined. Comparing these data with those of previous studies, it was possible to verify that different types and times of enrichment lead to different esterification results.

Acknowledgements

This work was partially supported by project OCTOPHYS, Ref. AGL 2010-22120-CO3 funded by Spanish Government. A.V. Sykes (SFRH/BPD/36100/2007) and D.B. Reis (SFRH/BD/76863/2011) wish to thank Fundação para a Ciência e a Tecnologia for their grants.

References

Christie W.W. 1982. Lipid Analysis. (Second revised edition). Pergamon Press, Oxford. 207p.

Navarro J.C., R.J. Henderson, L.A. McEvoy, M.V. Bell, and F. Amat. 1999. Lipid conversion during enrichment of *Artemia*. Aquaculture 174:155-166.

Rodríguez C., J.A. Pérez, and J.R. Henderson. 2002. The esterification and modification of n-3 and n-6 polyunsaturated fatty acids by hepatocytes and liver microsomes of turbot (*Scophthalmus maximus*). Comp. Biochem. Phys. Part B 132:559-570.

Sorgeloos P. 1977. Decapsulation of *Artemia* cysts: a simple technique for the improvement of the use of brine shrimp in aquaculture. Aquaculture 12:311-315.

Sorgeloos P., P. Dhert, and P. Candreva. 2001. Use of the brine shrimp, *Artemia* spp., in marine fish larviculture. Aquaculture 200:147-159.

Tocher D.R. and D.G Harvie. 1988. Fatty acid compositions of the major phosphoglycerides from fish neural tissues: (n-3) and (n-6) polyunsaturated fatty acids in rainbow trout (*Salmo gairdneri L.*) and cod (*Gadus morhua*) brains and retinas. Fish Physiol. Biochem. 5:229-239

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

ASSESSMENT OF PROTEIN DIGESTIVE CAPACITY AND UTILISATION DURING ONTOGENY OF SENEGALESE SOLE LARVAE: A TRACER STUDY USING IN VIVO PRODUCED RADIOLABELLED PEPTIDE FRACTIONS

N. Richard[1*], S. Engrola[1], P. Palma[1], W. Pinto[2], D. Simes[1], and L. Conceição[2]

[1] CCMAR, Centro de Ciências do Mar do Algarve, Universidade do Algave, Campus de Gambelas, 8005-139 Faro, Portugal. (*E-mail: nrichard@ualg.pt)
[2] Sparos Lda, Centro Regional para a Inovação do Algarve (CRIA), Universidade do Algave, Campus de Gambelas, 8005-139 Faro, Portugal

Fish seem to have a limited capacity to digest complex proteins during early life stages. A better understanding of fish larvae protein digestive capacity and subsequent metabolic utilisation (amino acid oxidation and retention), depending on the molecular weight of the dietary proteins and also depending on the different larval developmental stages, is essential for the development of inert diets fitting larval nitrogen requirements along ontogeny. The purpose of this work was to develop a method allowing the in vivo production of radiolabelled peptides and proteins of different molecular weights using *Artemia* metanauplii, and apply these tools to study the development of the nitrogen digestive capacity and evaluate the nitrogen metabolic utilisation during the ontogeny of fish larvae. This study was conducted on Senegalese sole, a high-commercial-value Mediterranean flatfish species with very good farming potential.

Molecular weight distribution of the *Artemia* proteins was first characterised at different hours after hatching (hatching, 15h, 24h, and 36h after hatching). After liquid nitrogen freezing and lyophilisation, *Artemia* were homogenised in extraction buffer (100mM 1,4-piperazine-*N,N*'-bis(2-ethanesulfonic acid), 1mM EGTA, 1mM $MgCl_2$, pH 6.5, and 200µg of protease inhibitors cocktail. Protein extract was concentrated on centrifugal filter and applied on two different size exclusion chromatography systems: high molecular weight proteins were fractionated on a HiLoad 26/60 Superdex 200 prep grade column (GE Healthcare) and low molecular weight polypeptides were separated on a Superdex peptide 10/300 GL column (GE Healthcare).

The methodology above mentioned was also used afterwards to obtain radiolabeled proteins of different molecular weights, after a 15h enrichment of newly hatched *Artemia* nauplii (0.5×10^6 individuals in 2.5 l of seawater, in duplicates)

with 10ml of L-[U-^{14}C] free amino acids mixture (1mCi, ARC, Scopus Research, The Netherlands).

Digestibility and metabolic utilisation (protein retention and catabolism) of radiolabelled proteins of various molecular weights (free amino acids, 1 and 7kDa) produced as described previously, were assessed during ontogeny of Senegalese sole larvae (at 12, 21, and 34 days after hatching) fed on live feed. Fish larvae digestibility and metabolic utilisation of the radiolabelled proteins was evaluated using the controlled tube-feeding methodology.

The elution pattern of *Artemia* high molecular weight proteins from hatching to 24h after hatching was characterised by a major peak with a molecular weight range above 500kDa, followed by several weaker and less resolved peaks among the molecular weight range of 10kDa-500kDa. At 36h after hatching, a net decrease in the abundance of the highest molecular weight fraction of *Artemia* proteins and a simultaneous increase in the abundance of lower molecular weight proteins were observed. Concerning the distribution pattern of *Artemia* polypeptides, the same profile was obtained from hatching to 36h after hatching. This pattern presented two major peaks with a molecular weight range of approximately 2.5kDa-10kDa and 0.4kDa-2.3kDa, followed by two weaker peaks with a molecular weight range of 0.15kDa-0.3kDa and 0.06kDa-0.13kDa.

Free amino acids and 1kDa peptide fractions presented a high digestibility at all developmental stages studies in sole larvae while digestive capacity for the 7kDa protein fraction appeared to increase along larval development. Furthermore, results showed that protein fraction of 7kDa tended to be more retained in larval body and catabolized at a smaller rate by the larvae along their development.

These results support the notion that Senegalese sole larvae have difficulties in digesting complex proteins. This method may assist in defining the most suitable protein-ingredient types and molecular weight range, for different developmental stages of Senegalese sole and other fish larvae.

N.R. and S.E. are supported by grants from the Portuguese Foundation of Science and Technology (FCT), SFRH/BPD/ 23514/2005 and SFRH/BPD/ 49051/2008, respectively. This work was funded by Project EPISOLE (FCT) [PTDC/MAR/ 110547/2009], by project HYDRAA (FCT) [PTDC/MAR/ 71685/2006] and by project MICALA – I&DT Co-Promoção Nº 13380 (Portugal, supported by POAlgarve 21, QREN and European Union).

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

RECENT ADVANCES IN *SERIOLA DUMERILLI* CULTURE

J. Roo*, A. Mesa-Rodriguez, C. Hernández-Cruz, M. Izquierdo, and H. Fernández-Palacios

Grupo de Investigación en Acuicultura: Instituto Canario de Ciencias Marinas & Universidad de Las Palmas de Gran Canaria, PO Box 56, E-35200 Telde, Las Palmas, Canary Islands, Spain. *E-mail: jroo@iccm.rcanaria.es

Major commercial aquaculture interest in *Seriola* species focuses on the greater amberjack *S. dumerili* (Risso, 1810), Japanese amberjack *S. quinqueradiata* (Temminck and Schlegel, 1845), yellowtail amberjack *S. lalandi*, (Valenciennes, 1833), and longfin yellowtail *S. rivoliana* (Valenciennes, 1833). The greater amberjack is the preferred species of this genus for aquaculture diversification in the Mediterranean and Atlantic regions in UE. Despite some authors having had occasional success in obtaining gonadal maturation and spontaneous spawnings in this species, frequently final ovary maturation is inhibited in captivity. In the present study, modification of several aspects for broodstock management and hormonal induction resulted in obtaining large number of spawns with high quality that would provide reliable numbers of juveniles for the sustainable development of great amberjack culture. The results showed that intramuscular injections of 20µg.kg^{-1} body weight GnRHa, in males and females maintained under natural temperature and photoperiod conditions in the Canary Islands produced a high induction efficiency. Thus, the number of spawns (22) and female fecundity (2.48 million eggs.female^{-1}.spawning season) were similar to those of free wild populations and higher than those previously obtained in captivity for this species. Besides, with the exception of the two first spawns, high spawning quality was obtained (98.92% fertilized eggs, 92.58% hatched eggs, 68.31% larval survival), being higher than those obtained until present for this species. In addition, eggs obtained from those spawns were utilized to perform different larval rearing trials either in semi-intensive (SIS: 4.5 eggs.l^{-1} in 40-m^{3}tanks) or intensive (IS: 75 eggs l^{-1} in 2-m^3 tanks) culture systems. In addition, the first studies to established essential fatty acid requirements at *Artemia* feeding stage were conducted. An average hatching rate of 77.7±37.7% was obtained, while larval survival at 30dah was significantly improved with the use of semi-intensive conditions, with values up to 25.8%. On the other hand, increasing either DHA and EPA content in *Artemia* enrichment was positively correlated with larval survival and growth. Additionally, major problems at weaning phase and pre-growing will be addressed.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

GENETIC VARIABILITY OF WILD HORSESHOE CRABS (*TACHYPLEUS GIGAS*) FROM WEST COAST OF PENINSULAR MALAYSIA

M. Rozihan[1], E. Ismail[2], and B. Akbar John[3]

[1] Department of Aquaculture, Faculty of Agriculture, 43400 UPM, Serdang, Selangor, Malaysia
[2] School of Bioscience and Biotechnology, Faculty of Science and Technology, 43600 UKM Bangi, Selangor Malaysia
[3] Department of Biotechnology, Kulliyyah of Science, International Islamic University Malaysia, 25200, Kuantan, Pahang, Malaysia

Introduction

Horseshoe crabs are xiphosuran arthropods distributed within a restricted geographical boundary. Only four species of horseshoe crabs have been recorded on the continental shelf of the world's oceans from temperate to tropical latitudes. Interestingly, three out of the four species inhabit the Malaysian coastline with the restricted distribution of *Tachypleus tridentatus* along the East Malaysia (Borneo Island), while the nesting grounds of *T. gigas* and *Carcinoscorpius rotundicauda* were observed along the Peninsular Malaysia (Zaleha et al., 2012). Field observation proved their dwindling population size due to various anthropogenic activities that leads to shrinking of breeding zones along the nesting grounds in Malaysia (Akbar John et al., 2012). In the last few decades, serious attention has been paid to various molecular markers for their application in predicting the genetic richness in wild populations. Recently, Li et al. (2009) and Faurby et al. (2010) studied population structure and genetic diversity of Japanese *T. tridentatus* and American *Limulus polyphemus* using microsatellite markers respectively, while others used mitochondrial AT-rich region (Nishida and Koike, 2009). In Malaysia, we have also conducted studies to determine the *T. gigas* population subdivision using mtDNA AT-rich region and microsatellite markers (Rozihan and Ismail, 2011, 2012). Almost all previous studies concluded that horseshoe crabs have limited migratory ability among geographically distant populations. Thus, the present study was aimed to explore the genetic richness and dispersal ability of Malaysian horseshoe crab (*T. gigas*) distributed along the west coast of Peninsular Malaysia.

Materials and methods

Forty three *T. gigas* were collected from wild populations on the west coast of Peninsular Malaysia (Kedah = 6; Langkawi = 6; Selangor = 11; Johor = 20). Genomic DNA were extracted using Promega® DNA Purification Kit. Eighteen microsatellite loci developed for *L. polyphemus* by King and Eackles (2004) were used. Amplification of DNA was performed based on King et al. (2004).

Allelic frequencies, number of alleles (N), A*r* (allelic richness), expected heterozygosity (H_e), and observed heterozygosity (H_o) were calculated (under Hardy-Weinberg equilibrium) using FSTAT 2.93v (Goudet, 1995). A multilocus estimate of the genetic population heterogeneity (Pairwise F_{ST}) values among sites and their significant variation (Analysis of Molecular Variance, AMOVA) were calculated using ARLEQUIN3.1v (Schneider et al., 2000). An un-rooted neighbor-joining tree (NJ tree: Saitou and Nei, 1987) using the NEIGHBOR program in PHYLIP 3.6v.

Results

All microsatellite loci were highly polymorphic with loci-specific allelic variability. Highest allelic frequency was observed in LpoA26, LpoA44, and LpoA315 loci (0.21). Mean allelic frequency and allelic richness per locus showed no significant difference among populations (P>0.05). The H_o was lower than those H_e for all populations. Significant departures from HWE in the direction of heterozygote deficiency were observed in 39 out of 72 single loci. Inbreeding coefficient (F_{IS}) value showed restricted migratory pattern of horseshoe crabs between Southwest and Northwest populations. Genetic distances (GD) measures ranged from 0.0164-0.2442 (Table I). Highest GD value was observed between Johor and Kedah (0.2442) followed by Selangor and Kedah (0.1905). This observation clearly indicated the restricted migratory ability of *T. gigas* in west coast populations. Neighbor-joining (NJ) tree showed two apparent clusters segregating southwest population (Johor and Selangor) from Northwest (Langkawi and Kedah) samples (Fig. 1).

Table I: Estimates of pairwise genetic distance (Nei, 1978; below diagonal) and genetic differentiation F_{ST} (above diagonal).

	Johor	**Selangor**	**Kedah**	**Langkawi**
Johor	-	0.0090*	0.0000*	0.0000*
Selangor	0.0996	-	0.0000*	0.1171
Kedah	0.2442	0.1905	-	0.7207
Langkawi	0.1522	0.0660	0.0164	-

Note: *indicates statistical significance at P < 0.05 level

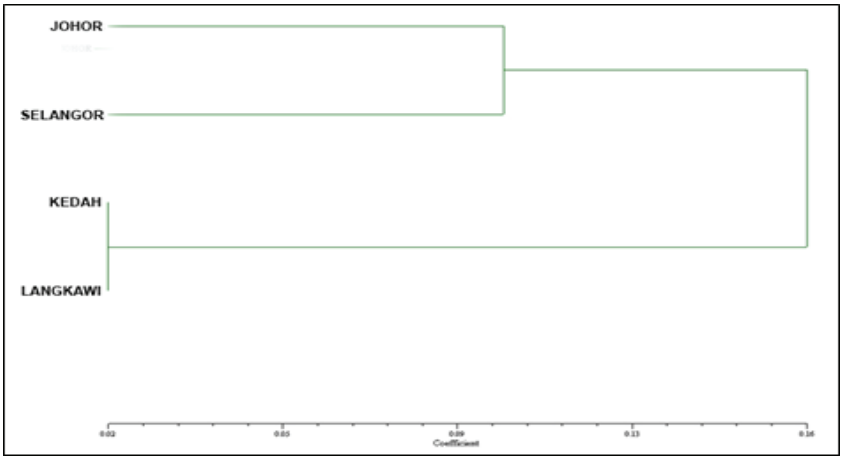


Fig. 1. Neighbor-joining tree shows genetic distance between four populations of *T. gigas* sampled from the west coast of Peninsular Malaysia.

Discussion

Results indicated restricted migratory ability of *T. gigas* along the west coast of Peninsular Malaysia. A similar observation was noted by King et al. (2005) who observed considerable genetic differentiation between the populations distributed in distant geographical region compared to little genetic variation among populations within a region. MtDNA and allozymes studies by Saunders et al. (1986) and Selander et al. (1970), respectively, revealed genetic differentiation among *L. polyphemus* population inhabiting Florida and north of Georgia. The observed less genetic distance between Johor vs. Selangor samples and Kedah vs. Langkawi population might be due to the migration of late spawning individuals between geographically closer populations that eventually reflected in GD data. Recently, mitochondrial AT-rich region analysis in *T. gigas* sampled from the Malaysian coastline (Rozihan and Ismail, 2011; 2012) and *T. tridentatus* from coastal waters of Japan (Yang et al., 2007) showed similar genetic distance pattern between populations. Faurby et al. (2010) also concluded that the horseshoe crabs have limited migratory capacity along the shores. Hence, knowledge of their wild population is essential for the strategic management and conservation of wild population in Malaysian coasts.

References

Akbar John B., B.Y. Kamaruzzaman, K.C.A. Jalal, and K. Zaleha. 2012. Sediment Profiling of the Nesting Grounds of Horseshoe Crabs at East Peninsular Malaysia. *International Journal of Biology* 4(2):159-165.

Faurby S., T.L. King, M. Obst, E.M. Hallerman, C. Pertoldi, and P. Funch. 2010. Population dynamics of American horseshoe crabs—historic climatic events

and recent anthropogenic pressures. *Molecular Ecology* 19(15):3088-3100. doi: 10.1111/j.1365-294X.2010.04732.x

Goudet J. 1995. Fstat Version 1.2: a computer program to calculate F-statistic. Journal of Heredity 86:485-486.

King T.L. and M.S. Eackles. 2004. Microsatellite DNA markers for the study of horseshoe crab (*Limulus polyphemus*) population structure. Molecular Ecology Note 4(3):394-396.

King T.L., M.S. Eackles, A.P. Spidle, and H.J. Brockman. 2005. Regional differentiation and sex biased dispersal among populations of the horseshoe crab *Limulus polyphemus*. Trans. Am. Fish. Soc. 134:441-465.

Li Q.-Z., Q. Li, J. Liu, L.-H. Ni, and J.-F. Kong. 2009. Isolation and characterization of microsatellite loci in the horseshoe crab (*Tachypleus tridentatus*). *Conservation Genetics* 10(6):1879-1881. doi: 10.1007/s10592-009-9844-x.

Nishida S. and H. Koike. 2009. Genetic Structure of Japanese Populations of *Tachypleus tridentatus* by mtDNA AT-Rich Region Sequence Analysis Biology and Conservation of Horseshoe Crabs. In J. T. Tanacredi, M. L. Botton, and D. Smith (Eds.), (pp. 183-196): Springer US.

Rozihan M. and E. Ismail. 2011. Genetic Structure and Haplotype Diversity of *Tachypleus gigas* Population along the West Coast of Peninsular Malaysia-Inferred through mtDNA AT Rich Region Sequence Analysis. *Biotechnology*.doi: 10.3923/biotech.2011.

Rozihan M. and E. Ismail. 2012. Impact of Malaysian Continental Drift on the Genetic Diversity of Horseshoe Crab Inferred through mtDNA Sequence Analysis. International Journal of Biology 4(1):104-110.

Saitou N. and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4(4):406-425.

Saunders N.C., L.G. Kessler, and J.C. Avise. 1986. Genetic variation and geographic differentiation in mitochondrial DNA of the horseshoe crab, *Limulus polyphemus.* Genetics 112:613-627.

Schneider S., D. Roessli, and L. Excofier. 2000. Arlequin: A Software for Population Genetics Data Analysis. Genetics and Biometry Lab, Department of Anthropology, University of Geneva, Switzerland.

Selander R.K., S.Y. Yang, R.C. Lewontin, and W.E. Johnson. 1970. Genetic variation in the horseshoe crab (*Limulus polyphemus*), a phylogenetic "relic". Evolution 24:402-414.

Yang M.-C., C.A. Chen, H.-L. Hsieh, and C.-P. Chen. 2007. Population Subdivision of the Tri-Spine Horseshoe Crab, *Tachypleus tridentatus*, in Taiwan Strait. *Zoological Science* 24(3):219-224. doi: 10.2108/zsj.24.219

Zaleha K., B. Akbar John, H.E. Atika, B.Y. Kamaruzzaman, and K.C.A. Jalal. 2012. Spawning and nesting behaviour of *Tachypleus gigas* along the east coast of Peninsular Malaysia. *International Journal of Biology* 4(2):102-111.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

VIRULENCE GENES AND QUORUM SENSING OF *VIBRIO HARVEYI*

H.A.D. Ruwandeepika[1,2,3], T. Defoirdt[1], T.S.P. Jayaweera[3], I. Karunasagar[2], and P. Bossier[1]

[1] Laboratory of Aquaculture and Artemia Reference Center, Ghent University, Rozier 44, 9000 Gent, Belgium
[2] UNESCO MIRCEN for Marine Biotechnology, Karnataka Veterinary, Animal and Fisheries Sciences University, College of Fisheries, Mangalore-575002, Karnataka, India
[3] Department of Livestock Production, Faculty of Agricultural Sciences, Sabaragamuwa University of Sri Lanka, Belihuloya, Sri Lanka

Introduction

Vibrio harveyi is a luminescent Gram-negative marine bacterium which is a major pathogen of a variety of hosts including shrimp and fish. Several virulence factors, including proteases and haemolysins, have been associated with pathogenicity of *V. harveyi*. The expression of virulence genes in vibrios is controlled by different regulatory systems, including quorum sensing. Quorum sensing, the bacterial cell-to-cell communication system, involves the production, secretion, and detection of extracellular signal molecules to regulate the expression of certain genes (Waters and Bassler, 2005). Three different quorum sensing signal molecules acting synergistically in gene regulation have been identified in *V. harveyi*: Harveyi Autoinducer 1 (HAI-1), Autoinducer 2 (AI-2) and Cholerae Autoinducer 1 (CAI-1).The signals are detected at the cell surface by membrane-bound, two-component receptor proteins that feed a common phosphorylation/dephosphorylation signal transduction cascade. Central in this signal transduction cascade is the LuxO protein. Phosphorylated LuxO indirectly inhibits production of the transcriptional regulator protein LuxR, whereas unphosphorylated LuxO is incapable of inhibition of LuxR production because of a conformational change. LuxR directly activates the *lux* operon and directly or indirectly controls many other quorum sensing target genes (Waters and Bassler, 2005). At this moment it is not yet so clear about the quorum sensing regulation of virulence genes in *V. harveyi*. Hence in this study, we quantified the expression level of the virulence regulators *luxR* and *toxR* and the virulence factors chitinase (*chiA*), phospholipase (*pl*), metalloprotease (*vhp*), serine protease (*srp*), and haemolysin (*vhh*) in quorum sensing mutants and in wild type isolate they were derived in vivo and in vitro. In Addition, this study was aimed to investigate the quorum sensing regulation on expression of type three secretion system

(TTSS) genes (*vopD*: *V. harveyi* outer protein, *vcrD*: *V. harveyi* calcium response protein, and *vscP*: *V. harveyi* secretion protein) of *V. harveyi* in vitro.

Materials and methods

In order to test whether quorum sensing regulates the expression of the virulence genes under investigation, used the *V. harveyi luxO* mutants JAF483 and JAF548, which contain a point mutation in *luxO*, resulting in LuxO proteins locked in the high and low cell density conformation respectively. Hence, the quorum sensing system is completely inactive in mutant JAF548 (QS-) and maximally activated in mutant JAF483 (QS+), irrespective of cell density or signal molecule concentration. Bacteria were obtained from Laboratory of Aquaculture and Artemia Reference Centre, Ghent University, Belgium, are listed in Table I. Cultures were grown in Marine broth (Difco Laboratories, Detroit, USA) and incubated for 24h at 28°C. The strains were grown to late exponential phase, and virulence gene expression was measured by reverse transcriptase real-time PCR as described before (Ruwandeepika et al., 2011) and expressed relative to the expression in strain JAF548 (in which quorum sensing is inactive). RNA extraction, reverse transcription and real time PCR was adopted from Ruwandeepika et al. (2011). Virulence genes (*vhh* hemolysins, *vhp* metalloproteases, *srp* serine protease), gene regulators (*luxR* quorum sensing master regulator and *toxR* transcriptional activator), and type three secretion system (TTSS) genes (*vopD*: *V. harveyi* outer protein, *vcrD*: *V. harveyi* calcium response protein, and *vscP*: *V. harveyi* secretion protein) were quantified by using ABI PRISM 7300 Fast Real Time System thermal cycler (Applied Biosystems) as described by Ruwandeepika et al. (2011) using gene-specific primers. Real-time PCR data was analyzed using the $2^{-\Delta\Delta CT}$ method taking the *rpoA* as housekeeping gene for calculating the gene expression level. In order to obtain more relevant data on virulence gene expression, the expression of the virulence genes was studied during the infection of gnotobiotic brine shrimp larvae, using a recently developed experimental procedure (Ruwandeepika et al., 2011).

Results and discussion

The quorum sensing master regulator gene *luxR* was used as a positive control. As was expected, the expression of the *luxR* gene was significantly higher in JAF483 (QS+) than in JAF548 (QS-) (Table I). The wild-type strain BB120 showed approximately the same expression level as JAF483; both showed around threefold higher expression when compared with JAF548 (Table I). This finding is consistent with the work of Tu and Bassler (2007), who reported that *luxR* mRNA levels increase fivefold in the presence of signal molecules under in vitro conditions. There were also significant differences between the mutants in expression levels of the *vhp* metalloprotease, with approximately sevenfold higher levels in JAF483 (QS+) than in JAF548 (QS-) (Table 1), thus indicating

that the *vhp* gene is positively regulated by quorum sensing. The expression in the wild type was even higher than in JAF483, although the difference was not significant. This is in line with a previous study by Mok and colleagues (2003) who found that the *proAC* metalloprotease is positively regulated by quorum sensing in *V. harveyi*, with a 25-fold higher promoter activity after adding AI-2. The *vhh* haemolysin, *srp* serine protease and *toxR* genes showed no difference in expression levels between JAF548 (QS-) and JAF483 (QSc), indicating that it is not regulated by the three channel quorum sensing signal transduction cascade. Phospholipase gene shown to be down regulated by QS in this study and to date, QS regulation of phospholipase has only been reported in the opportunistic human pathogen *Pseudomonas aeruginosa*. Barker et al. (2004) identified an extracellular phospholipase C gene, directs twitching mobility of *P. aeruginosa* up a gradient of certain phospholipases. The authors noted that this gene is regulated by AHL quorum sensing. In addition, the *chiA* gene also showed the negative regulation by QS and it is also in line with the previous studies done by Chritensen et al., 2003. Hence, QS regulation is different for different virulence factors, with some being positively regulated, others negatively regulated and others independent as has also been seen in other aquaculture pathogens such as *Aeromonas* sp. and *Edwardsiella sp*. Further this study revealed that expression of all three genes of TTSS were negatively regulated by quorum sensing having significantly lower expression in QS+ (Fold-expression 1, 1.1 and 1.1 for *vopD*, *vcrD* and *vscP* respectively) compared to QS- mutant (Fold-expression 27.4, 19.7 and 13.4 respectively at $P < 0.05$). This might reflect the need to produce the different virulence factors at different stages during infection, with virulence factors that are negatively regulated by QS being predominantly needed early during infection and virulence factors that are positively regulated by QS being required at later stages. In general, the same trend could be observed both under *in vivo* conditions and in vitro for virulence regulators *luxR* and *toxR* and the virulence factors chitinase (*chiA*), metalloprotease (*vhp*), serine protease (*srp*) and haemolysin (*vhh*) although differences in expression were more pronounced in vivo (Fig. 1).

Table I. Expression of *luxR*, *toxR*, serine protease (*srp*), metalloprotease (*vhp*), hemolysin (*vhh*) chitinase (*chiA*), phospholipase (*pl*) and TTSS genes relative to *rpoA* mRNA in late log phase cultures of *V. harveyi* wild type and quorum sensing mutants*.

Strain	*luxR*	*toxR*	*srp*	*vhp*	*vhh*	*chiA*	*pl*	*vopd*	*vcrd*	*vscp*
BB120	3.5 ^{b}	1.4 ^{A}	1.6 ^{i}	13.4 ^{u}	1.7 ^{x}	1.6 ^{p}	0.4 ^{s}	7.1	4.3 ^{u}	1.9 ^{c}
JAF483	3.3 ^{b}	0.8 ^{A}	0.8 ^{i}	6.8 ^{z,u}	1.1 ^{x}	0.2 ^{q}	0.4 ^{s}	1.0	1.0 ^{u}	1.0 ^{c}
JAF548	1.0 ^{a}	1.0 ^{A}	1.0 ^{i}	1.0 ^{x}	1.0 ^{x,y}	1.0 ^{p}	1.0 ^{t}	26.3	18.2 ^{v}	12.2 ^{d}

*Values in the same column with a different superscript letter are significantly different from each other (P<0.01). BB120 is the wild type of *V. harveyi* from which strains JAF483 and JAF548 are derived. Jaf483 is QS maximally active mutant and JAF548 is QS inactive mutant.

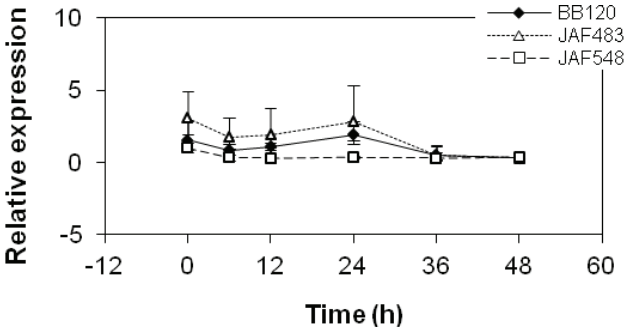


Fig. 1. In vivo expression of vhp metalloprotease mRNA in wild type *V. harveyi* BB120 and the quorum sensing mutants JAF483 and JAF548 (in which quorum sensing is maximally active and completely inactive, respectively) during infection of brine shrimp larvae.

Conclusion

This study concludes that *vhp* and *luxR* genes are positively regulated by the QS whereas *chiA*, *pl* genes, and genes of TTSS are negatively regulated. Meanwhile some genes such as *toxR*, *srp*, and *vhh* are shown to be independent from QS.

References

Barker A.P., A.I. Vasil, A. Filloux, G. Bai, P.J. Wilderman, and M.L. Vasil. 2004.. A novel extracellular phospholipase C of Pseudomonas aeruginosa is required for phospholipid chemotaxis. *Mol. Microbiol*. 53:1089-1098.

Christensen A.B., K. Riedel, L. Eberl, L.R. Flodgaard, S. Molin, L. Gram, and M. Givskov1. 2003. Quorum-sensing-directed protein expression in *Serratia proteamaculans*. Microbiology 149:471-483

Mok K.C., N.S. Wingreen, and B.L. Bassler. 2003. *Vibrio harveyi* quorum sensing: a coincidence detector for two autoinducers controls gene expression. *EMBO J* 22:870-881.

Ruwandeepika H.A.D., T. Defoirdt, P.P. Bhowmick, I. Karunasagar, I. Karunasagar, and P. Bossier. 2011. *In vitro* and *in vivo* expression of virulence genes in *Vibrio* isolates belonging to the Harveyi clade in relation to their virulence towards gnotobiotic brine shrimp (*Artemia franciscana*) *Environmental Microbiology* 13(2):506-517

Waters C.M., and B.L. Bassler. 2005. Quorum Sensing: Cell-to-Cell Communication in Bacteria. *Ann Rev Cell Dev Biol* 21:319-346.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

LIPID DIGESTION IN FIRST FEEDING LARVAE – VISUALIZATION IN VIVO

Ø. Sæle[1]*, K.E.L. Rød[1], and S. Farber[2]

[1] NIFES, Strandgaten 229, 5004 Bergen, Norway (*oystein.saele@nifes.no)
[2] Carnegie Institution for Science, Department of Embryology, 3520 San Martin Drive, Baltimore, Maryland 21218, USA.

The study of digestion in fish larvae presents major challenges because of their small size. Cod larvae are not longer than ~4.5mm standard length and a zebrafish larva is ~4mm. As a consequence, we have tried to describe digestive capabilities with studies of digestive enzyme activity via mRNA expression in homogenates of whole larvae. These methods have great limitations when the goal is to shed light on larval ability to digest a nutrient. Enzyme activity in a whole animal is difficult to interpret as there are often several enzymes in multiple tissues that hydrolyze the same substrate.

Experiments where newly hatched larvae are fed with radiolabeled lipids have been very valuable in the study of lipid digestion, but this method is limited because one cannot distinguish between undigested lipids in the gut and lipids which are digested, absorbed, and re-synthesized in the animal. We have therefore, in cooperation with Avanti Polar Lipids, Inc. (USA), developed fluorescent lipids that make it possible to characterize lipid digestion in living larvae, regardless of size. These lipids (triacylglycerol (TG) and phospholipid (PL)) may be labeled with a fluorochrome on two fatty acids (FA).

When a fish larva is fed TG or PL with two labeled FAs, digests and absorbs the free FAs and then re-synthesizes TG and PL in the intestine, the vast majority of the re-synthesized lipids will have only one labeled FA. This is because the diet consists of a large amount of unlabeled TG and PL in comparison to labeled TG and PL. Since TG and PL with only one labeled FA have a different character than lipids with two labeled FAs, they can be separated and quantified with HPLC, thus quantifying digested and re-synthesized lipids. The lipids are detected with fluorescence which makes it a very sensitive method. Both TG and PL can be quantified below 1pmol.

By feeding zebrafish larvae emulsions with different TG and PL compositions we have characterized the ability of the larvae to digest, absorb, re-synthesize, and metabolize both TG as well as PL.

To measure digestion of TG we have also developed a quenched TG molecule that increases fluorescence when it is digested. By using this molecule as a substrate in “traditional” in vitro lipase assays, the sensitivity has increased many folds. This is an advantage when working with larvae.

Results using these methods will be presented and discussed.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

THE SELF-FERTILIZING MANGROVE KILLIFISH *KRYPTOLEBIAS MARMORATUS* AS A MODEL FISH FOR AQUACULTURAL STUDY

Y. Sakakura[1], K. Suga[1], A. Kanamori[2], and A. Hagiwara[1]

[1] Graduate School of Fisheries Science and Environmental Studies, Nagasaki University, Nagasaki 852-8521, Japan
[2] Graduate School of Science, Nagoya University, Nagoya 464-8602, Japan

The mangrove killifish *Kryptolebias marmoratus* is Cyprinid fish, which is the only known self-fertilizing vertebrate. This species is small in body size (up to about 5cm), and has a relatively short generation time (about 100days). It is found in tropical mangrove swamps in a harsh environment where water temperature ranges 18-36°C and salinity fluctuates 0-70ppt. *K. marmoratus* is so tolerant to handling stress that it can thrive in captivity and can be reared in a small jar (about 100ml volume) individually. This species is not an aquaculture or ornamental species, however, it has been used for cancer research and ecotoxicology. *K. marmoratus* also has many advantageous traits as a model animal for ontogeny, growth, fecundity, behavioral, and breeding studies because it is easy to establish clonal lineages via selfing, and we can track all individual records.

Feeding success and nutrition at the first feeding phase of marine fish larvae are very important factors to achieve satisfactory results of larviculture. Copepods have been recognized as the most suitable feed for early stages of fish larvae because of their nutritional advantage compared with other live feeds such as rotifers and *Artemia*. However, less emphasis has been given on the effects of copepods on the behavioral development of fish, particularly on their feeding and swimming behavior. Behavioral observations are useful in understanding patterns of prey selection and have important implications on metabolic energy costs. Therefore, we conducted small-scale rearing experiments and behavioral observations of the mangrove killifish individually using different live feeds and artificial diets. Nine types of live feeds; 3 morphotypes of rotifers (L, S, SS), first instar *Artemia franciscana*, *Fabrea salina*, copepods (*Acartia tsuensis*, *Tigriopus japonicus*), cladocerans (*Diaphanosoma celebensis*, *Moina mongolica*), and commercial pellets (400 and 700μm) were used for 10-day individual rearing of the mangrove killifish, and feeding behavior and growth of fish were compared. With rotifers (L, S, and SS types) and newly hatched *Artemia* nauplii as food, all the larvae showed maximum feeding success throughout the experi-

mental period. Significantly greater growth was observed after 10days rearing not only with L type rotifer and *Artemia* nauplii, but also copepods and cladocerans that showed significantly lower feeding success throughout the experiment. It is noteworthy that the 'hard to catch' live feed can enhance growth performance of fish larvae. When 3 types of diets (*Acartia tsuensis* 104-732μm, unenriched and DHA-enriched *Artemia franciscana* nauplii 656-906μm), were fed for 20days to *K. marmoratus* larvae, *Acartia*-fed larvae had significantly lower growth than fish fed DHA-enriched *Artemia*. Feeding success was highest in larvae fed *Acartia* on day 1. Ingestion rate and satiation time did not differ among fish fed different types of feeds until day 20. Swimming activity before feeding was significantly lower in larvae fed *Acartia* compared with larvae fed *Artemia* until day 10. Higher growth in *Acartia*-fed fish on day 10 is probably due to the suitable size and high DHA content of *A. tsuensis*, and lower swimming activity of the larvae. However, on day 20, lower growth observed in *Acartia*-fed fish may be attributed to the shift in the food size preference of the fish.

We have been keeping two lineages of the mangrove killifish, namely PAN-RS strain from Panama and DAN strain from Belize. Each strain is homozygotic (clonal), and these 2 strains are genetically different each other, which is confirmed by AFLP, microsatellites, and SNP. Furthermore, these 2 strains have significantly different traits, where the PAN-RS strain shows higher growth, aggression, and fecundity than the DAN strain. Expression of growth-related genes in PAN-RS such as growth hormone, IGF-families, and their receptors are significantly higher than those of DAN, indicating that these 2 strains can be quite a simple model to interpret the differences in life history traits. Recently, we successfully produced a hybrid individual of these 2 strains whose descendants are genetically heterozygotic and have a wide variety of traits from parental strains. Since these descendants reproduce by selfing, it is easy to obtain recombinant inbred strains, which is major approach for plant and animal breeding. We found a particular lineage from this hybrid showing high growth and low aggression, which are valuable traits for aquaculture. We have been tracking the mode of inheritance of traits in the hybrid lineages for more than 5 generations using morphometric characters, behavioral observation, and co-dominant genetic markers (SNP).

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

OPTIMUM PHOSPHOLIPIDS AND ANTIOXIDANT LEVELS TO DEVELOP NOVEL MICRODIETS FOR GILTHEAD SEA BREAM LARVAE

R. Saleh[1,2*] and M. Izquierdo[1]

[1] Grupo de Investigación en Acuicultura (IUSA & ICCM), University of Las Palmas de Gran Canaria, Carretera de Taliarte, s/n, 35200 Telde, Gran Canaria, España
[2] Oceanography Department, Faculty of Science, Alexandria University, 21515 MoharramBek, Alexandria, Egypt

Phospholipids (PL) are particularly important in larval fish production for their essential function as necessary components for cellular biomembrane and organelle formation, as well as for being an endogenous energy source during early development. Dietary PL has also been related to digestive system maturation, may promote digestive enzymes activities, and consequently play an important role on larval digestive physiology and the metabolic pathways of the assimilated nutrients. The quantitative requirements of PL for larval fish range from about 2–12% of diet, being lower in the larvae of common carp (2% chicken egg), 5% soybean lecithin (SBL) for red sea bream, 7.4% SBL for knife jaw, 7% SBL for Japanese flounder, with the highest reported value being for sea bream 15% SBL. However, for the latter species, our recent studies have found an optimum inclusion of about 10% using krill PL as a PL source and 8% using SBL. Inclusion of SBL in early weaning diets improves survival, growth and stress resistance in sea bream, European sea bass and pikeperch larvae. Also, it increases the digestive enzyme activities, particularly PLA2 and alkaline phosphatase, in sea bream and sea bass. In addition, inclusion of dietary SBL for sea bream and sea bass enhances the gut and liver lipid transport activity, improving the absorption of several nutrients from the lumen. This results in high incorporation of n-3 HUFA, particularly DHA into larval polar lipids.

PL include a large group of compounds and their functions in larval development could be related to their specific composition, both in type of lipid classes and fatty acids contents. For instance, inclusion of marine-origin PL like bonito eggs and krill PL, rich in n-3 HUFA and PC, improves growth, resistance to stress, and survival of larval ayu, Atlantic cod, and sea bream more effectively than PL from a vegetal source. Recently, sea bream growth, hepatic utilization of dietary lipids, and gut health were all improved when 2.5% dietary SBL was substituted by krill PL. Moreover, dietary MPL reduces skeletal malformations compared to dietary SBL in sea bream larvae, which could be related to a higher

resistance to vertebral deformities by a stronger mineralization as suggested by the up-regulating of the skeletal extracellular matrix proteins BMP-4, Runx2, ALP, osteocalcin, and osteopontine. This up-regulation caused by the higher larval n-3 HUFA content, especially DHA, has been observed in other vertebrates. On the contrary dietary inclusion of SBL markedly increased the oxidative risk of seabream larvae compared to MPL, as denoted not only by the higher MAD values obtained, but also by the higher gene expression of enzymes involved in the oxidative metabolism such as SOD, CAT, and GPX. This peroxidation is highly deleterious, resulting in damage to cellular biomembranes and to molecules of high biological importance such as lipids, proteins, carbohydrates, and/or DNA and may retard growth and survival and increase deformities and occurrence of pathological conditions in fish. Thus, to avoid adverse effects and improve high dietary n-3 HUFA performance, it is necessary to supplement antioxidants such as α-tocopherol to larval diets. Thus, increasing dietary α-tocopherol improves marine fish larval growth in terms of body weight, in relation to dietary n-3 HUFA levels, illustrating its protective role against oxidation. As well, peroxidation products in the liver of sea bream fed oxidized oil supplemented with vitamin E are reduced. Increased α-tocopherol levels in inert diets or rotifers for sea bream larvae reduces MAD content and oxidative enzyme gene expression, alleviating muscular lesions caused by excessive dietary DHA. Vitamin E is important for proper skeletal development since it associates with the lipid bilayer of bone cells as the first line of defense against free radicals. The inclusion of complementary antioxidative factors such as selenium (Se) could also counteract the high oxidation risk in early weaning diets high in HUFA. In fish, Se is also involved in protecting lipid components and membranes at both the cellular and subcellular level from oxidative damage. Se deficiency can lead to oxidative stress in organs, reduced growth, and increased mortality in several fish species. Increase in Se up to 11.65mg.kg^{-1} diet for sea bream significantly improved larval survival and resistance to stress, whereas it did not affect larval growth. The degree of larval lipid oxidation, as indicated by malondialdehyde (MDA) content and AOE genes expression, was significantly lower in those larvae fed diets high in Se (8.47 and 11.65mg.kg^{-1} diet) levels compared with those of lower Se levels (1.73 and 3.91mg.kg^{-1}). Also, a reactive response as a result of Se inclusion was observed by the increase in osteocalcin, osteonectin osteopontin, alkaline phosphatase, and matrix gla protein gene expression in larval tissues, suggesting its implication in skeletal development. In conclusion, not only is necessary to include PL in larval diets, but also the type of PL is important as well as the ratio with antioxidant nutrients such as α-tocopherol and Se.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EUROPEAN SEA BASS LARVAL EARLY WEANING DEVELOPMENT USING GREENWATER AND SYNBIOTIC IN ALEXANDRIA, EGYPT

Ahmed. Md. Salem[1,2*], A.M. Nour[3], S.S. Assem[1], T.M. Srour[2], H.S. El-Sayed[1], and H.A. Ibrahim[4]

[1] Fish Reproduction & Spawning Lab., Aquaculture Division, (NIOF), Alexandria, Egypt
[2] Animal and Fish Production Dep., Faculty of Agriculture (Saba basha), Alexandria University (*E-mail: Email: ahmedmdsalem@yahoo.com)
[3] Animal and Fish Production Dep., Faculty of Agriculture (El-Shatby), Alexandria University
[4] Marine Microbiology Lab., Marine Environment Division, (NIOF), Alexandria, Egypt

Introduction

FAO aquaculture species increased to 541 species in 2010, including 327 finfishes (FAO, 2012). Turkey is the major producer of European sea bass (*Dicentrarchus labrax*), Egypt is the main producer of mullets (*Mugilidae* spp.) (FAO, 2013) and is the 8th largest world aquaculture producer and the top Mediterranean Sea, Arab, and African aquaculture producer (FAO, 2012). Egyptian aquaculture reached 986 820 tons (72.44% of the total fisheries production) in 2011, and is divided among tilapia (61.88%), mullets (11.55%), carps (20.64%), catfishes (1.34%), sea bass (1.80%), sea bream (1.43%), meagre (1.23%), and shrips (0.08%) (GAFRD, 2011). Egypt's European sea bass production in 2011 was 3084 tons: 1313 tons from capture fisheries (7%) and 17 714 tons from aquaculture (97%). Although Egypt has six marine hatcheries, there is still a reliance on wild fry collection – e.g., 63 million mullet and shrimp – and total marine fry production from hatcheries in 2011 was 15.8 million fry, though only 1.6 million fry were European sea bass (GAFRD, 2011). The rapid growth of fish culture was initiated by the technological breakthrough of seed production in 1980s. Since then it has been accompanied by increased disease problems. Disinfectants and antimicrobial drugs have had little effect in controlling diseases (Qi, 2008). Few studies have focused on combining probiotics and prebiotics into the same synbiotic, and information on their impacts in live feeds (rotifers and *Artemia*) and European sea bass larval rearing and weaning is limited and scattered. For this reason, this study was carried out in the Fish Reproduction and Spawning Lab, Aquaculture Division, NIOF, Alexandria, Egypt.

Materials and methods

Three experiments were conducted to study the effects of using greenwater (G) and greenwater plus synbiotic (S) on rotifers (*B. plactilis*), *Artemia* (*A. fransiscana*) enrichment for 4 and 6h, respectively, and European sea bass (*D. labrax*) larvae until 40dph early weaning using treatments enriched with *Artemia* and rotifers. Larval rearing and feeding protocol were: 50 fertilized eggs l^{-1} were stocked in well-aerated 30-l duplicate aquaria, larval first-feeding was on 7-15dph using enriched rotifers, 10-35dph larvae were co-fed on *Artemia* nauplii and metanauplii, and from 25-40 larvae were co-fed using O.Range, INVE microdiets and protocols. Water was exchanged 10% daily and siphoned. The greenwater used *Nanochlorapsis salina* as positive control, Synbiotic was used in multi-bacterial powder phase containing 20 billion CFU 500 g^{-1} (*Lactobacillus acidophilus, L. casei, L. plantarum, Enterococcus faecium, Bacillus subtilis,* and *B. licheniformis,* fungal Probiotic: bakery yeast *Saccharomyces cerevisiae,* probiotics in 200 billion CFU.$500g^{-1}$, fructooligosaccharides phytobiotic in $10g.500g^{-1}$ and mannaoligosccharides in $10g.500g^{-1}$ prebiotic, and enzymes. The synbiotic dose in these experiments was 50 million CFU per tank. The quality and development in *Artemia* and rotifers was measured according to fatty acid content, bacterial counts, (Total bacterial counts (TCB), *Aeromonas* counts (ABC), *Staphylococcus* counts (SBC), *Vibrio* counts (VBC), and *Bacillus* counts (BBC)), and in early weaned 40-dph larvae using length, fatty acids, water quality, and bacterial counts. Statistical analysis was done according to Basic Statistics ANOVA, STATISTICA for Windows (StatSoft, Inc. 1995).

Results and discussions

Greenwater plus synbiotic recorded the best significant ($P<0.05$) final total length (FTL), total length gain in mm (LG), total length average daily gain in mm d^{-1} (LADG), total length specific growth rates % d^{-1} (LSGR %), total length gain % d^{-1} (LG %), and survival % while the smallest significance ($P<0.05$) was noticed by greenwater (Table I). Greenwater plus synbiotic showed great and promising results in most microbiological parameters increasing TBC and BBC and reducing potential pathogenic bacteria (ABC, SBC, and VBC) compared with greenwater control but not all had significant differences in rotifers and *Artemia*, and in early weaned larval tanks, water quality were great in both treatments. *Lactobacillus bulgaricus, L. acidophillus, L. sporogenes, L. casei, L. plantarum, Bacillus* sp., and *Streptococcus thermophillus* are commonly used as probiotic in animal nutrition (Ringo and Gatesoupe, 1998; Jacobsen et al., 1999; Venkat et al., 2004; Wang and Xu, 2006 and Suzer et al., 2008). The beneficial effects of these probiotics include higher growth and feed efficiency, prevention of intestinal disorders, and pre-digestion of anti-nutritional factors present in the ingredients. Also, probiotics had resistance to some aquatic pathogens such as *Vibrio* spp. (Villamil et al., 2003 and Planas et al., 2006). Algal probiotics im-

proved growth and quality of Gilthead sea bream newly hatched larvae (Salem and Hebalah, 2012).

Total saturated, mono-unsaturated FA, total n-6 HUFA, LA C18:2 FA, eicosadienoic acid C20:2, mead acid C20:3, ARA C20:4, DHA:EPA, and ARA:EPA:DHA of enriched rotifers showed no significant differences and the highest achieved by (G). The total n-3 HUFA, ALA C18:3, EPA C20:5, DHA C22:6, n-6:n-3, EPA:DHA, and ARA:EPA showed no significant differences and the highest detected by (G) as showed in Table II. Total saturated, total n-6 HUFA, LA C18:2, ARA C20:4, ALA C18:3, n-6:n-3, and ARA:EPA of enriched *Artemia* showed no significant differences and the highest achieved by (S), total mono unsaturated FA, eicosadienoic acid C20:2, mead acid C20:3, total n-3 HUFA, EPA C20:5, DHA C22:6, DHA:EPA, and the EPA:DHA showed no significant differences and the highest achieved by (G) and docosadienoic acid C22:2 and ARA:EPA:DHA showed the highest significance ($P<0.05$). Total saturated, total mono unsaturated, total n-6 HUFA, LA C18:2, ARA C20:4, total n-3 HUFA, ALA C18:3, EPA C20:5. and EPA: DHA of early weaned larvae showed no significant differences and the highest achieved by (S). Docosadienoic acid C22:2 and DHA C22:6 showed no significant differences and the highest achieved by (G). The n-6:n-3 ratio, the ARA: EPA, DHA:EPA, and ARA:EPA:DHA showed the highest significant ($P<0.05$) achieved by (S). The "greenwater" technique is part of the commonly applied techniques for rearing larvae of gilthead sea bream *Sparus aurata* (Lavens and Sorgeloos, 1996). Supplying microalgae directly to the larval rearing tank contributes towards maintaining the nutritional quality of rotifers up to their ingestion by the larvae. Rotifers can also be enriched to perform experiments in larval fish nutrition, such as vitamins, iodine, or selenium (Gimenez et al., 2007; Hamre et al., 2008; Conceicao et al., 2009). Essential fatty acids have important functions on larval development. EPA is effective in promoting larval survival while DHA appears to be particularly important for promoting larval growth (shortening larval duration) and development of neural tissues such as the brain and retina. ARA promotes growth, survival, and improves resistance to acute stress in marine larvae and post-larvae (Figueiredo et al., 2012). Naz (2008) revealed that in sea bass fertilized eggs, HUFA were higher than saturated fatty acids, as found by Bulut et al. (2004). In addition, the fertilized eggs and yolk-sac larvae were rich in the n-3, EPA, and DHA.

Table I. Effect of using greenwater (G) and greenwater plus Synbiotic (S) on European sea bass (*D. labrax*) newly hatched larvae length gains between 7dph as initial and 40dph as final lengths in mm.

Parameter	FTL	LG	LADG	LSGR%	LG%	Survival %
G	9.500^{b}	5.318^{b}	0.161^{b}	2.481^{b}	127.291^{b}	7.592^{b}
S	11.383^{a}	7.222^{a}	0.219^{a}	3.046^{a}	173.693^{a}	20.111^{a}

Letters in the same row are for effects difference significance ($P<0.05$).

Conclusions

Greenwater plus synbiotic in enriched *Artemia* and rotifers showed great and promising results in sea bass larvae not only in growth and quality but also in improving potentially useful bacteria and controlling potentially pathogenic bacteria throughout critical larval rearing and weaning tanks. These could be one of the first steps for marine hatchery development in lesser-developed countries leading to marine aquaculture development.

Table II. The effects of greenwater (G) and greenwater plus Synbiotic (S) on 4h-enriched rotifers, 6h-enriched *Artemia*, and 40dph larvae fatty acids % of total lipids.

Fatty Acid		Rotifers		*Artemia*		40dph larvae	
		G	S	G	S	G	S
∑ Saturated		17.613	12.111	27.825	29.912	16.103	26.286
∑ Mono unsaturated		4.285	2.7613	13.651	12.843	9.002	15.415
LA n-6	C18:2	0.953	0.737	25.488	30.642	2.903	6.824
Eicosadienoic acid	C20:2	0.706	0.00	1.022	0.00	0.00	0.00
Mead acid	C20:3	0.338	0.00	0.544	0.00	0.00	0.00
ARA n-6	C20:4	0.666	0.144	0.539	1.362	0.00	1.441
Docosadienoic acid	C22:2	1.071	0.394	0.243^{b}	1.052^{a}	0.343	0.338
∑ n-6 HUFAs		3.734	1.275	27.835	33.056	3.246	8.604
ALA n-3	C18:3	0.111	0.122	0.534	0.542	0.00	0.008
EPA n-3	C20:5	0.680	0.297	0.605	0.311	0.545	1.098
DHA n-3	C22:6	0.702	0.00	1.033	0.761	1.735	1.599
∑ n-3 HUFAs		1.492	0.419	2.172	1.614	2.279	2.704
∑ HUFAs		5.225	1.695	30.007	34.670	5.526	11.308
∑ Unsaturated		9.510	4.456	43.658	47.513	14.527	26.724
n-6:n-3		2.335	2.872	12.609	20.824	1.287^{b}	3.162^{a}
DHA:EPA		0.616	0.00	7.615	2.535	3.503	1.716
EPA:DHA		0.406	0.00	1.299	0.408	0.292	0.648
ARA:EPA		1.067	1.154	2.858	4.474	0.00^{b}	1.274^{a}
(ARA:EPA):DHA		0.442	0.00	2.136^{b}	5.883^{a}	0.00^{b}	0.816^{a}

Letters in the same row in the same sub table are for effects difference significance ($P<0.05$).

References

Conceicao L., M. Yufera, P. Makridis, S. Morais, and M.T. Dinis. 2009. Live feeds for early stages of fish rearing. Aquaculture Research 41(5):613-640.

Figueiredo J., J. Lin, J. Anto, and L. Narciso. 2012. The consumption of DHA during embryogenesis as an indicative of the need to supply DHA during early larval development: A Review. Journal of Aquaculture Research and Development 3:140.

Lavens P. and P. Sorgeloos.1996. Manual on the production and use of live food for aquaculture. FAO Technical Papers 361. FAO, Rome, Italy, 295pp.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

AN ASSESSMENT OF BIOSECURITY AND HAZARD MANAGEMENT PRACTICES IN THE LARVICULTURE OF SHRIMP (*PENAEUS MONODON*), BANGLADESH

S.M. Sharifuzzaman and S. Adhikari

Institute of Marine Sciences and Fisheries, University of Chittagong, Chittagong- 4331, Bangladesh

Introduction

The aquaculture of black tiger shrimp (*Penaeus monodon*; locally known as 'bagda chingri') plays an important role in the economy of Bangladesh by fetching valuable foreign exchange, generating employment, and supplying the protein requirements. However, in addition to frequent growout production losses due to white spot syndrome virus (WSSV), a major bottleneck toward reliable shrimp farming is inadequate supply of post-larvae. This is due to higher outbreaks of diseases in larviculture caused by viruses, bacteria, and other undiagnosed and emerging pathogens (Austin, 2005; Bondad-Reantaso et al., 2005) and also due to ever declining catches of wild larvae. Therefore, to meet the post-larval demand and to sustain profitable farming, the application of probiotics and the improvement of husbandry, hygiene, and biosecurity practices are currently viewed as extremely important, principally as an alternative to antibiotics, to promote health management (i.e., disease prevention and control) and production of healthy and good quality fry.

Biosecurity measures prevent the introduction of pathogens and their subsequent proliferation in the rearing system and to the environment, in the event of diseases (Lotz, 1997). Thus, biosecurity in aquaculture involves some of procedures related to disinfection, sanitary measures, personnel hygiene, and hazard identification and disease monitoring. Non-compliance with these measures remains the key factor affecting the success of coastal shrimp culture in Bangladesh. For example, the imported post-larvae, which were transported from Thailand in 1994 and subsequently stocked for growout without quarantine, were believed to introduce viral disease into the country. Since then, shrimp farming is low-yielding and less profitable due to prevalence of WSSV in broodstock and post-larvae. Additionally, water intake and wastewater drainage points for many hatcheries used to be in the same location, generating a contaminated/polluted water source for the remaining users. This study aims to assess biosecurity main-

tenance and hazard management practices in the larviculture of shrimp at Kalatoly hatchery zone of Cox's Bazar, Bangladesh.

Materials and methods

Twenty four shrimp larviculture facilities, which were located at the Kalatoly hatchery zone of Cox's Bazar along the Bay of Bengal, were chosen and 10 of them randomly selected for this study, assuming a similar production capacity for the hatcheries. Biosecurity and hazard management practice data (i.e., quarantine facility, disinfection, waste treatment, sanitary measures, personnel hygiene, pollution free water source, microbiology, and PCR test) and other related information were collected through a semi-structured questionnaire survey and interviews, examination of records, and systematic observation of hatchery establishments. After three months, a follow-up questionnaire survey and interviews were done to revise the initial information provided by the interviewee and to enhance the reliability of the data as well as to address any existing gaps.

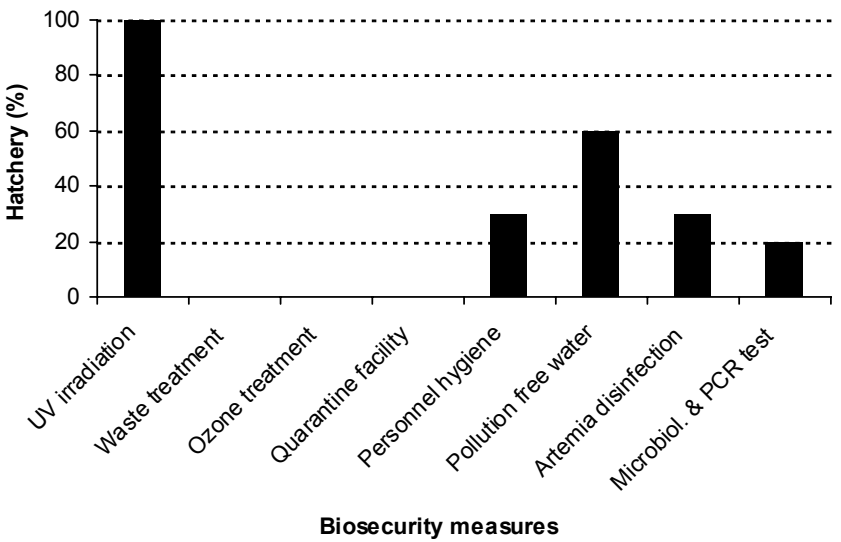


Fig. 1. Compliance of biosecurity measures by the shrimp hatchery.

Results and discussion

The results indicate that 40% hatcheries did not have access to pollution-free water sources, and surprisingly water intake/discharge points for many hatcheries were located in same site. Not a single hatchery used ozone treatment for disinfection and quality improvements of system waters, and they discharged wastewater into the environment without any treatment. Although a separate broodstock maturation room was noted for 70% hatcheries, the quarantine unit was totally absent among the hatcheries. Only 20% hatcheries were equipped

with on-site disease diagnostic laboratory for microbiology and PCR testing (i.e., for screening of spawners, eggs, and larvae for WSSV and other microbial pathogens), and 30% hatcheries were using disinfected *Artemia* nauplii as larval feed. There was a lack of awareness about personnel hygiene, and the workers and technicians of many hatcheries were ignoring simple rules of hand disinfection, foot-bath use, and wearing gloves, rubber boots, and protective clothing at the workplace (Fig. 1). These facts suggest that the shrimp hatcheries in Cox's Bazar, Bangladesh do not comply with standard biosecurity and hazard controlling measures, thus remain at high risk of disease outbreaks.

Ozone applications have been extensively examined as a means of disinfection and control wide-ranging diseases. Of relevance, Chang et al. (1998) demonstrated that a 10-min dose of $0.5\mu g.ml^{-1}$ ozone was effective enough to control white spot syndrome baculovirus (WSBV) infection in juvenile *P. monodon*. Moreover, *P. monodon* post-larvae fed probiotics and further treatment with residual ozone concentration at $0.35mg.l^{-1}$ for 30min inhibited 3 log units of *V. harveyi* for 24h, and thus significantly increased shrimp survival over controls (Meunpol et al., 2003). The quarantine measures and screening of broodstock or larvae for potential pathogens are viewed as extremely important to prevent vertical/horizontal transmission of pathogens from infected spawners to stocked post-larvae, or from batches of infected post-larvae in a growout pond with possible spread out to a neighboring pond or even to another farm (see Esparza-Leal et al., 2009), and to restrict trans-boundary movement of pathogens, such as the introduction of WSSV in Bangladesh due to importing of shrimp seeds without quarantine. *Artemia* nauplii, which are routinely used in penaeid shrimp larviculture, are reported as one of the major vectors to introduce microbial pathogens to the culture system (Lopez-Torres and Lizarraga-Partida, 2001). Moreover, marine microalgae have been identified as carriers of WSSV pathogen and may play an important role in the horizontal transmission of WSSV, especially during disease outbreaks (Liu et al., 2007). Therefore, the use of disinfected live foods in hatcheries should be achieved to avoid disease problems. Wastewater discharges without any treatment into the sea and neighbouring areas, as in the case of Kalatoly hatchery zone of Cox's Bazar, has the potential for spreading contamination/pollution and diseases. To handle this problem, the solid (including morbid shrimp) or liquid wastes contaminated with bacterial or viral pathogens can be heated, treated with chemicals (i.e., formic acid, sodium/calcium hydroxide, chlorine, iodine, and calcium oxide), and disinfected with ultraviolet light and/or ozone (Gill, 2000). The implementation of strict personnel sanitation and hygiene, including washing of hands and feet, and feed preparation with good hygienic standards, is also precondition in ensuring hatchery biosecurity. In light of the above considerations, it is essential to improve overall biosecurity and hazard controlling measures in shrimp hatcheries of Bangladesh for greater yields and economic returns.

Conclusions

The results of this study points out inadequate biosecurity and poor hazard management practices in the shrimp hatcheries of Cox's Bazar, Bangladesh. As a result, viruses and other pathogens are entering the hatchery systems via infected broodstock, intake of contaminated water or other sources, eventually generating poor quality post-larvae with low survival and frequent disease outbreaks, hindering the sustainability of this sector.

References

Austin B. 2005. Bacterial pathogens of marine fish. p.391-413. In: Oceans and Health: Pathogens in the Marine Environment. Belkin S., and R.R. Colwell (Eds.). Springer, New York. 464p.

Bondad-Reantaso M.G., R.P. Subasinghe, J.R. Arthur, K. Ogawa, S. Chinabut, R. Adlard, Z. Tan, and M. Shariff. 2005. Disease and health management in Asian aquaculture. Veterinary Parasitology 132:249-272.

Chang P-S., L-J. Chen, and Y-C. Wang. 1998. The effect of ultraviolet irradiation, heat, pH, ozone, salinity and chemical disinfectants on the infectivity of white spot syndrome baculovirus. Aquaculture 166:1-17.

Esparza-Leal H.M., C.M. Escobedo-Bonilla, R. Casillas-Hernández, P. Álvarez-Ruíz, G. Portillo-Clark, R.C. Valerio-García, J. Hernández-López, J. Méndez-Lozano, N. Vibanco-Pérez, and F.J. Magallón-Barajas. 2009. Detection of white spot syndrome virus in filtered shrimp-farm water fractions and experimental evaluation of its infectivity in *Penaeus* (*Litopenaeus*) *vannamei*. Aquaculture 292:16-22.

Gill T.A. 2000. Risk assessment and biosecurity. In: Waste from processing aquatic animals and animal products: implications on aquatic animal pathogen transfer. FAO Fisheries Circular No. 956. Rome, Italy. 33p.

Liu B., Z. Yu, X. Song, and Y. Guan. 2007. Studies on the transmission of WSSV (white spot syndrome virus) in juvenile *Marsupenaeus japonicus* via marine microalgae. Journal of Invertebrate Pathology 95:87-92.

Lopez-Torres MA, and M.L. Lizarraga-Partida. 2001. Bacteria isolated on TCBS media associated with hatched *Artemia* cysts of commercial brands. Aquaculture 194:11-20.

Lotz J.M. 1997. Disease control and pathogen status assurance in an SPF-based shrimp aquaculture industry, with particular reference to the United States. p.243-254. In: Diseases in Asian Aquaculture III. Flegel T.W., and I.H. MacRae (eds.). Fish Health Section, Asian Fisheries Society, Manila.

Meunpol O., K. Lopinyosiri, and P. Menasveta. 2003. The effects of ozone and probiotics on the survival of black tiger shrimp (*Penaeus monodon*). Aquaculture 220:437-448.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

THE EFFECTS OF DIETARY POLY-B-HYDROXYBUTYRATE ON GROWTH, FEED UTILIZATION, AND SURVIVAL OF NILE TILAPIA (*OREOCHROMIS NILOTICUS*) FRY

M.L. Situmorang, K. Dierckens, and P. Bossier

Laboratory of Aquaculture & Artemia Reference Center (ARC), Department of Animal Production, Ghent University, Rozier 44, 9000 Ghent, Belgium

Introduction

Poly-β-hydroxybutyrate (PHB) is a natural polymer that is intracellularly accumulated as a carbon and energy reserve by a wide variety of bacteria and can be depolymerised into water-soluble short-chain fatty acid (SCFA) monomers. It has shown the potential to be used as an alternative anti-infective strategy for aquaculture rearing (De Schryver et al., 2009). Several studies on PHB were performed using different aquaculture species such as European sea bass *Dicentrarchus labrax* juveniles (De Schryver et al., 2009), giant river prawn *Macrobrachium rosenbergii* larvae (Nhan et al., 2010), and Siberian sturgeon *Acipenser baerii* juveniles (Najdegerami et al., 2011). Results indicated that PHB increases the weight gain, survival, and SCFA concentration in the gastrointestinal tract. TCBS counts as a measure for *Vibrio* spp. were found to be significantly lower in PHB treated giant river prawn, indicating that the PHB addition also had a growth inhibitory effect towards these potentially pathogenic microorganisms. However, further research is needed in order to determine the exact mode of action. As there is only limited information on PHB function as an ingredient in fish starter diets, the present study was conducted to assess the potential effects of several dietary levels of PHB on feed utilization, survival and growth of Nile tilapia *Oreochromis niloticus* fry.

Materials and methods

Six hundred tilapia fry (26±2mg) were randomly distributed into twelve 38-l flow-through aquaria (three replicates per treatment) at an initial fish density of 50 fish per tank. Semi-purified microdiets at the size of 300-500µm were prepared containing 0 (control), 0.5, 2.5, and 5% (w/w) PHB. The control diet was formulated containing 50% gluten, 20% fishmeal, 13% maize meal, 7.5% soybean oil, free amino acids, vitamins, and mineral mixtures. Fishes were fed to satiation 3-4 times.day^{-1}. After four weeks of feeding, the specific growth rate (SGR), feed conversion ratio (FCR), survival percentage, fish lipid content and

digestive enzyme activity of each treatment group was measured and ANOVA analysis was applied to determine differences between treatments.

Results and discussion

During the experiment, water temperature was maintained at 28±1°C and pH ranged from 8.2-8.8. NH_4, NO_2, and NO_3 levels never exceeded 0.05, 0.5, and 2.5mg.l^{-1}, respectively. The initial average fish weight of 26±2mg increased to 218±101, 290±61, 269±68, and 257±48mg for the 0, 0.5, 2.5, and 5% PHB treatment, respectively. As the frequency distributions of the fish final body weight values of all treatment groups were significantly skewed (Fig. 1), a non-parametric Kruskal-Wallis statistical analysis was performed on the data of fish final body weight values of all treatments.

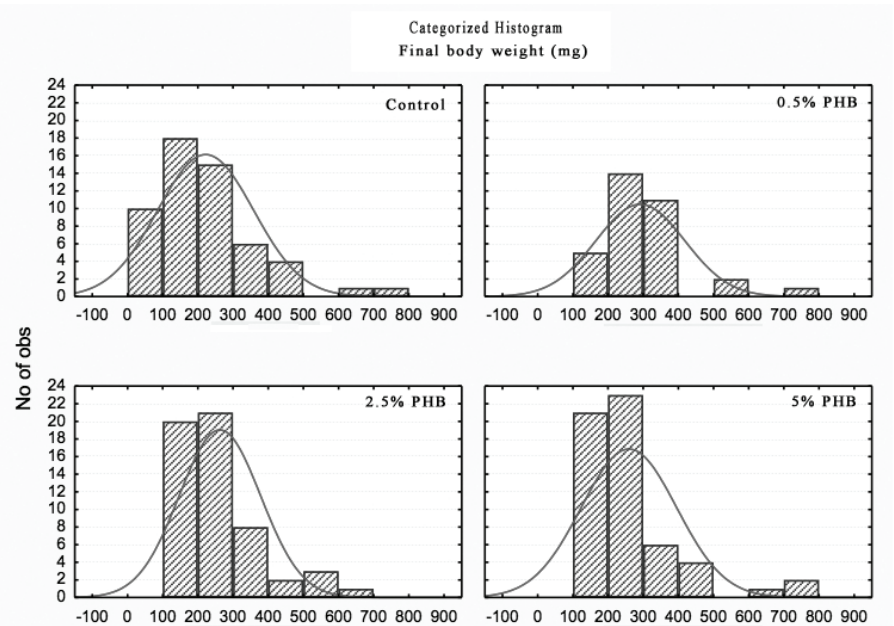


Fig. 1. Frequency distributions of the fish final body weight values (in mg) of treatment groups fed different experimental PHB-supplemented diets

It can be concluded that at the end of the experiment the fish fed with 0.5% PHB diet reached the highest fish body weight values that were significantly higher (P<0.05) compared to the group offered the control diet. The highest specific growth rate of 5.4±0.1% body weight gain.day^{-1} was also obtained by the 0.5% PHB treatment group (Table I). There were no treatment effects on either FCR or survival percentage (P>0.05). The total lipid content (% on DW) of the fish from the treatment groups were significantly higher compared to the control

group, indicating that dietary PHB supplementation increased the lipid absorption by the fish (Table II).

Table I. Summary of growth, feed utilization, and survival of fry fed different experimental PHB-supplemented diets

No	Diet	Final body weight (mg)	Survival (%)	SGR (%BW.day^{-1})	FCR (WW.DW^{-1})
1	Control	218±101^{a}	90±9^{a}	5.3±0.0^{a}	1.0±0.3^{a}
2	0.5% PHB	290±61^{b}	77±7^{a}	5.4±0.1^{b}	0.9±0.0^{a}
3	2.5% PHB	269±68^{ab}	96±7^{a}	5.2±0.0^{a}	0.9±0.1^{a}
4	5.0% PHB	257±48^{ab}	87±7^{a}	5.2±0.0^{a}	0.8±0.1^{a}

Values are mean ± SD (n=3). Different letter within column denote significant differences (P<0.05)

As in other vertebrates, the ability of fish to utilize ingested nutrients depends on the presence of appropriate enzymes in appropriate locations in the wall and along the lumen of the intestinal tract. Generally, distribution and intensity of intestinal enzyme activity along the gut varies with feeding habits and intestinal morphology. In this experiment, the increased lipid content in the fish and general growth in the PHB treatment groups might be related to the higher fish lipase activity compared to the control group (Table II).

Table II. Total lipid (fish and diet) and lipase activity of fish groups fed with different experimental PHB-supplemented diets

Treatment	Total lipid (% on DW)		Fish lipase activity (U mg protein^{-1})	
	Diet	Fish	Week 2	Week 4
Control	14.6±0.4^{a}	25.4±0.3^{a}	0.018±0.006^{a}	0.021±0.003^{a}
0.5% PHB	15.3±0.7^{a}	28.3±0.1^{b}	0.026±0.007^{ab}	0.025±0.004^{ab}
2.5% PHB	16.0±2.4^{a}	27.7±1.1^{b}	0.028±0.006^{b}	0.029±0.004^{b}
5%PHB	16.1±0.2^{a}	27.9±0.1^{b}	0.027±0.005^{b}	0.029±0.001^{b}

Values are mean ± SD (n=3). Different letter within column denote significant differences (P<0.05)

It is generally accepted that dietary manipulation modulates the gut microbiota and several studies have observed that dietary fatty acids affect the attachment sites for the gastrointestinal microbiota, possibly by modifying the fatty acid composition of the intestinal wall (Ringo et al., 2002, Dimitroglou, 2011). While intestinal microbiota clearly impact the host's energy balance, their role in intestinal absorption and extraintestinal metabolism of dietary fat is less clear. A recent study by Semova et al. (2012) however demonstrated that the gut microbiota also promote dietary lipid absorption, providing mechanistic insight into how microbiota-diet interactions regulate host energy balance. Taken together, dietary PHB supplementation seems to positively impact tilapia fry culture performance. The increase in growth, lipase activity and fish lipid content in fish groups fed with PHB-supplemented diets might be correlated with changes in

fish intestinal microbiota following dietary PHB supplementation. Intestinal microbial community will need to be further examined by making use of physiological and molecular techniques in order to explore the suggestion of the intestinal microbiota modulation by PHB supplementation, which may be important in manipulating the intestinal host-microbe interactions.

References

De Schryver P., A.K. Sinha, P.S. Kunwar, K. Baruah, N. Boon, W. Verstraete, G. De Boeck, P. Bossier. 2009. Poly-β-hydroxybutyrate (PHB) increases growth performance and intestinal bacterial range-weighted richness in juvenile European sea bass, *Dicentrarchus labrax*. Applied Microbiology & Biotechnology 86:1535–1541.

Dimitroglou A., D.L. Merrifield, O. Carnevali, S. Picchietti, M. Avella, C. L. Daniels, D. Güroy, and S.J. Davies. 2011. Microbial manipulations to improve fish health and production - a Mediterranean perspective. Fish & Shellfish Immunology 30:1-16.

Najdegerami E.H., T.N. Tran, T. Defoirdt, M. Marzorati, P. Sorgeloos, N. Boon, and P. Bossier. 2011. Effects of poly-β-hydroxybutyrate on Siberian sturgeon (*Acipenser baerii*) fingerlings performance and its gastrointestinal tract microbial community. FEMS Microbiology Ecology 79:25-33.

Nhan D.T., M. Wille, P. De Schryver, T. Defoirdt, P. Bossier, and P. Sorgeloos. 2010. The effect of poly β-hydroxybutyrate on larviculture of the giant freshwater prawn *Macrobrachium rosenbergii*. Aquaculture 302:76–81.

Ringø E., J.B. Lødemel, R. Myklebust, L. Jensen, V. Lund, T.M. Mayhew, and R.E. Olsen. 2002. Effect of soybean, linseed and marine oils on aerobic gut microbiota of Arctic charr *Salvelinus alpinus* L. before and after challenge with *Aeromonas salmonicida* ssp. *salmonicida*. Aquaculture Research 33:591-606.

Semova I., J.D. Carten, J. Stombaugh, L.C. Mackey, R. Knight, S.A. Farber, and J.F. Rawls. 2012. Microbiota Regulate Intestinal Absorption and Metabolism of Fatty Acids in the Zebrafish. Cell Host & Microbe 12(3):277-288.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

QUANTITATIVE CHARACTERISTICS OF ATLANTIC HALIBUT (*HIPPOGLOSSUS HIPPOGLOSSUS* L) EGG PRODUCTION THROUGHOUT THE REPRODUCTIVE SEASON AND THEIR RELATIONSHIP TO EMBRYO AND LARVAL QUALITY

T.H. Skaalsvik, S.L. Bolla, and I. Babiak

Faculty of Biosciences and Aquaculture, University of Nordland, N- 8049 Bodoe

Introduction

Assessment of egg quality is an important aspect of finfish hatchery management, but guidelines for such assessments are scarce, especially for marine batch spawners such as the Atlantic halibut (*Hippoglossus hippoglossus*). To date, laborious operations are frequently carried out on batches of egg that later will be lost or discarded due to insufficient quality. As indicated by Bromage et al. (1994), a good assessment of egg quality should be easy to perform and should be possible to carry out as soon as possible after stripping, so that low quality batches can be discarded at an early stage. In the present work, potential indicators of egg and larval quality as well as several performance parameters were studied in Atlantic halibut.

Materials and methods

The 39 batches of eggs were collected throughout the reproductive season from 17 fish kept in the research station of the Universtity of Nordland, Bodoe, Norway. The eggs were inseminated under dim red light and incubated in cold room at 5.4 C. The paternal effect on offspring was minimized by using the same cryopreserved semen for all inseminations. Parallel controls were fertilized with fresh semen. The advancement of the spawning season and four ovarian fluid parameters were registered: pH, electrical conductivity (EC), osmolality, and the amount of fluid. The behavior of the broodfish (handling resistance) at egg collection was registered and the eggs were analyzed for cortisol content. Fertilization and hatching rates and larval survival were calculated, and larvae standard length (SL) and myotome height (MH) were measured at the end of the yolk-sac stage. Further, the occurrence of three major types of larval deformities was registered: jaw deformity, yolk-sac oedema, and spinal deformities.

Results and discussion

Offspring performance parameters were not significantly different between the half-sibs produced with use of fresh or cryopreserved semen.

There was a significant and negative effect of successive batch number on MH (p=0.039). This could be related to the reduced amount of material allocated to the eggs over the course of the spawning season and decreasing dry weight and size of the eggs in subsequent batches of eggs, as previously observed by Evans et al (1996).

There was a significant and negative effect of the batch collection date on the cortisol content of the eggs (p=0.010) and the handling resistance of the brood-fish was significantly related to the cortisol content of the eggs (p=0.041). Generally, the batches of eggs with the lowest content of cortisol were collected from the fish with the highest resistance against handling during stripping.

The cortisol content of eggs correlated significantly and positively with the occurrence of yolk-sac oedema (p=0.005). Increased rate of abnormal larvae from stressed fish has previously been demonstrated in several studies, e.g., by Morgan et. al. (1999), who concluded that chronically stressed Atlantic cod (*Gadus morhua*) was able to spawn successfully, but a negative impact on particular developmental abnormalities were observed.

The EC of the ovarian fluid correlated significantly and negatively with the fertilization rate (r=-0.58). The batches with the best fertilization rates were found in the EC range between 1.5 and 3mS.cm^{-1}, and decreasing rates were associated with higher EC values. The frequency of yolk-sac oedema correlated significantly and positively with the ovarian fluid EC (ρ=0.40). It has previously been hypothesized that the EC would indirectly measure ovarian fluid protein levels from proteolytic breakdown and release of the yolk proteins during overripening (Barnes et. al., 2003).

The pH of ovarian fluid correlated significantly and positively with fertilization rate (r=0.63) and hatching rate (r=0.44). Within the pH ranges observed in the study, the higher the pH was, the higher was the fertilization rate, up to approximately pH=8.25. Significant correlations between ovarian or coelomic fluid pH and fertilization rate have previously been observed in a number of fish species and have been directly linked to reduced egg quality due to overripening of the eggs (Fauvel et. al.,1993; Lahnsteiner 2000).

The ovarian fluid quantity correlated significantly and negatively with fertilization and hatching rates (ρ=-0.36 and ρ=-0.35), while there was a significant positive correlation with yolk sac oedema (ρ=0.49). The amount of ovarian or coelomic fluid has been poorly investigated as a possible indicator of egg quality

in marine fish. It has been mentioned as a useful indicator of egg quality in general (Pavlov et. al. 2007), but specific descriptions at species levels are scarce. Increased occurrence of yolk sac oedema have previously been linked to environmental parameters, such as temperature (Lein et. al., 1997; Ottesen and Bolla, 1998) and salinity (Bolla and Ottesen, 1998), but to our knowledge this is the first time that a link to egg quality before fertilization have been demonstrated.

The relative hatching rate correlated significantly with the fertilization rate (r=0.51, n=38). A further exploration of this relationship revealed that fertilization rate correlated significantly with the relative hatching rate for batches with a low (<50%) fertilization success (r=0.64, n=12), while for the batches with a high (>50%) fertilization success there was no correlation (r=-0.08, n=24). These results support previous suggestions that the fertilization rate is a good predictor of hatching success only when the fertilization rate is low (Pavlov et. al., 2007).

There was a significant negative correlation between the fertilization rate and SL (r=-0.33, p=0.050) and between the hatching rate and spinal deformity (ρ=-0.46, p=0.005). MH and SL of larvae correlated significantly and positively (r=0.40, p<0.001, MH=0.4769+0.0236×SL*)*.

A cumulative effect of ovarian fluid pH, EC, osmolality, and quantity explains over 75% of the total variation in fertilization rate

The findings from the present study indicate that measured parameters, in particular pH and EC, might have a potential for future use as eggs quality indicators in hatchery management.

References

Barnes M. E., W. A. Sayler, R. J. Cordes and R. P. Hanten. 2003. Potential indicators of egg viability in landlocked fall chinook salmon spawn with or without the presence of overripe eggs. North American Journal of Aquaculture 65(1):49-55.

Bolla S. and O. H. Ottesen. 1998. The influence of salinity on the morphological development of yolk sac larvae of Atlantic halibut, *Hippoglossus hippoglossus* (L.). Aquaculture Research 29(3):203-209.

Bromage N., M. Bruce, N. Basavaraja, K. Rana, R. Shields, C. Young, J. Dye, P. Smith, M. Gillespie, and J. Gamble. 1994. Egg quality determinants in finfish: The role of overripening with special reference to the timing of stripping in the Atlantic halibut Hippoglossus hippoglossus. Journal of the World Aquaculture Society 25(1):13-21.

Evans R.P., C.C. Parrish, J.A. Brown, and P.J. Davis. 1996. Biochemical composition of eggs from repeat and first-time spawning captive Atlantic halibut (*Hippoglossus hippoglossus*). Aquaculture 139(1-2):139-149.

Fauvel C., M. H. Omnes, M. Suquet, and Y. Normant. 1993. Reliable assessment of overripening in turbot (*Scophthalmus maximus*) by a simple pH measurement. Aquaculture 117(1-2):107-113.

Lahnsteiner F. 2000. Morphological, physiological and biochemical parameters characterizing the over-ripening of rainbow trout eggs. Fish Physiology and Biochemistry 23(2):107-118.

Lein I., I. Holmefjord, and M. Rye. 1997. Effects of temperature on yolk sac larvae of Atlantic halibut (*Hippoglossus hippoglossus* L.). Aquaculture 157(1-2). p 123-135.

Morgan M.J., C.E. Wilson, and L.W. Crim. 1999. The effect of stress on reproduction in Atlantic cod. Journal of Fish Biology 54(3): 477-488.

Ottesen O. H. and S. Bolla. 1998. Combined effects of temperature and salinity on development and survival of Atlantic halibut larvae. Aquaculture International 6(2) 103-120.

Pavlov D., E. Kjørsvik, T. Refstie, and Ø. Andersen. 2007. Brood stock and egg production, in Culture of cold-water marine fish. Blackwell Publishing Ltd. Oxford, UK. p 129-203.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

MICROBIAL CHARACTERIZATION OF ENRICHED *ARTEMIA* SP. AT TWO DIFFERENT TEMPERATURES AND ENRICHMENTS

F. Soares*, S. Castanho, M. Moreira, A.C. Mendes, and P. Pousão-Ferreira

IPMA, Aquaculture Research Station, Av. 5 de Outubro s/n, 8700-305 Olhão, Portugal.
*Email: fsoares@ipma.pt

Introduction

In aquaculture larvae survival rates are higher than in nature but mortality is still very important. Pathogen loads are believed to be an important factor in larval survival given that in this development stage, the fish immune system is still limited. Larval mortality is often caused by opportunistic bacteria introduced mainly by the provided life feeds (Planas and Cunha, 1999).

Artemia sp. is widely used as live food in larviculture to feed marine fish and penaeid shrimps and a known vector for introducing bacteria into systems, despite the enrichment process used (Makridis et al., 2010).

The process of preserving the enriched *Artemia* at low temperatures is used in some hatcheries, although no scientific data about microbiological quality are available. Candeias-Mendes et al. (2011) showed that essential fatty acids can be preserved during a 12h period at 5ºC and this is a procedure to consider as a way for reducing labour and costs in hatcheries.

In this work, the bacteria associated with *Artemia* enrichment was characterized, after a normal enrichment protocol and after being starved at normal and low temperature. Two different enrichment products were tested.

Materials and methods

A continental strain of *Artemia* sp. from the same batch was divided into two enrichment products per treatment – GD, an experimental emulsion based of several freeze-dried microalgaes supplemented with decosahexaenoic acid (DHA) and RP, a standard commercial product Red Pepper®, Bernaqua™. After the enrichment process, metanauplii from the two enrichments (GD and RP) were randomly distributed in two groups: the (+) group maintained at usual marine fish larvae culture temperature (19±1ºC) and the (–) group kept at low tem-

perature (5±1ºC). Therefore the trial had a 4×3 experimental design, with 4 treatments – GD-, GD+, RP-, RP+ – kept in triplicate tanks without food.

Artemia for bacteriological analysis were sampled after the enrichments (GDi and RPi) period and after 24h at two different temperatures. 10ml of *Artemia* and water were sampled and homogenized. After, that the homogenate was sequentially diluted ten-fold with 1.5% sterile saline solution, and 100µl of each dilution was spread in triplicate on agar plates. Tryptic soy agar (TSA, Merck) was used to obtain the total number of aerobic bacteria, and thiosulfate-citrate-bile salts-sucrose agar (TCBS, OXOID) was used to isolate and count the bacteria. Plates were incubated at 22ºC for 7 days and counting was made at 2 and 7 days after incubation.

The isolates obtained were examined using phenotypic tests. Routine tests for determining biochemical characteristics of the isolates were carried out according with Holt (1994) and Buller (2004).

Non-parametric (Wilcoxon Rank test) statistical analysis was made with IBM™ SPSS Statistics 21.0.

Results and discussion

After enrichment, *Artemia* with GD treatment had a lower proportion of *Vibrionaceae* when compared with the RP treatment (Fig. 1). This may be the effect of antimicrobial molecules produced by the microalgae, present in GD composition, since phytoplankton species are capable of producing substances that are toxic to other bacteria (Srinivasakumar and Rajashekhar, 2009) and some appear to be naturally bacteriostatic (Kellam and Walker, 1989).

After 24h at 19±1ºC, the total number of bacteria and *Vibrionaceae* cfus (colony forming units) present in *Artemia* increased exponentially when compared with the cold treatment (Fig. 1). Although, in the *Artemia* kept at 5ºC, the total bacterial decreased and the proportion of vibrionic cfus increased in both treatments. The fact that the proportion of *Vibrionaceae* increased in cold condition can be supported by Høj et al. (2009), who showed that vibrio cells were relatively more resistant to antimicrobial treatment, namely cold temperatures. These results indicate that this preservation method can be routinely used at hatcheries with clear advantage in routine management. This is an improvement in larval rearing because bacteria introduced by live feed are an important mortality cause.

Bacterial identification indicates that some specific *Vibrionaceae* appear in each treatment. In the GD *Artemia*, *Moritella marina*, *Vibrio aestuarians*, *V. coralliilyticus*, and *V. vulnificus* were identified, while in the RP *Artemia*, *V. Pelagius* was identified. *V. alginolyticus* was common in the two treatments, as reported

by Snoussi et al. (2006) and this species has been described as a common bacteria of the intestinal microflora in several marine fish species, with some strains identified as a pathogen of different marine species (Austin et al., 1993). The *Vibrionaceae* identified are similar with the species found in other studies (Høj et al., 2009).

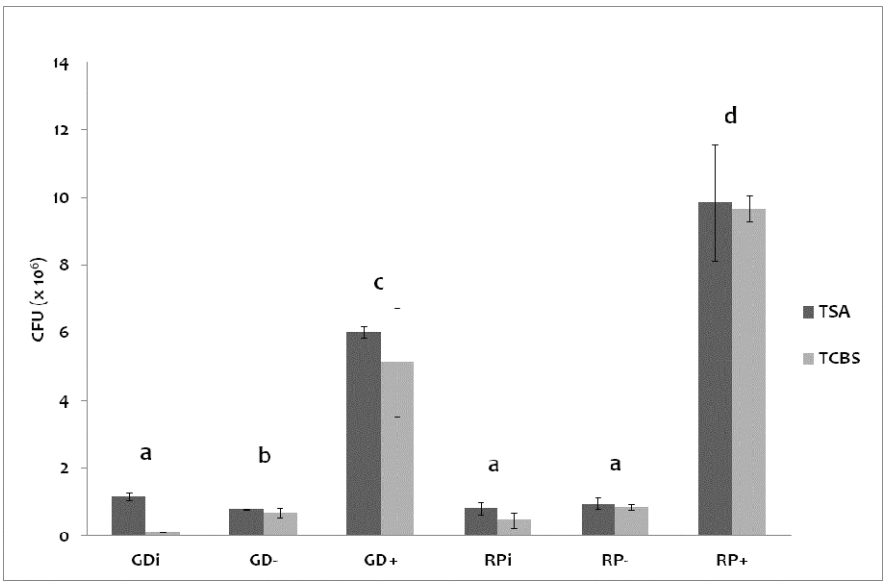


Fig. 1. Total bacteria (dark grey bars) and *Vibrionaceae* (grey bars) present in the different treatments.GD- enriched microalgae, RP- Red Pepper®, i- initial sample, (-) 5ºC, (+) 19ºC. Letters a, b, c, indicate different groups with statistical significant differences (Wilcoxon Rank test, $P<0.05$).

Conclusions

In live feed production, cold preservation (5±1ºC) seems to be a viable methodology to restrain total bacterial proliferation, regardless of the enrichment.

Species diversity present in the enriched *Artemia* is related with the different treatments and *V. alginolyticus* was common in both treatments.

References

Austin B., M. Stobie, P.A.W. Robertson, H.G. Glass, J.R. Stark, and M. Mudarris, 1993. *Vibrio alginolyticus* – the cause of gill disease leading to progressive low-level mortalities among juvenile turbot, Scophthalmus maximus L., in a Scottish aquarium. Journal of Fish Diseases 16:277-280.

Buller N.B. 2004. Bacteria from fish and other aquatic animals: a practical identification manual, 1st Edition, CABI Publishing, pp.171-176.

Candeias-Mendes A., S. Castanho, J. Coutinho, N.M. Bandarra, and P. Pousão-Ferreira. 2011. Methodology to Preserve the Nutritional Value of Enriched Artemia in Hatcheriy. World Aquaculture 2011, Natal, Brasil, 6-11 Junho, Book of Abstracts:206.

Holt J.G. 1994. Bergey's Manual of Determinative Bacteriology, 9th Edition, Williams & Wilkins.

Høj L.; D.G. Bourne, and M.R. Hall. 2009. Localization, abundance and community structure of bacteria associated with Artemia: Effects of nauplii enrichment and antimicrobial treatment. Aquaculture 293:278-285.

Kellam S.J. and J.M. Walker. 1989. Antibacterial activity from marine microalgae in laboratory culture. British Phycological Journal 24:191-194.

Makridis P., L. Ribeiro, M.T. Dinis. 2010. Influence of microalgae supernatant, and bacteria isolated from microalgae cultures, on microbiology, and digestive capacity of gilthead Seabream (*Sparus aurata*) and senegalese sole (*Solea senegalensis*) larvae. Journal of the World Aquaculture Society 41(5):780-790.

Planas M. and I. Cunha. 1999. Larviculture of marine fish: problems and perspectives. Aquaculture 177:171-190.

Snoussi M., K. Chaieb, R. Mahmoud, and R. Bakhrouf. 2006. Quantitative study, identification and antibiotics sensitivity of some Vibrionaceae associated to a marine hatchery, Annals of Microbiology 56(4):289-293.

Srinivasakumar K.P. and M. Rajashekhar. 2009. In vitro studies on bactericidal activity and sensitivity pattern of isolated marine microalgae against selective human bacterial pathogens. Indian Journal of Science and Technology 2(8):16-23.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EVALUATION OF PROBIOTIC BACTERIA AGAINST AEROMONADS SYNDROME IN COMMON CARP (*CYPRINUS CARPIO* L.) IN SIMPLE AXENIC LARVICULTURE

G. Suantika*, P. Aditiawati, D.I. Astuti, A. Sjarmidi, N. Lim, and Z.F. Khotimah

Microbial Biotechnology Research Group, School of Life Sciences and Technology, Institut Teknologi Bandung, Jl. Ganesha 10 Bandung 40132, Indonesia, Tel. +62-22-251-1575, *E-mail: gsuantika@sith.itb.ac.id

Abstract

Evaluation of probiotics, *Bacillus firmus* and *B. coagulans* against *Aeromonas hydrophila* in axenic common carp larviculture was conducted. The highest egg hatching rate was obtained from the axenic system + probiotic bacteria (AP) (98.33%), followed by axenic system (A) (96.67%); axenic system + probiotic + *A. hydrophila* (AC) (93.33%); non-axenic system (NA) (93.33%); and axenic system + *A. hydrophila* (AH) (83.33%). 100% survival rate (SR) was obtained from all treatments, except AH (90%). The highest weight (0.013g) was obtained from the A treatment, followed by AC (0.0127g), AP (0.0123g), AH (0.012g), and NA (0.005g). In conclusion, the axenic system can be used to improve common carp larviculture, and further use of *B. coagulans* and *B. firmus* was able to control Aeromonads syndrome during the larviculture stage.

Introduction

One bottleneck of common carp larviculture is low production and high mortality due to bacterial infections mainly caused by *A. hydrophila*. Antibiotic use has been applied to solve this problem. However, this method increases production costs and triggers bacterial resistance. One of the solutions offered is the use of probiotic bacteria – referred to selected, adapted, and cultured bacteria – which positive influences larviculture by degrading organic wastes in pond bottom and competing with pathogenic bacteria for nutrition, thus helping host immunity or producing antimicrobial compounds. Application of axenic system simultaneously with addition of certain probiotic bacteria could be used as a tool to evaluate the effect of probiotic in order to increase larval fish production (Marques et al., 2006). Therefore, this research used an axenic system to evaluate the effectiveness of probiotic bacteria (*B. firmus* and *B. coagulans*) in hampering *A. hydrophila* infection and increasing carp (*Cyprinus carpio*) larviculture productivity.

Materials and methods

Inhibitory test of probiotic bacteria against Aeromonas hydrophila
The Kirby Bauer (disk paper) method was used to show inhibitory activity of *B. coagulans* and *B. firmus* toward *A. hydrophila*.

Axenicity test
Bacterial contamination was checked by sampling 100µl water from the system and plating on NA medium. The plates were incubated at 28^{o}C and bacterial counts performed after five days.

Egg incubation and common carp larviculture in axenic system
Carp fish larvicultures were classified into five treatments: (i) Non-axenic system (NA), (ii) Axenic system (A), (iii) Axenic system + probiotic bacteria (AP), (iv) Axenic system + with probiotic and *A. hydrophila* (AC), and (v) Axenic system + *A. hydrophila* (AH). Each bottle culture was stocked with 20 fish eggs, supplemented with 0.15% vv^{-1} of bacteria inoculum (OD=0.5) according to the treatment and kept at room temperature *(*24±1°C*)* during 9 days. Larvae which were hatched from the eggs and then fed on sterilized *Artemia* sp. nauplii every two days during culture period.

Biological parameters
Biological parameters such as hatching rate (HR), larval survival rate (SR), and larval growth were calculated at the end of culture period.

Water quality parameter
Water quality parameters such as temperature, pH level, dissolved oxygen, ammonia, nitrite, and nitrate concentrations were measured every two days during the culture period.

Bacterial sampling
Microbiological analyses were performed by sampling 1ml culture water every two days. Serial dilution (10^{-1} to 10^{-7}) was prepared in sterile NaCl (0.85%) from the homogenized samples and 0.1ml was plated on NA. The plates were incubated at 25°C and bacterial counts were performed after 24-48h.

Statistical analyses
All data were analyzed using one-way ANOVA to obtain significant differences among treatments (p>0.05).

Results and discussion

Inhibitory activity of probiotic bacteria against Aeromonas hydrophila
The transparent zone which formed by the inhibitory activity of *B. coagulans* (0.5mm) was bigger than that of *B. firmus* (0.3mm), resulting from variation in

bacterial specific growth rate (SGR) of these two probiotic bacteria. The SGR of *B. coagulans* was 0.0213cells.ml^{-1}h, higher than *B. firmus* which was 0.211cells.ml^{-1}h (Andriani, 2006). Rosario et al. (2005) stated that *Bacillus* bacteria produce antibiotic compounds such as polymixin, colistin, and circulin which are able to inhibit growth of *A. hydrophila*.

Axenicity system test

The system maintained 66% axenicity level. Contaminants still existing in the system probably resulted from culture conditions which were not sterile enough and contaminated water samples when taken from the system.

Biological parameters

Biological parameters are summarized in Table I. Hatching rate, survival rate, and growth of larvae in the axenic system were higher than the non-axenic system, indicating a positive effect of the axenic system.

Table I. Biological parameters measured.

Treatment	Hatching rate (%)	Survival Rate (%)	Weight (g)	Length (cm)
Non axenic	93.33 ± 0.33	100 ± 0.33	0.0050	0.84
Axenic	96.67 ± 0.67	100 ± 0.88	0.0130	1.08
+ Probiotic bacteria	98.33 ± 0.33	100 ± 0.33	0.0123	0.98
+ Probiotic bacteria + *A. hydrophila*	93.33 ± 0.88	100 ± 0.88	0.0127	1.07
+ *A. hydrophila*	83.33 ± 1.7	90 ± 1.73	0.0120	1.1

Generally, addition of probiotic bacteria into the axenic culture also gave higher hatching rate, survival rate and larvae weight compared to the addition of *A. hydrophila* treatment, due to pathogenic bacteria activity in producing chemical coumpunds which are able to break egg layer and cause the death of fish embrio (Hansen and Olafsen, 1999). The presence of probiotic bacteria in the culture reduces pathogenic effect of *A. hydrophila* due to their competition in obtaining nutrition and space. Verschuere (2000) stated that *Bacillus* sp. produce chemical compounds such as antibiotic, lisozym, protease, organic acid and hidrogen peroxide which are able to inhibit the growth of pathogenic bacteria. There was not any significant difference of eggs hatching rate, whereas there were significant differences of survival rate and larvae growth among these five treatment groups.

Water quality

All water quality parameters were still in the suitable range for common carp larviculture and there was not any significant difference among each culture treatment ($p>0.05$). It shows that there was not any influence of treatments on-water quality parameters.

Microbiological parameters

Calculation of total bacteria showed that *B. firmus*, *B. coagulans*, and *A. hydrophila* existed in each treatment during the culture period. Number of bacteria calculated from each culture was not significantly different ($p>0.05$).

Conclusion

It can be concluded that axenic system application can be used to increase common carp larviculture productivity in term of eggs hatching rate, survival rate, swimming activity, and growth of larvae. In addition, *B. firmus* and *B. coagulans* were able to inhibit the growth of *A. hydrophila* and it further improves the productivity of common carp larviculture.

References

Andriani. 2006. Optimasi Bakteri Probiotik dalam Penghambatan Pertumbuhan *Aeromonas* sp. pada Kultur Larva Ikan Mas (*Cyprinus carpio*, L). Bandung: Skripsi Sarjana SITH-ITB.

Hansen G.H. and J.A. Olafsen. 1999. Bacterial Interactions in Early Life Stages of Marine Cold Water Fish. Microbial Ecology 38:1-26.

Marques A., F. Ollevier, W. Verstraete, P. Sorgeloos, and P. Bossier. 2006. Gnotobiotically Grown Aquatic Animals: Opportunities to Investigate Host-Microbe Interactions. Journal of Applied Microbiology 100:903-918.

Rosario S., C. Cruz, V. Faustino, and A.O. Tuazon. 2005. In Vitro Study on the Antimicrobial Activity of Probiotic Milk Against Common Pediatric Community Acquired Respiratory Pathogens. Pediatric Infectious Disease Society of the Philippines Journal 9(2):25-29.

Verschuere L., G. Rombaut, P. Sorgeloos, and W. Verstraete. 2000. Probiotic Bacteria as Biological Control Agents in Aquaculture. Microbiology and Molecular Biology Reviews 64(4):655-671.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EFFECT OF POLY-HYDROXYBUTYRATE ON GROWTH AND ENZYMATIC ACTIVITY OF CHINESE MITTEN CRAB, *ERIOCHEIR SINENSIS*, JUVENILE

L. Sui[1], G. Chang[2], Y. Liu[1], and Y. Deng[1]

[1] Tianjin University of Science & Technology, Tianjin 300450, China
[2] Huaiyin Normal University, Jiangsu 223300, China

Introduction

Poly-β-hydroxybutyrate (PHB) is a bacterial storage compound, which can be partially degraded into water-soluble β-hydroxybutyric acid in the gastrointestinal tract of animals. Apart from being an energy resource, PHB is also considered as an alternative bio-control compound due to its antibacterial activity (Defoirdt et al. 2009; 2011). Recently it has been proven that PHB beneficially influences the colonic microflora of marine fish and crustaceans, and consequently improves the survival and growth of these aquatic animals (De Schryver et al. 2010; Nhan et al. 2010). Our previous studies on *Eriocheir sinensis* zoeal larvae also indicated that PHB-enriched rotifer and *Artemia* nauplii significantly improved the molting, survival, and tolerance to vibrio challenge (Sui et al., 2011; 2012). This paper studied the effect of PHB on enzyme activities of hematopancreas and haemolymph of juvenile *E. sinensis*, aiming to investigate the physiological modulation of PHB on crustaceans.

Materials and methods

E. sinensis juveniles with an initial body weight (BW) of 0.7-0.8g were separately reared in a recirculation system for 60 days at 28-30°C. The crabs were fed dry feeds supplemented with 0 (control), 0.5, 1, 3, and 5% PHB at a daily ration of 10% of BW. Each group contained 60 juvenile crabs. At the end of the experiment, the molting frequency (interval between successive molting), relative BW gain, and hepatosomatic index (HSI) of the crabs were recorded as follows:

$$relative\,BW\ gain = \frac{final\ BW - initial\ BW}{initial\ BW} \times 100\%$$

$$HSI = \frac{hepatopancreas\,WW}{body\,WW} \times 100\%$$, where WW=wet weight

The hepatopancreas and hemolymph collected from 2-3 crabs in the same group were pooled and stored frozen. Four replicates were analyzed for each parameter. The activities of digestive enzymes (i.e., pepsin, trypsin, lipase, and amylase) of hepatopancreas, alkaline phosphatase (ALP), acid phosphatase (ACP), and superoxide dismutase (SOD) of hemolymph were analyzed using enzyme activity assay kits (Nanjin Jiancheng Biotechnology, China).

Data are presented as mean±standard error. Homogeneity of variance was tested with Levene's test, using arcsine-square root or logarithmic transformation when necessary. Statistical analyses were conducted using one-way analysis of variance (ANOVA) and compared with Duncan's multiple range test. $P<0.05$ was regarded statistically significantly different, and statistics were determined using SPSS package (version 11.0).

Results and discussion

In a period of 60 days rearing, most of the crabs molted three times with shorter 1^{st}-2^{nd} molting intervals (16.2-18.7 days) and longer 2^{nd}-3^{rd} molting intervals (24.5-28.4 days). Molting, BW gain, and HSI of *E. sinensis* juveniles were improved with increasing PHB supplementation in feed. At the 2^{nd}-3^{rd} molting interval, 3% and 5% PHB supplementation resulted significantly shorter molting intervals than 0.5% PHB and (control) without PHB supplementation ($P<0.05$). Correspondingly, 5% PHB supplementation resulted in a better BW gain compared to the control. Meanwhile 3% and 5% PHB supplementation also significantly increased HSI of the crabs compared to the control ($P<0.05$). The above results confirmed our previous observations that PHB containing diets benefited the molting of *E. sinensis* zoeal larvae (Sui et al., 2011; 2012).

Table I. Molting intervals and growth of *E. sinensis* juvenile fed diets containing different levels of PHB during 60 days rearing. Means with different letters in the same column were significantly different ($P<0.05$, n=50-55)

PHB level (%)	Molting intervals (day)		Growth of body weight (%)	Hepatosomatic index (HSI) (%)
	1^{st}-2^{nd}	2^{nd}-3^{rd}		
0	18.7±3.7 ^{a}	28.3±3.9^{a}	441.6±76.9^{c}	6.91±1.21^{b}
0.5	18.1±4.4 ^{a}	28.4±3.2^{a}	444.3±60.1^{bc}	7.30±1.29^{ab}
1	17.2±6.4 ^{a}	27.9±3.4^{ab}	463.9±80.4^{abc}	7.29±1.04^{ab}
3	16.2±2.9 ^{a}	24.5±3.5^{c}	501.7±70.9^{ab}	7.74±1.06^{a}
5	16.4±4.5 ^{a}	25.7±3.3^{bc}	505.8±61.8^{a}	7.96±0.99^{a}

SOD activity increased significantly with elevating PHB level in feed ($P<0.05$), indicating that PHB induced response of SOD in *E. sinensis* hemolymph. It is

well known that invertebrates do not have the capacity to mount humoral and adaptive immune responses, thus haemocytes play an important role in their defense systems because of their ability to phagocytose, encapsulate, and kill microbes (Yao et al., 2006). In this study, the activities of both ALP (enriched in plasma membrane) and ACP (associated with phatocytic lysosomes) decreased significantly ($P<0.05$) with elevating PHB level. The reduced ACP and ALP activity corresponding with increased SOD activity may indicate that PHB supplementation benefits the immunity health status of the crabs.

Table II. Superoxide dismutase (SOD), alkaline phosphatase (ALP), and acid phosphatase (ACP) activity in hemolymph of *E. sinensis* juvenile fed diets containing different levels of PHB. Means with different letters in the same column were significantly different ($P<0.05$, n=4)

PHB level	Enzyme activities (U/g protein)		
(%)	SOD	ALP	ACP
0	296.0 ± 17.3^{c}	24.4 ± 3.2^{a}	3.2 ± 0.3^{a}
0.5	327.2 ± 2.5^{b}	19.3 ± 3.6^{ab}	3.2 ± 0.1^{ab}
1	371.3 ± 13.6^{a}	14.2 ± 3.2^{bc}	2.9 ± 0.2^{abc}
3	389.9 ± 14.1^{a}	10.6 ± 1.6^{cd}	2.6 ± 0.3^{bc}
5	373.6 ± 10.2^{a}	5.1 ± 1.6^{d}	2.4 ± 0.3^{c}

Increasing PHB supplementation significantly enhanced the response of pepsin, trypsin and lipase, whereas amylase activity reduced significantly ($P<0.05$). The increased enzyme activity of protease and lipase in hepatopancreas facilitate the digestion of dietary protein and lipid, and thus enhance the molting and weight gain of the crabs. On the other hand, reduced enzyme activities at elevated PHB level suggest that the digestive capacity of crabs for carbohydrates becomes weak. This could be explained that dietary carbohydrate maybe less used in presence of PHB, which can be used as energy resource (Topping and Clifton, 2001).

Table III. Pepsin, trypsin, lipase, and amylase activity in hepatopancreas of *E. sinensis* juvenile fed diets containing different levels of PHB. Means with different letters in the same column were significantly different ($P<0.05$, n=4)

PHB level	Enzyme activities ($U.mg^{-1}$ protein)			
(%)	Pepsin	Trypsin	Lipase	Amylase
0	14.1 ± 1.5^{c}	950.2 ± 122.7^{c}	4.4 ± 0.7^{c}	113.6 ± 13.6^{a}
0.5	18.7 ± 0.9^{bc}	901.0 ± 145.0^{c}	6.3 ± 0.8^{c}	102.5 ± 13.7^{a}
1	23.2 ± 0.9^{ab}	1613.4 ± 17.8^{b}	10.57 ± 1.3^{b}	96.8 ± 6.7^{b}
3	25.9 ± 1.7^{ab}	1923.6 ± 106.2^{a}	14.6 ± 2.2^{a}	93.5 ± 7.3^{b}
5	29.6 ± 0.9^{a}	2107.1 ± 90.0^{a}	10.5 ± 1.6^{b}	77.7 ± 8.2^{c}

Conclusion

PHB supplementation significantly improved the growth performance of molting frequency, body weight gain, and hepatosomatic index (HSI) of *E. sinensis* ju-

veniles. The beneficial effects of PHB are closely related to the enhanced anti-oxidant enzyme activity, immune system, as well as protease and lipase activity of the crabs. The current study together with our previous studies shows that PHB is a potential feed additive in *E. sinensis* larviculture and grow-out.

Acknowledgement

This study was supported by the Natural Science Foundation of China (grant number 31172427).

References

Defoirdt T., N. Boon, P. Sorgeloos, W. Verstraete, and P. Bossier. 2009. Short-chain fatty acids and poly-β-hydroxyalkanoates: (New) Biocontrol agents for a sustainable animal production. Biotechnol. Advances 27:680-685.

Defoirdt T., P. Sorgeloos, and P. Bossier. 2011. Alternatives to antibiotics for the control of bacterial disease in aquaculture. Current Opinion in Microbiology 14:251-258.

De Schryver P., A.K. Sinha, P. S. Kunwar, K. Baruah, W. Verstraete, N. Boon, G. De Boeck, and P. Bossier. 2010. Poly-β-hydroxybutyrate (PHB) increases growth performance and intestinal bacterial range-weighted richness in juvenile European sea bass, *Dicentrarchus labrax*. Appl. Microbiol. Biotechnol. 86:1535-1541.

Nhan D.T., M. Wille, P. De Schryver, T. Defoirdt, P. Bossier, and P. Sorgeloos. 2010. The effect of poly-β-hydroxybutyrate on larviculture of the giant fresh water prawn *Macrobrachium rosenbergii*. Aquaculture 302:76-81.

Sui L.Y., J.L. Cai, H.X. Sun, M. Wille, and P. Bossier. 2012. Effect of poly-β-hydroxybutyrate on Chinese mitten crab *Eriocheir sinensis* larvae challenged with pathogenic *Vibrio anguillarum*. J Fish Diseases 35:359-364.

Sui L.Y., Y. Liu, H.X. Sun, M. Wille, P. Bossier, and P. De Schryver. 2012. The effect of poly-β-hydroxybutyrate on the performance of Chinese mitten crab (*Eriocheir sinensis* Milne-Edwards) zoea larvae. Aquacult. Res., doi: 10.1111 /are.12077.

Topping D.L. and P.M. Clifton. 2001. Short-chain fatty acids and human colonic function: role of resistant starch and non-starch polysaccharides. Physiological Reviews 81:1031-1064.

Yao C.L., Z.Y. Wang, and J. H. Xiang. 2006. Crustacean haemocytes and their function in immune responses. Zool. Res. 1:549-557.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EGG PRODUCTION, EGG HATCHING SUCCESS, AND POPULATION GROWTH OF THE CALANOID COPEPOD *ACARTIA GRANI* (CALANOIDA, ACARTIIDAE) FED WITH EIGHT DIFFERENT DIETS

B. Sumares and N. Nogueira

Centro de Maricultura da Calheta, Governo Regional da Madeira, Vila da Calheta, 9370-133 Calheta email: bernardosumares@gmail.com; phone: +351 291820420

Introduction

Calanoid copepods, including species of the genus *Acartia*, are commonly used for larval diets of marine finfish.

Most calanoid copepods are filter-feeders, but there is little information on their natural food preference and on the individual food value of the species present in natural phytoplankton. For this reason, the identification of microalgal diets that allow good survival, fast rates of somatic growth and of reproduction, as well as high copepod yields is of paramount importance for larval fish culture.

Food quality and quantity are probably the most important factors regulating the productivity of copepod culture, and different microalgal diets can affect egg production, egg hatching, and quality.

To date, there have been no studies on how diet composition may affect population growth, egg production, and egg hatching success of *A. grani*. Several authors have suggested that monospecific diets may cause nutritional deficiencies, because of the inadequate content of one or more essential nutrients. To reduce this risk, the use of mixed diets has been suggested, because their combined nutrient contents are more likely to meet the nutritional requirements of the target species. It has been shown that the development rate of the calanoid *A. sinjiensis* was significantly better with a mixture of two microalgae than with monospecific diets. The nutritional content of heterotrophic protists is equally, if not more, important as that of algae in regulating zooplankton growth and production, as some heterotrophic protozoa appear to provide essential copepod growth compounds that are not always found in phytoplankton. Previous studies have snown that heterotrophic protists as a trophic link between poor nutritional quality algae and copepods resulted in higher egg production and egg hatching success.

This research was conducted to investigate the effects of different diets on the population growth, egg hatching success and egg production on the population of *A. grani*. The main objective is to optimize their diet that would maximize culture potential.

Materials and methods

All of the microalgae utilized in present experiments are commonly used algal species in aquaculture, therefore relatively easy to culture, with the exception of *Rhodomonas marina*. Three algal species were used in this study: *R. marina* (Rho); Tahitian strain *Isochrysis* sp. (T-Iso); and *Tetraselmis suecica* (Tet).

Algae were inoculated by starter cultures supplied by IPIMAR (Olhão) and were grown by batch method with Nutribloom medium (Necton), at 20±1ºC, salinity 25psu, 24h light in 1-l carboys, with continuous aeration. Seawater was 1-µm filtered and UV-irradiated. The algal cultures were in their exponential growth phase when used for feeding copepods. *Oxyrrhis marina* (Oxy) inoculate came from IPIMAR and was kept in our laboratory in 300-ml Erlenmeyer flasks in a controlled room similar to microalgae. O. *marina* were fed with 10-20ml of *R. marina* supplied once a week and every month the cultures were replicated to new Erlenmeyers to initiate new cultures.

Copepod eggs used in this work were collected in Algarve during the winter of 2010 and were maintained at 4ºC in the dark. Parental culture was started in a clean 500-l tank, with filtered, UV-treated seawater (38psu). Approximately 250 000 storage eggs were placed in the culture tank without aeration in the first 24h and continuous light (24L:0D) to promote the hatching of the nauplii.

Three separate experiments were carried out to assess the influence of various diets and their combinations on major parameters related to *A. grani* culture productivity, i.e., (1) egg hatching rate, (2) egg production rate, and (3) population increase over a 12-day culture period.

Diets used in the experiments:

- Diet 1: *R. marina* (Rho)
- Diet 2: *R. marina* (Rho) + Tahitian strain of *Isochrysis* sp. (T-Iso)
- Diet 3: *T. suecica* (Tet)
- Diet 4: *T. suecica* (Tet) + Tahitian strain of *Isochrysis* sp. (T-Iso)
- Diet 5: *R. marina* (Rho) + *O. marina* (Oxy)
- Diet 6: *O. marina* (Oxy)
- Diet 7: Tahitian strain of *Isochrysis sp*. (T-Iso)
- Diet 8: Tahitian strain of *Isochrysis* sp. (T-Iso) + *O. marina* (Oxy)

Results and discussion

Based on our results, the use of *Rhodomonas* algae positively affected all of the analyzed parameters. Moreover, our results show that the use of the binary diets seems to be more appropriate, positively affecting population dynamics of *A. grani*. Relative to hatching rate, the dietary treatment Rho+T-Iso clearly presented a superior egg hatching rate over the 72-hour experiment.

Average 24-h egg production was favorable again for the treatment that had *Rhodomonas* included. In monoalgal treatment (Rho) the average egg production was 4 eggs.female^{-1}.day for *A. grani*, similar to values observed for *Pseudocalanus elongates* (5 eggs.female^{-1}day).

Analysis of population growth is probably more pertinent to improving productivity of copepod culture for hatcheries because it provides a summary of the dietary effects on a range of interrelated parameters, including egg production, egg hatching rates, nauplii and copepodite development time, and survival. Diet significantly affected the population growth of *A. grani* in our study. With an initial number of only 8 females and 4 males, after 12 culturing days the treatment Rho+Oxy had the highest value of 1795 individuals (all stages included), followed by the treatment Rho+T-Iso that produced a total of 1275 eggs with a final mean population of 1783.0±560.6 individuals. It is generally assumed that by preying on heterotrophic protests, a copepod can diversify its diet and obtain a more balanced nutrition.

Table I Mean number of four life stages (eggs, nauplii, copepodites, and adults) within the population of *Acartia grani* cultured for 12 days with the first four diets (Rho; Rho+T-Iso; Tet; Tet+T-Iso) from an initial number of 12 adults. (*K*) is the specific population growth rate, and (D_t) is the doubling time. Different letters indicate significant differences ($p<0.05$). Data are represented as mean ±SD.

Treatment	Eggs	Nauplii	Copepodites	Adults	*K*	D_t
Rho	1029.0±339.4^{a}	107.0±42.1^{a}	249.8±78.8^{a}	83.0±47.3^{a}	0.39±0.02^{a}	1.73±0.07^{a}
Rho+T-Iso	1275.0±385.6^{a}	285.8±136.7^{ab}	164.5±23.7^{a}	57.8±19.0^{ab}	0.41±0.01^{a}	1.66±0.05^{a}
Tet	283.8±57.6^{b}	32.5±37.1^{ac}	6.0±5.7^{b}	2.5±1.7^{b}	0.27±0.01^{b}	2.52±0.08^{b}
Tet+T-Iso	286.0±181.0^{b}	260.0±138.2^{ab}	179.3±74.0^{a}	72.0±36.2^{a}	0.35±0.02^{c}	1.99±0.11^{c}
	p=0.000	p=0.011	p=0.000	p=0.015	p=0.00	p=0.00

Table II Mean number of four life stages (eggs, nauplii, copepodites, and adults) within the population of *Acartia grani* cultured for 12 days whit the second four diets (Rho+Oxy; Oxy; T-Iso; T-Iso+Oxy) from an initial number of 12 adults. (*K*) is the specific population growth rate, and (D_t) is the doubling time. Different letters indicate significant differences ($p<0.05$). Data are represented as mean±SD.

Treatment	Eggs	Nauplii	Copepodites	Adults	*K*	D_t
Rho+Oxy	1050.3±514.4^{a}	541.3±514.8^{a}	127.8±184.4^{a}	76.0± 64.8^{a}	0.41±0.03^{a}	1.68±0.14^{a}
Oxy	641.3±209.8^{ab}	221.3±183.7^{a}	35.5±32.5^{a}	48.3±17.2^{a}	0.36±0.01^{b}	1.91±0.08^{a}
T-Iso	348.8±94.3^{b}	17.0±10.0^{a}	44.3±32.9^{a}	28.3±8.2^{a}	0.29±0.01^{c}	2.32±0.11^{b}
T-Iso+Oxy	795.5±132.0^{ab}	198.8±118.9^{a}	75.0±110.3^{a}	33.3±27.9^{a}	0.38±0.01^{ab}	1.84±0.07^{a}
	p=0.032	p=0.116	p=0.642	p=0.296	p=0.00	p=0.00

Conclusion

Of the many algal species that have been used in aquaculture, *Rhodomonas* and *Isochrysis* have been conspicuously successful as a food for rearing copepod species. The diets with *Rhodomonas* and *O. marina* clearly positively affect the development of *A. grani.* To achieve improved productivity of *A. grani* we suggest using a binary diet was the best option. However and for further studies, a tri-algal diet (Rho+Oxy+T-Iso) will probably improve the production of *A. grani*, being more complete for all stages, due to their different requirements.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

CURRENT STATUS OF CRAB LARVICULTURE IN THAILAND AND DEVELOPMENT OF A DIET FOR DOMESTICATED BROODSTOCK

M. Tamtin[1,2], M. Wille[2], J. Saeton[3], K. Kademuan[4], V. Tanasomwang[5], P. Sobhon[3], and P. Sorgeloos[2]

[1] Phetcha Buri Coastal Aquatic Feed Research Center, Coastal Aquatic Feed Research Institute, Phetcha Buri, 76100, Thailand
[2] Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Rozier 44, B-9000, Belgium
[3] Department of Anatomy, Faculty of Science, Mahidol University, Bangkok, Thailand
[4] Thung Tala Forest Royal Development Study and Crab Conservation Center, Krabi, Thailand
[5] Coastal Fisheries Research and Development Bureau, Department of Fisheries, Bangkok, 10900, Thailand

Research on sustainable culture of blue swimming crab (*Portunus pelagicus*) and mud crab (*Scylla* spp.) began in DOF in 1956, and commercial culture started in 1987. A breeding research program was initiated in 1978 with limited success. In 2000 the production of blue swimming crabs peaked at 43 871 tons and 6921 tons for mud crabs. The production decreased drastically to 22 836 tons and 2130 tons, respectively in 2010. To further stimulate production, hatchery techniques have been developed. Initially, wild berried females were used as broodstock. Larval rearing may be intensive (indoors) or extensive (outdoor earthen ponds). For early stages, rotifers and *Artemia* are the most widely used live food, combined with greenwater culture (*Chlorella* or *Tetraselmis* spp.). In later stages, a wide variety of food is used. Around 5 million crablets, 2.5 million megalopa, and 150 million zoea can be obtained annually, with survival rates of 10% and 25-30% for mud crab and blue swimming crab, respectively. For reasons of sustainability, broodstock domestication techniques have been developed and a breeding program is conducted.

The lipid dynamics during ovarian maturation of wild-caught (collected from the Gulf of Thailand) female blue swimming crabs (*P. pelagicus*) at different stages of gonad maturation and egg development were analyzed for total lipid, lipid classes (LC), and fatty acid (FA) composition in the hepatopancreas, ovary, muscle, and extruded eggs. Total lipid accumulation in the hepatopancreas decreased with ovarian maturation until full maturation, with a concomitant increase in the ovary. Total lipid level of eggs significantly decreased through embryonic development. Neutral lipid (NL) was abundant in the hepatopancreas

with free fatty acids (FFA), triacylglycerols (TAG), and cholesterol+diacylglycerols (DAG) as major LC. Ovarian lipid was composed mainly of TAG, cholesterol+DAG, and phosphatidylcholine (PC). The same LC plus FFA were found in the lipid fraction of the eggs. The major fatty acids in ovary and hepatopancreas were 16:0, 16:1n-7, 18:0, 18:1n-9, 20:4n-6, 20:5n-3, and 22:6n-3. Their level increased in the hepatopancreas up to development stage 3, whereas in the ovary they were highest at full maturation, except for 20:4n-6, which was quite constant throughout maturation. The same major fatty acids were found in spawned eggs as in ovaries, but the level of 20:4n-6 decreased beyond stage 2. All tissues and eggs had higher n-3 HUFA levels relative to n-6 PUFAs, and with the exception of muscles, all tissues had higher DHA than EPA levels. The lipid requirements of developing ovaries is met by both ingested dietary lipids and from stores in the hepatopancreas, and 20:4n-6 appears to be utilized during egg development.

The dietary total lipid level requirements of pond-raised blue swimming crab (*P. pelagicus*) broodstock were determined. Five isonitrogenous diets containing five different dietary total lipid levels of 6.98, 9.40, 11.19, 12.46, and 15%, respectively, were compared in a feeding trial. The dietary lipid level significantly influenced the gonadosomatic index (GSI), fecundity, and reproductive performance of the crabs and the hatching rate and osmotic shock tolerance of the crab larvae. The optimum dietary total lipid level was determined based on the performance of broodstock and the egg and larval quality. The highest dietary total lipid levels had adverse effects on fecundity and hatching rate. Besides the gross quantitative indicator gonadosomatic index (GSI), more precise assessment methods, namely histological techniques and yolk protein (vitellogenin) determination, were used to evaluate the effect of total lipid on ovarian maturation.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

ONTOGENETIC DEVELOPMENT OF THE DIGESTIVE SYSTEM IN REARED FAT SNOOK (*CENTROPOMUS PARALLELUS*) LARVAE

A. Teles[1], W.M. Costa[1], D. Ammar[2], E.M. Nazari[2], Y.M.R. Muller[2], and V.R. Cerqueira[1]

[1] Universidade Federal de Santa Catarina, [a]Laboratório de Piscicultura Marinha, CCA, Departamento de Aquicultura, CCB, CP 476, 88.040-900 Florianópolis, Santa Catarina, Brasil
[2] Universidade Federal de Santa Catarina, Laboratório de Reprodução e Desenvolvimento Animal, CCB, CP 476, 88.040-900 Florianópolis, Santa Catarina, Brasil

Introduction

Knowledge about developmental changes in the digestive tract associated with feed assimilation processes is essential to understanding the nutritional physiology of fish larvae (Gisbert et al., 2004), since one of the main concerns in marine fish hatcheries is proper timing of changing from live feed to artificial diets.

An important tool to assess the potential of different diets is the study of organogenesis by histomorphology. Several studies have focused on the development of digestive enzymes and the histochemistry of the digestive tract, however, few studies used immunohistochemistry as a tool to identify digestive enzymes in fish larvae. Several studies over the past 20 years have focussed on the ontogeny of the digestive tract of marine fish larvae (Hachero-Cruzado et al., 2009), however such studies have not been done for fat snook *Centropomus parallelus*.

Snooks are carnivorous fish found from the coastal waters of the southeastern United States to southern Brazil in marine, estuarine, and fresh waters and are considered important for marine fish farming and restocking of estuarine and inland waters (Cerqueira, 2010).

The purpose of this paper is to describe the development of the digestive tract of laboratory-reared fat snook larvae, as well as to identify the onset of trypsin production, aiming to improve larviculture outcomes.

Materials and methods

Captive broodstock of fat snook were used for induced spawning with LHRH-a hormone. Eggs were collected from the breeding tank, incubated, and transferred

to larval rearing tanks (5000 l), with a density of 50 eggs.l^{-1}, temperature at 25.1±1.0°C, oxygen 6.4±0.65mg.l^{-1}, and salinity 33-35g.l^{-1}.

After hatching and during the first eight days, the rearing tanks were maintained in a static system. Water renewal started on day 8 (10%) and increased gradually to 100% on day 50. Larvae were initially fed rotifers (*Brachionus rotundiformis*) cultivated with microalgae (*Nannochloropsis oculata*) and a commercial diet (Culture Selco® Plus and Protein Selco®, INVE Aquaculture). From day 19 to day 53, larvae were fed *Artemia franciscana* nauplii and metanauplii enriched with a commercial emulsion (DHA Selco®, INVE Aquaculture). On day 42 co-feeding started with an artificial diet (NRD®, INVE Aquaculture), which was the only food item from day 54 onwards.

Samples of larvae (n=20) were taken from hatching until day 60, anesthetized in benzocaine solution (50mg.l^{-1}), then standard length (SL) was measured. They were then fixed in Bouin's aqueous solution and preserved in 70% ethanol. Fixed larvae were embedded in paraffin blocks, sagittally sectioned between 5-8µm, and stained with Mallory's trichrome to follow larval development. Immunohistochemistry was performed to identify the expression of trypsin, using antitrypsin rabbit IgG polyclonal antibody 1:100 (Santa Cruz Biotechnology). The endogenous peroxidase activity was stopped with 5% hydrogen peroxide in methanol. The sections were washed in 0.1M PBS (pH 7.4) Triton X-100 0.3% and blocked with 5% fetal bovine serum in PBS. The sections were incubated overnight at 4°C with primary antibody, followed by washes with PBS before being incubated for 90min with peroxidase-conjugated anti-rabbit IgG 1:300 (Sigma). They were then washed in PBS, and antibody binding sites were revealed with 3-3-diaminobenzidine (DAB) (Sigma). Negative controls were treated in the same manner as described above, except that the primary antibody was replaced by PBS buffer 0.1M (pH 7.4).

Results and discussion

From hatching to day 7 (yolk-sac larva, 1.7-3.0mm SL)
Differentiation of the digestive tract in oropharynx, esophagus, stomach and rudimentary intestine was established at the time of mouth opening, coincident with first feeding. At the same time, mucus-secreting goblet cells were absent or scarce in the esophagus and oropharynx, and appear a few days later. It was also observed formation of the pancreas and hepatocytes with large nuclei and homogeneous cytoplasm. On day 3, the intestinal valve between anterior and posterior intestine was formed. It is on day 4 that vacuolated cells appear in the posterior intestine, indicating the beginning of protein digestion and pinocytosis. At this age, the stomach begins to exhibit folds, the gallbladder is present, the liver is already well delimited with vacuolated cells, and pancreatic cells already have an acinar arrangement.

On day 5, trypsin, considered a key enzyme, since it activates other pancreatic proteases such as chymotrypsin, is marked for the first time. On day 7, there are numerous zymogen granules in the pancreas; trypsin marking is pronounced; the pyloric sphincter is formed; the intestinal valve is well developed; and there is a narrowing of the intestine middle portion, which now presents three quite distinct compartments.

From day 9 to day 15 (pre-flexion larva, 3.1-3.5mm SL)
On day 9, the presence of pharyngeal teeth is observed; microvilli appear in the foregut; and muscle cells are present in the walls of the esophagus and stomach. On day 11, the oral valves are observed; there is pancreatic diffusion through the abdominal cavity and the differentiation of two regions in the stomach. Mucous cells of the esophagus are observed on day 13. On day 15, there are taste buds in the pharynx. The twisting of the digestive tract becomes more evident at this age, just as there is a greater abundance of intestinal villi and a significant increase in the liver volume.

From day 18 to day 27 (flexion larva, 4.0-6.0mm SL)
On day 18 there are lots of muscle fibers around the esophagus. At day 21, it is observed a perpendicularity of the midgut in relation to the stomach; there are numerous goblet cells in the mucosa of the esophagus; and a reduction of vacuoles in the hindgut.

From day 30 to day 60 (post-flexion larva, 7.6-16.9mm SL)
On day 30, there are lipid vacuoles in the anterior and middle intestine (Fig. 1A), and the first appearance of the gastric glands (Fig. 1B). On day 35, the hindgut vacuoles are no longer observed. The appearance of gastric glands and the disappearance of supranuclear vacuoles in the hindgut indicate digestion capability. The coexistence of pinocytosis and extracellular digestion in fish larvae is considered a compensation mechanism for the incomplete digestion of macromolecules, until there is proliferation of gastric glands (Govoni et al. 1986). On day 55, goblet cells are visible in the esophagus (Fig. 1C arrowheads). On day 40, there are no more goblet cells in the stomach, which has three well defined regions: cardiac, pyloric, and fundus (Fig. 1D). In the fundus there are many gastric glands (arrows) with an evident and clear inner cavity, and a duct that opens into the organ lumen. Thereafter, no marked morphological changes are found in the stomach. On day 45, the hepatocyte cytoplasm is filled with lipid vacuoles. On day 40, pharyngeal teeth inserted in the cartilage (Fig. 1E arrow), and on day 60, teeth are forming in the mouth (Fig. 1F arrow and right-bottom detailed picture); well-developed pyloric caeca are observed, and pancreas invades the liver tissue.

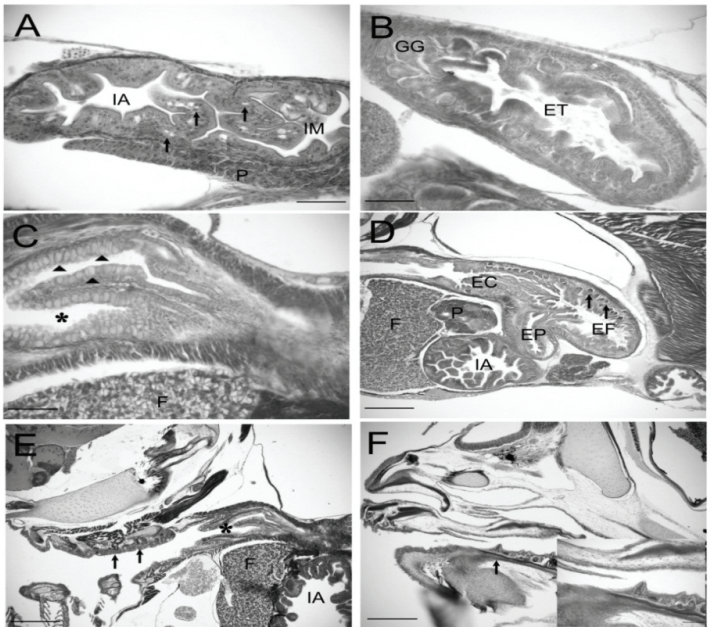


Fig. 1. Sagittal sections of snook larvae. Abbreviations: EC cardiac stomach, EF fundic stomach, EP pyloric stomach, ET stomach, F liver, GG gastric glands, IA foregut, IM midgut, P pancreas; * esophagus. Bars: 50 μm (A, B, C), 200 μm (D, E, F).

Conclusion

The present data showed that fat snook larvae are able to be weaned to artificial diets on day 30, due to the appearance of gastric glands. This means a gain of 15 days compared to most of the previous studies, and can significantly improve fat snook larviculture.

References

Cerqueira V.R. 2010. Cultivo do robalo-peva, *Centropomus parallelus*. p. 489-520. In: Espécies nativas para a piscicultura no Brasil. Baldisserotto, B., Gomes, L.C. (Eds.). Editora da UFSM, Santa Maria, Brasil. 608p.

Gisbert E., R.H. Piedrahita, and D.E. Conklin. 2004. Ontogenetic development of the digestive system in California halibut (*Paralichthys californicus*) with notes on feeding practices. Aquaculture 232:455-470.

Govoni J.J., G.W. Boehlert, and Y. Watanabe. 1986. The physiology of digestion in fish larvae. Environmental Biology of Fish 16:59-77.

Hachero-Cruzado I., J.B. Ortiz-Delgado, B. Borrega, M. Herrera, J.I. Navas, and C. Sarasquete. 2009. Larval organogenesis of flatfish brill *Scophthalmus rhombeus* L: Histological and histochemical aspects. Aquaculture 286: 138-149.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

CHOLECYSTOKININ AND TRYPTIC ENZYME ACTIVITY IN SEA BASS (*DICENTRARCHUS LABRAX*) LARVAE: A REGULATORY LOOP AND THE IMPACT OF FEEDING REGIMES

R. Tillner[1*], I. Rønnestad[2], P. Dhert[3], and B. Ueberschär[1,4]

[1] GEOMAR Helmholtz Centre for Ocean Research, Duesternbrooker Weg 20, 24105 Kiel, Germany. [*]E-mail: rtillner@geomar.de
[2] Department of Biology, University of Bergen, Pb 7800, 5020 Bergen, Norway
[3] Skretting, Akkerhage 4, 9000 Gent, Belgium
[4] GMA – Society/Association for marine aquaculture Ltd., Hafentörn 3, 25761 Buesum, Germany

Introduction

Although there is growing information on fundamental processes controlling digestive functions in marine fish larvae, this rarely translates into practical tools for feed formulations or feeding regimes. While fish larvae possess an effective battery of alkaline enzymes deriving from the pancreas for digestion in the intestine, low levels of these enzymes have been observed in several species during ontogeny, which may contribute to the overall high mortality usually observed in marine fish larvae after first-feeding (Ueberschär, 1995). Trypsin is considered to be a key enzyme in the digestive process as it is the most significant protease in the early larval stages and the hormone cholecystokinin (CCK) is considered one of the most important stimulators of pancreatic enzyme secretion in mammals. High tryptic enzyme capacity in the gut acts as a negative feedback control for the release of CCK, and vice versa, suggesting a regulatory loop.

Phytohemagglutinin (PHA) is known to trigger the endocrine mechanisms involved in digestion and eventually to enhance maturation of the gut and digestive enzyme capacity. Sea bass larvae were reared with live feed, a commercial micro-diet, and the same diet enriched with different concentrations of PHA. Individual larvae were analyzed for CCK concentration and tryptic enzyme activity on a short-term (hourly) and in addition tryptic enzyme activity on a long-term scale in order to evaluate the effect of the trigger PHA on the development of the digestive enzyme capacity. Apart from disclosing the postulated feedback loop among CCK and tryptic enzyme activity, the results may provide valuable information on the diurnal digestive capacity in sea bass larvae related to different diets and practical feeding conditions.

Materials and methods

Sea bass larvae were reared under standard conditions until 35 dph and assigned to the following feeding regimes: (1) *Artemia*, (2) *Artemia* + Gemma Micro (Skretting, Norway), (3) Gemma Micro, (4) Gemma Micro incl. 0.01% PHA, (5) Gemma Micro incl. 0.02% PHA. *Artemia* were fed three times daily at 9h00, 14h00 and 19h00 during the experiment. Gemma Micro was provided hourly in excess between 8h00-22h00 by hand and automated feeders. Gemma Micro was provided 60 and 30min before the administration of *Artemia* in the *Artemia* + Gemma Micro group. A 17 hour experiment was conducted at 23 dph. Larvae from the *Artemia* and Gemma Micro treatment were transferred to one respective starving tank at 8h00 and starved for the whole day ("ArtS" and "GMS"). Five larvae were sampled from each tank every full hour between 6h00 and 23h00. Individual samples were analyzed for CCK and tryptic enzyme activity according to Rojas-García et al. (2001).

Results and discussion

Both groups fed either *Artemia* ("Art") or co-fed *Artemia* and Gemma Micro ("ArtGM") grew faster compared to the three groups fed solely on Gemma Micro. In addition, larvae fed solely on *Artemia* showed the highest standard length compared to co-fed larvae after 19dph until the end of the experimental period. Among the three Gemma Micro groups, the inclusion of 0.01% PHA led to higher growth at 13 and 15dph, whereas the Gemma Micro group without PHA revealed a higher growth from 15dph until the end of the experimental period. The inclusion of 0.02% PHA resulted in the lowest values in standard length after 13dph among the three Gemma Micro groups. All groups revealed an increase in tryptic enzyme activity during ontogeny with increasing individual variability with increasing size (Fig. 1). The development of CCK revealed a largely fluctuating pattern during the day in all groups with significant differences between groups at 7h00, 9h00, 16h00, 17h00, 19h00, and 21h00. Large differences were also recorded for tryptic enzyme activity which clearly responded to feeding events (Fig. 2).

The higher tryptic enzyme activity in the groups fed Gemma Micro between 7 and 8.5mm fits to the higher protein content in microdiets compared to live feed. Our results underline the option to manipulate the maturation of the digestive tract by specific components in the diets and indicate that Gemma Micro sufficiently stimulates the digestive system compared to *Artemia*. The inclusion of 0.02% PHA in Gemma Micro resulted in lower enzyme activity; this result may indicate that the concentration of PHA was above a threshold at which PHA was supposed to have a negative effect on the digestive system.

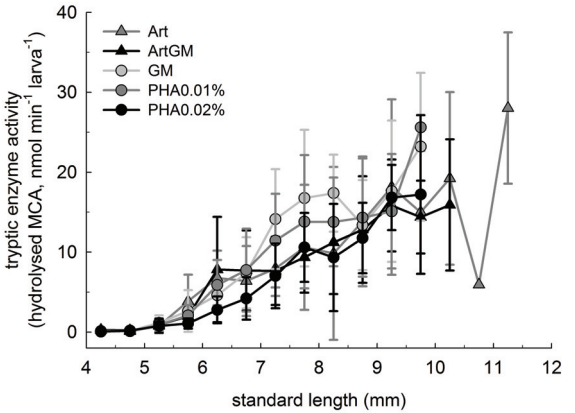


Fig. 1. Tryptic enzyme activity for length classes (standard length, 0.5mm precision, 4.0-4.5, 4.5-5.0, etc.) of larval sea bass (excluding head). Feeding treatments: (Art) *Artemia*, (ArtGM) *Artemia* + Gemma Micro, (GM) Gemma Micro, (PHA0.01%) Gemma Micro incl. 0.01% phytohemagglutinin (PHA), (PHA0.02%) Gemma Micro incl. 0.02% PHA. Data are presented as mean ± standard deviation (1-33 larvae per length class per treatment).

Remarkably, CCK revealed a relatively similar pattern among treatments in the experiments on the diurnal rhythm. This similarity is even more pronounced among the *Artemia*-derived treatments (ArtS, Art, ArtGM) and may indicate that the response of CCK follows a pre-programmed, endogenous rhythm independent of feed intake. The three peaks in tryptic enzyme activity at or shortly after the scheduled feeding events in the *Artemia*-starving larvae indicate an imprinting concerning feeding times and synchronization with the availability of digestive enzymes ready for hydrolysis of incoming feed. It has to be noted, that the larvae were adapted to the administration of food at the same hour for a long period. Actual uptake of food, such as *Artemia*, further enhances the release and activation of trypsin into the gut beyond the values which were assumed to be a result of the imprinting only. Among the groups fed with Gemma Micro with or without PHA the development of CCK was less comparable, which may be a consequence of the less advanced developmental stage of the groups fed only with micro diets (see growth above). The absence of a clear diurnal pattern in tryptic enzyme activity in the groups fed with Gemma Micro only could be explained by a continuous availability of feed particles and consequently continuously new protein as a substrate for trypsin. The results from the one-day experiment indicate that the dose of PHA was beyond the threshold where the positive effect may turn into a harmful impact. Future experiments will consider the enrichment with lower doses.

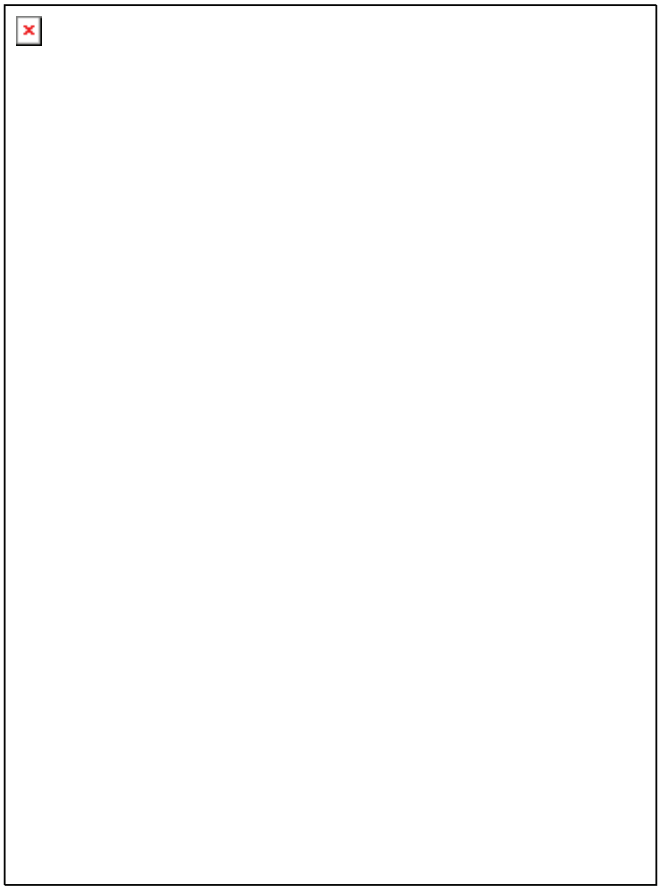


Fig. 2. CCK and tryptic enzyme activity in larval sea bass (excluding head) between 6h00 and 23h00 at 23dph. See text for treatment details. Grey bars represent the feeding period of Gemma Micro every full hour, grey arrows the administration of *Artemia* nauplii. Different superscripts represent significant differences between feeding treatments at each time point (nested one-way ANCOVA, Tukey-HSD, $p<0.05$). Data are represented as mean ± standard deviation ("ArtS" and "GMS" n=5 individuals, all feeding treatments n=3 tanks).

References

Rojas-García C.R., I. Rønnestad, et al. 2001. "Combined sensitive analytical methods for cholecystokinin levels and tryptic activity in individual fish larvae." Journal of Experimental Marine Biology and Ecology 265(1):101-115.

Ueberschär B. 1995. "The use of tryptic enzyme activity measurement as a nutritional condition index: laboratory calibration data and field application." ICES Mar Sci Symp 201:119-129.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

REPRODUCTION OF EUROPEAN EEL AND LARVAL CULTURE – STATE OF THE ART

J. Tomkiewicz[1*], J.G. Støttrup[1], G. Corraze[2], S. Kausik[2], L.K. Holst[3], F. McEvoy[4], S. Dufour[5], A.G. Lafont[5], J. Asturiano[6], S.R. Sørensen[1], H. Tveiten[7], P. De Schryver[8], I.A.E. Butts[1], P. Munk[1], J. Zambonino-Infante[9], S.N. Politis[1], M. Krüger-Johnsen[1], and P. Lauesen[10]

[1] Technical University of Denmark, National Institute of Aquatic Resources. Kavalergården 6, 2920 Charlottenlund, Denmark (*jt@aqua.dtu.dk)
[2] Inra - UR 1067 Nutrition Métabolisme Aquaculture, F-64310 Saint Pée-sur-Nivelle, France
[3] BioMar, Mylius Erichsensvej 35, 7330 Brande, Denmark
[4] Copenhagen University, Department of Small Animal Clinical Sciences, Faculty of Life, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark
[5] Muséum National d'Histoire Naturelle, Research Unit BOREA, Biology of Aquatic Organisms and Ecosystems, CNRS 7208- IRD207- UPMC, 75231 Paris Cedex 05, France.
[6] Grupo de Acuicultura y Biodiversidad. Instituto de Ciencia y Tecnología Animal. Universitat Politècnica de València. Camino de Vera s/n 46022 Valencia, Spain.
[7] Nofima, Muninbakken 9-13 Breivika, NO-9291 Tromsø, Norway
[8] Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Rozier 44, 9000 Gent, Belgium
[9] Ifremer, Unit of Functional Physiology of Marine organism, BP 10070, 29280 Plouzané, France.
[10] Billund Aquaculture Service Aps., Kløvermarken 27, Billund, 7190, Denmark

Aquaculture of European eel (*Anguilla anguilla*) relies exclusively on wild-caught glass eels. Hence, the long-term goal of reproduction of eels in captivity is to provide a stable production of fry. Breeding of eels in captivity is hampered by complex hormonal control mechanisms that inhibit eel reproduction while being in European waters and aquaculture. In the EU project PRO-EEL, we induced maturation and gonadal development in captive and wild-caught eels through hormonal treatment of broodstock in order to produce viable gametes. Fertilization was made in vitro from stripped eggs and milt to obtain embryos and larvae. Using an inter-disciplinary approach, egg quality and larval development competence was improved and larval longevity and survival gradually enhanced to reach feeding stages. Reproduction experiments included farmed female eels on diets with improved lipid profiles and wild female silver eels. Farmed female broodstocks were fed three different diets varying primarily in levels of arachidonic acid (ARA), eicosapentaenoic acid (EPA), and docosahex-

aenoic acid (DHA) prior to experiments. Eggs were obtained from female farmed and wild broodstocks treated with Salmon Pituitary Extract (SPE) to induce maturation and dihydroxy-progesterone (DHP) to induce oocyte maturation and ovulation, while milt was obtained from farmed male broodstocks treated with human chorion gonadotropin, hCG. After fertilization, eggs were incubated at 20°C in filtered natural seawater adjusted to a salinity of 36ppt. Prior to hatch (~45h post-fertilization) embryos were transferred to rearing systems with seawater (as above) containing 40ppm penicillin and 65ppm streptomycin to reduce microbial interference. Reproduction success of stripped farmed female eel was influenced by diet in terms of fertilization, embryonic and larval production. Females fed a diet high in ARA and enhanced EPA:DHA ratio had higher likelihood, producing viable offspring than eels fed the standard feed, reaching the same success rate as wild eels. However, the quantity and quality of the eggs and larvae produced was generally higher in wild female eels. The ARA, EPA, and DHA in farmed eel eggs reflected the feed and approached wild eel egg composition, but did not reach the same levels. Furthermore, yolk-sac size of larvae produced by farmed females was lower on average,. Larvae were obtained from all broodstocks, however, changing the female treatment scheme from constant to increasing SPE dose enhanced larval production. Thus, wild female eels subjected to increasing dose produced more viable offspring than those on constant dose. Eel fecundity is high and some batches comprised several hundred thousand eggs and larvae, allowing the initiation of larval culture and feeding trials. These results are promising for production and establishment of captive reproduction of European eel for a self-sustained aquaculture. Present focus is on developing new and innovative techniques and technology for larval rearing and feeding.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

APPLYING OF FRESHWATER ROTIFER (*BRACHIONUS ANGULARIS*) IN REARING NEWLY HATCHING FRIES OF MARBLE GOBY (*OXYELEOTRIS MARMORATUS*)

Tran Suong Ngoc[1], Nguyen Van Hoa[2], and Vu Ngoc Ut[1]

[1] Department of Applied Hydrobiology, College of Aquaculture and Fisheries, Cantho University, Vietnam
[2] Department of Coastal Aquaculture, College of Aquaculture and Fisheries, Cantho University, Vietnam

Introduction

Sand goby (*Oxyleotris marmoratus*) is one of the largest gobies in the world with a good taste, non-bony composition, and high protein value. Their high value and increasing market demand have resulted in expansion of sand goby production in Vietnam. The growth and survival rate of larval and juvenile fish are determined by various environmental factors, as well as types of feeding. The food supply during larval stage is an important factor to ensure high growth and survival rates, especially during the first few days as newly hatched sand goby larvae. Prey selection is affected by characteristics of both the larvae and the prey, including fish mouth size, prey size, and prey density (Pham Thanh Liem, 2001). The total length of *O. marmoratus* on the second day after hatching was ~4mm with a mouth size of 0.1mm (Tavarutmaneegul and Lin, 1988), therefore they certainly require small prey. Rotifers are widely used as live food in rearing both marine and freshwater larval fish, crabs, prawn, shrimp, and molluscs (Sarma, 1991). *Brachionus angularis* is among the smallest freshwater rotifer at 110-150µm in size (Hu and Xi, 2008) that could be suitable prey for sand goby larvae. The purpose of this study was to investigate feed and feeding scheme in the culture of larval sand goby from newly hatched to 10 days old.

Materials and methods

Sand goby eggs were collected from the fish hatchery in Hau Giang province and incubated at 28°C. Newly hatched larvae (2 days old) were used for experiments. *B. angularis* was isolated from catfish nursery ponds and were fed with *Chlorella* sp. at a concentration of 60 000 cells.rotifer^{-1}.day^{-1}(Ngoc et al., 2010). The single-celled green algae *Chlorella* sp. was cultured on Walne medium and harvested in the log phase of growth. The experimental design for sand goby consisted of 12 composite tanks (80ml capacity) and stocked at 10ind.ml^{-1}. The

first experiment was to determine the suitable rotifer density for feeding. This experiment was set up with four triplicate treatments, in which rotifers were stocked at 5, 8, and 11ind.ml^{-1} while the control treatment was fed by heated chicken-egg and Soya meal slurry (Hung and Khanh, 2003). The second experiment was performed to detect an appropriated density of *Chlorella* supplemented as substrate in greenwater system. It included four treatments with *Chlorella* density at 0 (control), 0.5, 1, and 1.5×10^6cells.ml^{-1}. Feeding of rotifers was 11ind.ml^{-1}. Fish larvae were fed four times per day at 8h, 12h, 16h, and 20h, respectively. Rotifer density was determined at first and adjusted based on eating by fish larvae. Water exchanges of 30% in volume were done every three days. Temperature, pH, and DO was measured every day. TAN, N-NO_2^-, and N- NO_3^- was collected every three days. Total length and survival of fish was measured at the end of experiments.

Results and discussion

Water conditions included: temperature, 27.9-28.7°C; pH, 8.16-8.27; DO, 4.80-5.54mg.l^{-1}; concentration of TAN, 0.075-0.425mg.l^{-1}; NO_2^-, 0.019-0.035mg.l^{-1}; and NO_3^-, 0.093-0.903 mg.l^{-1}. Water conditions were suitable for fish larvae growth.

The average length of fish larvae at the beginning of the experiment was 3.21±0.18mm and reached 5.07-5.11mm after nine days with no significant differences among treatments ($p>0.05$).

Survival rate of larvae at the end of the first experiment varied from 19.3-35.3%. Feeding of egg and soya-meal slurry to fish larvae gave the lowest results because of sinking of food. According to Tavarutmaneegut and Lin (1988), *O. marmoratus* larvae were poor feeders due to their passive feeding behaviour, so they could not catch food effectively. Besides, locomotory organs after two days post-hatching were not yet well-developed, but rather only "swim up, sink down" behavior (Senoo et al., 1994) caused inefficient feeding. As a result of a mucus layer from uneaten food at the bottom tank, some of fish larvae were trapped and became weak and died. Figure 1a shows freshwater rotifer *B. angularis* were proper prey for *O. marmoratus* larvae. The importance of the prey organism size in food selection by larval fish have been demonstrated. According to Pham Thanh Liem (2001), the mouth size of *O. marmoratus* at the 3rd day after hatching was 298µm and the ratio between prey size and mouth height ranged from 21.5-31.8% at first-feeding. The suitable size for fish larvae in this stage was 64-94µm while the Vietnamese strain of *B. angularis* is 68.2±10.76µm wide and 81.9±9.74µm long.

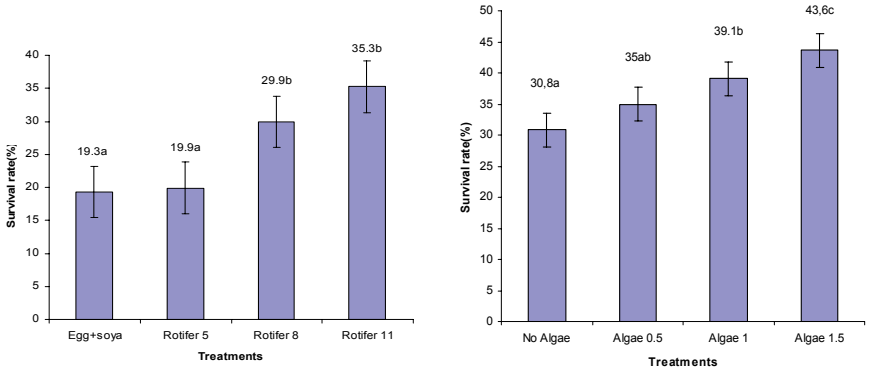


Fig. 1. Survival of fish larvae at the end of the 1st (left) and 2nd (right) experiments.

According to Tavarutmaneegul and Lin (1988), Van der Meeren (1991), and Morales-Ventura et al. (2004), the large variation in fry survival rates was most likely caused by different population densities of food organisms, particularly rotifers. Low survival rate of larvae fed at 5 rotifers.ml^{-1} was significantly different from higher rotifer densities. The prey might not adequately meet the demand of fish larvae when feeding at 5 rotifers.ml^{-1}. With the higher densities of rotifers, survival rates of *O. marmoratus* larvae were much improved, as fishes could catch prey much easier.

In the second experiment, survival rates of larvae were significantly different among treatments (Fig.1b). According to Pham Thanh Liem (2001), on day 2 post-hatching, *O. marmoratus* larvae commenced feeding on phytoplankton at 95% on day 2 to 100% on day 3 after hatching. Therefore, fish larvae could eat *Chlorella* on day 2 post-hatching while larvae had no food in the control treatment on the same day. Kailasam et al. (2007) showed survival rate was higher when sea bass larvae were initially fed at 48h when compared to 72h and 96h. On the other hand, *Chlorella* was the best microalgae in terms of quality, with high protein content (50%), lipid (20%), vitamin A, B_1, B_2, B_6, B_{12}, C, D, K, nicotinic acid, and pentatonic acid (Tran Van Vy, 1995) and kept the prey in high nutrition value due to the organisms feeding continuously. Besides, the algae could help to maintain water quality in the nursery tanks. Addition of algae to rearing tanks influenced the light regime in the tanks, consequently affecting the feeding behaviour of the larvae (in optimal light condition). These reasons explain why survival rate of fish supplemented by *Chlorella* was higher than control treatments.

Conclusion

Feeding experiments to improve survival of sand goby larvae from newly hatched to 10 days old stage indicate optimal densities of freshwater rotifer *B.*

angularis from 8-11 rotifers.ml^{-1}. The survival rate of fish will be improved if *Chlorella* is applied in densities from 1-1.5×10^{6}cell.ml^{-1}.

References

Coutteau P. 1996. Micro-algae. p.9-61. In: Manual on the production and use of live food for aquaculture. Patrick Lavens and Patrick Sorgeloos (Eds). Published by Food and Agriculture Organization of the United Nations.

Hu H. and Y. Xi. 2008. Demographic parameters and mixis of the three Brachionus angularis Gosse (Rotatoria) strains fed on different algae. Limnologica 38:56-62p

Hung N.M and P. Khanh. 2003.Ky thuat nuoi ca bong tuong. NXB Nong Nghiep.46p

Kailasam M., A.R. Thirunavukkarasu, S. Selvaraj, and P. Stalin. 2007. Effect of delayed initial feeding on growth and survival of Asian sea bass Lates calcarifer (Bloch) larvae. Aquaculture 271:298-306

Liem P.T. 2001. Studies on the early development and larval rearing of rearing of Oxyeleotris marmoratus (Bleeker). Degree of Master of Science in the Faculty of Science and technology. Kolej Universiti Terengganu. Universiti Putra Malaysia

Morales-Ventura J., S. Nandini, and S.S.S. Sarma. 2004. Functional response during the early stages of the charal fish Chirostoma riojai (Pisces: Atherinidae) fed rotifers and cladocerans. Journal of applied Ichthyology 20:417-421

Ngoc T.S., N.T. Duc, N.T Khuong, and V.N. Ut. 2010. Effect of Chlorella and yeast on population growth of freshwater rotifer (Brachionus angularis). Scientific journal of Can Tho University. Vol 14:66-75

Sarma S.S.S., N. Iyer, and H.J. Dumont. 1991. Rotifers and aquaculture (Review). Environmental Ecology. 9:414-428.

Senoo S.; M. Kaneko; S.H. Cheah, and K.J. Ang. 1994. Egg development, hatching and larval development of marble goby Oxyeleotris marmoratus under artificial rearing conditions. Fisheries Science 60(1):1-8

Tavarutmaneegul P and C.K. Lin, 1988. Breeding and Rearing of Sand Goby (Oxyeleotris marmoratus Blk.) fry. Aquaculture 69:299-305

Tran Van Vy. 1995. Thuc an tu nhien cua ca. NXB Nong nghiep. 292p

Van der Meeren T. 1991. Selective feeding and prediction of food consumption in turbot larvae (Scopthalmus maximus L.) rearing on the rotifers Brachionus plicatilis and natural zooplankton. Aquaculture 93:35-55.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

COPEPODS ENHANCE GROWTH AND DEVELOPMENT IN ATLANTIC COD (*GADUS MORHUA* L.) LARVAE

T. van der Meeren[1], Ø. Karlsen[1], A. Mangor-Jensen[1], I. Rønnestad[2], and K. Hamre[3]

[1] IMR, Austevoll Research Station, NO-5392 Storebø, Norway
[2] University of Bergen, Dept. of Biol., PO Box 7803, NO-5020 Bergen, Norway
[3] NIFES, PO Box 2029 Nordnes, NO-5817 Bergen, Norway

Introduction

Copepods have a superior feed quality and result in both high growth and survival rates combined with low incidence of malformations when fed to fish larvae. This is often linked to their contents of essential nutrients (van der Meeren et al., 2008; Hamre et al., 2008), although the mechanisms of how copepods as feed may affect the larval development are still unclear. With the new and rapidly developing molecular tools, including high-throughput transcriptomic techniques, there is an increasing interest of research in this area. In particular, interactions between the phenotype, gene expression, and environmental factors are in focus, and feed represents an important environmental input to the larva in this respect.

Copepods for larval fish rearing are in limited use, mainly because of the difficulties of producing copepod nauplii in sufficient amounts at a specific time. These difficulties are related to a long generation time in copepods as compared to rotifers. To overcome this, a production unit for copepods needs to be large in order to enable harvesting of sufficient amounts of copepod nauplii for the young larval fish. In this study we established a large-scale production system for copepods and performed a subsequent startfeeding experiment with larval Atlantic cod fed either copepods or rotifers/*Artemia*.

Materials and methods

Copepods were produced in "Svartatjern", a 25 000m^3 sea-water pond system (for details, see Naas et al., 1991) that was restarted early in 2011 after 10 years closure. One full year cycle of copepod production was performed to prime the pond with copepod resting or diapauses eggs. This enabled us to collect sufficient amounts of copepod nauplii (1.8×10^9 ind.) in the spring 2012 during the 5-weeks period which they were needed for start-feeding. Hydrography and water

visibility was monitored weekly, and agricultural fertilizer (22-3-10 NPK) was added to start and maintain the primary production (assessed as Secchi disc readings to be maintained at ~2m depth). Weekly tube samplings in the centre of the pond were used to monitor copepod concentrations. A UNIK-900 wheel filter was used to fractionate, concentrate, and harvest the copepods (van der Meeren and Naas, 1997).

At three days post-hatch, 50 000 cod larvae were transferred from egg incubators to each of 12 black 500-litre PEH tanks. Temperature was gradually increased from 6°C at transfer to 12°C at day 9 post-hatch. The rearing was carried out under a photoperiod of 16 hours light with smooth transition to and from darkness (0.5 hour). The tanks were illuminated each by 2×24W halogen light bulbs. Larvae in six tanks were fed rotifers grown in continuous cultures on baker's yeast and algal paste (Rotifer diet, Reed Inc.) and enriched with Multigain (Biomar) and selenium as described by Nordgreen et al. (2013). The rotifers were switched with *Artemia* between days 32 and 35 post-hatch. Larvae in the remaining six tanks were fed copepod nauplii (80-150µm fraction). To include an increasing portion of larger plankton (copepodids), the plankton fraction fed to the cod larvae was extended upwards to 180, 212, and 250µm at day 11, 18, and 23 post-hatch, respectively. The live feed was administered into the tanks three times a day, and algal paste (Nanno 3000, Reed Inc.) was added prior to each feeding. The larvae were weaned according to developmental stage (approximated from size), at day 37 post-hatch for the copepod fish and at day 56 post-hatch for the rotifer/*Artemia* fish. The experiment was terminated when the fish was 49 mm (day 73 and 84 post-hatch for the copepod and rotifer/*Artemia*-fed fish, respectively).

Results and discussion

A total of 288 millions copepod nauplii and 141 millions copepodids/copepods were harvested and used over the whole experiment. Similarly, 642 and 301 millions rotifers and *Artemia* was used, respectively. The cod larvae in the two feeding treatments showed almost similar growth until day 22 post-hatch, where after the copepod group quickly increased growth rate and departed from the rotifer/*Artemia* group. At day 36 post-hatch, the copepod group had reached a standard length of 12.3mm while the rotifer/*Artemia* group was 9.3mm. The copepod-fed larvae were also more pigmented than the larvae fed rotifers.

The average specific growth rate in length (SGR_L) from day 4 to 22 post-hatch was 2.51 and 2.43% daily increment in length for the copepod and rotifer/*Artemia* groups, respectively. From day 22 until weaning, SGR_L was 4.48% per day for the copepod larvae while it was only 2.41% per day for the rotifer/*Artemia* larvae. After weaning growth resumed in the rotifer/*Artemia* group, which then had a SGR_L slightly ahead of the copepod group (4.02 versus 3.81%

per day, respectively. The difference in size between the groups was also maintained after 9 months.

Larval cod feeding on copepods has the capability to grow very fast (up to 37% increase in weight per day in the late larval and early juvenile stages, see Finn et al., 2002). The boost in growth among the cod larvae after feeding on copepods for about three weeks in the early larval stages has been reported previously, both at low and high larval densities (Koedjik et al., 2008). According to Busch et al. (2011), the larger prey size of the copepods could not explain the discrepancy between the rotifer/*Artemia* group and the copepod group. Thus, the most plausible explanation is that this difference in growth is related to the nutritional quality of copepods.

Biochemical analyses indicate that the only nutrients that were suboptimal in rotifers in the present experiment were protein, taurine, and perhaps arachidonic acid (ARA), eicosapentaenoic acid (EPA), and phospholipids. Vitamins and minerals were present at sufficient levels (Hamre et al., 2013; Peglase et al., 2013). The copepod-fed larvae contained more taurine and ARA, but less EPA than larvae fed rotifers/*Artemia*. After the *Artemia* feeding period, larvae fed rotifers/*Artemia* also contained more lipid than those fed copepods, even though the diets had similar levels of lipid. Molecular, metabolic and histological analyses will be conducted to describe the underlying mechanisms of the differences in performance between copepod and rotifer/*Artemia* fed larvae. As the cod genome has been mapped, data from RNA sequencing will be of particular interest to investigate effects of the larval feed on development.

Acknowledgements

Thanks to technical staff at IMR-Austevoll for skilled assistance. Funded by The Research Council of Norway (CODE knowledge platform project www.uib.no/rg/mdb/projects/code-cod-development; grant. 199482/S40).

References

Busch K.E.T., S. Peruzzi, F. Tonning, and I.-B. Falk Petersen. 2011. Effect of prey type and size on the growth, survival and pigmentation of cod (*Gadus morhua* L.) larvae. Aquaculture Nutrition 17:595-603.

Finn N., I. Rønnestad, T. van der Meeren, and H.J. Fyhn. 2002. Fuel and metabolic scaling during the early life stages of Atlantic cod *Gadus morhua*. Marine Ecology Progress Series 243:217-234.

Hamre K., A. Srivastava, I. Rønnestad, A. Mangor-Jensen, and J. Stoss. 2008. Several micronutrients in the rotifer *Brachionus plicatilis* may be limiting for growth, survival and normal development of cod larvae. Aquaculture Nutrition 14:51-60.

Hamre K., M. Yufera, I. Ronnestad, C. Boglione, L. Conceição, and M. Izquierdo. 2013. Fish larval nutrition and feed formulation - knowledge gaps and bottlenecks for advances in larval rearing. Reviews in Aquaculture. (in press).

Koedijk R., A. Folkvord, A. Foss, K. Pittman, S.O. Stefansson, and A.K. Imsland. 2008. The influence of first-feeding diet on the Atlantic cod *Gadus morhua* phenotype: survival, development and long-term consequences for growth. Journal of Fish Biology 77:1-19.

Naas K.E., T. van der Meeren, and D.L. Aksnes. 1991. Plankton succession and responses to manipulations in a marine basin for larval rearing. Marine Ecology Progress Series 74:161-173.

Nordgreen A., S. Penglase, and K. Hamre, 2013. Increasing the levels of the essential trace elements Se, Zn, Cu and Mn in rotifers (*Brachionus plicatilis*) used as live feed. Aquaculture 380–383:120-129.

Penglase S., T. Harboe, Ø. Sæle, S. Helland, A. Nordgreen, and K. Hamre. 2013. Iodine nutrition and toxicity in Atlantic cod (*Gadus morhua*) larvae. PeerJ 1:e20; DOI 10.7717/peerj.20

van der Meeren T. and K.E. Naas. 1997. Development of rearing techniques using large enclosed ecosystems in the mass production of marine fish fry. Reviews in Fisheries Science 5:367-390.

van der Meeren T., R.E. Olsen, K. Hamre, and H.J. Fyhn. 2008. Biochemical composition of copepods for evaluation of feed quality in production of juvenile marine fish. Aquaculture 274: 375-397.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

A GNOTOBIOTIC MODEL SYSTEM: THE CASE OF *ARTEMIA FRANCISCANA*

S. Vanmaele*, T. Defoirdt, and P. Bossier

Laboratory of Aquaculture & *Artemia* Reference Center, Faculty of Bioscience Engineering, Department of Animal Production, Ghent University, Rozier 44, B-9000 Gent, Belgium *E-mail: sofie.vanmaele.sv@gmail.com

Introduction

Diseases still cause unpredictable and high mortalities in intensive crustacean culturing, leading to major economical losses. Controlling diseases has traditionally been done using antibiotics; however, in recent years resistance has become a major problem. Therefore, new strategies are being developed for a more sustainable production, one of which is immunostimulation (Bachere, 2003; Smith et al., 2003). However, the efficacy of these immunostimulants is not yet sufficiently validated, as are possible long-term effects.

Understanding host-microbe interactions is crucial to develop and evaluate these new strategies and gnotobiotic systems have demonstrated to be very instrumental here (Marques et al., 2006a). A gnotobiotic system is a system in which the animals are cultured in axenic conditions or with a known microflora. An excellent candidate for such a gnotobiotic model system is *Artemia franciscana*, being a small and robust animal with a short life cycle.

An axenic setup allows understanding the complex interactions between the microbial community and its host, by allowing control over the variables as well as allowing for better reproducibility of the results (Marques et al., 2006a). However, the immune system of the axenic animals is often underdeveloped compared with non-gnotobiotic animals. This study is focussing on this specific problem. The gut morphology of non-gnotobiotic *Artemia* nauplii was thoroughly studied by Hootman and Conte (1974) using transmission electron microscopy and one descriptive study is available illustrating the digestive tract morphology of gnotobiotic *A. franciscana* nauplii (Gunasekara et al., 2011). However, only the internal morphology of the early stages of the life cycle of *A. franciscana* is being considered here. Preliminary results of this study showed there was a difference in the immune responses of the animal before and after day 5 of development. Therefore a comparison is being made between the mor-

phology of gnotobiotic *Artemia* and corresponding non-gnotobiotic animals of the same age covering the first 10 days of development.

Materials and methods

The animals were fed with axenic and non-axenic *Tetraselmis suecica* 66/4 obtained from the Culture Collection of Algae and Protozoa Department (CCAP, Dunstaffhage Marine Laboratory, Scotland). Each strain was cultured according to Marques et al. (2006b), using 10% inocula, 0.22-pm filtered aeration, continuous light, a standard Walne medium (Walne, 1967), and filtered and autoclaved seawater (FASW, 0.2pm). All handlings were performed in a laminar-flow hood to maintain axenity. Experiments were performed with *A. franciscana* cysts, originating from Great Salt Lake, Utah, USA (EG@ Type, M E Aquaculture NV, Belgium). Axenic cysts and nauplii were obtained via decapsulation according to the procedures described by Marques et al. (2004a,b). Each treatment consisted of three replicates. The daily feeding schedule was adapted from Marques et al. (2004b) and was intended avoid unbalanced feeding to affect the water quality in the test bottles.

Axenity of feed, decapsulated cysts, and *Artemia* cultures were checked at the end of the experiments using plating on MA (n=2) followed by an incubation at 28°C during 5 days, according to the procedures of Marques et al. (2004a,b). Contaminated culture bottles were not considered for further analysis and the experiment was repeated. Axenity of the microalgae was also confirmed by flow cytometry (Fig 1).

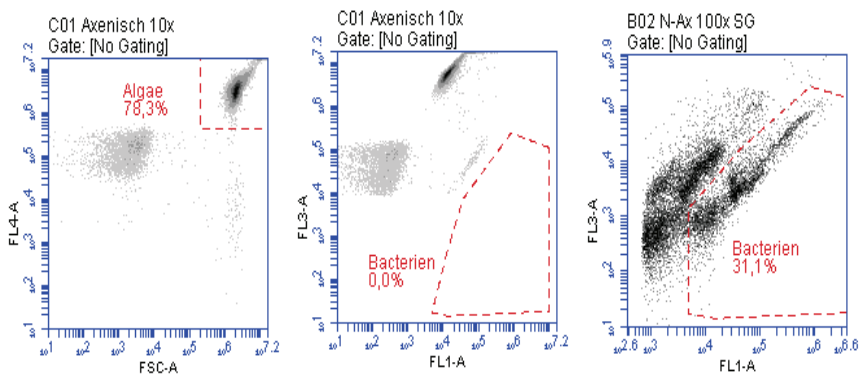


Fig. 1: Results from the flow cytometry. The graph on the left shows the presence of the microalgae in the axenic sample of *Tetraselmis suecica*, the graph in the middle shows the absence of bacteria in this same axenic sample and the graph on the right shows the presence of bacteria in the non-axenic sample of the same strain of *T. suecica*.

Sampling was done at 2, 4, 6, 8, and 10dah for light microscopy and transmission electron microscopy (five replicates for each). Animals sampled were fixated according to procedures described by Gunasekara et al. (2011). The dry weight and length of the sampled *Artemia* was also determined at every sampling point.

Results and discussion

The experiments are still being performed while writing this paper so only some preliminary results can be shown here.

In previous experiments we observed that the dry weight of axenic *Artemia* fed with axenic microalgae was only 1/3 of the dry weight of non-axenic nauplii fed with non-axenic algae (two strains of *Dunaliella tertiolecta* 19/27 and 19/6B were used in this experiment). This time the animals will be fed with *T. suecica* 66/4. During previous experiments it was also observed that challenge tests performed with 3 different Vibrios showed mortality up to day 5, however after day 6, mortality was no longer observed, this also being the case for two-week-old and three-week-old *Artemia*. These two factors lead up to the current experiments being performed to verify on a morphological level which internal changes are occurring around day 5, if there is a significant difference between axenically reared animals and non-axenically reared animals, and whether or not axenic animals fed with axenic microalgae are suitable for evaluating immune responses.

References

Bachere E. 2003. Anti-infectious immune effectors in marine invertebrates: potential tools for disease control in larviculture. Aquaculture 227:427-438

Gunasekara R.A.Y.S.A., A. Rekecki, P. Cornillie, M. Cornelissen, P. Sorgeloos, P. Simoens, P. Bossier, and W. Van den Broeck. 2011. Morphological characteristics of the digestive tract of gnotobiotic *Artemia franciscana* nauplii as a prerequisite to study effects of micro-organisms. Aquaculture 321:1-7

Marques A., J. Franqois, J. Dhont, P. Bossier, and P. Sorgeloos. 2004a. Influence of yeast quality on performance of gnotobiotically grown *Artemia*. J. Exp. Mar. Biol. Ecol. 310(2):247-264.

Marques A., J. Dhont, P. Sorgeloos, and P. Bossier. 2004b. Evaluation of different yeast cell wall mutants and microalgae strains as feed for gnotobiotically-grown brine shrimp *Artemia franciscana*. J. Exp. Mar. Biol. Ecol. 312(I):115-136.

Marques A., F. Ollevier, W. Verstraete, P. Sorgeloos, and P. Bossier. 2006a. Gnotobiotically grown aquatic animals: opportunities to investigate host-microbe interactions. Journal of Applied Microbiology 100:903-918

Marques A., T.H. Thanh, P. Sorgeloos, and P. Bossier. 2006b. Use of microalgae and bacteria to enhance protection of gnotobiotic *Artemia* against different pathogens. Aquaculture 258:116-126

Smith V.J., J.H. Brown, and C. Hauton. 2003. Immunostimulation in crustaceans: does it really protect against infection? Fish & Shellfish Immunology 15:71-90.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

MUSCLE GROWTH OF TRIPLOID ATLANTIC COD LARVAE (*GADUS MORHUA*)

C. Vargas Cecilia[1*], Ø. Hagen[1], S. Peruzzi[2], and C. Solberg[1]

[1] University of Nordland, Faculty of Biosciences and Aquaculture
[2] University of Tromsø, Faculty of Biosciences, Fisheries and Economics

Introduction

The production of Atlantic cod (*Gadus morhua*), a species of economic importance in Norway, is facing several challenges hampering its commercial potential. Early sexual maturation, which leads to reduced growth, survival, and flesh quality, represents a major commercial issue. In addition, the negative consequences of farmed escapees on wild stocks or spawning in sea cages, increasing the risk of genetic pollution (Jensen et al., 2010). This is of great concern for the Norwegian Ministry of Fisheries and Coastal affairs which encourages new technologies and methods to solve issues associated with early puberty. The production of triploid (3n) cod may be one solution in solving some of these problems as their gonadal development is impaired and are functionally sterile (Peruzzi et al., 2009; Piferrer et al., 2009; Taranger et al., 2010). In this study, the growth and muscle development of triploid cod larvae are compared to their diploid (2n) counterpart.

Materials and methods

Fish origin and husbandry
Gametes were obtained from broodstock of the 2nd generation held at CodFarmers' Hatchery in Bodø (North Norway). Fertilization and triploidy induction were conducted at the Mørkvedbukta Research Station, - University of Nordland in November 2012. The triploidization was performed according to Trippel et al. (2008), where cod eggs were subjected to hydrostatic pressure shocks of 8500psi after 180 minute degrees eggs (i.e., 30min post-fertilization at 6°C) for 5min. Diploid and triploid eggs were incubated separately at 6.0±0.2°C in 280-l cone-bottomed incubators until hatching and the eggs were disinfected in a solution of Pyceze (0.8ml.l^{-1} seawater) for 6min.

Newly hatched larvae were transferred to 200 l water volume, with three tanks per ploidy group. Water exchange rate was kept at 6 exchanges per day until 29 days post-hatching (dph) and increased to 10 exchanges per day during the co-

feeding dry feed period. Continuous light (600lux) regime was applied and temperature was gradually increased from 6-10±0.3°C and dead larvae were removed daily. A solution of Neptune (Skretting AS) was added daily to the tanks as green water technique. The larvae were fed on rotifers (*Brachionus plicatilis*, Cayman strain), 2 hours – short-term enriched with OriGreen (Skretting AS) until day 22, and enriched with *Artemia* (19-31dph) and a commercial microdiet (Gemma Micro 150-300, Skretting AS) from 26dph until the end of the experiment.

Analytical methods
The larvae were sampled randomly from each tank and anaesthetized with 3-aminobenzoic acid ethyl ester (MS-222, Tamro 257675) immediately after sampling and rinsed in distilled water before treated as described for the respective analytical methods.

Two-day-old larvae from each ploidy groups were individually placed into disposable test tubes, deep frozen and prepared for propidium iodide (PI) flow cytometric analysis as described by Peruzzi et al. (2007).

For dry weight measurements and muscle cellularity, larvae from each ploidy group were sampled on day 1, 8, 19, 29, 36, and 44dph. Samples were transferred to pre-weighed tin capsules, dried at 60°C for 24 hours and the dry weight was measured to the nearest 0.1µg using a microbalance (Mettler Toledo UMX2, Columbus, OH, USA). The specific growth weight (mg, $\%day^{-1}$) of cod larvae was calculated as: $SGR\% = (\ln W_1 - \ln W_0) / (t_1 - t_0)$.

For muscle morphometric measures, larvae were cut at the end of the abdominal cavity and the posterior part was mounted in a micro aluminium container containing cryomatrix (Shandon Cryomatrix, Thermo). The sample was immediately frozen in 2-methyl butane cooled to near its freezing point (-159°C) in liquid nitrogen, wrapped in pre-labelled aluminium foil and stored in a -80 freezer. The samples were processed in a cryostat at -20°C (Microm HM 550, MICROM International GmgH) and 3-µm sections were mounted on poly-L-lysine treated slides, air dried and stained with heamotoxyline (Sigma-Aldrich). The total cross-sectional area (TCA) of the larvae and the measurement of 600 fast muscle fibres per larvae were performed using a light microscope (Axioskop 2 mot plus, Carl Zeiss). Pictures were taken from three standardized locations within the deeper layer of the myotomal muscle.

Results and discussion

Flow cytometry analysis showed that the protocol applied for triploidization resulted in 100% triploid.

3n larvae were slightly heavier than 2n larvae at hatching and grew slightly better during the first days of exogenous feeding, although the differences were not statistically significant (8dph, 3n: 0.19±0.11mg; 2n:0.13±0.04). On the contrary, during the weaning period from *Artemia* to dryfeed, 3n larvae experienced a stagnant growth compared to 2n. During this period (29-36dph), 3n larvae only had 0.3% of SGR while 2n larvae showed 2.8%. These results suggest that triploid cod during the larval stages may have a lower digestion capacity and/or absorption of nutrients.

By 44 days post hatching, ploidy larval groups of similar average body total length (2n: 11.1mm; 3n: 11.4mm) showed no differences in the total fast fibre number, fibre density, fibre diameter and/or fibre cross-sectional area. In addition, the fibre size distribution of triploid larvae was similar to that of diploids. Our findings differ from earlier reported results from other fish species where 3n fish have been reported having a lower number, but larger muscle fibres than 2n fish. However, the effect of triploidy on muscle growth dynamic might be species specific and/or may vary at different developmental stages. In the case of cod, differences between the ploidy groups could appear at later stages, during the adult phase. It is also possible that larval rearing conditions (i.e., feeding regime, temperature, communal rearing) have a marked effect on growth and hence on muscle development.

Conclusions

Triploid larvae were slightly heavier than diploids at hatching and during the first days of exogenous feeding. Thus, the growth of triploid larvae was negatively affected during the weaning onto dryfeed.

At 44dph, 3n larvae had similar fast fibre number, fibre diameter, cross-sectional area, fibre density and overall muscle fibre distribution compared to their 2n siblings.

The digestion and absorption capacity of nutrients in triploid cod larvae may be differ from their diploid siblings and more research in this area should be therefore be conducted.

References

Jensen O., T. Dempster, E.B. Thorstad, I. Uglem, and A. Fredheim 2010. Escapes of fishes from Norwegian sea-cage aquaculture: causes, consequences and prevention. Aquaculture Environment Interactions 1(1):71-83.

Peruzzi S., G.. Rudolfsen, R. Primicerio, M. Frantzen, and G. Kaurić 2009. Milt characteristics of diploid and triploid Atlantic cod (*Gadus morhua* L.). Aquaculture Research 40:1160-1169.

Peruzzi S., A. Kettunen, R. Primicerio, and G. Kaurić. 2007. Thermal shock induction of triploidy in Atlantic cod (*Gadus morhua* L.). Aquaculture Research 38(9):926-932.

Piferrer F., A. Beaumont, J-C.Falguiere, M. Flajshans, P. Haffray, and L. Colombo. 2009. Polyploid fish and shellfish: Production, biology and applications to aquaculture for performance improvement and genetic containment. Aquaculture 293(3-4):125-156.

Taranger G.L., M. Carrillo, R.W. Schulz, P. Fontaine, S. Zanuy, A. Felip, and T. Hansen. 2010. Control of puberty in farmed fish. General and Comparative Endocrinology 165(3):483-515.

Trippel E. A., T.J. Benfey, S.R.E. Neil, N. Cross, M.J. Blanchard, and F. Powell. 2008. Effects of continuous light and triploidy on growth and sexual maturation in Atlantic cod, *Gadus morhua*. Cybium 32(2):136-138.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

STUDIES ON THE CHARACTERISATION OF BIOMARKERS OF NUTRITIONALLY-DERIVED STRESS IN PARALARVAL CULTURES OF THE COMMON OCTOPUS (*OCTOPUS VULGARIS*)

I. Varó[1], J.C. Navarro[1], J. Iglesias[2], J.J. Otero[2], J. Sánchez[2], E. Almansa[3], Ó. Monroig[1], F. Hontoria[1], A.E. Morales[4], and G. Cardenete[4]

[1] Instituto de Acuicultura Torre de la Sal. (IATS-CSIC), 12595 Ribera de Cabanes, Spain
[2] Instituto Español de Oceanografía, Centro Oceanográfico de Vigo, Apartado 1552, 36200 Vigo, Spain
[3] Instituto Español de Oceanografía, Centro Oceanográfico de Canarias, Avd. Tres de Mayo 73, 38005 Santa Cruz de Tenerife, Spain
[4] Departamento de Zoología, Universidad de Granada, Campus de Fuentenueva, 18071 Granada, Spain

Introduction

Nowadays, due to the high mortality within the first 30 days of life, octopus paralarval culture represents the main obstacle for commercial production of this species. The causes of such mortality are not yet well defined and understood. As a part of a broader project aimed at characterising the causes of such mass mortality, we envisaged the study of nutritionally-derived stress, through the selection of biomarkers capable of its detection and quantification.

Materials and methods

Paralarval cultures starved and fed *Nannochloropsis* sp - and *Isochrysis galbana*-enriched *Artemia,* and *Artemia* plus crustacean zoeae (*Maja* sp.), were raised in 500-l tanks until the incidence of mass mortalities. Two experiments were carried out that lasted 14 and 30 days, respectively. Samples were taken at 0, 5 and 14 days in experiment 1 and at 0, 4, 16, and 30 days in experiment 2. The following biomarkers were analysed: RNA/DNA, stress protein Hsp70, superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), Se-dependent glutathione peroxidase (Se-GPX), the isozymic pattern of SOD, and the levels of malondialdehyde (MDA). Assays related with antioxidant defenses were performed only in samples from experiment 2 at hatching and at days 16 and 30 due to problems of availability.

RNA and DNA were quantified following the procedure described in Varó et al. (2007), using RiboGreen ™ RNA Quantitation Kit and PicoGreen ™ DNA Quantitation Kit respectively (Molecular Probes). HSP70 were separated by 1D-SDS-PAGE using a Mini-Protean Tetra cell system (Bio-Rad), and transferred onto PVDF membranes in a Trans-Blot^{R} Turbo Blotting system (Bio-Rad). Blots were visualized on a VERSADOC Imaging system (Bio-Rad) using ELC-PRIME reagent (Amersham), and quantified by densitometry. Specific activities of antioxidative enzymes and MDA levels were determined according to Pérez-Jiménez et al. (2009), as well as SOD isoforms, determined by PAGE (MiniProtean II, Bio-Rad). CuZn-SODs were inhibited by CNK (50 mM) and types of SOD were detected by the photochemical nitroblue tetrazolium (NBT) staining method. Data were compared with ANOVA followed by Tukey's test (more than 2 groups), and with Student t-tests (Significant differences: $p<0.05$).

Results and discussion

After hatching, 4 to 5 days of starvation are evident in a decrease in RNA:DNA ratio and HSP70 values as a consequence of nutritional stress (Table I). From days 4 to 30 the RNA:DNA remained unchanged irrespective of diet. The levels of HSP70 were higher in the dietary groups, and especially higher in the paralarvae fed *Artemia* up to 5 days. At the end of experiment 1 (14 days), lower values were found, likely as consequence of mortality. Paralarvae fed zoeae in the second experiment showed higher HSP70 values, possibly reflecting a better nutritional status that correlated with higher growth (data not shown), and pointing at this biomarker as a sensitive indicator.

Table I. RNA:DNA ratio and levels of HSP70 of *Octopus vulgaris* paralarval cultures starved and fed *Nannochloropsis* sp - and *Isochrysis galbana*-enriched *Artemia* and crustacean zoeae raised up to 30 days of age. Different letters denote significant differences between diets for each age. Values are means ± std

Exp. nº	Age (days)	RNA:DNA ratio				HSP70 (arbitrary units.ng^{-1} protein)			
		Diet				Diet			
		Hatching	Starving	Zoeae	*Artemia*	Hatching	Starving	Zoeae	*Artemia*
1	0	7.05± 0.44				3.31± 1.48			
2	0	5.82± 0.55				3.25± 1.16			
1	5		2.95± 0.73	3.56± 0.32	3.21± 0.54		1.78± 0.02^{a}	6.57± 0.32^{b}	11.71± 0.33^{c}
2	4		3.24± 0.55	3.88± 1.62	4.55± 1.98		1.94± 1.00^{a}	3.65± 1.22^{a}	7.89± 0.64^{b}
1	14			4.15± 0.85	4.49± 0.94			2.91± 1.32	2.82± 1.22
2	16			5.46± 0.59	5.50± 1.05			5.46± 1.68^{a}	1.33± 0.81^{b}
2	30			3.72± 0.47	4.00± 1.06			4.86± 1.05^{a}	2.89± 0.88^{b}

Enzymatic results show that paralarvae possess a complete enzymatic pool, with high activity (especially Mn-SOD) as compared to other cephalopods (Table II). There is an increase in CAT and Se-GPX activity during development. There is no oxidative damage associated to the feeding regimes, probably due to the anti-oxidant properties of the diets. Besides, there are not clear changes in enzymatic activities associated to growth or survival, except Se-GPX activities that were higher in the paralarvae fed zoeae than in those fed *Artemia*; in agreement with a better growth and survival and reflecting the differences in such enzyme activities between live preys. Also, the SOD isozymic profiles reflect those of live preys.

Table II. Specific activities of antioxidant enzymes (U mg protein^{-1}) and levels of MDA (nmol MDA g tissue^{-1}) of *Octopus vulgaris* paralarval cultures fed *Nannochloropsis* sp- and *Isochrysis galbana*-enriched *Artemia* and crustacean zoeae raised up to 30 days of age. Different letters denote significant differences between diets for each age. Asterisk indicates differences with hatching. Values are means ± std

	Hatching	**Day 16**		**Day 30**	
		Zoeae	***Artemia***	**Zoeae**	***Artemia***
SOD	9.88 ±0.82	8.35±3.22	7.50±0.76	7.54±0.49	11.41±0.79
CAT	3.59±0.26	4.35±0.21	5.74±0.26*	6.73±0.45*	6.49±0.20*
Se-GPX	2.37±0.69	3.59±0.35	n.d.	5.26±1.19*^{b}	3.41±0.23*^{a}
GPX	1.25±0.41	1.27±0.17^{a}	2.97±0.30^{b}	1.55±1.36	1.06±0.27
t-GPX	3.62±0.34	4.86±0.18^{b}	2.97±0.30^{a}	6.80±0.17^{b}	4.04±0.43^{a}
GR	2.95±0.2	2.08±0.07	2.61±0.02	2.30±0.19	1.92±0.05*
MDA	4.41±0.38	4.09±0.45	4.48±0.23	3.82±0.71	4.67±0.45

Conclusions

The results point at the RNA:DNA ratio as an indicator of starvation, and at the levels of HSP70 and Se-GPX activity as more sensitive biomarkers of the nutritional status of paralarvae. There is no evidence of a pro-oxidative status promoted by diets, but a detailed analysis of oxidative damage to proteins could help to associate oxidative damage to mortalities and growth.

Acknowledgements

This research was supported by "Ministerio de Ciencia e Innovación" through the OCTOPHYS Project (AGL-2010-22120-C03-02). OM was supported by a Marie Curie R.G. (PERG08-GA-2010-276916, LONGFA) and by a Juan de la Cierva postdoctoral contract. The study was also carried out under the scope of a PROMETEO Project (2010/006) from Generalitat Valenciana.

References

Pérez-Jiménez A., C. Hidalgo, A.E. Morales, M. Arizcun, E. Abellán, and G. Cardenete. 2009. Antioxidant enzymatic defenses and oxidative damage in *Dentex dentex* fed on different dietary macronutrient levels. Comparative Biochemistry and Physiology, Part C 150:537-545

Varó I., J.C. Navarro, B. Nunes, and L. Guilhermino. 2007. Effects of dichlorvos aquaculture treatments on selected biomarkers of gilthead sea bream (*Sparus aurata* L.) fingerlings. Aquaculture 266:87-96

Zielinski S. and H.O. Pörtner, 2000. Oxidative stress and antioxidative defense in cephalopods: a function of metabolic rate or age?. Comparative Biochemistry and Physiology, Part B 125:147-160

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EFFECTS OF FEEDING TIME, RATES, AND FREQUENCIES ON SURVIVAL RATE OF STRIPPED CATFISH FRY (*PANGASIANODON HYPOPHTHALMUS*) FED BY FRESHWATER ROTIFERS (*BRACHIONUS ANGULARIS*)

Vu Ngoc Ut, Nguyen Phi Long, and Tran Suong Ngoc

College of Aquaculture and Fisheries, Can Tho University, Vietnam

Introduction

Live food plays an important role in production of many fish and shellfish. In fish species which have small size of mouth gape, live food is very crucial for their early life stages. Among the live food, rotifers have been known as the most essential live food for most small fish species. In addition to the brackish water rotifer, *Brachionus plicatilis* which has been widely used in marine fish larval production, many other freshwater rotifers have been recently studied to serve as initial feed for some important freshwater fish species.

Stripped catfish (*Pangasianodon hypophthalmus*) has been known as one of the most important aquaculture species of Vietnam, especially in the Mekong Delta. High production of this species is not only supporting the economic growth of the country but also contributing to the world fish production. Although high production has been maintained throughout the years, survival rates of fingerlings has been low. The most prominent associated reason for this could be lacking of an appropriate feeding regime including feeding types, rate, time, and frequencies. In previous study, freshwater rotifers (*B. angularis*) was found to be the most essentially initial feed for stripped catfish during the first 3 days after hatching compared to home-made feed and water fleas (*Moina* sp.) (Vu Ngoc Ut et al., 2012). The present study aimed to investigate the effects of feeding rates, times and frequencies on survival rate of stripped catfish pre-fingerlings fed with freshwater rotifer, *B. angularis* to serve as base for further improving the production of this species in the Mekong Delta, Vietnam.

Materials and methods

The study was studied with three experiments conducted at Cantho University. Fish fry used in the experiment were 20h post-hatch obtained from Cantho University hatchery. Freshwater rotifers used to feed the fish were *B. angularis* with a size range of 90-100µm in length, which had been isolated and cultured by the

University Live food production wet lab. Water fleas (*Moina* sp.) used during the later stage of experiments were obtained from an ornamental fish store. Their average length was 598±250µm.

The experiments were designed in a 20-l bucket system with 18 l of water and slight aeration provided. The experiment system was set up in a room with controlled temperature. 20h-post-hatch fry were distributed in the system at density of 10 ind.l^{-1}. In all experiments, fry were fed with rotifers during the first 3 days and then with water fleas during the rest of the rearing period (7 days).

In the first experiment, six treatments with 3 replicates each were designed in which the fries were first fed at 24h, 30h, 36h, 42h, and 48h after hatching and the control in which fish were fed with water fleas at 36h to investigate the best time of start feeding to result in highest survival rate of the fish during 10 days of the rearing period. Yolk sac of the fries was continuously observed from the beginning to after 3 days.

In the second experiment, different feeding densities of rotifers including 3, 5, 8, and 11 ind.ml^{-1} were investigated. Fish were fed 4 times per day at 7 am, 11 am, 3 pm, and 7 pm. In this experiment, the first feeding time was determined from the first experiment.

The third experiment was designed with different feeding frequencies. Fish fries were fed 1 (at 7 am), 2 (at 7 and 11 am), 4 (at 7 am, 11 am, 3 pm and 7 pm), and 6 (7 am, 11 am, 3 pm, 7 pm, 11 pm, and 3 am) times per day. Feeding density was the best density obtained from the second experiment. Different feeding regimes equaled to different treatments with 3 replicates each.

Water temperature and pH were monitored daily both in the morning (7 am) and in the afternoon (2 pm) whereas dissolved oxygen (DO), total ammonium (TAN), and nitrite (NO_2^-) were measured every 3 days. Sediment at the bucket bottom was siphoned whenever necessary and replacement of lost water was done right after siphoning.

Fish were sampled every 3 days to measure length and weight. Mouth gapes were also monitored in every 3 days. Relative growth rate of fry was determined. Survival rate of the fish was assessed at the end after terminating the experiments. The mouth gapes were determined by the length of the upper jaw using method of Shirota (1970) and calculated by the following equation:

$D(90°) = AB \times \sqrt{2}$, Where D is width of the mouth (µm); AB is length of the upper jaw (µm)

Results and discussion

Water parameters

Water parameters monitored in all experiments were mostly in suitable ranges. Temperature ranged from 26.3-7.8°C in the morning and afternoon, respectively. pH was also appropriate with a range of 7.4-7.5. As the system was slightly aerated, DO concentrations were always high ranging from 4.8-5mg.l^{-1}. TAN and nitrite concentrations were ranging from 0.3-0.4mg.l^{-1}. According to Timmons et al. (2002) the suggested level of nitrite in aquaculture ponds should be less than 1mg l^{-1}. However, Truong Quoc Phu (2006) recommended that NH_3 and NO_2^- should not exceed 0.1mg l^{-1}. Nevertheless, fish in the experiments did not show any signs of effect during the rearing period.

Sizes of mouth gape of fish fries from day 1 to 10 of rearing

The sizes of mouth gape of fry at day 1 (when fish set up in the experiments) were similar in all 3 experiments with average of 232±48µm, 231±48µm, and 236 ± 41µm, respectively. After 3 days of rearing, the mouth gape sizes increased to 496±27µm, 505±14µm, and 450±51µm, respectively. The results indicated that at the first 3 days, their mouth size could be too small to feed on larger prey like water fleas (approximately 600µm), while they could easily ingest the rotifers (<200 µm). This could be the reason why survival rates of fish were significantly higher when fed with rotifers compared to water fleas or homemade feed (Vu Ngoc Ut et al, 2012). After day 3 their mouth became bigger (>500 µm), and water fleas could therefore be suitable.

Survival rates of stripped catfish fries at day 10 of rearing

In the first experiment, fish fry were fed with rotifers during the first 3 days at different first feeding times including 24, 30, 36, 42, and 48h after hatching. Fish fries fed at 24h after hatching had highest survival rate (27.8%) which was significantly higher ($P<0.05$) than that of those being fed at 30, 36, 42, and 48h after hatching (24%, 19%, 16%, and 16%, respectively). It was also significantly higher than those fed at 36h but with water fleas (16%) (Fig. 1). The results indicated that fry, after absorbing the yolk sac if supplemented with external feed (especially rotifers) could yield higher survival rate due to reduced cannibalism. The later the start feeding is, the lower the survival rates of fry as consequence of cannibalism and starvation.

In the second experiment, highest survival rate (32%) was obtained from fish fed with 8 ind.ml^{-1} It was significantly higher than that of 3 and 5 ind.ml^{-1} (13% and 23%, respectively). At 11 ind.ml^{-1}, survival rate was 31%, not significantly different to that of those being fed 8 ind.ml^{-1} (Fig. 2).

In the third experiment, fry fed 6 times per day had significantly highest survival rate, up to 66% compared to fish fed more infrequently (Table I). Survival rates

of fish fed 1, 2, and 3 times per day were 9, 11, and 28%, respectively. This result indicated that if fish are fed more frequently, provision of both quantity and quality of food would meet the demand and result in higher survival rates of fish.

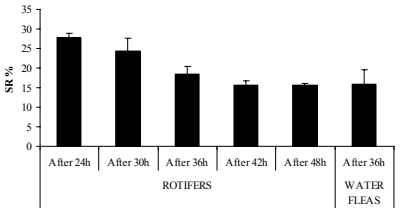


Fig. 1:Survival rate of fish fry at different first feeding times.

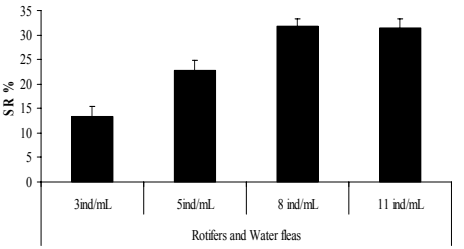


Fig. 2: Different densities of rotifers (first 3 days) and water fleas (the rest of duration).

Table 1: Survival rate of stripped catfish fries under different feeding frequencies

Treatments	Survival rates (%)
Feeding 1 time per day	9
Feeding 2 times per day	11
Feeding 4 times per day	28
Feeding 6 times per day	66

Conclusions

Survival rates of stripped catfish fry during the first 10 days of rearing could be significantly improved if fed with rotifers in the first 3 days and then water fleas at 24h after hatching with rotifer density of 8 ind.ml^{-1} continuously 6 times per day at 4-h intervals.

References

Timmons M.B, M.E., James, W.W. Fred, T.S. Steven, and J.V. Brian. 2002. Recirculating Aquaculture Systems (2nd Edition). NRAC Publication No. 01–002.

Truong Quoc Phu 2006. Water quality management. Reference book for undergraduate, 220 pp. Can Tho University.

Vu Ngoc Ut, Pham Thi Hong, Nguyen Thanh Hung, and Tran Suong Ngoc. 2012. Effect of rotifers as initial feed on the survival rates of stripped catfish fires (*Pangasianodon hypophthalmus*). International Fisheries Symposium: Sharing Knowledge for Sustainable development of Aquaculture and Fisheries in South East Asia. Book of Abstract, p. 290.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

THYROID REGULATION IN TELEOST EMBRYONIC AND LARVAL DEVELOPMENT – CAN IT BE A PROMISE FOR AQUACULTURE?

C.N. Walpita[1,2*], A.D. Crawford[3], and V.M. Darras[1]

[1] Laboratory of Comparative Endocrinology, Katholieke Universiteit Leuven, Naamsestraat 61, 3000 Leuven, Belgium (*E-mail: walpitacn@yahoo.com, walpitacn@sab.ac.lk
[2] Department of Livestock Production, Faculty of Agricultural Sciences, Sabaragamuwa University of Sri Lanka, PO Box 02, Belihuloya 70140, Sri Lanka
[3] Department of Pharmaceutical Sciences, Katholieke Universiteit Leuven, Herestraat 49, 3000 Leuven, Belgium

Abstract

The role of thyroid hormones (THs) in fish development is often overlooked and particularly neglected for the embryonic and larval stages of fish. We used a set of experiments to elucidate the importance of TH in embryonic to early larval development in zebrafish. In the first set of experiments we used morpholino antisense oligonucleotides to knock down TH downstream genes and showed that the depletion of TH in early development resulted in severe deformities, developmental delay, and pigmentation, which could be quantitatively recovered by subsequent TH treatments. In the second set of experiments, blocking TH deposition into zebrafish eggs by treating parental fish with goitrogens resulted not only initial low TH content in eggs, but also subsequent loss of egg laying ability. Overall, these data strongly suggest a key role of TH in early development in fish, proving its worth to be investigated for effective use in reproduction and larviculture.

Introduction

Beyond the physical parameters of the egg quality, endocrine status of eggs is believed to be crucial in successful embryonic and larval development, yet only little attention has been given to date with regard to enhancing the endocrine status of eggs during common aquaculture operations. Although thyroid hormones (TH) are well known endocrine regulators of growth and development in fish (Tanaka et al, 1995; Yamano 2005) and significant amounts of maternally derived TH are deposited in fish eggs (Walpita et al, 2007), its influence on embryonic and subsequent larval development is yet to be uncovered in fish. Aiming to deplete bioactive TH levels in developing fish embryos in order to understand its possible roles in these early stages, we used two approaches. First,

morpholino antisense technology was used to block TH activation in early fish embryos. In the second set of experiment we used parental hypothyroidism as a mean to deprive TH deposition into fish eggs.

Materials and methods

Morpholino knock-down of TH activating deiodinase in zebrafish embryos
A group of zebrafish embryos collected at single cell stage were injected with a pre-tested volume and concentration of a morpholino oligo (5'-TCCACACTAAGCAAGCCCATTTCGC-3'), designed to block the translation of type II iodothyronine deiodinase enzyme (D2) which is known as the major activator of TH (D2MO group). A D2-specific mismatch MO (5'-TCGACAGTAAGGAAGC GCATTTCCC-3') was used as a control (injected control group), alongside un-injected control embryos (uninjected control group). The D2MO group was treated with 50nM T_3 as an attempt of rescuing the effects caused by D2MO. All embryos were reared in 0.3× Danieau's solution and each treatment group contained 30-40 embryos. Embryos were allowed to develop at 28±1°C. At 31 hours post-fertilization (HPF) embryos were imaged after manual removal of the egg casing. Images were analyzed for development related parameters, i.e., otic vesicle length (OVL), head-trunk angle (HTA), and pigmentation index (PI).

Prolonged exposure of maternal fish to goitrogens
Environmentally relevant concentrations of goitrogens, i.e., propylthiouracil (PTU) 0.003%, ammonium perchlorate (AP) 0.0018% and phenylthiourea (PTUrea) 0.003% were used as treatments in three independent experiments, and adult fishes were continuously exposed for more than 4 weeks. Reproductive behavior was observed and embryos were collected at 2 hours post-fertilization (HPF) and after. Subsequent TH quantification in developing embryos was done by radioimmunoassay (RIA). Transcript levels of TH-downstream indicator genes in embryos were analyzed by real-time quantitative PCR technique (RTQPCR).

Results and discussion

Morpholino knock-down of TH activating deiodinase in zebrafish embryos
Relative quantification of morphological and developmental effects of D2 morpholino injection to zebrafish embryos revealed that blocking the TH activation using the said technology has a profound negative effects on growth, development and pigmentation of embryos ($p<0.05$, Scheffé test), as measured by highest OVL, lowest HTA, and lowest PI, corresponding to the least developmental progression (Fig. 1).

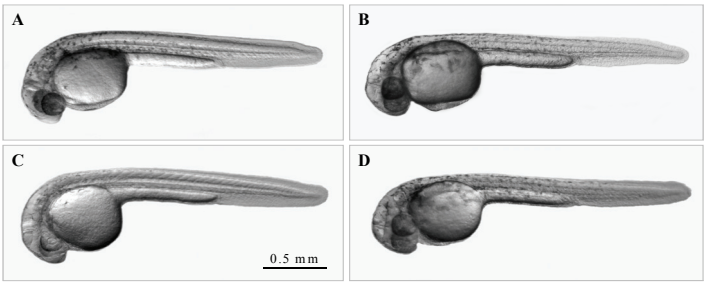


Fig. 1 Representative photos of morpholino injected and uninjected zebrafish embryos at 31hpf. (A) uninjected control group; (B) mismatch-injected control group; (C) D2 morpholino injected group; (D) D2 morpholino injected group treated with 50nM T_3.

A second morpholino targeting the same gene, but with a splice blocking action (5'-GTCTTATGCTGTCAGCTTACCTGTT-3'), produced similar results on growth and development related parameters, suggestive of the said effects are purely due to the knock-down action of the target gene, but not an aspecific action. However, the antisense-induced delays in developmental progression and pigmentation were reversible through treatment with T_3, suggesting that these phenotypic effects are most likely due to the depletion of intracellular T_3 levels.

Our results suggest that a depleted thyroid status of zebrafish embryos can cause a significant delay in growth and development of the embryos, which can eventually lead to unsynchronized larval growth. Further, TH enhancement as in the case of rescue group can be used to overcome the effects, suggesting indeed the phenotypic effects in the treated group are due to target gene knock-down. This shows that TH plays an important role during embryonic and larval development in fish and that a depleted thyroidal status in fish eggs can delay this development. TH supplementation, on the other hand, can be used to accelerate growth, development, and hatching of fish larvae.

Prolonged exposure of maternal fish to goitrogens

In this study, abrupt stopping of egglaying by zebrafish was observed in PTU and AP treated groups after 4 and 8 weeks respectively, In PTUrea treated experiment, after 8 weeks of exposure, fish showed signs of lowering egg production. However, before they completely stopped laying eggs at ten weeks, few samples were collected sufficient enough for RIA and RTQPCR for testing only one development stage. PTUrea treatment caused a significant reduction ($p<0.05$) in triiodothyronine deposition in the embryos (Fig. 2).

This shows that environmentally relevant levels of goitrogens can effectively block TH deposition into zebrafish eggs, rendering hormonally imbalanced eggs. This hormonal imbalance might be the reason for abrupt stopping of spawning. It is suggested that a certain critical level of TH is needed to be deposited for the

subsequent normal embryonic development and all these tested chemicals may have reduced the TH deposition below the critical level. Taken together, these two studies suggest that TH play a vital role in early embryonic development in zebrafish. Its depletion in the embryos can cause severe growth retardations, which in larviculture would be leading to irregularities and high losses. Similarly, TH supplementation could, at least in part, revoke the effects caused by lack of internal TH. Low goitrogen levels on the other hand can have strong negative effects on egg laying ability of zebrafish. These data therefore suggest that, maternal and embryonic TH status have a greater influence on early development in fish, hence, opening a new window to investigate its potential applications on larval development in aquaculture.

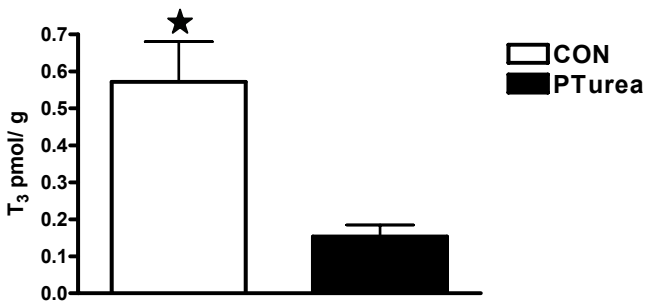


Fig. 2. T_3 concentration (pmol.g^{-1} eggs) in zebrafish embryos collected at 2 HPF. Embryos were collected from untreated fish or fish exposed for 8 weeks to 0.003% PTUrea. Data are presented as mean ± SEM (n=6 pools of about 200 embryos). Statistically significant differences are indicated by * ($p<0.05$, Wilcoxon rank-sum test)

In conclusion, this study suggests that TH status of zebrafish embryos plays a decisive role in embryonic development.

References

Tanaka M., J.B. Tanangonan, M. Tagawa, E.G. de Jesus, H. Nishida, M. Isaka, R. Kimura, and T. Hirano. 1995. Development of the pituitary, thyroid and interrenal glands and applications of endocrinology to the improved rearing of marine fish larvae. Aquaculture 135:111-126.

Walpita C.N., A.D. Crawford, E.D.R .Janssens, S. Van der Geyten, V.M. Darras. 2008. Type 2 iodothyronine deiodinase is essential for thyroid hormone-dependent embryonic development and pigmentation in zebrafish. Endocrinology 150: 530-539.

Yamano K. 2005. The role of thyroid hormone in fish development with reference to aquaculture. Japan Agriculture Research Quarterly 39:161-168.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

WATER MANAGEMENT AND BIOCONTROL – SELECTION OF PROBIOTIC STRAINS

B. Weber*, G.A. Santos, M. Mohnl, and G. Schatzmayr

BIOMIN Research Center, Technopark 1, 3430 Tulln, Austria
*E-mail: barbara.weber@biomin.net.

Introduction

In high-density rearing of larvae, water quality is important because of its effect on larval mortality, a major restriction in juvenile production (Tinh et al., 2007). Mortalities are due to poor water quality or to infectious diseases. Water quality deteriorates when organic debris from dead larvae or food, and toxic substances like nitrogen compounds or hydrogen sulfide accumulate. High levels of ammonia nitrogen (NH_4^+, NH_3) are toxic. Upon pH increase the equilibrium favors NH_3 that diffuses across cell membranes, and disturbs respiration and osmoregulation (Barbieri, 2010). Prolonged exposure to nitrite (NO_2^-) or high levels of NO_2^- are lethal, especially under oxygen-limited conditions. Permanent exposure to high levels of nitrate (NO_3^-) reduces growth rate, and renders animals more susceptible to infectious disease (Camarga et al., 2005). Infectious diseases occur when high larval density and poor water quality select for growth of opportunistic pathogens, like *Vibrio* sp. or *Aeromonas* sp. that reside permanently in the water or are introduced via live feed. To prevent disease, good husbandry techniques are a prerequisite. Vaccination and antibiotics are means to control disease. Vaccination however is not applicable for larval cultures and routine usage of antibiotics promotes antibiotic resistances in bacterial pathogens and is thus, not a safe approach (Kesarcodi-Watson et al., 2008).

One way to control water quality and pathogen level is the addition of probiotics to rearing water or to live feed. Bioremediation is the application of microorganisms to remove waste products and toxic compounds. To remove nitrogen compounds, probiotic bacteria have to perform nitrification (oxidation of NH_4^+, NH_3 to NO_3^-) and denitrification (reduction of NO_3^- to nitrogen gas) (Chávez-Crooker and Obreque-Contreras, 2010). Pathogen antagonism by probiotics is mediated with non-specific substances (hydrogen peroxide or short-chain fatty acids) or bacteriocins (Kesarcodi-Watson et al., 2008).

Before probiotic strains are applied in vivo, they undergo careful in vitro assessment. We determined the potential of strains from the genus *Bacillus*, the

lactic acid bacteria (LAB), and chemolithoautroph bacteria to bioremediate nitrogen compounds and to antagonize pathogens.

Materials and methods

To measure nitrification and dentrification in vitro, all strains, except *Thiobacillus* sp. were cultured in Brunner mineral medium (DSMZ number #462) lacking $(NH_4)_2SO_4$, but supplemented with $1g.l^{-1}$ C and $1g.l^{-1}$ yeast extract and with either $1.5mg.l^{-1}$ nitrite, $60mg.l^{-1}$ nitrate, or $16mg.l^{-1}$ ammonium. Cultures were incubated at 30°C either with or without aeration for 48 hours. Samples were taken after 0, 24, and 48hours to determine the concentration of the respective compound. *Thiobacillus* sp., was cultured in DSMZ#113 medium with CO_2 and samples were withdrawn during nine days. Nitrite, nitrate and ammonium concentrations in cell-free supernatants were determined with specific test kits from MERCK (Germany) that rely on colorimetric absorbance readings.

To test pathogen antagonism in vitro, agar diffusion- and microtiter dish assays were employed. For agar diffusion, agar plates were first inoculated with a probiotic, and then covered with softagar seeded with different aquaculture pathogens. After 24 and 48h, plates were scored for clear halos around the probiotic inoculation point. For microtiter dish assays, pathogens were grown in spent probiotic culture supernatants and observed for growth over 48hours.

Results and discussion

Bioremediation of nitrogen compounds was observed for *Bacillus* sp., *Paracoccus* sp. (Table I), and *Thiobacillus* sp. (Fig.1), but not for the LABs *Enterococcus* sp., *Lactobacillus* sp., and *Pediococcus* sp.. Ammonium nitrogen concentration maintained unchanged under anaerobe conditions, because this process requires oxygen. *Bacillus* sp. produced ammonium up to 280% of the initial level, followed by a decrease to 216% at 48hours. *Paracoccus* sp. efficiently removed ammonium and only 7% were detected at 48hours. Nitrite is an intermediate ion during nitrification and denitrification. Both *Bacillus* sp. and *Paracoccus* sp. removed excess nitrite. *Paracoccus* sp. Was, however, more efficient, 7% were left under aerobic conditions and 2.2% were left under anaerobic conditions, compared to 31% and 14% for *Bacillus* sp.. Nitrate is reduced to 10% of the initial concentration by *Bacillus* sp. under anaerobic conditions. *Paracoccus* sp. is unique because it performs anaerobe denitrification, but also aerobic denitrification (Robertson and Kuenen 1984), with 7.8% and 11.7% nitrate left in the medium, respectively. *Thiobacillus* sp. often dominates nitrate-rich environments and is applied to clear nitrate from contaminated water in water treatment plants. In vitro, denitrification was analyzed in a closed system, where inorganic nitrate

served as electron donor. Nitrate was degraded and this degradation was accompanied by an increase of nitrite and ammonium over time (Fig.1).

Table I. Bioremediation of nitrogen compounds.

PROBIOTIC	% compound left in the culture after 48 hours incubation					
	NH_4^+		NO_2^-		NO_3^-	
	+ O_2	- O_2	+ O_2	- O_2	+ O_2	- O_2
Bacillus	216	100	31	14	73	10
Enterococcus	100	100	100	100	100	100
Pediococcus	100	100	100	100	100	100
Lactobacillus	100	100	100	100	100	100
Paracoccus	7.4	100	7	2.2	11.7	7.8

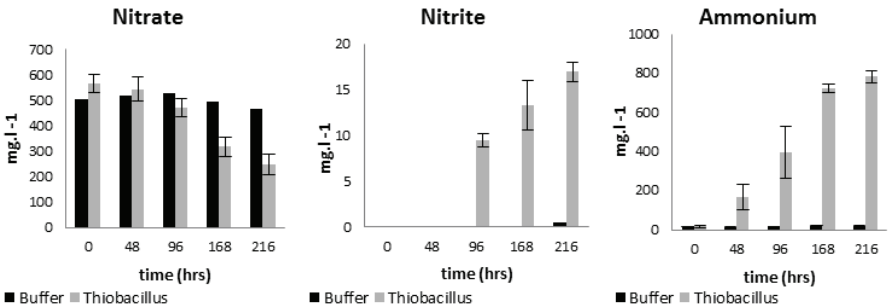


Fig. 1. *Thiobacillus* sp – denitrification. Nitrate, nitrite, and ammonium ion concentrations in the supernatants were determined at 0, 48, 96, 168, and 216 hours.

Additionally, *Paracoccus* sp. and *Thiobacillus* sp., encode proteins involved in sulfur oxidation metabolism and are thus able to convert hydrogen sulfide into a nontoxic form (Quentmeier et al., 2004).

Pathogen antagonism was determined for *Bacillus* sp., the three LABs, *Enterococcus* sp., *Lactobacillus* sp., and *Pediococcus* sp., as well as *Paracoccus* sp. (Table II). *Bacillus* sp. and the LABs antagonized a range of pathogens, but the sensitivity of each pathogen towards a certain probiotic strain differed. For example, *A. salmonicida* was susceptible to *Bacillus* sp. and *Pediococcus* sp., but less affected by *Enterococcus* sp., and *Lactobacillus* sp. In contrast, *Paracoccus* sp. and *Thiobacillus* sp. did not counteract any of the tested pathogens.

Table II. Pathogen antagonism.

	PROBIOTIC				
PATHOGEN	***Bacillus***	***Enterococcus***	***Pediococcus***	***Lactobacillus***	***Paracoccus***
A. hydrophila	+++	++	+++	+	-
A. salmonicida	+++	+	++	+	-
E. tarda	+	+	+	+	-
F. indologenes	(+)	+	+	+	-
S. agalactiae	++	+	++	+	-
V. harveyi	+	++	+	++	-
V. parahaem.	-	(+)	+	(+)	-
Y.ruckeri	++	++	++	++	-

Paracoccus sp. and *Thiobacillus* sp., are well suited for water quality management, but not for biocontrol of pathogens. The opposite is true for the LABs, they have great potential to control pathogens that reside in the water or on the live food, but not for bioremediation. *Bacillus* sp. is very versatile and suitable for both applications.

References

Barbieri E. 2010. Acute toxicity of ammonia in white shrimp (*Litopenaeus schmitti*) at different salinity levels. *Aquaculture* 306: 329-333.

Camargo J.A., A. Alvaro, and A. Salamanca. 2005. Nitrate toxicity to aquatic animals: a review with new data for freshwater invertebrates. *Chemosphere* 58: 1255-1267.

Chávez-Crooker P. and J. Obreque-Contreras. 2010. Bioremediation of aquaculture wastes. *Curr Opin Biotechnol*. 21:313-317.

Kesarcodi-Watson A, H. Ksapr, M.J. Lategan, and L. Gibson. 2008. Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes. *Aquaculture* 274: 1-14.

Quentmeier A, P. Hellwig, F. Bardischewsky, R. Wichmann, and C.G. Friedrich. 2004. Sulfide dehydrogenase activity of the monomeric flavoprotein SoxF of *Paracoccus*. *Biochemistry* 43:696-703.

Robertson L.A. and J.G. Kuenen. 1984. Aerobic denitrification--old wine in new bottles? *Antonie Van Leeuwenhoek.* (5-6):525-44.

Tinh N.T.N., K. Dierckens, P. Sorgeloos, and P. Bossier. 2007. A review on the functionality of probiotics in the larviculture food chain. *Marine Biotechn.* 10:1-12.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

LOW *ARTEMIA* CONSUMPTION STRATEGIES IN LARVAL SHRIMP REARING

R. Wouters and E. Naessens

INVE Technologies nv/sa, Hoogveld 93, B-9200 Dendermonde, Belgium

Introduction

In the past decade, shrimp hatcheries and feed companies have worked very hard toward the development of *Artemia* replacement protocols. In 2009, a review of research papers indicated that replacing more than 50% of *Artemia* nauplii with formulated feeds generally resulted in reduced survival and growth (Wouters et al., 2009), while commercial hatcheries experienced that the possible economic benefit (depending on *Artemia* cyst pricing) of 65% *Artemia* replacement protocols (i.e., in dry matter calculations, formulated feeds represent a share of 65% and *Artemia* a share of 35%) did not justify the risk of culture failure. In a hatchery cycle from nauplii stage to post-larval stage PL10, a 65% *Artemia* replacement protocol corresponds to an *Artemia* cyst consumption of 2.5-4kg per million produced post-larvae PL10 (note: in this paper we have made all calculations based on a cysts hatching percentage of 80%). Besides, there is a trend of hatcheries to harvest their tanks earlier than PL10, and in most cases feeding of *Artemia* nauplii stops after stage PL5, as white-legged shrimp post-larvae then become benthic and can feed on sinking microbound diets.

INVE Technologies N.V. (the R&D company of INVE Aquaculture) has carried out lab-scale and commercial-scale trials to evaluate the effect *Artemia* reduction on hatchery culture parameters of *Litopenaeus vannamei* shrimp with commercial dry feeds and/or improved experimental formulations.

Materials and methods

In a laboratory scale experiment in Thailand, nauplii of *L. vannamei* were stocked at 200 l^{-1} in 175-l fiberglass tanks with 3 replicate tanks per dietary treatment. The used experimental feeds were based on the commercial FRIP-PAK (FP) range. In treatment FP Normal-A, a protocol with normal *Artemia* consumption was applied (approximately 3kg cyst per million PL10). In treatment FP Low-A, a protocol with low *Artemia* consumption was used (approximately 1.5kg *Artemia* cysts per million PL10).

FRIPPAK FRESH (experimental formula) #1CAR, #2CD and #3CD and FRIPPAK PL+ Ultra crumbles (standard commercial formula) were fed to the animals and the amount was adjusted according to the different feeding protocols. High concentrations of *Chaetoceros* algae (60 000 to 100 000cells.ml^{-1}) were fed from stage Z1 till PL3 in all treatments. Evaluation parameters were registered in stage PL10. A larval development index was calculated based on staging at the moment of metamorphosis to PL1.

In a large-scale trial done at a commercial hatchery in Mexico, 30-ton tanks were stocked with 6.6 million *L. vannamei* nauplii and cultured until PL4 stage (hatchery phase). At PL4, they were transferred to 60-m³ raceway tanks at densities of 4 million PL per tank (raceway phase). In the hatchery phase, the animals received experimental FP feeds, similar to those tested in the previously mentioned lab-scale experiment, and high concentrations of live algae *Chaetoceros* sp. and *Thalassiosira weissflogii*. From PL6 onward, all tanks received 100% dry formulated feed. Three different levels of *Artemia* consumption were tested (1.6kg , 0.8kg, and 0kg cysts per million PL10, respectively, in treatments named FP Normal-A, FP Low-A, and FP Zero-A). In the Western hemisphere, tank shape and hatchery protocols are generally adjusted toward lower use of *Artemia* cysts than in the Eastern hemisphere. Mexico is a country with particularly low cyst consumption protocols compared to other shrimp producing countries worldwide, hence a consumption of only 1.6kg is here referred to as "normal". The Student's t test (one-sided) was used on data to compare the effect of the different treatments.

Results and discussion

The lab-scale experiment showed no differences ($p>0.05$) between treatments in terms of larval development (LDI) and survival (Table I). There are only few published reports (Jones et al. 1997; D'Abramo et al. 2006; Gallardo et al. 2013) on tests with successful development and survival applying high *Artemia* replacement levels (in casu 85%). This confirms preliminary results obtained in earlier trials (not presented) indicating the effectiveness of the experimental FRIPPAK feed formula.

Table I. Evaluation criteria of the lab-scale experiment in 175-l tanks. Larval development index (LDI) at metamorphosis M3/PL1, survival and biometrics at PL10. Treatments: FP Normal-A (approximately 3 kg cyst consumption per million PL10 + dry feed + live algae); FP Low-A (approximately 1.5 kg cyst consumption per million PL10 + dry feed + live algae)

Treatment	LDI[1] (-)		Survival (%)		Individual Dry weight (µg)		Length (µm)		Count[2] (#.g^{-1})	
	Mean	Std	Mean	Std	Mean	Std	Mean	Std	Mean	Std
FP Normal-A	6.70	0.08	81.5	6.5	841	22	8652	321	266	9
FP Low-A	6.67	0.13	82.7	5.1	691	18	8442	229	314	34

[1]LDI : 6 = mysis 3 stage, 7 = postlarva 1 stage; [2]Count = number of animals per 1 gram wet weight

Growth, length, and PL-count of the tested treatments were not significant different ($p>0.05$), however, the individual dry weight (IDW) of the post-larvae in treatment FP low-A was slightly lower than the IDW of the post-larvae of treatment FP Normal-A ($p<0.05$). These results indicate that the hatchery cycle need to be prolonged with one or more days to obtain seedstock (late post-larval stage) of a similar size than those obtained under normal *Artemia* use.

Table II. Evaluation criteria of the large-scale trial at a commercial hatchery. Larval development index (LDI) at metamorphosis M3/PL1, survival and biometrics at PL3 and PL12 stage respectively. Treatments: FP Normal-A (approximately 1.6 kg cyst consumption per million PL10 + dry feed + live algae); FP Low-A (approximately 0.8 kg cyst consumption per million PL10 + dry feed + live algae); FP Zero-A (dry feed + live algae).

Hatchery phase (PL3)					
Treatment	LDI[1] (-)	Survival (%)	Individual dry weight (µg)	Length (µm)	Count[2] (#/g)
FP Normal-A	6.9	67,7	209	4370	739
FP Low-A	6.9	77,1	224	4534	761
FP Zero-A	6.7	75,6	145	4045	1063
Raceway phase (PL12)					
		(%)	(mg)		(#/g)
FP Normal-A		59,8	6,6		151
FP Low-A		66,8	7,3		138
FP Zero-A		55,1	3,8		263

[1]LDI : 6 = mysis 3 stage, 7 = postlarva 1 stage; [2]Count = number of animals per 1 gram wet weight

The results of the large-scale experiment show that, with the experimental FRIPPAK larval feeds, it was possible to reduce *Artemia* consumption to approximately 0.8 kg cysts without affecting the culture parameters. With zero *Artemia* consumption, however, growth was clearly affected in a negative way. It was also observed that without *Artemia* consumption, the pigmentation of the postlarvae was less intense and also PL size was less uniform than in the remaining treatments. These data prove that under commercial culture conditions, with up-to-date feed formulation and technology, significant *Artemia* reduction can

be achieved. Optimized larval rearing techniques are necessary to obtain this success. In particular, the use of live algae and microbial management (e.g. use of probiotics) are thought to play an important role. Preliminary observations indicate that the quality and growth potential of the seedstock are compromised when *Artemia* is completely replaced. The long-term performance of PL produced under low *Artemia* feeding protocols is not documented, hence still needs to be evaluated during prolonged and controlled grow-out experiments.

Conclusion

Recent trials and testimonials with *L. vannamei* larvae and postlarvae give first indications that the consumption of *Artemia* can be reduced to 1.5kg and even 0.8kg cysts per cycle without affecting survival and growth. Adjusted feeding protocols and newly-developed artificial feeds are required in order to obtain cost-effective results. At total replacement levels (zero *Artemia*), nevertheless, the growth of postlarvae is reduced, represented by a lower PL size.

References

D'Abramo L.R., E.I. Perez, R. Sangha, and A. Puello-Cruz. 2006. Successful culture of larvae of *Litopenaeus vannamei* fed a microbound diet exclusively from either stage PZ2 or M1 to PL1. Aquaculture 261(4): 1356-1362.

Gallardo P., G. Martínez, P. Gabriela, A. Paredes, and G. Gaxiola. 2013. Replacement of *Artemia franciscana* Nauplii by Microencapsulated Diets: Effect on Development, Digestive Enzymes, and Body Composition of White Shrimp, *Litopenaeus vannamei*, Larvae. J. WAS 44(2): 187-197 .

Jones D.A., A.B. Yule, and D.L. Holland. 1997. Larval nutrition. Pages 353-389 in D'Abramo, L.R., D.E. Conklin, and D.M. Akiyama, editors. Crustacean Nutrition. Advances in World Aquaculture, Vol. 6. World Aquaculture Society, Baton Rouge, LA, USA.

Wouters R., M.L.Cobo, J. Dhont, and M. Wille. 2009. Developments in feed formulations, feeding practices and culture techniques for marine shrimp larvae. Pages 79-91 in C. Browdy and J.E. Darryl (editors). The rising tide. Proceedings of a Special Session on Shrimp Farming. World Aquaculture 2009. The World Aquaculture Society, Baton Rouge Louisiana, USA.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

ORTHOGONAL ANALYSIS OF ELEMENTS AFFECTING THE FORMATION OF CYSTOCARPS ON FEMALE GAMETOPHYTE OF *GRACILARIOPSIS LEMANEIFORMIS*

D. Xu, L.L. Wang, X.Z. Zhang, J. Wang, and X.N. Zang

College of Marine Life Sciences, Ocean University of China, Yushan Road 5, Qingdao, 266003, China

Introduction

Recently having been used as complementary food for abalone during summer when other food is lacking, *Gracilariopsis lemaneiformis* is commonly known as agarophyte in China. The current large-scale cultivation method for this seaweed is suspended on raft while it grows vegetatively usually from autumn to the following early summer in the south part of China. Huge amounts of seedlings are needed each autumn when the cultivation starts, so the seedling preservation and supplement is always difficult for farmers. Some phycologists have tried the spore germinating method for culturing another agarophyte *Gracilaria chilensis* using tetraspores released from the tetrasporaphyte (Alveal et al., 1997; Halling et al., 2005). Furthermore, environmental factors (Sukumaran and Kaliaperumal, 2001) as well as different levels of its own branches (Ye et al., 2006) affecting the release rate and survival rate of tetraspores for *Gracilaria* have been tested. However, the gametophytes germinated from tetraspores include male and female and the ratio is 1:1. Actually, in nature the ratio for tetrasporophyte and female:male gemetophytes should be 2:1:1, but usually male gametophyte is not easily found since it rots easily after sperm release. So, if tetraspore is used for large-scale cultivation, half of the production is fragile and easily lost. Therefore carpospores, which will germinate to tetrasporophyte, maybe the better choice for spore cultivation of *Gracilaria*.

In this experiment, we tested the environmental factors affecting cystocarp formation which release carpospores by crossing the male and female gametophytes of *G. lemaneiformis* using an orthogonal experimental design.

Materials and methods

One strain of male gametophytes and one strain of female gametophytes of *G. lemaneiformis* were used for all the experimental groups. Each group had two branches of male and female gatetophytes which was 2-3cm long.

Orthogonal design was conducted using 6 factors and 5 levels (Table 1) with light:dark=12:12. The number of cystocarp per cm will be countered on surface of female gametophytes for each group after 20 days co-cultured with male gametophytes.

Table 1. The six environmental factors and five levels used in the orthogonal design.

	1	**2**	**3**	**4**	**5**
light (lux)	100	500	1000	3000	5000
Temperature (°C)	10	15	20	25	30
N (mM)	0.1	0.4	0.8	1.5	2.0
P (mM)	0.01	0.02	0.05	0.1	0.2
Fe^{3+} (mM)	0.001	0.002	0.005	0.01	0.02
VB12($\mu g.l^{-1}$)	0.5	5	50	500	5000

Results and discussion

According to the results, the consequences of range of these six factors were Temperature> P> Light> N> Fe> VB12 (Table II).

Table II. Analysis for orthogonal experiments

Exp	**Light lux**	**°C**	**N mM**	**P mM**	**Fe^{3+} mM**	**VB12 $mg.l^{-1}$**	**cystocarp $individual.cm^{-1}$**
1	1	1	1	1	1	1	0
2	1	2	2	2	2	2	0
3	1	3	3	3	3	3	1.758
4	1	4	4	4	4	4	0
5	1	5	5	5	5	5	0
6	2	1	2	3	4	5	0
7	2	2	3	4	5	1	0
8	2	3	4	5	1	2	1.27
9	2	4	5	1	2	3	0
10	2	5	1	2	3	4	0
11	3	1	3	5	2	4	0
12	3	2	4	1	3	5	0
13	3	3	5	2	4	1	3.729
14	3	4	1	3	5	2	0
15	3	5	2	4	1	3	0
16	4	1	4	2	5	3	0
17	4	2	5	3	1	4	0
18	4	3	1	4	2	5	0.216
19	4	4	2	5	3	1	0
20	4	5	3	1	4	3	0
21	5	1	5	4	3	2	0
22	5	2	1	5	4	3	0
23	5	3	2	1	5	4	0.682
24	5	4	3	2	1	5	1.209
25	5	5	4	3	2	1	0
Average 1	0.352	0.000	0.043	0.136	0.496	0.746	
Average 2	0.254	0.000	0.136	0.988	0.043	0.254	
Average 3	0.746	1.531	0.593	0.352	0.352	0.352	
Average 4	0.043	0.242	0.254	0.043	0.746	0.136	
Average 5	0.378	0.000	0.746	0.254	0.136	0.285	
Range	0.703	1.531	0.703	0.945	0.703	0.610	

Temperature had the most important effect on cystocarp formation which showed the obvious differences among groups and also consistent to previous studies. The 20°C group was the only group having cystocarp growing but all the other different temperature group had no cystocarp in this experiment.

The next important factor was P content. Our results showed that although higher N content was preferred by the formation of cystocarps, P did not have the same pattern. On the contrary, the 0.02mM group had the best effects.

According to the statistical analysis, only temperature had the obvious difference and for the other factors tested, although some effects were present but were not statistically significant.

References

Alveal K, H. Romo, C. Werlinger, and E.C. Oliveira. 1997. Mass cultivation of the agar-producing alga *Gracilaria chilensis* (Rhodophyta) from spores. Aquaculture 148(2-3):77-83

Halling C, Aroca G, Cifuentes M, Buschmann AH, Troell M. 2005, Comparison of spore inoculated and vegetative propagated cultivation methods of *Gracilaria chilensis* in an integrated seaweed and fish cage culture. Aquaculture International 13(5):409-422

Sukumaran S. and N. Kaliaperumal. 2001. Sporulation in *Gracilaria crassa* Harvey ex. J. Agardh at different environmental factors. Seaweed Res. Utilisat. 23:1-2.

Ye N.H., H.X. Wang, G. Wang, et al. 2006. Formation and early development of tetraspores of *Gracilaria lemaneiformis* (*Gracilaria*, Gracilariaceae) under laboratory conditions. Aquaculture 254: 219-226

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

MICROPARTICLES DELIVERY SYSTEM IN GNOTOBIOTIC EUROPEAN SEA BASS LARVAE (*DICENTRARCHUS LABRAX*, L. 1758)

E.N. Yaacob[1,2], B. De Geest[3], D. Vanrompay[4], K. Dierckens[1], and P. Bossier[1]

[1] Laboratory of Aquaculture & Artemia Reference Center, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium
[2] Department of Aquaculture Science, Faculty of Fishery and Aqua Industry, Universiti Malaysia Terengganu, Terengganu, Malaysia
[3] Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium
[4] Department of Molecular Biotechnology, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

Introduction

Rearing of marine fish larvae is one of the most challenging phases in aquaculture practice due to rapid development changes of the larvae within a short time period. Recently, a major concern to improve survivability of the larvae has focused on the use of immunostimulants or antimicrobial peptides to steer the innate and/or adaptive immunity of marine fish larvae. Thus, increasing the immune protection and prepare the larvae to face environmental stress or pathogen invasion. Oral administration of immunostimulants such as peptides can be delivered via encapsulation in microparticles. The use of smaller particles size is important due to the larvae mouth opening post hatching. In order to achieve optimal uptake, microparticles should assert certain size and density. The use of inert microparticles to study the particles size selection could avoid several factors that might affect selection such as particles' surface characteristics, feeding attractants and nutritional quality (Baer et al., 2008). In this study, we examined maximum uptake of inert uniform-size microparticles with different sizes and the uptake of alginate microparticles in a gnotobiotic larval European sea bass system. Data obtained from this study could provide insight into an optimal microparticle delivery system using biodegradable microparticles loaded with protein of interest in axenic sea bass larvae.

Materials and methods

Axenic European sea bass larvae (Dierckens et al., 2009) were stocked in vials with 9ml sterile artificial sea water at a salinity of 35g l^{-1} at 6 days after hatching (DAH) with 12 larvae per vial in triplicate. Temperature of the room was set

constant at 16±1°C and vials were mounted on a horizontal rotor with four rotations per minute (rpm). At 7DAH, larvae were fed once with uniform-size Fluoresbrite® polystyrene latex microparticles (Polyscience Inc., Warrington, Pennsylvania, USA) of 2, 10, 20, and 45µm. All Fluoresbrite® microparticles were prepared at a constant volume of $4.0\times10^9 \mu m^3$ particles.ml^{-1}. Alginate microparticles loaded with fluorescein isothiocyanate bovine serum albumin (FITC-BSA) used for the study of biodegradable microparticles had a size range of 1-25µm at a density of 10^6 particles.ml^{-1}. Larvae were collected at 4, 8, 12, 24, and 48h post-feeding, gently washed on 100-µm mesh, anesthetized with 0.1% benzocaine, and fixed for counting or observation using epifluorescent microscopy. Data for maximum volume of the inert microparticles uptake at five different time points were compared using student's t-Test at $\alpha=0.05$.

Results and discussion

In general, smaller 2-µm microparticles showed the highest number of particle uptake per larva, increasing until 48h compared to the rest of the test sizes (Fig. 1a). Total volume of microparticles in the gastrointestinal tract of larvae fed with 2-µm particles showed significant difference ($p<0.05$) only after 24h post-feeding compared to 10-µm particles. Most probably, 2-µm microparticles at early feeding were taken up passively into the gut by drinking. However, when fed with 10-µm particles, the volume of microparticles in the gut increased significantly ($p<0.05$) throughout the experiment. After four hours, larvae that were fed with 20- and 45-µm microparticles showed maximum volume of microparticles taken up with 10^4 and $10^6.\mu m^3$ observed per larva, respectively (Fig. 1b). No significant difference of microparticle volume in guts at different times was found ($p>0.05$). Thus, feeding fish larvae with microparticles of 20µm and 45µm can reach maximal feeding in shorter time compared to smaller sized microparticles. Apparently, feeding sea bass larvae with biodegradable alginate microparticles showed a relatively higher uptake per fish larva comparing to the inert microparticles. At four hours post-feeding, accumulation of fluorescent color from FITC-BSA loaded in alginate microparticles was observed to be scattered in the mid and hindgut (Fig. 2a), while a very intense fluorescent color was observed along the gastrointestinal tract of larvae after 12, 24, and 48h (Fig. 2b). Additionally, no intact microparticles were observed after 12h, suggesting degradation of microparticles in the gut. In an earlier study on European sea bass weaning using microparticulate compound diets by Cahu and Zambonino (1994), diets ranging from 60-120µm were used from larval mouth opening from 6-13DAH, 120-200µm from 14-25DAH, and 200-400µm from 26DAH onwards. Proper size of particle is particularly important and a study using all-protein-membrane microcapsules in Asian sea bass larvae (*Lates calcarifer* L.) at the age of 4DAH showed that smaller microparticles (less than 50µm) are not easily detected by the larvae. Once ingested, it can be easily moved through the gut. However, larger microparticles (>100µm) are difficult to ingest and had dif-

ficulty passing through the intestino-rectal valve (Walford et al., 1990). In this study, 24 and 48h post-feeding, total mortality occurred when fed with 45-µm inert particles which may be due to similar difficulty. Furthermore, it is impossible to simulate an accurate natural foraging environment for marine fish larvae (e.g., light, temperature, turbulence, type and nutritional quality of prey) in a laboratory experimental set-up. Thus, extrapolating experimental results to the marine environment seems difficult (Suthers, 2000).

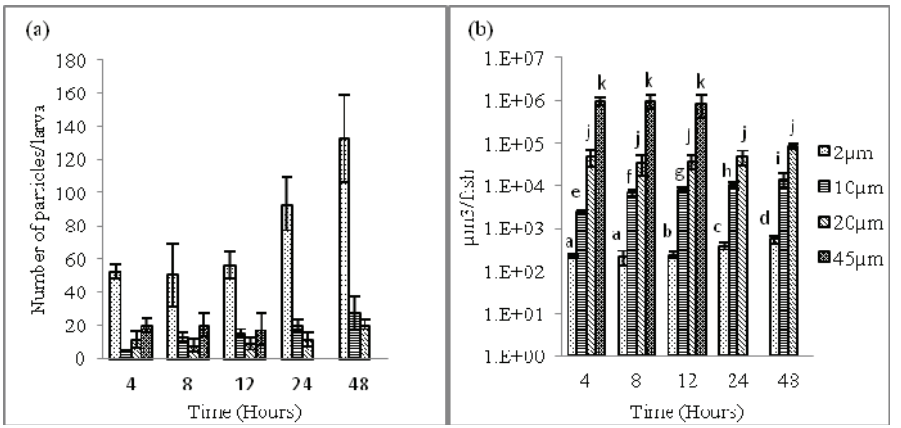


Fig. 1. Values are means with bars as standard deviation (a) smaller microparticles shown increasing number of particles being taken up per larva throughout the experiment compared the rest of the test sizes. (b) letters indicate significant differences in the volume of the same particle size that had been taken up between each time point. Student's t-Test ($p<0.05$).

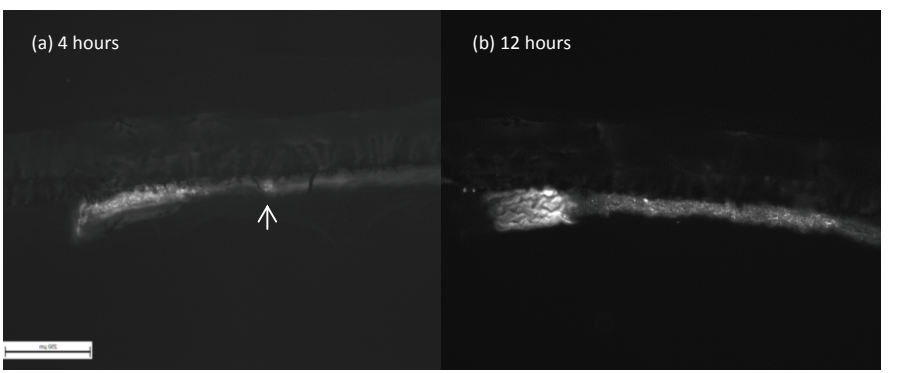


Fig. 2. Epifluorescent microscopy (×10) image of sea bass (DAH7) gastrointestinal tract post-feeding with FITC-BSA alginate microparticles. (a) arrow shown aggregation of FITC-BSA alginate microparticles in midgut 4h post-feeding. (b) higher intensity of FITC-BSA was observed 12h post-feeding and gastrointestinal tract of larvae were filled with alginate microparticles.

Conclusion

Encapsulation of protein or drug of interest using biodegradable microparticles such as alginate microparticles at the size range from 2-25µm might be appropriate for protein delivery in gnotobiotic sea bass larvae at 7DAH. Furthermore, larger sizes of alginate microparticles (up to 100µm) also may be possible to be used since inert microparticles do not have the same characteristics as natural food for fish larvae. Thus, extrapolating uptake of inert microparticles to the uptake of biodegradable particles such as alginate microparticles or formulated microdiet should be evaluated carefully.

References

Baer A., C. Langdon, S. Mills, C. Schulz, and K. Hamre. 2008. Particle size preference, gut filling and evacuation rates of the rotifer Brachionus "Cayman" using polystyrene latex beads. Aquaculture 282(1-4):75-82. doi:10.1016/j.aquaculture.2008.06.020

Cahu C.L. and J.L. Zambonino. 1994. Early weaning of sea bass (Dicentrarchus labrax) larvae with a compound diet: effect on digestive enzymes. Comparative Biochemistry and Physiology 109(2):213-222.

Dierckens K., A. Rekecki, S. Laureau, P. Sorgeloos, N. Boon, W. Van Den Broeck, and P. Bossier. 2009. Development of a bacterial challenge test for gnotobiotic sea bass (Dicentrarchus labrax) larvae. Environmental Microbiology 11(2):526-533.

Suthers I. 2000. Significance of larval condition: comment on laboratory experiments. Canadian Journal of Fisheries and Aquatic Sciences 57(7):1534-1536.

Walford J., T.M. Lim, and T.J. Lam. 1990. Replacing live foods with microencapsulated diets in the rearing of seabass (Lates calcarifer) larvae: do the larvae ingest and digest protein-membrane microcapsules? Aquaculture 92:225–235.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

DEVELOPMENT OF AN NNV-FREE LARVAE REARING SYSTEM AND PRODUCTION OF SPR GROUPER FINGERLINGS

H.L. Yang, H.Y. Lin, and C.C. Lin

Institute of Biotechnology, National Cheng Kung University, and Merit Ocean Biotech., Taiwan, 70101 ROC

Grouper is a popular fish among Orientals and Arabians. Culture of grouper has developed rapidly due to the success of rearing larvae in earthen ponds or semi-indoor hatcheries, and the depletion of wild grouper resources. Recently, grouper larvae production has suffered from severe Nervous Necrosis Virus (NNV) infections coming from using virus-contaminated water, commercial fertilized eggs, live starting feeds, and infected hatcheries.

We have achieved a stable larvae production without NNV infection by constructing an isolated indoor virus-free hatchery, developing an oral NNV vaccine, and using virus-free starting feeds.

The stable production of larvae allowed a step-by-step analysis of the chemical and physical parameters and nutritious requirements of grouper larval development. This information facilitated the development of a digitalized standard operating procedure (SOP). With this hatchery system, over 60 consecutive fingerling production cycles were attained without any NNV infection, and have increased the rearing efficiency from 2-30 individuals.ton^{-1} water in the earth pond to 1000 individuals.ton^{-1} water. This platform larval rearing system has also been used successfully to culture larvae of *E. coioides*, *E. lanceolatus*, *E. fuscoguttatus,* and *E. bruneus.*

In order to prevent the fish from encountering disease in the grow-out period and to relieve farmers from expensive and tedious injection requirements, the NNV-free fingerling were pre-immunized with a multivalent vaccine for NNV, iridoviruses, and *Vibrio* species before delivery. Five field trials were implemented with over 15 000 fish in each trail for 8-12 months at three regions. These SPR fingerlings were demonstrated to evade diseases and increase average survival rate to 80% as compared to the current 30-50% using other fingerlings.

Our results demonstrated the success of using this indoor rearing system to grow grouper larvae without NNV infection, and the pre-immunized SPR fingerlings could prevent diseases and increase survival rate at the grow-out farm.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

THE IMPACT OF QUORUM SENSING ON MOTILITY IN *VIBRIO HARVEYI*

Q. Yang, P. Bossier, and T. Defoirdt

Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Rozier 44, B-9000 Gent, Belgium

Introduction

Quorum sensing (QS) is a cell-to-cell signaling mechanism of bacteria, which controls the expression of a number of target genes (Ruwandeepika et al., 2011). Bacterial motility is essential for pathogenic bacteria during the initial stages of infection and is considered to be an important virulence factor in many pathogens (Rui et al., 2008). Flagella are the organelles responsible for motility. Some *Vibrio* species possess dual flagellar systems – polar flagellum and lateral flagella – which are suited for movement under different circumstances. To date, approximately 50 polar and 40 lateral flagellar genes have been identified (McCarter, 2004). In this work, we investigated the impact of QS on the motility and expression of motility-related genes in *V. harveyi*.

Materials and methods

Bacterial strains, growth conditions and reagents
The bacterial strains used in this study are listed in Table I. The bacteria culture and reagents used were according to Ruwandeepika et al. (2011).

Table I *Vibrio harveyi* strains used in this study.

Strains	Relevant characteristics	References
BB120	ATCC BA-1116;Wild type strain from which strains JAF483, JAF548 and JMH 634 were derived	Bassler et al. (1997)
JAF483	LuxO locked in maximally active QS (QS+)	Freeman and Bassler (1999)
JAF548	LuxO locked in minimally active QS (QS-)	Freeman and Bassler (1999)
JMH634	Triple autoinducer synthase mutant	Henke and Bassler (2004)

Swimming motility assays
The swimming motility of all strains was assayed according to Rui et al. (2008).

Quantification of the expression of motility-related genes

The expressions of ten genes (Table II) related with flagella were assayed and calculated according to Ruwandeepika et al. (2011) with reverse transcriptase real-time PCR. The RNA extraction, reverse transcription, and real-time PCR were performed by SV Total RNA Isolation System (Promega, USA), RevertAidTM H minus First strand cDNA synthesis kit (Fermentas Gmbh, Germany), and Maxima® SYBR Green/ROX qPCR Master Mix (Fermentas, Cambridgeshire), respectively. For the real-time PCR, ten pairs of primers were designed using the software Primer Premier Version 5.00 (Premier Biosoft International, Palo Alto, CA) with predicted product sizes in the 100 to 200bp range (Table II) and the reaction was performed in StepOne™ Real-Time PCR System thermal cycler (Applied Biosystems).

Statistical analyses

All data were presented as mean±S.D. One-way analysis of variance (ANOVA) and Tukey's multiple comparison tests were used to determine whether significant differences existed between treatments using a significance level of 5%.

Results

As can be seen in Figure 1, QS significantly influenced the swimming motility of *V. harveyi*. The swimming motility of BB120 was significantly lower than that of JAF483 (a mutant with maximal QS activity), but was significantly higher than that of JMH548 and JMH634 (mutants with inactive QS) ($p<0.05$). The expression of motility related genes was consistent with the motility data (Table II)

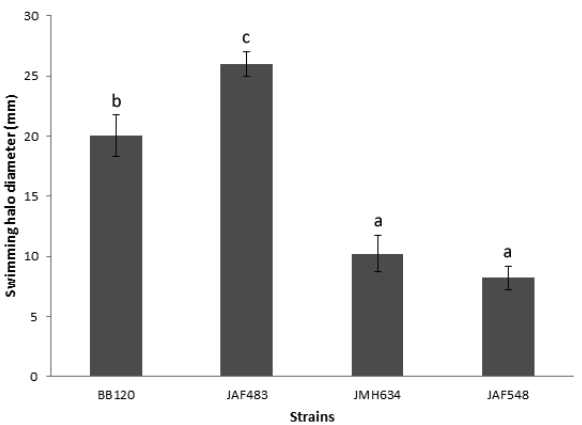


Fig.1. Swimming motility of *V. harveyi* BB120 (wild type), JAF 483 (QS+), JAF548 (QS-), and JMH634 (triple autoinducer synthase mutant) on soft Marine Agar plates (0.3% agar) after 24h of incubation at 28°C. Error bars indicate the SD of 3 replicates. Different letters indicate significant differences ($p<0.05$).

Table II Relative expression of motility-related genes relative to *rpoA* mRNA in late log phase cultures of *V. harveyi* wild type and quorum sensing mutants. For each gene, the expression in the mutant JAF548 (in which quorum sensing is completely inactive) was set at 1 and the expression in all other strains was normalized accordingly using the $2^{-\Delta\Delta CT}$ method.

Gene name	Descriptions	Sequences of forward and reverse primers (5'-3')	Relative expression (fold)[a]		
			JAF548	BB120	JAF483
flaA	Polar flagellin	CTGCGGGTCTTCAAATCTC GTTAGTGGTCTCGTTCATTGC	1a	1.64b	2.54c
flaC	Polar flagellin	GCTTGATGTCGCCTTGAGAA GCTGCCATTTGCTGCTTG	1a	2.04b	7.02c
flaK	Polar flagellar regulator	ATTGCCCGTTGATGATTTG CTTCTGTGCCCGATACTTGT	1a	1.27b	1.71c
fliA	Flagellar biosynthesis sigma factor	CGCCGAGTGTTCAGGTAGA CCGATGGGTCACGATTTAGT	1a	2.50b	4.93c
fliS	Polar flagellin specific chaperone	CTCCGCACAAAGTCATTCAA CAATGTCACCACCATCTTCC	1a	2.22b	3.42c
flgB	Flagellar basal body rod	AAACACGCCTGGCTACAAA ACGCTCTAAATCCAAATCTACC	1a	1.20b	1.90c
cheA	Chemotaxis protein	AGCCTGTGATTCCTGAGCC AGTGATGTCGCCGCTGTC	1a	2.00b	3.90c
cheR	Chemotaxis protein	ATGCGATGACGACTAACGA ACGCTTGGCAATAAACCTG	1a	1.40b	2.50c
lafA	Lateral flagellar flagellin	TAACTTCGCATCGCTTGTAAC TCGTCTGCTGCTGAGTTGATA	1b	0.60a	0.59a
lafK	Lateral flagellar regulator	GAGCCAAATGAACACCTCG AACAATCGCAATCACCACA	1b	0.63a	0.65a

[a]: Values in the same row with a different letter are significantly different ($P < 0.05$)

Conclusion

The present work proves that QS regulates motility and the expression of 10 motility-related genes in *V. harveyi.*

References

Bassler B.L., E.P. Greenberg, and A.M. Stevens. 1997. Cross-species induction of luminescence in the quorum sensing bacterium *Vibrio harveyi*. *J Bacteriol* 179:4043-4045.

Freeman J.A. and B.L. Bassler. 1999. A genetic analysis of the function of LuxO, a two-component response regulator involved in quorum sensing in *Vibrio harveyi*. *Mol. Microbiol.* 31,:665-677.

Henke J.M. and B.L. Bassler. 2004. Quorum sensing regulates type III secretion in *Vibrio harveyi* and *Vibrio parahaemolyticus*. *J Bacteriol* 186:3794-3805.

McCarter L.L. 2004. Dual flagellar systems enable to motility under different circumstances. *J Mol Microbiol Biotechnol*. 7:18-29.

Rui H., Q. Liu, Y. Ma, Q. Wang, and Y. Zhang. 2008. Roles of LuxR in regulating extracellular alkaline serine protease A, extracellular polysaccharide and mobility of Vibrio alginolyticus. FEMS Microbiol Lett. 285:155-162.

Ruwandeepika H.A., P.P. Bhowmick, I. Karunasagar, P. Bossier, and T. Defoirdt. 2011. Quorum sensing regulation of virulence gene expression in *Vibrio harveyi in vitro* and *in vivo* during infection of brine shrimp (*Artemia francsiscana*). *Environ. Microbiol. Rep.* 3: 597-602.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM

C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

MULTIPLE VITELLOGENIN YOLK PRECURSORS IN EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*)

O. Yilmaz[1], F. Prat[2], A.J. Ibáñez[3], H. Amano[4], S. Koksoy[5], and C.V. Sullivan[6]

[1] Faculty of Fisheries, Akdeniz University, Antalya 07070, TURKEY
[2] Departamento de Biología Marina y Acuicultura, Instituto de Ciencias Marinas de Andalucía (CSIC), Avda. República Saharaui 2, 11510 Puerto Real, Cádiz, SPAIN
[3] Electron and Confocal Microscopy Service, University of Valência–SCIE, Dr Moliner, 50-Research building, Lab-1.63, 46100 Burjassot-Urjassot, Valência, SPAIN
[4] School of Marine Biosciences, Kitasato University, 1-15-1 Kitasato, Minami, Sagamihara, Kanagawa 252-0373, JAPAN
[5] Central Research and Immunology Laboratories, Akdeniz University, Faculty of Medicine, Antalya, 07070, TURKEY
[6] Department of Biology, College of Agriculture and Life Sciences, North Carolina State University, Raleigh, NC 27695-7617, USA

Introduction

Vitellogenin (Vtg) is synthesized by the estrogenized liver, secreted into the blood, taken up by growing oocytes, and processed into the major yolk proteins (YPs) and lipids. The major YPs are lipovitellin (Lv; heavy chain [LvH], light chain [LvL]), phosvitin (Pv), β′-component (β′c), and C-terminal peptide (Ct). In acanthomorph teleosts, a tripartite Vtg system may include two complete Vtg paralogs (VtgAa and VtgAb) bearing all YP domains, and an incomplete Vtg (VtgC) lacking Pv and much of the C-terminus (β′c /Ct). In some marine teleosts spawning pelagic eggs, virtually all YPs derived from VtgAa are proteolytically cleaved into free amino acids (FAAs) during final maturation, whereas major products of VtgAb (LvHAb) and VtgC (LvC), remain largely intact. FAAs are osmotic effectors of oocyte hydration and are diffusible nutrients selectively utilized by early embryos. Large lipoproteins derived from VtgAb and VtgC are utilized by late stage larvae. Thus, the multiple Vtg system provides for acquisition of proper egg buoyancy and delivers the appropriate types of nutrients for each stage of early development. The degree to which dysfunction of this system is involved in poor egg quality, which is widespread in farmed fishes, has not previously been explored. The objectives of this study were to (1) obtain complete cDNA sequences encoding each form of sea bass Vtg and characterize the Vtg proteins encoded by these cDNAs, (2) confirm the presence and quantity of each form of Vtg and YP in the liver, blood plasma, and ovary of females with

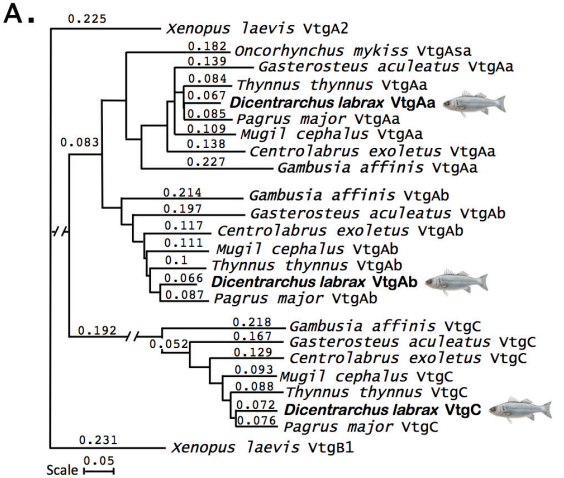
fully-grown oocytes, and 3) deliver a methodology to assess involvement of the multiple Vtg system in the egg quality of farmed fish.

Materials and methods

Partial cDNA sequences encoding each type of sea bass Vtg were obtained by sequencing liver PCR products generated using an array of degenerate and gene-specific primers obtained from published works and other investigators or designed de novo. Partial cDNAs were assembled into contiguous sequences. Vtg polypeptide sequences deduced from the assembled cDNAs were classified by comparison to published Vtg sequences for other teleosts spanning a broad array of taxa. The presence and relative quantity of the Vtgs and their product YPs in liver, blood plasma and ovary of post-vitellogenic (PV) sea bass were assessed by HPLC and tandem mass spectrometry (nanoLC-MS/MS), with the sea bass Vtg sequences embedded in the stickleback (*Gasterosteus aculeatus*) proteome as the reference library and employing ProteoIQ software to generate normalized spectral counts for each Vtg and YP. Western blotting performed using Vtg-type specific antisera against purified grey mullet (*Mugil cephalus*) LvAa, LvAb, and LvC (Amano et al., 2008) was employed to confirm the presence of the Vtgs and Vtg-derived YPs in blood plasma and ovary, respectively, and to examine the degree to which each form of Lv was degraded during oocyte maturation.

Results and discussion

Complete cDNA sequences encoding three different sea bass Vtg genes were obtained and posted to Genbank (*SbsvtgAa*, JQ283441; *SbsvtgAb*, JQ283442; *SbsvtgC*, JQ341410). Alignments and comparisons of the deduced Vtg peptide sequences with those from other teleosts allowed definitive identification of these Vtgs as sea bass VtgAa, VtgAb, and VtgC, respectively. A representative comparison of the three types of sea bass Vtg to those from several acanthomorph species, including two *Moronidae* species, for which full-length sequences are available is shown in Figure 1. The relative abundance and proportional ratio of the three Vtgs or their product YPs present in liver, plasma and ovary of females just completing oocyte growth was revealed via nanoLC-MS/MS to generate spectral counts normalized by ProteoIQ (Fig. 2). VtgAb spectra were 2- to 5-fold more abundant than VtgAa spectra, depending on the sample type, and VtgC spectra were very limited, except in ovary, where they were ~threefold lower in abundance than for VtgAb. Western blotting performed using Vtg-type specific antisera raised against grey mullet Lvs also detected all three forms of Vtg in extracts of blood plasma from these postvitellogenic (PV) females and from estrogenized males (data not shown). Corresponding blots of ovary extracts (Fig. 3) revealed that the Lvs derived from all three forms of Vtg undergo partial proteolysis during oocyte maturation, as evidenced by comparison of results for PV oocytes with those for ovulated eggs (OV).



B.

	Dicentrarchus labrax		
Moronidae Ssp.	VtgAa	VtgAb	VtgC
M. americana	93% (95%)	93% (96%)	95% (97%)
M. saxatilis	94% (96%)	93% (96%)	95% (97%)

Fig. 1. A. Clustal W-formatted dendrogram illustrating the relationship of sea bass Vtgs to those of other acanthomorph teleosts. Numbers represent p-distances between sequences. The Vtgs from sea bass (*D. labrax*) were classified as VtgAa, VtgAb, and VtgC. B. Percent identity and (similarity) of European sea bass Vtgs with those of other species of the family *Moronidae*.

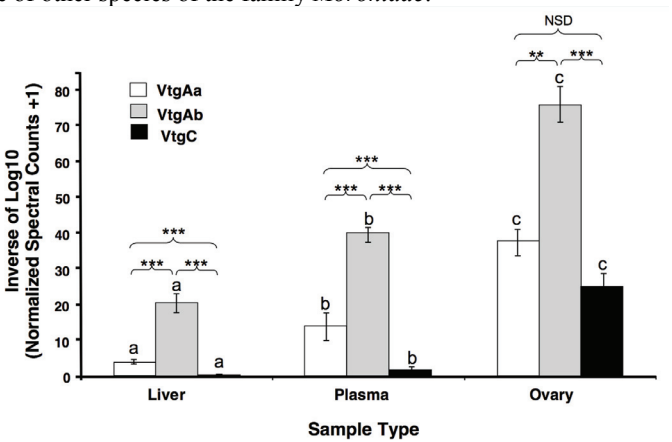


Fig. 2. Relative abundance of Vtgs and Vtg-derived yolk proteins in postvitellogenic sea bass liver, blood plasma and ovary as determined by tandem mass spectrometry. The inverse of log transformed normalized spectral counts of tryptic peptides derived from each form of Vtg is shown. Vertical bars indicate the mean for 4 fish and vertical brackets indicate SEM. Horizontal brackets indicate statistical comparisons between means with the significance level set at P≤0.05 (*), P≤0.01 (**), or P≤0.001 (***). NSD = not significantly different. For comparisons of mean

values for each form of Vtg between tissue types, bars bearing different letter superscripts indicate are significantly different (P≤0.001), except for the comparison for VtgAa between liver and plasma (P≤0.01) and for the comparisons for VtgAa between plasma and ovary, and for VtgC between liver and plasma (P≤0.05). Two-way, mixed-model ANOVA followed by one-way ANOVAs and Tukey's HSD post-hoc tests were used.

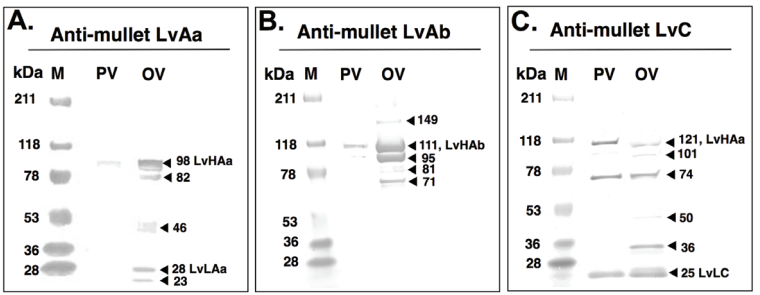


Fig. 3. Western blotting of ovary samples from female sea bass. Blots were made using rabbit antisera to purified grey mullet LvAa (A.), LvAb (B.), and LvC (C.). Samples included prestained molecular weight markers (M), extracts of ovary from postvitellogenic females (PV), and ovulated egg extracts (OV). Numbers to the left of each panel indicate the mass of molecular weight markers (kDa). Small numbers with arrows pointing to individual bands on the blots indicate the estimated mass (kDa) of the protein in that band. Selected bands are tentatively identified with the abbreviation for the corresponding sea bass Lv heavy chain (LvHAa, LvHAb, or LvHC) or light chain (LvLAa or LvLC) present in that band based upon estimated masses of the LvH or LvL primary peptides.

Conclusions

European sea bass produces all three forms of Vtg described for acanthomorph teleosts and each type of Vtg contributes significantly to the store of YPs deposited in growing oocytes and eggs. VtgAb is the dominant Vtg in this species. All three types of sea bass Lv undergo significant proteolysis during oocyte maturation. The obtained Vtg sequences and the nanoLC-MS/MS methods employed in this study set the stage for evaluation of the role(s) that the multiple Vtg system plays in determining egg quality in farmed sea bass.

References

Amano H., T. Fujita, N. Hiramatsu, H. Kagawa, T. Matsubara, C.V. Sullivan, and A. Hara. 2008. Multiple vitellogenin-derived yolk proteins in grey mullet (*Mugil cephalus*): disparate proteolytic patterns associated with ovarian follicle maturation. Molecular Reproduction and Development 75:1307-1317.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

EFFECT OF LIGHT CONDITIONS ON THE POPULATION GROWTH OF ROTIFERS

T. Yoshimatsu

Graduate School of Bioresources, Mie University, 514-8507, Japan)

Introduction

Rotifer culture techniques have been developed under batch culture, continuous culture or semi-continuous culture, and the high-density culture developed in Japan, which saves hatchery space needed for live food culture, and increases the larval fish production and reduces production costs. In compact high density culture systems, it is easy for people to precisely control its environmental conditions like water temperature, light condition, etc. Vitamin B_{12} (VB_{12}) is a water-soluble vitamin containing cobalt as an important component element. In aquaculture, the function of VB_{12} for enhancing growth performance of rotifer was studied and reported previously.

Recently, we found cobalt compound (cobalt (II) sulfate heptahydrate: $CoSO^4 \cdot 7H_2O$) supplementation to rotifer feed also effectively enhanced rotifer population growth. We also found recently that lighting of rotifer culture tank stabilized the culture of rotifer and improved its production performance. In this paper we report the effect of lighting with different wavelengths from LEDs (Light Emitting Diodes) to rotifer culture tanks to enhance VB_{12} productivity.

Materials and methods

Rotifer culture
So called S and L-type rotifer *Brachionus plicatilis* sp. complex was used for the experiment. The rotifer stock-cultured with commercial *Chlorella vulgaris* (Chlorella V12, Chlorella Industry Inc., Fukuoka, Japan) was harvested and inoculated into 200-ml glass bottles (triplicated) at densities of 50 ind.ml^{-1}, respectively. The rotifer was cultured for four days under 28°C for S-type and 20°C for L-type with ordinary aeration. Culture water was prepared after diluting sand-filtered seawater (32-34psu) with freshwater to adjust salinity to 25 and 20psu, optimum for culturing S-type and L-type rotifers, respectively.

As a rotifer feed, a VB_{12}-free *C. vulgaris* K-22 cultured in the laboratory was used. The feed was fed once in the morning at an estimated optimum feeding

ratio of 150 000 and 300 000 algal cells.rotifer^{-1}.day, calculated based on the optimum feeding ratio for S-type and L-type rotifers, respectively. Cobalt (II) sulfate heptahydrate ($CoSO_4 \cdot 7H_2O$) was supplemented to each illumination groups to enhance the bio-production of VB_{12} in the culture media of rotifer. The concentration of cobalt compound was decided based on previous experimental results: i.e., 0.1mg.l^{-1} of culture water per day. Cobalt compound was dissolved into freshwater in advance and trickled into culture water when feeding was done in the morning.

Lighting condition

Rotifer culture vessels (Fig. 1) in an incubation chamber were exposed to a photoperiod of L:D=12:12 using auto-timer controlled LED panels (15cm×15cm, CCS Inc., Japan). LED panels emitting four different color lights with single peak wave-length (Red: 660nm, Green: 525nm, Blue: 470nm, White: mixed wavelength) were used for the experiment with three different photon flux densities of 10, 20, and 40μmol.m^{-2}.s. The LED panels were set about 12cm below the culture vessels with the greatest care applied to not affect the temperature of culture water. The dark-conditioned group (D group, L:D=0:24) were subjected by covering vessels with a black vinyl-sheet.

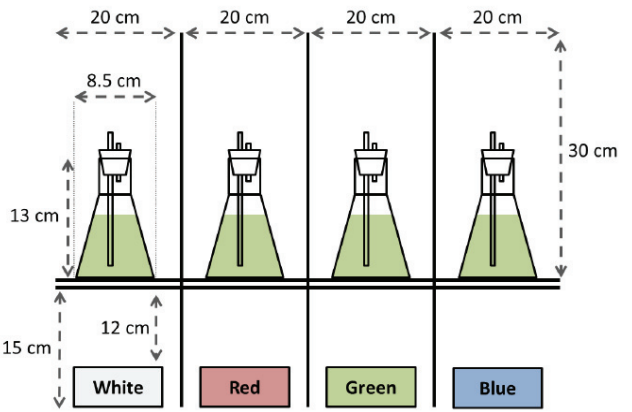


Fig. 1. Culture system using LED device set in an incubation chamber. For rotifer culture, an air stone with vinyl tube was installed instead of glass pipe

Vitamin B_{12} determination

Total VB_{12} contents of each collected sample were detected from 100-ml culture medium by bioassay using *Lactobacillus delbrueckii lactis*. Firstly, the culture water was filtered with a plankton net to remove rotifers and large suspended particles. As a next step, the sample water was centrifuged and rinsed with clean seawater several times, and the sedimentary fraction containing bacte-

ria/bacterial flocks and tiny suspended particles was measured with clean seawater and provided for the bioassay for VB_{12} determination.

Results and discussion

In the present culture trial, the population growth of rotifer was improved by lighting with the supplementation of Co compound, like we reported previously. In the dark condition group very poor population growths were observed in both S-type and L-type rotifer groups, compared to LED lighted groups. The population growth was enhanced by increasing light intensity and the best growth performance was obtained in 20 and 40 μmol. m^{-2}. s^{-1} groups for S-type and L-type rotifer, respectively (Fig. 2).

As for the VB_{12} production after four days' culture, distinctive increase of VB_{12} production was detected in light groups, especially in the Red LED group with long wavelength. Production trend of VB_{12} in each group was identical to those of the rotifer production, and the best production performance was obtained in 20 and 40μmol.m^{-2}.s Red LED groups for S-type and L-type rotifer, respectively. On the other hand, in dark-condition groups, very low contents of VB_{12} were detected in both S-type and L-type rotifer culture water.

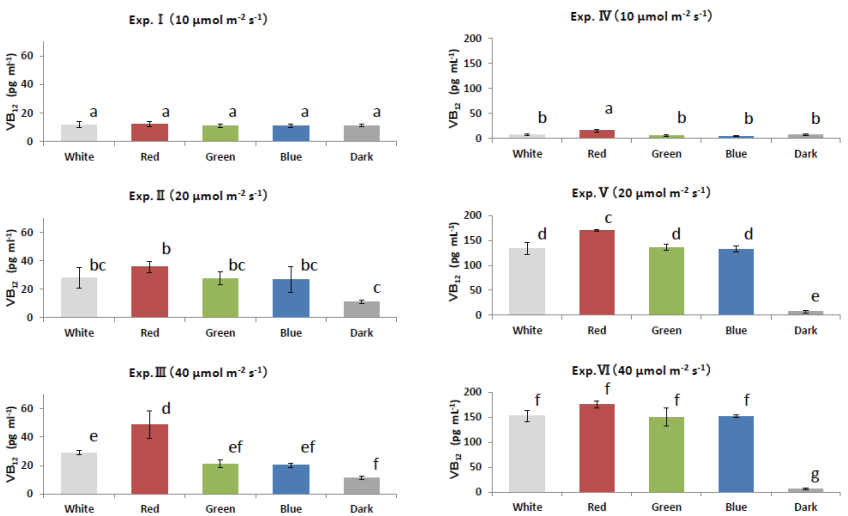


Fig. 2. Production of vitamin B_{12} in each color group under different light intensities.

We still do not know the mechanism how VB_{12} was synthesized utilizing the cobalt compound and what kind of bacteria are working for it in the culture water. Nevertheless, from the present experimental results, it is suggested that light-

ing, especially red light with long wavelength, was important and effective for producing VB_{12} in rotifer culture and the cobalt (II) sulfate heptahydrate supplementation was also very effective for VB_{12} synthesis in the rotifer culture tank, and consequently the newly synthesized VB_{12} enhanced growth performance of rotifer. Cobalt (II) sulfate heptahydrate is a cheap compound, easy to obtain, and a good source of cobalt for animals. This sulfate salt is also available and used as a common trace mineral food additive for many livestock and cultured fish in many countries. Therefore, the results from the this research can be useful information for hatcheries where expensive VB_{12} can be replaced with small amounts of cheap and safe cobalt compound, i.e., cobalt (II) sulfate heptahydrate, for enhancing the production performance of rotifer under a proper lighting condition.

References

Tanaka K., H. Takahashi, Y. Hamasaki, and T. Yoshimatsu. 2009. High density culture of marine zooplankton, *Brachionus plicatilis* as initial food organism for aquaculture. *Current Topics Biotech*. 4:41-46.

Yoshimura K., A. Hagiwara, T. Yoshimatsu, and C. Kitajima. 1996. Culture technology of marine rotifers and implication for intensive culture of marine fish in Japan. *Mar. Freshwater Res.* 47:217-222.

Yoshimura K., K. Usuki, T. Yoshimatsu, C. Kitajima, and A. Hagiwara, 1997. Recent developments of a high density mass culture system for the rotifer *Brachionus rotundiformis* Tschugunoff. *Hydrobiologia* 358:139-144.

Yoshimura K., K. Tanaka, and T. Yoshimatsu. 2003. A novel culture system for the ultra-high-density production of the rotifer, *Brachionus rotundiformis*—a preliminary report. *Aquaculture* 227:165-172

Yoshimatsu T., T. Higuchi, D. M. Zhang, N. R. Fortes, K. Tanaka and K. Yoshimura. 2006. Effect of dietary cobalt supplementation on the population growth of rotifer *Brachionus rotundiformis*. *Fisheries Science* 72:214-216.

Yoshimatsu T., T. Higuchi, Y. Hamasaki, K. Tanaka. 2008. Preliminary trials on the effect of lighting for the population growth of the rotifer, *Brachionus plicatilis*. *JARQ* 42(2):131-136.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

ONTOGENY OF THE DIGESTIVE TRACT OF THE OMNIVOROUS FISH *CHELON LABROSUS*

M. Yúfera[1], V. de las Heras[1], J.A. Martos-Sitcha[1,2], C. Sarasquete[1], G. Martínez-Rodríguez[1], and J.B. Ortiz-Delgado[1]

[1] Instituto de Ciencias Marinas de Andalucía (CSIC), E-11510 Puerto Real, Spain
[2] Dept. of Biology, Faculty of Marine and Environmental Sciences, University of Cádiz, E-11510 Puerto Real, Spain

Introduction

Thick-lipped grey mullet, *Chelon labrosus* (Mugilidae), is a euryhaline marine teleost very common in estuaries, coastal lagoons, intertidal marshes. and earthen ponds in the northeastern Atlantic and Mediterranean coasts. This species, together with other mugilids, is currently of local importance in extensive aquaculture in some of these areas (Anras et al., 2010; Yúfera and Arias, 2010). Its omnivorous-detritivorous feeding habits make thick-lipped grey mullet a very promising species for diversification in the sense more sustainable marine aquaculture with less dependence on fish meal for food. The larval rearing of *C. labrosus* is relatively easy under the same standard protocols for altricial marine species (Boglione et al., 1992; Ben Kemish et al., 2012). The aim of the present study was to examine larval development of *C. labrosus* at a histological level with emphasis on the digestive tract.

Materials and methods

Mullet larvae were reared in 200-l tanks at 19ºC, 35g.l^{-1} salinity, and a photoperiod of 12h light:12h dark. The larvae were fed on rotifers (*Brachionus plicatilis*) from 5-13 days after hatching (dah), and on *Artemia* nauplii from 11dah onwards. Weaning on commercial feed occurred between 26-29dah. Microalgae were supplied from mouth opening. Samples for histological analysis were taken periodically from 2-78dah. The sampled larvae were fixed in 4% v/v buffered formaldehyde (pH 7.2) and embedded in paraffin blocks. Sagittal and transverse histological sections of whole specimens (5-7µm thickness) were stained with Haematoxylin-Eosin (H-E) and Haematoxylin-VOF (VOF: light green-orange G – acid fuchsin).

Results and discussion

Larvae were 3.5mm total length at hatch and ~22mm at the last sampling (85dah). In recently hatched larvae, the digestive tract appeared as a straight tube dorsal to the yolk-sac but undifferentiated into specific gut regions (Fig. 1A). The single-layer epithelium consisted of cuboidal and columnar cells. The yolk-sac had a homogenous acidophilic vitelline matrix surrounded by a squamous epithelium. At the end of the yolk-sac stage the remaining matrix became heterogeneous. A progressive differentiation of the bucopharyngeal cavity, oesophagus, and intestine was detected from 3dah onwards. The first digestive differentiation event occurred with the opening of both mouth and anus at 4dah. The mouth differentiated into upper and lower functional-movable jaws in which mandibular and pharyngeal teeth were already observed. The first taste buds appeared at 5dah. In the oesophagus, longitudinal folds and branching increased progressively from 6dah onwards (Figs. 1B and C). The first goblet cells were detected on 5dah, increasing in number and size over time.

A dilatation in the posterior region of the oesophagus, lined by a simple, cuboidal epithelium, as well as a thickening of mucosal layer, was observed by 14-16dah, indicating the primordial stomach. At this time, the pyloric sphincter and oesogaster also appeared. Gastric glands were first seen in the developing stomach at 16dah, together with a thick muscular gastric layer (Fig. 1E). From 22dah, an evident proliferation of gastric glands in the cardial and fundic regions was detected as well as the transition from multi-striated to smooth muscle (Fig. 1F). The three regions – cardial, fundic, and pyloric – are clearly observed from 25dah. During the second month, the musculature surrounding the pyloric region of the stomach became progressively more evident, forming the gizzard (Fig. 2).

From 4dah, two regions – anterior and posterior – were observed in the intestine. During the following days, the number and length of the mucosa folds increased. From 10dah, the lipid absorption was evident in the enterocytes of the anterior and median intestine. Likewise, acidophilic supranuclear protein inclusions were observed in the enterocytes of the posterior intestine (Fig. 1D). These inclusions appeared during the whole study period. The liver was evident by 4dah, increasing in size with development, and became bilobulated at 25dah. The pancreas was also evident by 4dah and showed acidophilic zymogen granules. The exocrine pancreas extended dorsally along the digestive tract.

The development of the digestive system of this species is of particular interest because the juveniles and adults exhibit omnivorous feeding habits. The larvae showed a relatively fast growth at 19ºC and, accordingly, the sequence of appearance of the different structures and organs was also fast. The oesophagus presented multiple folds and was covered with mucous cells one week after the start of feeding. The first gastric glands were observed by two weeks after hatch-

ing and the stomach was completely formed at 22dah. The still-developing gizzard, characteristic in juveniles and adults mugilids, was clearly observed from the end of the second month of life.

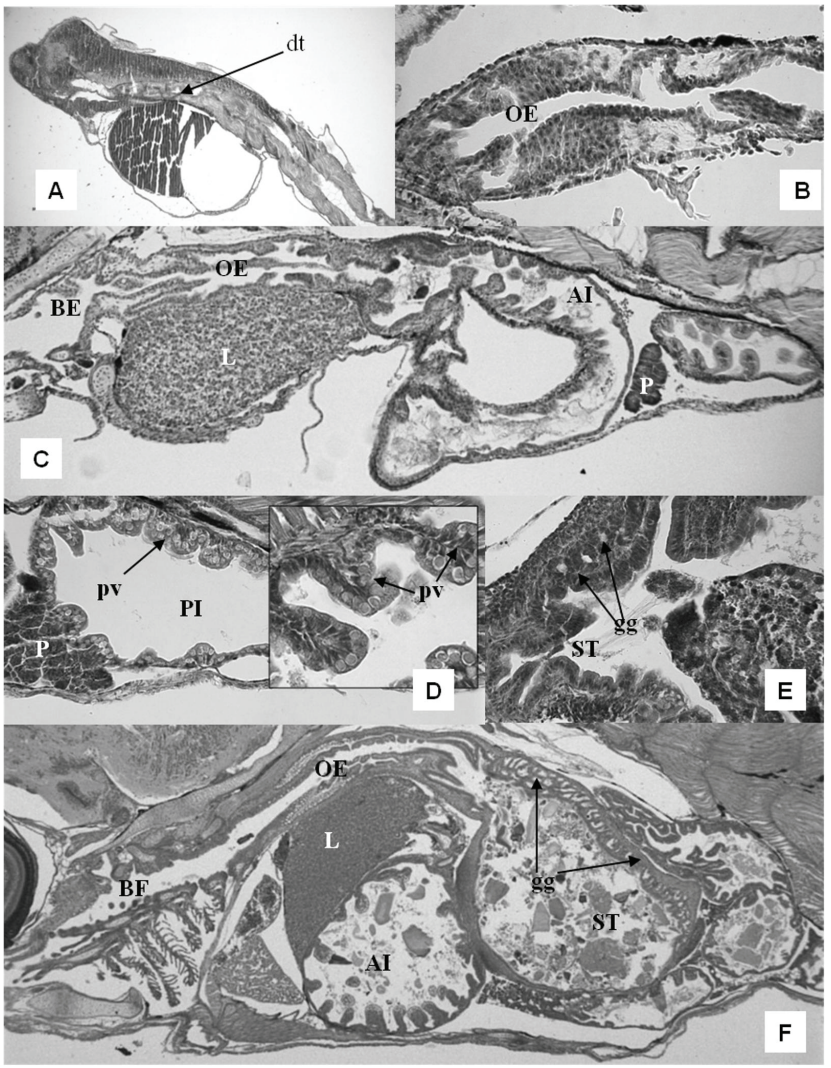


Fig. 1. (A) Full view of 3-dah yolk-sac larvae. (B) Oesophagus in 6-dah larvae. (C) Digestive tract in 16-dah larvae. (D) Hindgut with protein vacuoles in 16-dah larvae. (E) Details of first gastric glands in 16-dah larvae. (F) Digestive tract in 29-dah larvae. BF Bucofarynge; OE oesophagus; ST stomach; AI anterior intestine; PI posterior intestine; L liver; P pancreas; pv supranuclear protein vacuoles; gg gastric glands; dt digestive tract.

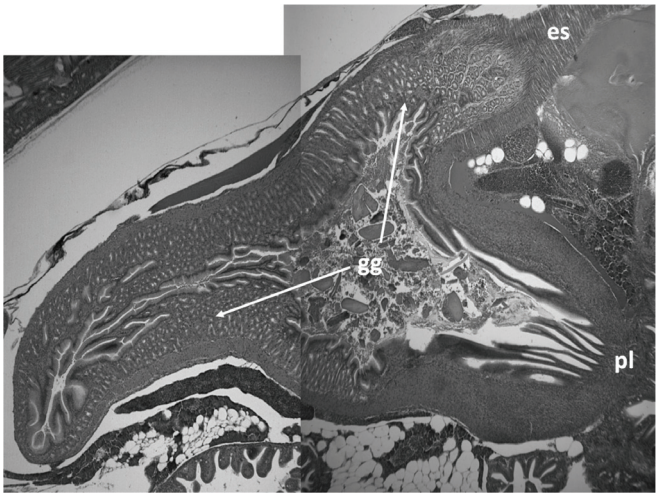


Fig. 2. Stomach in 78-dah larvae showing the development of the musculature in the pyloric region. gg gastric glands; es oesogaster; pl pylorus.

Acknowledgements

Project "Optimization of the maturation and spawning in the mugilid *Chelon labrosus*", co-financed by Spanish Ministry of Education and European Social Fund and executed in collaboration with formation centres C.I.F.P. Marítimo Zaporito (Cádiz), I.E.S. Mutriku (Guipuzkoa), I.E.S. "Els Alfacs" (Tarragona) and I.E.S. "Manuel Tárraga Escribano" (Murcia). Publication benefits from participation in LARVANET COST action FA0801.

References

Anras L., C. Boglione, S. Cataudella, M.T. Dinis, S. Livi, P. Makridis, G. Marino, A. Ramalho, and M. Yúfera. 2010. The current status of extensive and semi-intensive aquaculture practices in Southern Europe. Aquaculture Europe 35(2):12-16.

Ben Khemis I., E. Gisbert, C. Alcaraz, D. Zouiten, R. Besbes, A. Zouiten, A.S. Masmoudi, and C. Cahu. 2013. Allometric growth patterns and development in larvae and juveniles of thick-lipped grey mullet *Chelon labrosus* reared in mesocosm conditions. Aquaculture Research (DOI: 10.1111/j.1365-2109.2012.03192.x).

Boglione C., B. Bertolini, M. Russiello, and S. Cataudella. 1992. Embryonic and larval development of the thick-lipped mullet (*Chelon labrosus*) under controlled reproduction conditions. Aquaculture 101:349–359.

Yúfera M. and A.M. Arias. 2010. Traditional polyculture in "esteros" in the Bay of Cádiz (Spain). Hopes and expectancies of a unique activity in Europe. Aquaculture Europe 35 (3): 22-25.

LARVI '13 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry (Editor)
Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, 2013

APPLICATION OF A MULTI-STRAIN PROBIOTIC IMPROVES UTILIZATION OF MICROALGAE IN *LITOPENAEUS VANNAMEI* POST-LARVAE

J. Zwielehner[1*], T. Suyawanish[2], G.A. Santos[1], and P. Encarnação[3]

[1] BIOMIN Holding GmbH, Industriestrasse 21, 3130 Herzogenburg, Austria (*Corresponding author: jutta.zwielehner@biomin.net; phone: +43 2782 8030)
[2] Aquatic BioLAbs Co., Ltd., 50/107 Soi 125/1 Senanikom Rd., Lat Phrao, Bankok 10230, Thailand
[3] Biomin Singapore PTE., LTD., 3791 Jalan Bukit Merah #08-08, Singapore 159471

Introduction

Shrimp larvae are reared intensively using microalgae, which improves feeding, growth, and survival of the larvae (Salvesen et al., 2000; Robinson et al., 2005). Opportunistic pathogens can proliferate in larval culture due to high concentrations of organic matter. Algae, rotifers, and *Artemia* cultures can therefore harbour high concentrations of pathogenic bacteria (Eddy et al., 2002; Munro et al., 1995; Asok et al., 2012). Severe losses in shrimp larviculture are caused by infection with opportunistic pathogenic bacteria, including several members of the *Vibrionaceae* family (Flegel, 2012; Ruwandeepika et al., 2011). Prophylactic treatment of larvae with antibiotics can reduce the pathogen load, but has to be avoided since it leads to emergence of antibiotic-resistant pathogens, and it impedes the establishment of a normal non-pathogenic microbiota (Crab et al., 2010; Asok et al., 2012). This scenario has led to an ever-growing interest in research and development of alternative strategies for disease control within the frame of good hatchery practices, including adequate hygiene conditions and the use of probiotics, prebiotics, and immunostimulants. We tested freeze-dried algae, either alone or together with a multi-strain probiotic, for their influence on survival and stress resistance in *Litopenaeus vannamei* post-larvae (PL).

Materials and methods

Experimental design
Five different treatments were tested, using 5 replicates per treatment as follows: (Control) live fresh algae (*Chaetoceros*) + commercial diets; (T1) *Chaetoceros* + freeze dried algae (*FD algae*) + commercial diets; (T2) *Chaetoceros* + freeze dried algae (FD algae) + commercial diets + 3g probiotic AquaStar® Hatchery.day^{-1}); (T3) *Chaetoceros* + commercial diets + 3g.day^{-1} probiotic AquaStar®

Hatchery; (T4) *Chaetoceros* , commercial diets and 30g.day^{-1} probiotic AquaStar® Hatchery.

L. vannamei nauplii were stocked in 70-l aquaria filled with 50 l saltwater at 30ppt, pH 8.18, alkalinity 110mg $CaCO_3$.l^{-1}, temperature ~25.8-28.8°C, stocking density ~167-201 nauplii per aquarium. The larval zoea-P2 were fed 7 meals per day and post-larvae (PL) PL2-PL15 were fed 6 meals per day. Phytoplankton was fed to shrimp larvae once they were going to zoeal stage and *Artemia* was fed from mysis to PL10.

Commercial powder feed was used until PL15. Water exchange was done on alternate days for 10%, 20%, and 30% for mysis, early PL, and PL stages, respectively. High concentration of *Chaetoceros* from the commercial laboratory was suspended and sub-cultured for three days and filtered (50-µl net) before feeding. The commercial dry microalgae (GreenStim™ Shrimp Zoea, Mysis and PL, SBAE Industries) and probiotic AquaStar®-Hatchery (Biomin®, Austria) were prepared following the manufacturers' instructions. The algal cell concentrations for both products were compared by measuring turbidity.

Shrimp were tested for ammonia stress tolerance (20ppm of ammonium chloride solution for 96 hours), *Vibrio* spp. count on TCBS media (no enrichment) after 18h incubation, and survival (zoea 3, PL2, and PL15).

Data were analyzed by one-way ANOVA and variances were checked for homogeneity by the Levene tests. When the variances were not homogeneous, a non-parametric Kolmogorov Smirnov test was applied. Data were considered significantly different at $p<0.05$.

Results and discussion

Using freeze-dried algae (T1) had no clear benefit compared to control: survival rate was 49.6% (density of 97 PL.l^{-1}) vs. 47.1% (density of 79 PL.l^{-1}). Better survival rates were achieved when probiotics were added with the microalgae blend (T2) with 56.1% with a density of 112 PL.l^{-1}. Group T3 survival was 51.0% at 103 PL.l^{-1} density, and for T4: 45.8% at 82.6 PL.l^{-1}.

Although T4 had the lowest survival, the PL size of T4 receiving the multi-strain probiotic at 10× the recommended dosage was the biggest (data not shown); they had a very strong appearance and the highest ammonia stress tolerance at 12h and 24h (Fig. 1).

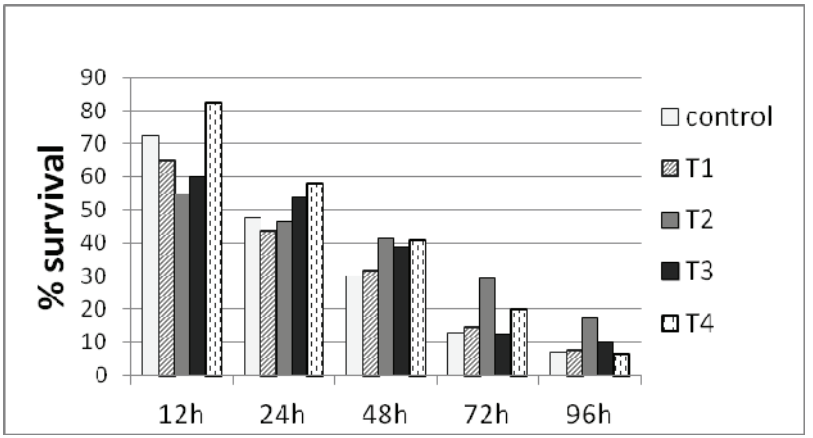


Fig. 1. Survival rates (%) of healthy PL15 stressed with 20ppm NH_4Cl for 96h.

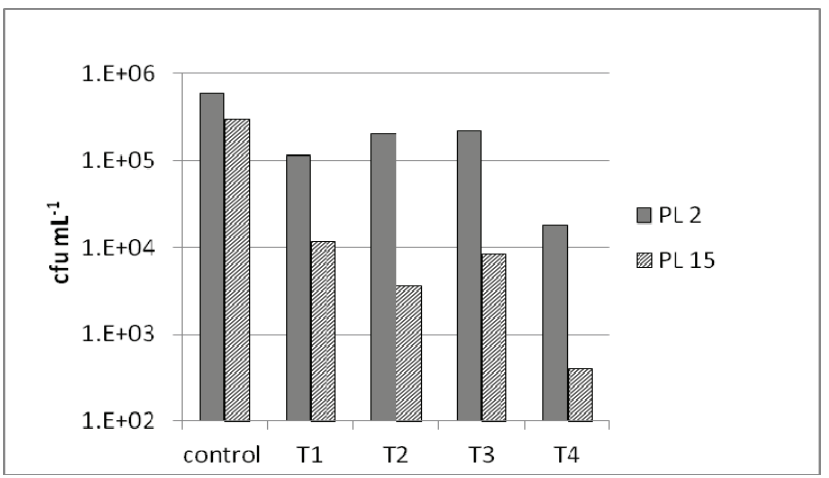


Fig. 2. *Vibrio* spp. cell counts (TCBS medium) in PL culture water during the *Litopenaeus vannamei* feeding trial.

Control shrimp were the least active during the ammonia stress test and they were the most susceptible to ammonia stress and *Vibrio* spp. pathogenic load. At PL2 and PL15, the *Vibrio* spp. pathogen abundance was significantly lower in T4 than in control (Fig. 2). At PL15 the multi-strain probiotic was able to enhance the beneficial effects of live algae on vibriosis prevention, showing that the multi-strain probiotic was effective in reducing the vibrio load in the water.

Conclusion

Application of freeze-dried algae has good potential for application in shrimp hatcheries and reducing dependence on live algae. It seems that the use of probi-

otics with freeze-dried algae improves the performance of the larvae with regards to homogeneity, color, health, and activity, probably through improved algae digestibility. The multi-strain probiotic (AquaStar® Hatchery) had beneficial effects on shrimp growth performance, stress tolerance, and pathogen resistance.

References:

Asok A., E. Ashad, C. Jasmin, S.S. Pai, I.S. Singh, A. Mohandas, and A. Anas. 2012. Reducing Vibrio load in Artemia nauplii using antimicrobial photodynamic therapy: a promising strategy to reduce antibiotic application in shrimp larviculture. Microb. Biotechnol. 5(1):59-68.

Bruwiere S., T. Suyawanish, M. Stael, and P. Encarnacao. 2011. A combination of dry microalgae and probiotics can effectively replace live microalgae in shrimp hatcheries. In proceedings, World Aquaculture 2011, June 6-10, Natal, Brazil, pp. 189.

Crab R., A. Lambert, T. Defoirdt, P. Bossier, and W. Verstraete. 2010. The application of bioflocs technology to pretoect brine shrimp (Artemina franciscana) from pathogenic Vibrio harveyi. J. Appl. Microbiol. 109(5):1643-9.

Eddy S.D. and S.H. Jones. 2002. Microbiology of summer founder *Paralichthys dentatus* fingerling production at a marine fish hatchery. Aquaculture 221:9-28.

Flegel TW. 2012. Historic emergence, impact and current status of shrimp pathogens in Asia. J. Invertebr. Pathol. 110(2):166-73.

Munro P.D., A. Barbour, and T.H. Birkbeck. 1995. Comparison of the growth and survival of larval turbot in the absence of culturable bacteria with those in the presence of *Vibrio anguillarum, Vibrio alginolyticus,* or a marine *Aeromonas* sp. Appl. Environ. Microbiol. 61:4425-4428.

Robinson C.B., T.M. Saocha, J.M. Fox, R. Gandy, and D.A. Mckee. 2005. The use of inert artificial commercial food sources as replacements of traditional live food items in the culture of larval shrimp, *Farfantepenaeus aztecus.* Aquaculture 245(1-4):135-147.

Ruwandeepika H.A., T. Defoirdt, P.P. Bhowmick, I. Karunasagar, I. Karunasagar, and P. Bossier. 2011.. In vitro and in vivo expression of virulence genes in Vibrio isolates belonging to the Harveyi clade in relation to their virulence towards gnotobiotic brine shrimp (Artemia franciscana). Environ. Microbiol. 13(1):506-17.

Salvesen I, K.I. Reitan, J. Skjermo, and G. Oie. 2000. Microbial environments in marine larviculture: Impacts of algal growth rates on the bacterial load in six microalgae. Aquacult. Int. 8:275-287.

INDEX OF AUTHORS

Page numbers refer to the first page of the author's article.

Acosta N.G., 392
Adhikari S., 417
Aditiawati P., 433
Adjei-Boateng D, 2
Adriaens D., 52
Agh N, 6
Ahmadian E, 6
Akbar John B., 399
Ako H., 194
Alami-Durante H., 151
Alfredsen J.A., 10
Alix M, 12
Almansa E., 392, 473
Álvarez A.C., 135
Álvarez-González C.A., 93
Alver M.O., 10
Amano H., 505
Ammar D., 447
Andrade J.P., 392
Andree K.B., 43
Ansquer D., 362
Antony Jesu Prabhu P., 125
Aragão C., 265
Arfuso F., 265
Armesto P., 91
Arndt C, 15
Asih S., 165
Assem S.S., 413
Asturiano J., 455
Astuti D.I., 433
Attramadal K., 17, 23, 241
Attramadal Y., 169
Ayala-Borboa E.G., 19
Babiak I., 296, 425
Bakke I., 17, 23, 241
Balasubramanian M., 157
Bandarra N., 271, 290, 300, 378
Bannister J., 211
Barón-Sevilla B., 249
Barr Y., 184
Barros C., 354
Baruah K., 25
Battaglene S.C., 27, 81, 186
Bautista R., 91
Bégout M.-L., 91
Bell J.G., 180
Belmonte A., 39
Benítez-Santana T., 29
Benzekri H., 91
Bequé E., 33
Bergh Ø., 37, 101
Berkovich N., 39
Betancor M.B., 29, 41
Biggs J.C.G., 81
Biłas M., 338
Biswajit M., 204
Boglino A., 43
Boglione C., 382
Bolla S., 45, 328, 425
Bonacic K., 48
Bones A.M., 241
Borchert P., 157
Borisov R.R., 223
Bossier P., 25, 161, 190, 198, 245, 308, 316, 324, 330, 346, 372, 403, 421, 465, 496, 501
Bouilliart M., 52
Bouyssiere B., 125
Brekke O.-L., 45
Bridges C., 39
Bueno M., 125
Butts I.A.E., 54, 455
Caballero M.J., 41
Cabrita E., 58, 334
Cadena-Roa M.A., 19, 66
Calderón J., 85
Campos C., 60
Canada P., 62
Candeias Mendes A., 290
Cardenete G., 473
Carmona-Contreras J., 66
Carneiro D.J., 376
Carter C.G., 27
Castanheira F., 290, 300
Castanheira M.F., 334
Castanho S., 97, 271, 378, 429
Casteleyn C., 161
Cataudella S., 382

Cecconi P., 108
Cerqueira V.R., 447
Chaffa F., 262
Chakrabarti R., 70
Chang G., 437
Chang Y.H., 73
Chardard D, 12
Chen S.-Q., 384
Chen Y.M., 73
Chevalier A., 362
Chiappi L., 87
Christou M., 143, 147
Christou M., 77
Civera-Cerecedo R., 19, 66
Claros M.G., 91
Clavijo-Ayala J.A., 376
Cobcroft J.M., 81, 186
Cobo M.L., 85
Colin S.P., 186
Conceição L.E.C., 60, 62, 115, 271, 290, 300, 368, 396
Cordeiro N., 97
Corraze G., 455
Corriero A., 39
Corteel M., 87
Costa W.M., 447
Cousin X., 91
Coutinho J., 271, 290, 378
Coward E., 23
Crawford A.D., 481
Cuenca-Soria C.A., 93
Cunha M.E., 97
Cuny M., 328
D'Alvise P., 101
Dabrowski K., 102
Dal Pai-Silva M., 237
Darias M.J., 43
Darras V.M., 481
Das P.C., 279
Dauga C., 362
Davie A., 104, 334
De Geest B., 496
De La Gándara F., 39
de la Peña M., 253
de las Heras V., 513
De Moro G., 153
De Schryver P., 106, 324, 455
De Wolf T., 87, 108
Defoirdt T., 245, 346, 403, 465, 501
Deng Y., 437
Deptuła R., 342
Dhert P., 451
Dias J., 265, 290, 368
Dierckens K., 245, 421, 496
Dinis M.T., 334
Diogo P., 368
Divanach P., 143, 219, 259
Douxfils J., 262
Drillet G., 176
Dufour S., 455
Dumas S., 358
Dupont-Nivet M., 157
El-Bermawi N., 111
Elizur A., 39
El-Sayed H.S., 413
Encarnação P., 517
Engrola S., 60, 62, 115, 265, 300, 396
Eriksen T.I., 10
Estefanell J., 119
Estévez A., 48
Estévez A., 43
Evjemo J.O., 169
Faggio C., 265
Farber S., 407
Farmer T., 102
Fernandes J.M.O., 60, 296
Fernández I., 123
Fernández-Díaz C., 202
Fernández-Palacios H., 275, 398
Fielder D.S., 81
Fitzgibbon Q.P., 27
Fontagné-Dicharry S., 125
Fontaine P, 12
Forberg T., 129
Fragkoulis S., 131
Franchi G., 87
Freitas T.M., 376
Fushimi H., 283
Galaviz M.A., 135
Gamboa M., 97, 271
García Gasca A., 135
García-Aguilar N., 358
García-Manchón J., 139
Gavaia P., 58, 97, 123, 368
Georgakopoulou E., 143
Georgiou A.N., 147

Georgiou S., 151
Gerdol M., 153
Geurden I., 157
Girisha S.K., 204
Gisbert E., 43, 93, 123, 135
Godin S., 125
Gómez-Arbonés X., 48
Gomułka P., 227
Gonçalves R., 97
Gonzaga F., 372
Gordin H., 39
Goytortúa-Bores E., 19, 66
Gram L., 101
Grosso C.R.F., 237
Gunasekara R.A.Y.S.A., 161
Gustiano R., 165
Hachero-Cruzado I., 300
Hagemann A., 169
Hagen Ø., 469
Hagiwara A., 173, 409
Hamre K., 175, 461
Han Y., 384
Hansen B.W., 176
Harboe T., 294
Hart N.S., 186
Hauville M.R., 180
Hawkyard M., 184
Hernández-Cruz C.M., 29, 275, 398
Hernández-Rodríguez M., 249
Hilder P.I., 186
Hirai N., 173
Höglund E., 257
Holst L.K., 455
Holtz W., 215
Hontoria F., 286, 473
Hu B., 190
Hua N.T., 194
Huynh Thanh Toi, 198
Ibáñez A.J., 505
Ibrahim H.A., 413
Iglesias J., 473
Iliopoulou M., 77, 143
Infante C., 202
Ismail E., 399
Izquierdo M., 29, 41, 119, 275, 398, 411
Jalili R, 6
Jayasankar P., 279
Jayaweera T.S.P., 403
Jena J.K., 279
Jepsen P.M., 176
Jiménez-Fernández E., 202
Jomori R.K., 376
Kademuan K., 445
Kamiya T., 283
Kanamori A., 409
Karlsen Ø., 461
Karunasagar I., 204, 403
Katharios P., 143, 219
Kaushik S., 125, 157
Kausik S., 455
Kestemont P., 231, 262
Khotimah Z.F., 433
Khurshut E.E., 206
King J., 211
Kjørsvik E., 17, 23, 241
Kojima J.T., 376
Kokkinias P., 131, 147
Koksoy S., 505
Kolkovski J., 208
Kolkovski S., 208, 211
Komrakova M., 215
Kontara E.K., 165
Korzeniowska M., 102
Kostopoulou V., 219
Kotani T., 283
Koumoundouros G., 77, 131, 143, 147
Kovatcheva N.P., 223
Krejszeff S., 227, 233, 338, 342
Król J., 231
Krüger-Johnsen M., 455
Kucharczyk D., 227, 233, 338, 342
Kuembet U., 215
Kuo J.C., 73
Kupren K., 227, 233
Kusano T., 283
Kvalheim K., 294
Labaere I., 108
Labreuche Y., 362
Łączyńska B., 342
Lafont A.G., 455
Langdon C., 184
Lauesen P., 52, 54, 455
Laurel B., 184
Le Quang Nha, 304
Lebedev R.O., 223

Leclerq E., 104
Ledoré Y, 12
Leitão N.J., 237, 376
Lenzi S., 108
Li K., 241
Li X., 245
Lie K.K., 294
Lillebø S., 101
Lim N., 433
Lima J.V., 354
Lin C.C., 500
Lin H.Y., 500
Lippemeier S., 388
Liu C.-L., 384
Liu H., 125
Liu Y., 437
Loizides M., 143
Lopes A.F., 62, 115
Lopes T.S., 376
López L.M., 135
López-Peraza D.J., 249
Ludevese-Pascual G., 253
Ludsin S., 102
Lukram I.M., 70
Lund I., 257
Main K.L., 180
Makridis P., 259
Mamuris Z., 151
Manchado M., 91
Mandiki S.N.M., 262
Manfrin C., 153
Mangor-Jensen A., 461
Marschall E., 102
Martínez-Páramo S., 58, 265, 368
Martínez-Rodríguez G., 269, 513
Martins G., 368
Martos-Sitcha J.A., 513
Mata J.A., 269
Mateus M., 97
Mathieu C., 262
Mazurais D., 91
McEvoy F., 455
Médale F., 125, 157
Medeiros M.V., 104
Meiri-Ashkenazi I., 39
Mendes A.C., 271, 378, 429
Menossi O.C.C., 376
Mesa A., 29, 139
Mesa-Rodriguez A., 275, 398
Meyer S., 388
Migaud H., 104, 180, 334
Miliou H., 219
Minniti G., 17
Mitra G., 279
Miwa M., 296
Miyashima A., 283
Moguel-Hernández I., 358
Mohnl M., 485
Moison M, 15
Molina Domínguez L., 139
Monroig Ó., 286, 392, 473
Morais S., 48, 271, 290, 300
Morales A.E., 473
Moreira M., 429
Moren M., 294
Moura P., 97
Moutou K.A., 151
Moyano F.J., 269
Muller Y.M.R., 447
Munk P., 455
Mylonas C., 39
Naessens E., 489
Nagasawa K., 296
Natale M., 211
Navarro J.C., 286, 392, 473
Navarro-Guillén C., 300
Nazari E.M., 447
Nery R., 354
Nevejan N., 324, 372
Ngo Thi Thu Thao, 304
Nguyen Huu Hiep, 364
Nguyen Phi Long, 477
Nguyen Thi Hong Van, 308, 320
Nguyen Thi Ngoc Anh, 312
Nguyen Thi Xuan Hong, 316
Nguyen Van Hoa, 198, 308, 320, 457
Nguyen Van Hung, 324
Nicolaisen O., 45, 328
Nielsen K.F., 101
Niu Y., 330
Nogueira N., 441
Nolasco-Soria H., 19, 66
Nordgreen A., 294
Norouzitallab P., 25
Nour A.M., 413
Nowosad J., 227, 338

Øie G., 17, 169
Oliveira C.C.V., 334
Olsen Y., 17, 23, 241
Olsvik P.A., 175
Ortiz-Delgado J.B., 513
Ortiz-Galindo J.L., 93
Østensen M.-A., 17, 241
Otero J.J., 473
Otero-Ferrer F., 139
Pacchiarini T., 58
Palacios-Mechetnov E., 350
Palińska-Żarska K., 227, 338, 342
Pallavicini A., 153
Palma P., 396
Pande G.S.J., 346
Panserat P., 157
Papadakis I., 143
Papandroulakis N., 259
Parshin-Chudin A.V., 223
Paz-Raymundo B., 350
Pechonkin D.S., 223
Peixoto S., 354
Peña R., 358
Penglase S., 175
Pérez-Urbiola J.C., 350
Peruzzi S., 469
Peyramale A., 362
Pham D., 362
Pham Thi Tuyet Ngan, 364
Phuthongphan R., 33
Pinto W., 115, 368, 396
Planas J.V., 91
Plovie A., 372
Politis S.N., 54, 455
Ponce M., 91
Portella M.C., 237, 376
Pousão-Ferreira P., 97, 271, 290, 378, 429
Power D.M., 151
Prat F., 505
Presslauer C., 296
Prestinicola L., 382
Prusińska M., 233
Qi Z.-Z., 384
Quental-Ferreira H., 97
Quillet E., 157
Rakotomalla T.M., 262
Ramírez B., 119
Ramírez D.T., 93
Rasinger J., 175
Rehberg-Haas S., 388
Reis D.B., 392
Rekecki A., 87
Reyes O., 253
Ribeiro L., 97, 378
Richard N., 62, 115, 396
Robles V., 58
Rød K.E.L., 407
Rodrigues J.B., 237
Rodríguez C., 392
Roher N., 202
Rojas-Contreras M., 19, 66
Rombaut G., 33, 87, 108
Rønnestad I., 451, 461
Roo J., 119, 275, 398
Rosani U., 153
Rosenfeld H., 39
Rozihan M., 399
Ruwandeepika H.A.D., 403
Ruyra A., 202
Sæle Ø., 407
Saeton J., 445
Sakakura Y., 173, 409
Saleh R., 29, 411
Salem Ahmed. Md., 413
Sánchez J., 473
Sandoval G., 135
Santos G.A., 485, 517
Saputra A., 165
Sarasquete C., 58, 513
Sarropoulou E., 151
Sato N., 283
Schaerlinger B, 12
Schatzmayr G., 485
Schrama J., 157
Schulz C., 388
Segade Botella Á., 139
Sharifuzzaman S.M., 417
Simes D., 396
Simon C.J., 27
Situmorang M.L., 421
Sjarmidi A., 433
Skaalsvik T.H., 425
Skjermo J., 17, 23
Skov P.V., 257
Smith G.G., 27

Soares F., 97, 429
Soares R., 354
Sobhon P., 445
Socorro J., 119
Socorro-Cruz J., 139
Solberg C., 469
Sommer U, 15
Sonnenholzner S., 85
Sørensen S.R., 54, 455
Sorgeloos P., 25, 85, 190, 198, 308, 320, 445
Sparstad T., 241
Srour T.M., 413
Stańczak K., 227
Stephens F., 211
Støttrup J.G., 455
Suantika G., 433
Subagja J., 165
Suga K., 409
Sui L., 437
Sullivan C.V., 505
Sumares B., 441
Sun M.-M., 384
Suyawanish T., 517
Swain S.K., 279
Sxoinaraki M., 147
Sykes A.V., 392
Tacon P., 125
Talbot C., 104
Tamtin M., 445
Tanasomwang V., 445
Tang L., 384
Teles A., 447
Tillner R., 451
Tocher D.R., 286, 392
Tokmechi A, 6
Tomkiewicz J., 52, 54, 455
Torboli V., 153
Tornalejo J., 253
Tovar-Ramírez D., 19, 66, 350
Trąbska I., 342
Tran Huu Le, 320
Tran Ngoc Hai, 312
Tran Suong Ngoc, 364, 457, 477
Tran Thi Thanh Hien, 312
True C.D., 135
Trương Quoc Phu, 364
Tsopelakos A., 219
Tu Anh Vo, 23
Tveiten H., 455
Ueberschär B., 451
Vadstein O., 17, 23, 129, 241
Valcarce D.G., 58
Valente L.M.P., 60, 62
Valles R., 48
Van de Wiele T., 330
Van den Broeck W., 161
van der Meeren T., 461
Van Stappen G., 198, 308, 320
Vanmaele S., 465
Vanrompay D., 316, 496
Vargas Cecilia C., 469
Varó I., 286, 473
Vassallo Aguis R., 39
Veiseth R., 169
Venier P., 153
Venugopal M.N., 204
Vogeley J., 354
Vu Ngoc Ut, 457, 477
Wabete N., 362
Wahyuningsih H., 165
Walpita C.N., 481
Wang J., 493
Wang J.-P., 384
Wang L.L., 493
Watts N., 211
Weber B., 485
Wergeland H., 101
Wille M., 85, 445
Wilson JG, 2
Winge P., 241
Wishkerman A., 43
Wouters R., 33, 85, 489
Wullur S., 173
Xu D., 493
Yaacob E.N., 496
Yang H.L., 500
Yang Q., 245, 501
Yilmaz O., 505
Yoshimatsu T., 509
Yoshizaki G., 296
Yúfera M., 269, 513
Zakęś Z., 231
Zambonino J., 91
Zambonino-Infante J.L., 180, 455
Zang X.N., 493

Żarski D., 227, 233, 338, 342
Zhang H.-Y., 384
Zhang X.Z., 493
Zlatnikov V., 39
Zogopoulou E., 219
Zou A.-G., 384
Zuasti E., 202
Zwielehner J., 517

Dear Reader,

We are pleased to present you the digital version of the book of abstracts & short communications presented at the 6th International Fish and Shellfish larviculture symposium 'larvi 2013' organized at Ghent University, Belgium, September 2-5, 2013.

On this USB stick, you will find a compilation of the mini-papers of the poster contributions and of the extended abstracts of the oral papers. The "content table" (with direct link to the respective articles) and a "search engine" (searching in the titles as well as in the text) should allow you to quickly retrieve the information you are interested in.

Please send your comments or any suggestions you have to improve future versions of such conference material to larvi@UGent.be

Yours very sincerely,

Patrick Sorgeloos
larvi 2013 Conference Chairman