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EDITORIAL

This book contains the mini-papers of the poster contributions (experience papers) and the abstracts of the oral (review) papers, presented at the occasion of larvi 2005, the fourth symposium on fish & shellfish larviculture, organized on September 5-8, 2005 at Ghent University, Belgium.

As in the previous larvi conferences, this book 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 in this book, 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 Though not peer-reviewed, they have passed through a limited editing process in order to improve, where needed, compliance with the editors' scientific and technical guidelines and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.

A lot of support was received from the members of the scientific committee: Peter Bossier, Maria Teresa Dinis, Hiroshi Fushimi, Gonzalo Gajardo, Joan Holt, Sadasivam Kaushik, Patrick Kestemont, Elin Kjørsvik, Frans Ollevier, Yngvar Olsen, Kjell Inge Reitan, Mike Rimmer, Eugène Rurangwa, and Amos Tandler. We would like to express our sincere thanks to them.

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

Gent, July 14, 2005

The Editors

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European Aquaculture Society, Special Publication No. 36, Oostende, Belgium, 2005

APPLICATION OF BLOODWORMS (CHIRONOMID LARVAE, MIDGE) IN INITIAL FEEDING OF PERSIAN STURGEON (*ACIPENSER PERCICUS*) LARVAE

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Introduction

Persian sturgeon (*Acipenser percicus*) is one of the commercially important and predominant fish species in Iran. Many live foods such as *Artemia*, *Daphnia*, and Oligochaeta are normally used for sturgeon fish larvae. Chironomid larvae and pupae are highly nutritious and constitute one of the staple food items in the diet of many fishes in the natural environment. The present work intends to evaluate bloodworms (chironomids) as an initial feed of Persian sturgeon (*Acipenser percicus*) larvae and provide a comparison with *Artemia* nauplii and *Daphnia*.

Material and methods

A 3×3 factorial experiment was designed with three types of live foods (*Artemia*, chironomids, and *Daphnia*) and three levels of feeding percentages (20, 35, and 50% of body wet weight). All nine treatments were replicated.

Larvae were obtained from fertilization of a male and a female by normal propagation method (Dettlaff et al., 1993). They were fed with *Artemia* for 36h until reaching 57mg average weight. Then the larvae were randomly selected and distributed among the experimental containers. Experiments were conducted in 0.1-m^2 tanks with a capacity of 20 l, with ~100 larvae stocked in each basin and each basin equipped with an individual aeration system.

Larvae were raised in freshwater with temperature ranging from 18-20°C. Feeding was carried out 6 times a day. Larvae were captured and weighed after 96h and mortalities were recorded daily.

Data were analyzed by using two-way ANOVA with SPSS software and grouping of the mean values was carried out by Tukey's multiple comparison test at probability level of 95% (p= 0.05).

Results and discussion

The results showed that live food type and percentage of feeding had a significant effect on final weight, FCR, and survival rate ($p<0.05$) and, except for survival rate, there was significantly interaction ($p<0.05$) between the live food and feeding percentage (Table I).

Table I: Statistical analysis of effects of live foods and feeding percentages on the Persian sturgeon larvae

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Type of live food (T)	FCR	29.184	2	14.592	93.225	0.000
	Survival	604.519	2	302.259	27.759	0.000
	Final weight	4186.963	2	2093.481	209.348	0.000
Food percentage (F)	FCR	2.483	2	1.241	7.930	0.003
	Survival	295.407	2	147.704	13.565	0.000
	Final weight	2271.630	2	1135.815	113.581	0.000
Interaction (T×F)	FCR	4.477	4	1.119	7.151	0.001
	Survival	77.259	4	19.315	1.774	0.178
	Final weight	593.704	4	148.426	14.843	0.000

The three live feeds tested were effective on growth, FCR, and survival of the fish (Figs. 1-3, respectively). In the present study growth performances significantly ($p<0.05$) improved with increasing feeding percentage (20 to 50%). Using chironomids at 50% feeding percentage had notable performances in comparison with *Artemia* and can be a effective live food instead of *Artemia*. The importance of chironomid larvae as a live food for tropical fish culture is well known in Asians countries. It has been reported in the literature to be very adequate for growth in fishes (Johnson, 1929; Ling, 1966; Yashouv, 1970). It has been found that if carps are provided with bloodworms as a supplementary food they gain better weight and the growth rate is more uniform (Yashouv, 1956). Very young bloodworms have also been demonstrated to promote efficient growth in *Mugil capito* fish fry (Yashouv and Ben-Shackar, 1967).

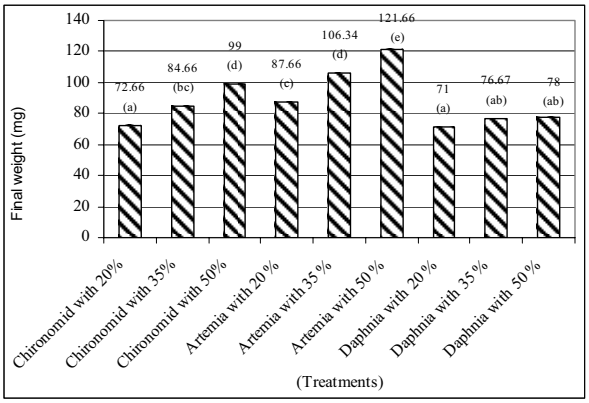

Fig. 1. Influence of different dietary treatments on the growth of Persian sturgeon larvae (Treatments shown with same letter(s) have no significant differences (p>0.05).

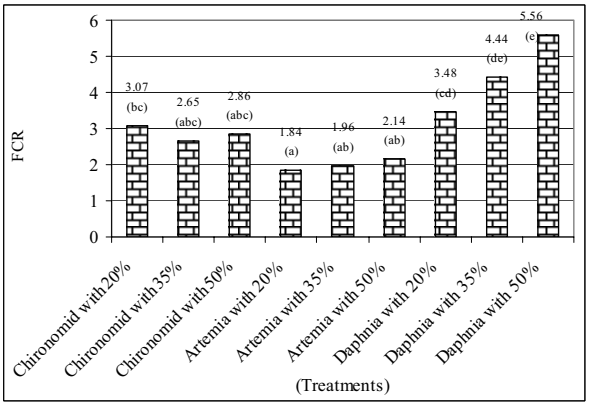

Fig. 2. Influence of different dietary treatment on the FCR of Persian sturgeon larvae (Treatments shown with same letter(s) have no significant differences (p>0.05).

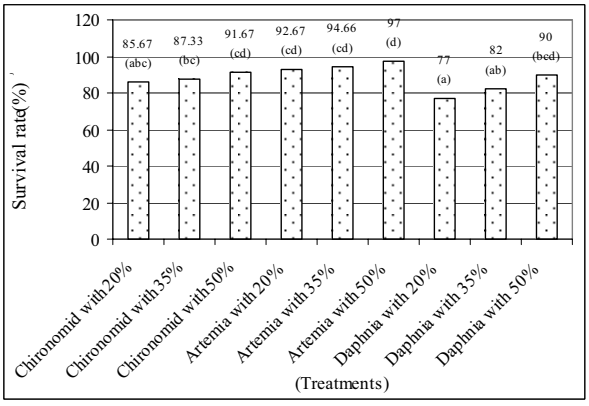

Fig. 3. Influence of different dietary treatment on the survival of Persian sturgeon larvae (Treatments shown with same letter(s) have no significant differences (p>0.05).

Conclusion

It is concluded that *Artemia*, Chironomid, and *Daphnia* (in decreasing order) are better live food for Persian sturgeon larvae. Feeding percentage of 50% had better results in comparison with other levels. Using chironomids at 50% feeding percentage can be a good replacement live food when availability of *Artemia* is limited.

Acknowledgements

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FEEDING IN *PENAEUS SEMISULCATUS* AT SETTLEMENT – THE POTENTIAL ROLE OF MICROBIAL MATS

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Introduction

Several studies have shown that *Penaeus semisulcatus* juveniles are associated with seagrass beds (e.g., Hill and Wassenberg, 1993; Loneragan et al., 1994). However, in the Arabian Gulf some studies have demonstrated that early-stage penaeid shrimp post-larvae are also found on mud flats and associated microbial mat habitats, especially in areas where seagrass beds and mangroves are absent (Jones et al., 2002). The aim of present study was to test the hypothesis that intertidal microbial mats may be an important source of nutrition for *P. semisulcatus* postlarvae at first settlement.

Materials and methods

In an initial series of growth experiments, laboratory-reared *P. semisulcatus* post-larvae at a series of post-metamorphic stages (1, 3, and 7d) were introduced into 7-l plastic aquaria (18 × 28.5 × 17cm), with three replicate tanks assigned to each dietary treatments. For day 1 post-larvae (PL1) (initial mean total length 5.57±0.14mm and mean weight 0.30mg), dietary treatments were (1) freshly collected and washed microbial mat alone, (2) microbial mat combined with 5 *Artemia* nauplii.ml^{-1}, and (3) *Artemia* nauplii alone (5 nauplii.ml^{-1}). For day 3 post-larvae (PL3) **(**initial total length 7.13 ± 0.17 mm, mean weight 1.0 mg) and day 7 post-larvae (PL7) (initial total length 8.97±0.15mm, mean weight 1.8mg), dietary treatments were either microbial mat or fresh mussel (*Mytilus edulis*), both offered to excess. To determine any feeding preference, gut contents of post-larvae after 2-3h of feeding on microbial mats were assessed by dissection under a binocular microscope.

To determine the relative importance of different components, freshly collected microbial mat was separated into four sizes fractions: 1000-500µm, 250-125µm, 125-38µm, and <38µm, by washing over a series of sieves. *P. semisulcatus* post-larvae (PL6, initial total length 9.1±0.21mm, mean weight 2.6mg) were grown in experimental tanks as above, with three replicate tanks fed each size fraction of

microbial mat, added to the tanks daily to excess. The δC and δN stable isotope ratios and total C and N content in tissue of shrimp fed complete microbial mat and those of the various microbial mat size fractions were determined.

Results and discussion

At day 1, post-larvae were observed not to be fully benthic in their behaviour and at this stage the treatments fed *Artemia* nauplii exhibited significantly better growth than those with only microbial mat as a food source. Mean survival in groups of post-larvae with microbial mat as sole food source was only 43% compared to 100% in the *Artemia*-fed control groups. In contrast, once the post-larvae became fully benthic (PL3 and PL7) they were able to feed successfully on the microbial mats with significantly higher growth rates than the control groups fed mussels (Fig. 1) and with very high survival after up to one month (93% and 100% from day 3 and day 7, respectively).

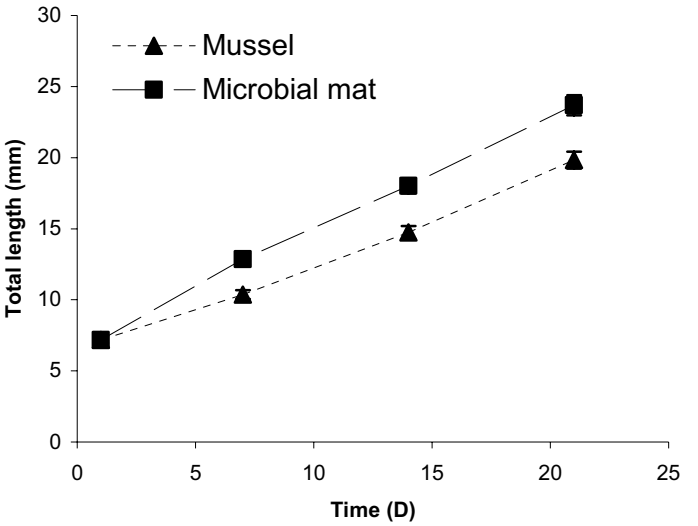

Fig. 1. Growth of *P. semisulcatus* postlarvae fed either microbial mat or mussel from day 6 after metamorphosis.

Examination of gut contents suggests that post-larvae feed indiscriminately on microbial mat, with the percentage occurrence of ingested food components largely reflecting the composition of the microbial mats themselves. This is consistent with studies of other penaeid species (McTigue et al., 1991). Gut contents were dominated by algae and blue-green algae (40-45%) and diatoms (20%), with a lesser contribution by faunal components including nematodes (5-15%), eggs/cysts (9-10%), and foraminifera (0-3%). Examination of faeces indicated that the faunal components were most readily digested, with intact algae pre-

dominant in faecal samples. Although PL6 post-larvae were observed to ingest all size fractions of microbial mat, those fed only the fractions below 500µm exhibited poor growth and survival. This was particularly true of the fractions below 125µm, which did not contain macrofauna (Fig. 2).

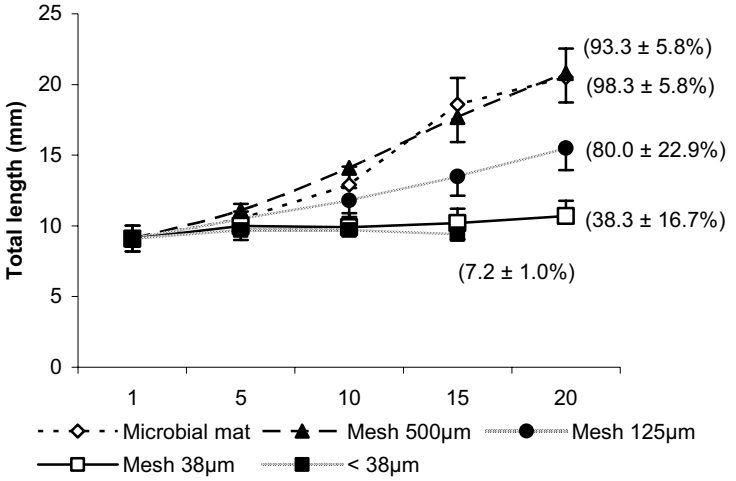

Fig. 2. Growth and survival of *P. semisulcatus* postlarvae fed either intact or size-fractions of microbial mat from PL6 onwards. Figures in brackets indicate mean survival ± s.d. in each treatment

In contrast, there was no significant difference in growth and survival between post-larvae feeding on either intact microbial mat or the 1000-500 µm fraction. The latter fraction effectively selected most of the fauna, principally nematodes, from the mat indicating that this component is the principle source of nutrition. This is confirmed by the δC and δN stable isotope ratios of shrimp tissue which are very similar to those of nematodes isolated from the microbial mats, but differ to any of the sieved size-fractions.

Conclusions

The present study confirms that microbial mats are able to support growth and survival of *P. semisulcatus* post-larvae once they are fully benthic at PL3. However, the very first postlarval-stages (PL1) are still dependent on zooplankton. Although microbial mats are ingested non-selectively, it is the associated infauna, principally nematodes, which support growth and survival of the shrimp, as opposed to the green and blue-green algae or the detritus components. This is consistent with earlier studies that have shown that free-living nematodes can support growth in penaeid shrimp larvae (Biedenbach et al., 1989; Kumlu and Fletcher, 1997).

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ESTIMATING LARVAL DENSITY IN COD (*GADUS MORHUA*) FIRST-FEEDING TANKS USING MEASUREMENTS OF FEED DENSITY AND LARVAL GROWTH RATES

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Availability of a reasonably accurate estimate of the larval biomass contained in cod larval rearing tanks is important for production management issues such as calculation of feed doses and planning of live feed production. The biomass development trend of larval groups may also provide important information for early detection of abnormal rearing conditions and prevention of high mortality loss. While direct measurement of the larval density is difficult, an alternative is to use a model-based estimator to estimate the larval density from related variables that are more accessible, such as feed concentration and larval size.

This technique was tested in a start-feeding experiment at Brattøra Forskningssenter in Trondheim during March 2005. Cod larvae were reared in nine 160-l tanks from hatching until 16 days post hatch (dph). The initial larval densities were 40 larvae·l^{-1} in three tanks denoted A1, A2, and A3; 20 larvae·l^{-1} in tanks D1, D2, and D3; and 80 larvae·l^{-1} in tanks E1, E2, and E3. Feeding with rotifers (*Brachionus plicatilis*, SINTEF strain) was initiated at 3 dph, and continued with 3 feedings per day for the duration of the experiment. The A and D tanks were fed up to 5000 rotifers·l^{-1}, and the E tanks up to 7000 rotifers·l^{-1}. All surviving larvae were counted at the end of the experiment. Dry weight was measured at days 0, 3, 5, 9, and 15 by extracting 12 larvae at days 0 and 3; 12 larvae per tank at day 5; and 6 larvae per tank at day 15. The larvae were dried and weighed according to standard procedures.

Four tanks – A1, D1, E1, and E2 – were equipped with an automated rotifer counter that provided measurements of rotifer densities at a rate of 3-4 observations per hour for each tank. The counter extracted water samples from the tanks using a computer controlled pump and valves, and calculated rotifer density by means of digital imaging and machine vision techniques.

The feed intake and growth of the cod larvae are modeled by a net assimilation dynamic energy budget model. Rotifer dynamics are described using a simple model taking into account the water exchange rate, predation by cod larvae, and growth. The combined model calculates expected values of larval growth and rotifer density, both of which can be compared to measurements from the experiment. Using the structure of an extended Kalman filter, the deviations between modeled and measured values are used to adjust the model state, with the magnitude of adjustments depending on measurement and model uncertainties.

If the system is *observable*, meaning that sufficient measurements are available, and the model reflects the real process with sufficient accuracy, the corrections will cause all model states to converge toward their true values. In this way, state values that are not directly measured will be estimated indirectly.

Estimator runs against data from the experiment confirm that the larval density can be estimated using the model based technique. The A, D, and E groups in this experiment were set up with significant differences in larval density, and with mean survival rates of 45.8, 60.2, and 52.4 percent, respectively, the differences prevailed throughout the period. The actual density differences between tanks A1, D1, E1, and E2 – even a small difference between tanks E1 and E2 – were clearly detected by the model based estimator. This is due to the fact that the rate of decrease of rotifer density after each feeding depends strongly on the total feed intake rate of the larvae. The estimator exploits this relation to assess whether the feed dynamics indicate that the estimate of larval density should be adjusted. Results indicate that the model based technique may serve as an important basis for the development of automated monitoring and control system solutions for marine fish hatcheries.

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MICROBIAL ENVIRONMENT IN A FLOW-THROUGH AND A RE-CIRCULATING SYSTEM FOR INTENSIVE REARING OF COD LARVAE (*GADUS MORHUA* L.)

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Introduction

Current favourable prices have made rearing of Atlantic cod (*Gadus morhua* L.) attractive. Although production of cod has been increasing in Norway, consistency through larval stages is still low, despite a great improvement of feed quality. This has drawn attention to the lack of control of tank water quality and microbial composition in the rearing systems. The open ocean has a stable water quality in terms of temperature, salinity, and low nutrient availability. In contrast, the water in intensive land-based systems is destabilised by fluctuations in organic load and other perturbations caused by daily production methods. Unstable conditions often select for bacterial types unwanted from a fish culture point of view (Vadstein et al., 1993). Several different types and levels of water treatment have been shown to have a positive effect on bacterial stability in aquaculture systems (Skjermo et al., 1997; Salvesen et al., 1999; Blancheton, 2000). Recirculation of culture water may allow a higher degree of control and stabilization of the microbial community compared to a flow-through system.

Materials and methods

Development of the microbial community (load and composition) in the culture water in a flow-through (FTAS) and a recirculating system (RAS) for intensive rearing of cod was studied from hatching to day 41. Water treatment included vacuum aeration and temperature control in the FTAS and temperature control, biofiltration, and protein skimming in the RAS. Present NTNU/SINTEF standard techniques for culture of early life stages of marine coldwater fish were

used. Colony forming units (CFU) on M-65 seawater agar and fluorescence microscopy of DAPI stained samples were used to quantify the bacterial load. In addition, CFU on TCBS (*Vibrio* spp.-selective) agar, denaturing gradient gel electrophoresis (DGGE), and terminal restriction fragment length polymorphism (TRFLP) were used to characterize and compare the qualitative microbial community development.

Results and discussion

Figure 1 shows the total number of CFU and *Vibrio* spp. fraction of the total CFU in samples from the RAS and the FTAS. CFU constituted 0.4±0.1% and 0.3±0.1% (Mean ± SD) of the number of bacteria counted directly in the microscope in the RAS and FTAS, respectively, and was stable in both systems through the experiment. Both systems experienced a peak in bacteria concentration, dominated by faster-growing species including *Vibrio* spp., immediately after transfer of larvae to tanks. The impact of this first bacterial peak might be reduced by maturing the seawater prior to stocking of tanks.

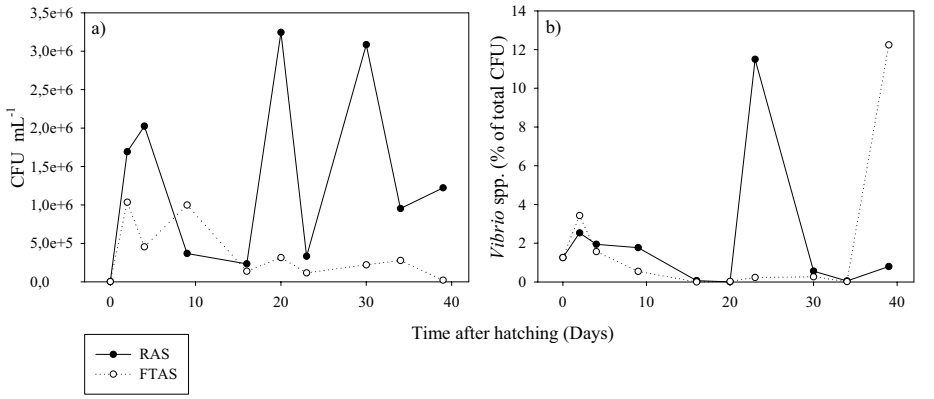

Fig. 1. Colony forming units in water samples from the two systems through the experimental period: a) CFU mL^{-1} on M-65 sea water medium agar plates after 14 days incubation and b) Percentage CFU on TCBS agar plates after two days incubation of total CFU numbers.

A high *Vibrio* spp. fraction in the RAS during the *Artemia* period (17-30dph) probably reflected the large amount of this type of bacteria added with the live feed. The massive *Vibrio* spp. influx did not, however, lead to any stable establishment of this bacteria type in the system. In the FTAS, the water exchange rate was high enough to avoid this bacterial accumulation. The waters of the two systems, both clearly reacted to the perturbation caused by a shift to dry feed (30dph), but in completely different ways. In the RAS the response seemed mainly to have been a quantitative increase in bacteria numbers. The FTAS, on the other hand, although maintaining relatively stable total bacterial concentra-

tions, showed a declining microbial diversity together with a shift in microbial composition to a community characterized, to a higher degree, by opportunistic types like *Vibrio* spp. In contrast to a traditional FTAS, a RAS may provide the retention time to allow the culture water to microbially mature in the system.

Table I gives the number of new operational taxonomic units (OTUs) in the two systems over the experimental period observed by TRFLP. The RAS water samples contained a higher number of OTUs than the FTAS, which indicated a higher diversity in the recirculation system. The occurrence of new OTUs was significantly higher in the FTAS during the *Artemia* period (27dph) and during the dry feed period (40dph). This indicated major shifts in the FTAS bacterial community following changes in the rearing regime, whereas the RAS retained to a higher degree the initial species composition.

Table I. Number of new operational taxonomic units (OTUs) observed by TRFLP with two different restriction digestion enzymes (Alu I and Hha I) on samples from the RAS and the FTAS at different times (days post-hatching) during the rearing period.

Number of new OTUs	**Alu I cut**				**Hha I cut**			
System	**RAS**		**FTAS**		**RAS**		**FTAS**	
Parallel	**I**	**II**	**I**	**II**	**I**	**II**	**I**	**II**
9 dph	18				37			
23 dph	13	12	13	12	22		12	23
27 dph	4	6	17	12	19	20	24	17
40 dph		8	6	9	11	10	9	21

Table II. Number of bands observed on four DGGE gels for samples from the RAS and the FTAS at different times (days post-hatching) during the rearing period. Results from parallel tanks are separated by a comma.

		Number of DGGE bands									
Gel	**System**	**4 dph**	**9 dph**	**13 dph**	**18 dph**	**20 dph**	**23 dph**	**27 dph**	**30 dph**	**34 dph**	**40 dph**
A	RAS	7	9				7,9	7,8			7,7
	FTAS	8	7				7,7	6,6			3,3
B	RAS	8	9	7,7	7,7	8,8					
	FTAS	9	7,7	8,9	6,6	5,5					
C	RAS	6	6	5	7	7	7	7	7	7	7
	FTAS	6	6	5	6	6	6	6	6	?	5
D	RAS						3,3	4,4	5,5	5,5	4
	FTAS						2,2	3,3	3,3	2,?	2

Table II gives the number of bands observed in samples from both systems on four denaturing gradient gel electrophoresis (DGGE) gels. A high number of bands indicate high species diversity in the sample. The bacterial diversity seemed to have been similar in the two systems from start. The DGGE profile

indicated a decrease in species diversity in the FTAS culture water towards 40dph, supporting the impression of a qualitative shift in this system at this point, while it seemed to have been more stable in the RAS. Shifts to a new diet did not seem to introduce new species that managed to remain in the water, nor did any species seem to disappear directly following these shifts. These results suggested that most of the bacteria species in question occurred in both fish culture water and in live feed culture water, but total amount, quantitative composition, and dominating species may have differed between the water types.

Conclusions

Recirculating system culture water had a higher concentration of bacteria, but seemed to be more stable with respect to bacterial composition. The recirculating system seemed to hold potential for further improvement of water quality stability through actions like introduction of an even and continuous organic load to the system, increased water exchange rates in larval tanks, better preconditioning of the biofilter (to the highest load expected), maturation of the start up water, and better control of live feed associated flora. The response of the bacterial communities to changes in organic load and/or water exchange rate were different in the two systems: The RAS responded to an increased organic load by adjusting the bacterial numbers without changing composition, while the FTAS seemed to alter the species composition rather than the absolute numbers as a reaction to the same challenge.

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INTENSIVE SHORT-TERM ENRICHMENT – AN EFFICIENT METHOD FOR ENHANCING PHOSPHOLIPIDS AND FREE AMINO ACIDS IN LIVE FEED

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Introduction

Enrichment of live feed, rotifers, and *Artemia* for improvement of their nutritional quality for fish and shrimp larvae has been a common practice for about 25 years. Yet, recent studies (Helland et al., 2003; van der Meeren, 2003) show that there is still a significant difference between the enriched live food's composition and the composition of the natural diet (i.e., copepods), such as higher content of phospholipids (PL) and free amino acids (FAA). Using the common enrichment procedure of 12-24h, it is difficult to produce a large increase of PL and FAA in live food, mainly due to transformation of dietary components and the limited diet concentration that can be used due to water quality deterioration in the enrichment tanks (McEvoy et al., 1998; Tonheim, 1999). A new enrichment boosting procedure was developed (Barr, 2001) that involved feeding high dietary concentrations for a relatively short time (1-2h), allowing the organisms to feed intensively, but not enough time to transform the dietary components or for water quality to deteriorate. Those results showed a substantial improvement of DHA content in *Artemia* nauplii, compared to standard enrichment, using the same diet.

The aim of the study was to improve the nutritional quality of live food, with emphasis on phospholipid (PL) and free amino acids (FAA), using the enrichment boosting procedure.

Materials and methods

Rotifers, *Brachionus plicatilis,* were cultured using the batch culture method (Ressem et al., 2001) and enriched with Algamac 2000 according to the producer recommendation. *Artemia* EG cysts (Inve, Belgium) were decapsulated and hatched and the nauplii were enriched with DHA Selco (Inve, Belgium),

according to the producer's instructions (except for an incubation time of 16 hours).

The experimental diet was liposome, using Soy lecithin (Epikuron 200SH, Degussa, Germany) as the phospholipidic membrane. For the experiment involving FAA enrichment, the liposomes were loaded with a mixture of 75% saturated amino acids as shown in Table I.

Table I. FAA content of the Epikuron liposome.

	Arg	**His**	**Met**	**Gly**	**Phe**	**Tau**	**Lys**	**Total**
Nmol.mg^{-1} PL	286.8	218.5	210.8	121.3	57.5	48.9	36.3	980.0
%	29.3	22.3	21.5	12.4	5.9	5.0	3.7	100.0

Before the boosting enrichment, enriched rotifers or *Artemia* nauplii were washed thoroughly and concentrated. Samples were taken for chemical analysis and dry mass (control treatment). The organisms were incubated in 1.5-l flasks in triplicate and immersed in a temperature-controlled water bath with aeration. The final density of the rotifers was 5×10^6.l^{-1} and 10^6.l^{-1} for *Artemia* nauplii. The incubation temperature was 23°C and 28°C and salinity was 20ppt and 33ppt for rotifers and *Artemia*, respectively, and oxygen concentration was maintained above 4ppm. The calculated diet concentrations were 1 and 2g.l^{-1}. The samples were washed in clean seawater and distilled water, filtered, frozen on dry ice, and stored at -80°C until analyzed. Samples were taken for dry mass and biochemical analysis.

Samples were analyzed for dry mass after freeze-drying. Total lipids were measured based on Folch (1957). Lipid classes and their fatty acids content were analyzed according to Thomassen et al. (1979). For FAA analysis, samples were vortexed with 6% TCA. Chloroform:methanol 2:1 was added for de-lipidation and the samples were mixed and centrifuged and sub-samples of the supernatant were analyzed. The samples were appropriately diluted in borate buffer (pH 10.4) and analyzed on a Gilson HPLC (fluorimetric OPA and FMOC reagents).

Data were analyzed using a Kruskal-Wallis test when several samples compared, or a Mann-Whitney U-test when two data sets were compared (Sokal and Rohlf, 1981).

Results and discussion

The results of liposome-boosting enrichment of rotifers and *Artemia* nauplii was a significant increase ($P<0.05$) in their PL content, about 140% and 123% increase for rotifers and nauplii, respectively (Fig.1). Furthermore, fatty acid analysis of the PL content of *Artemia* nauplii showed a significant increase ($P<0.05$) of stearic acid (C18:0) which is 86% of the Epikuron fatty acids.

Boosting enrichment with liposome loaded with FAA yielded significant increase of FAA found in the liposome, in rotifers, and *Artemia* nauplii. Furthermore, the changes in the individual FAA content reflected that of the FAA combination in the liposome, as shown for rotifers in Fig 2. The FAA changes in the nauplii were basically similar, but the effect was smaller (results not shown).

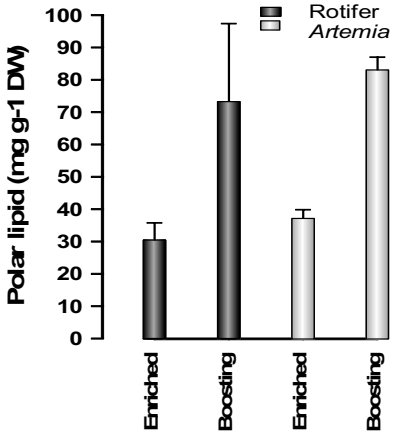

Fig. 1. Effect of liposome boosting enrichment, on the polar lipids fraction in rotifers, previously enriched with Algamac 2000 and *Artemia* nauplii, previously enriched with DHA Selco.

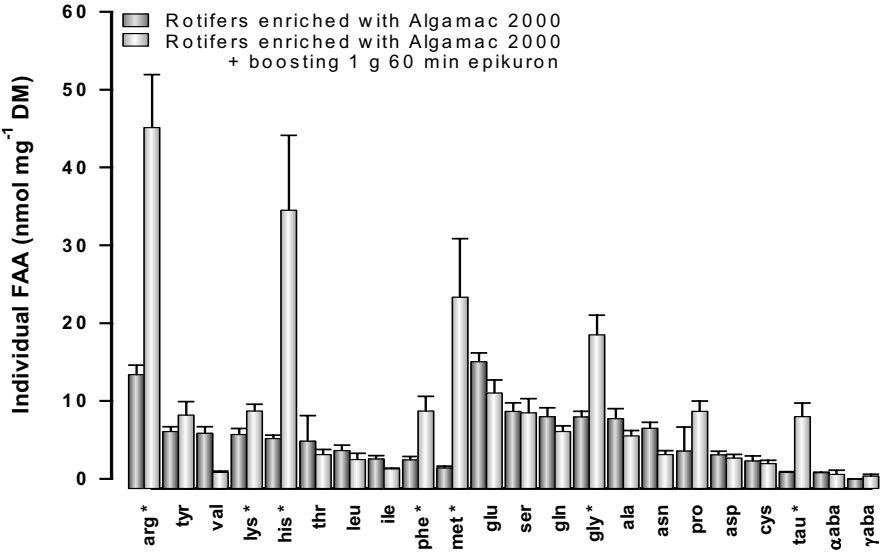

Fig. 2. Effect of boosting enrichment with liposome loaded with FAA as shown in Fig. 1 on FAA content in rotifers. *Amino acids which were included in the liposome.

This method using liposomes as diet for live food can be a versatile tool for nutritional studies in larvae. In regards to the effort for developing a diet that will replace the live food, we suggest to use this method to test the effect of dietary components in fish or shrimp larvae using live diet as a preliminary stage, thus

eliminating the possibility that the effect of the dietary component will be masked by other deficiencies in artificial diet.

Conclusions

To the best of our knowledge, we have achieved higher increase in PL and FAA in enriched live food than previously reported. Better results can be achieved in rotifers than in *Artemia* nauplii. This method can be very useful in fish and shrimp larval studies, as well as a tool in the ultimate goal of replacing live food with artificial diet. It is also a practical method that can be implemented in commercial hatcheries to improve live food using commercially available enrichment diets.

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A REVIEW OF NEW AND EMERGING TEMPERATE MARINE FINFISH SPECIES FOR FARMING IN AUSTRALIA: CHALLENGES IN LARVAL REARING

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Demand for farmed marine fish in Australia is growing both for local consumption and for export. Farming takes place in sea cages, intensive shore-based facilities, and saline groundwater. A major impediment to the development of new species for aquaculture in Australia has been the unreliable hatchery production of high-quality juveniles. Over 15 temperate species have been evaluated or are being farmed with ten main candidates.

An increasingly sophisticated research effort to support new species development is being coordinated through the Aquafin Cooperative Research Centre and the Fisheries Research and Development Corporation. Most states have marine research hatcheries and Western Australia, South Australia, New South Wales, and Queensland have one or more commercial fish hatcheries. Species such as black bream, Dhufish, King George whiting, mulloway, snapper, and striped trumpeter have been the focus of large grant-funded projects, while others like yellowtail kingfish have been successfully developed by industry. Research has been aided by investment in greater infrastructure for broodstock and the development of replicated and semi-automated larval rearing systems. The reliability, cost-effectiveness, and intensification of live feed production systems has been improved through the adoption of overseas technology and the importation of concentrated algal products. There has been increasing interest in developing alternative feeds for hatcheries, including alternative zooplankton and formulated diets.

Reliable supplies of high quality fertilized eggs are now available for a number of marine fish species. Some research and industry hatcheries still rely on wild-caught broodstock and hormone-induced ovulation. The temperature and photoperiod manipulation of spawning is becoming more common and is well understood for mulloway, snapper, and striped trumpeter. Larval rearing research in

the 1990s focused on better understanding the role abiotic factors have on development, growth, and survival of larvae. General descriptions of larval development, sensory organ ontogeny, and initial swim bladder inflation are available for some species, notably snapper and striped trumpeter. More recently the optimization of light intensity, photoperiod, temperature, and salinity has provided improved larval growth and survival.

Most species are now reared using some form of 'greenwater' culture. Early extensive culture has evolved and often involves an early intensive culture phase until after swim bladder inflation. There is now greater use of intensive systems using daily greenwater addition. The benefits of greenwater have been examined and linked to improved feed intake, better distribution, and microbial modification. Most hatcheries have seawater supplies of less than optimal quality and it is often drawn from estuaries. Control of the microbial environment in larval rearing tanks is a new area of research and involves determination of host-microbe interactions and research into the use of ozonation and probiotics.

For many species there remains a poor understanding of nutritional requirements during larval development. This is an area of increasing research and includes the assessment of an ever-increasing number of commercially available enrichment products for live feeds. The determination of specific lipid and vitamin requirements for selected species has been aided by advances in the production of specialty oils, purified sources of fatty acids and tailor-made enrichments. The use of dose response techniques to determine nutrient requirements, commonly used on juvenile fish, has now been extended to larvae.

Skeletal malformations including jaw, opercula, and spinal deformities remain problematic for many species and continue to hinder commercialization of species such as striped trumpeter. Research is currently underway to establish the possible role of early larval lipid and vitamin nutrition, and environmental factors. The detection and treatment of diseases is an important area of research. For example, the detection and control of *Kudoa neurophila,* which causes brain disease in striped trumpeter, has been achieved through ozonation of hatchery sea water. Viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) is another hatchery health issue for several species. Aside from economic losses due to mortality of infected fish, nodaviruses are of concern due to the potential for translocation of the virus and exposure of native fish species. Improvements in the control of nodavirus have been achieved through the development of detection tools, better hygiene, and the use of ozone to disinfect eggs prior to incubation.

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EPIDEMIC DYNAMIC OF THE WHITE SPOT DISEASE (WSD) IN *LITOPENAEUS VANNAMEI* SHRIMP

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OPTIMISATION OF DIETS FOR ATLANTIC COD (*GADUS MORHUA)* BROODSTOCK: EFFECT OF ARACHIDONIC ACID ON EGG AND LARVAL QUALITY

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The importance of three essential fatty acids (EFAs; docosahexaenoic (22:6n-3; DHA), eicosapentaenoic (20:5n-3; EPA) and arachidonic acids (20:4n-6; ARA)), in reproduction and egg and larval development has been demonstrated. The effects of sub-optimal EFA on fecundity, egg quality, hatching success, numbers of normal larvae, and incidence of deformity have been shown. ARA is a precursor of 2-series eicosanoids, an important group of compounds with a wide range of biological activities, which includes a role in final maturation of oocytes. Low levels of ARA have been shown to correlate with poor hatching success and other egg quality parameters in cod. ARA is therefore considered to be important in relation to egg quality, but to date no studies on the effects of cod broodstock diet supplementation appear to have been reported.

Marine fish have limited capacity for interconversion of EFAs so the fatty acid content of the diet is important to ensure optimal provision of EFA to allow normal growth and development. Supplementation of broodstock feeds with oils rich in specific highly unsaturated fatty acids (HUFA) can lead to an increase in levels of these fatty acids in the developing eggs and, in the case of sea bass and halibut, this has been shown to have a measurable impact on egg quality.

Recent results have shown that eggs from farmed cod have consistently lower ARA and ARA:EPA ratios than eggs collected from wild cod. However, in contrast with studies on other species, no consistent differences were noted in DHA, EPA, and DHA:EPA. Fatty acid analysis of several commercial feeds used for feeding cod broodstock have demonstrated that these contain low concentrations of ARA (typical of Northern Hemisphere fish oils) and high EPA:ARA ratios (generally > 10). Wild-caught cod tend to produce larger, better quality eggs and larvae than farmed broodstock. Reliance on wild fish creates risks with introduction of disease and prevents stock improvement by artificial selection, but because of the problems with egg quality in farm-reared broodstock, wild-caught

fish remain the main source of eggs for UK hatcheries. It seems likely that nutritional factors are at least partly responsible for the poor results from farmed broodstock. Manipulation of the nutrient composition of broodstock diets may offer a practical means to improve egg and larval quality.

Analysis of farmed and wild eggs has shown that eggs from farmed fish have lower ARA concentrations and ARA:EPA ratios which suggests that ARA concentrations in the broodstock diet may be sub-optimal. Vevodar® oil (DSM Food Specialities B.V., Delft, Holland) contains a high ARA concentration and is available in the volumes required for supplementation of cod broodstock feeds. This study assessed the practical value of ARA supplementation of cod broodstock diets in terms of egg and larval quality, and will investigate the nutritional basis for any observed differences. Four groups of 47 cod broodstock (27 males, 20 females**)** were placed in 4 circular fibreglass tanks (4m diameter, 12m^3 volume) supplied with fresh seawater in November 2004. Fish were weighed at the start of the study in November 2004, and weighed and examined by ultrasound to determine sex and state of maturation in January 2005.

Two diets were fed to duplicate tanks from November 2004 until May 2005, just prior to spawning. Feeding was to satiation (0.3-0.5% biomass/day). The diets were A) non-supplemented base diet and B) base diet supplemented with ARA. The base diet was 12-mm pellets of Ewos Marine 1500 (Ewos Ltd., Westfield, Scotland) and this was supplemented with ARA by addition of Vevodar® oil (11.4 $g \cdot kg^{-1}$**)** applied by spray coating the pellets in a cement mixer to provide diet B. A similar quantity of Northern hemisphere fish oil was added to diet A. Diet A provided 0.14% ARA and diet B 0.59% ARA as a % of dry diet.

Egg batches were collected between April and June 2005 using mesh egg collectors to trap floating eggs exiting from a surface outlet. Egg batch volume, mean egg diameter, and fertilisation rate were recorded. Egg batches meeting minimum volume and fertilisation rate criteria were incubated through to hatching and assessed for hatch rate and size at hatch. Samples of eggs, milt, and larvae were collected for lipid, fatty acid and eicosanoid analysis. As Vevodar® contains 35-40% ARA the biggest effect on dietary fatty acids was a 4-fold increase in dietary ARA and a 33% increase in total n-6 PUFA. As a result of the increased ARA the n-3:n-6 ratio was reduced from 3.5 in diet A to 2.5 in diet B and the EPA:ARA ratio was reduced from 15.6 in diet A to 3.7 in diet B.

Fatty acid analyses of eggs from wild and cultured cod broodstocks showed that wild eggs contained significantly higher ARA concentrations and significantly lower EPA:ARA ratios compared to eggs from cultured broodstocks.

In conclusion, supplementation of cod diets with ARA resulted in increased egg concentrations of ARA and reduced EPA:ARA ratios.

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EVOLUTION OF CONDITION INDICES DURING *CRASSOSTREA GIGAS* LARVAL DEVELOPMENT

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Introduction

Few studies are devoted to bivalve physiological indices in relation to growth (Pernet et al., 2003), survival (Gallager et al., 1986), and/or metamorphosis (Whyte et al., 1987) and their use as predictors have failed. Indeed, larval performances are highly variable between hatcheries for many reasons (Robert and Gérard, 1999). Beyond cultural practices, seawater quality, microalgae, genetics, origin of broodstock, and gamete quality constitute undoubtedly important sources of variation. Validation of such indices remains inefficient when such variability is not taken into consideration. We focused on three quality indices in relation to larval development indicators.

Materials and methods

Two and a half year-old broodstock, originating from Arcachon natural spatfall, was maintained in Aber Benoît (North Brittany) in 2004. Each month 100 oysters were harvested and placed in 800-l tanks for their conditioning under standard hatchery procedures. Six weeks later 30 oysters were harvested and stripped. To minimise genetic effects, oocytes obtained from 5 to 6 females were mixed with spermatozoa from 2-3 males. Larvae were reared in 150-450 l in cylindrical conical based tanks following proven techniques: 5 larvae.ml^{-1}, 24±1°C, 34ppm, 1-µm filtered seawater, (Robert and Gérard, 1999) and daily addition of 20-200 cells.μl^{-1} of a mixed microalgae diet matched to larval consumption.

From day 16, the whole population was considered competent to metamorphosis when the number of eyed larvae exceeded 50%. Pediveligers were retained on a 225-µm mesh sieve and distributed in 30-l tanks containing plastic disks used as spat collectors. Five to 7 days later, metamorphosis was indirectly evaluated by counting the number of remaining larvae. Larval development and metamorphosis of six different batches, reared from broodstock to postlarvae under strictly similar conditions to smooth sources of variation, were achieved from February to September 2004.

The evolution of the condition index ($100 \times \text{Organic Matter} \times \text{Dry Weight}^{-1}$) and structural index ($\text{Triacylglycerides} \times \text{Sterols}^{-1}$) were studied concurrently to evaluate its temporal variability under standardised conditions. Moreover, in toto neutral lipid stain techniques were perfected using high definition colour video as well as a dedicated image analysis software allowing quantitative analysis (and not exclusively qualitative) by individual measurements of neutral lipid area related to larval size.

Results and discussion

One batch of *Crassostrea gigas* larvae did not reach metamorphosis due to high bacterial mortality setting from day 14 (Table I). Larval harvest yield reached 49-70% on day 18-19 of which 65-75% of competent larvae were found. Gig 0409 batch was an exception with only 38% of mature larvae (Table I). Metamorphosis success appeared to be directly related to larval competency, excepted for the Gig 0406 batch with lower metamorphosis (40%) compared to larval competency (70%: Table I).

Table I. Characteristic of different batches of larvae used in the experiments and reared under similar experimental conditions. Values in parentheses indicate standard deviations.

Batch of larvae	Rearing period (day)	Larval length at the end of larval life (µm)	Competency: larvae ready to set (%)	Metamorphosis (%)
Gig 0404 (Ma)	16	292.9 (48.7)	64.01	62.29 (4.57)
Gig 0405 (Ap)	18	293.0 (40.9)	74.41	89.35 (0.89)
Gig 0406 (May)	19	278.1 (50)	69.30	39.64 (8.86)
Gig 0407 (June)	16: discard	221.9 (20.3)	-----	-----
Gig 0408 (Jul)	19	311.6 (30.1)	64.56	57.34 (11.96)
Gig 0409 (Au)	19	313.2 (25.5)	37.26	40.47 (24.08)

The larval condition index ($100 \times \text{MO} \times \text{PS}^{-1}$) was distinguished by a step increase throughout postlarval with a high variability from batch to batch (Fig. 1). At the end of embryogenesis (day 2) CI ranged from 10-25% and remained stable until day 7 with the exception of Gig 0405 batch (Fig. 1). Then, during the second

week, CI sharply increased to 20-45% on day 14, with a low progression thereafter (Fig. 1).

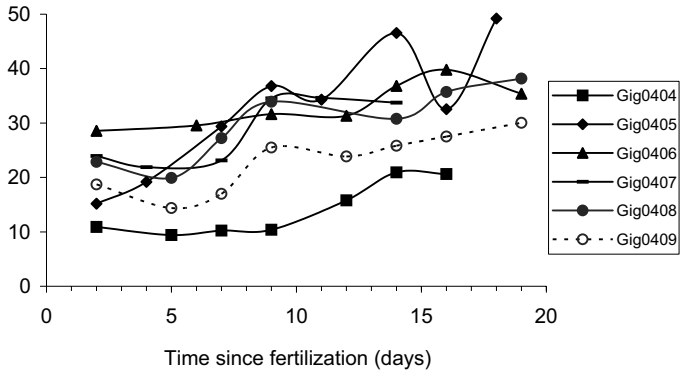

Fig. 1. Evolution of condition index ($100 \times OM \times DW^{-1}$) throughout larval development in six *C. gigas* batches run under similar experimental conditions.

The structural biochemical index $TAG \times ST^{-1}$ ranged from 4-8 on day 2, then remained constant or exhibited increasing or decreasing values after one week of rearing (Fig. 2). Therefore, on day 7 that index's amplitude variations were high, with values ranging from 3-13. A general decreasing trend was subsequently observed leading to tightening of values (6-10 on day 14 and 4-7 on day 18-19). Mean sterol and triacylglyceride contents followed a similar trend throughout larval development. Weak concentrations from day 2 ($0.22<ST<0.57$ and $1.11<TG<2.26$ng.larva^{-1}) to 7 ($0.55<ST<1.95$ and $2.48<TG<13.59$ng.larva^{-1}), were followed by a high accumulation until day 14 ($6.65<ST<20.04$ and $60.02<TG<134.10$ng.larva^{-1}) and slightly higher accumulation thereafter (day 18-19: $16.04<ST<27.38$ and $96.4<TG<187.8$ng.larva^{-1}) were noted.

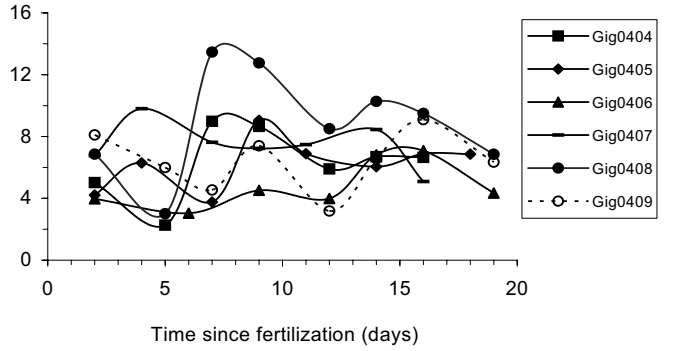

Fig. 2. Evolution of biochemical structural index ($TAG \times ST^{-1}$) throughout larval development in six *C. gigas* batches run under similar experimental conditions.

Nile red staining and epifluorescence analyses were performed on larvae issued from Gig 0409 batch. The evolution of neutral lipid area vs. size of larvae showed a slight decrease throughout larval development with values ranging from 23.45 (± 0.46) on day 7 to 19.52 (± 0.74) on D17.

Conclusion

The high variability of condition and biochemical structural indices in batches of larvae reared in standardised conditions will cause potential difficulties for its use as a predictive indicator for larval development and/or metamorphosis. In contrast, the first results on neutral lipid evolution via staining method seems to indicate that under normal conditions larvae maintain their neutral lipid reserves at a constant level corresponding to 20-25% of the total larval area (size).

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FISH IDENTIFICATION OF SCALLOP HYBRIDS (*CHLAMYS NOBILIS*♀×*C. FARRERI*♂) USING THE ITS-1 PROBE

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Introduction

As an approach to improving scallop aquaculture, crossbreeding has been performed among various scallop species (Bower et al., 1997; Cruz and Ibarra, 1997; Chang et al., 2002; Yang et al., 2002). Thus, identification of hybrids in progenies is highly desirable. In this study, we developed a simple and rapid way to identify scallop hybrids between *C. nobilis* and *C. farreri* by examining the numbers of FISH signals located in metaphase chromosomes and/or interphase nuclei.

Materials and methods

Sexually mature, parental scallops *C. nobilis*♀ and *C. farreri*♂ (two years old) were obtained from Xunshan Scallop Hatchery. The hybrids of *C. nobilis*♀ and *C. farreri*♂ were artificially induced and trochophore larvae sampled. Chromosomes were prepared following Wang and Guo (2004).

Genomic DNA was extracted using mantle tissue of *C. farreri* and *C. nobilis*. ITS-1 was amplified using universal primers (CTGCGTTCTTCATCGACCC and GGTTTCTGTAGGTGAACCTGC). PCR amplification, subcloning, and sequencing were performed according to Yu et al. (2001). Alignment was achieved by the program CLUSTAL X1.8. The probe was labelled with biotin-16-dUTP using PCR amplification.

In situ hybridization was performed as the following protocol. In a brief, slides were treated with 100μg.ml^{-1} RNase A in 2× SSC at 37°C for 30min, washed with 2× SSC at room temperature for 15min, and incubated at 50°C for about 3h. Chromosomes were denatured in a mixture containing 75% formamide and 2× SSC for 2-3min at 72°C, dehydrated through an ice-cold ethanol series(70%, 90%, and 100%; 5min each), and air-dried. The probe hybridization mix (containing 5ng.μl^{-1} of probe, 1μg.μl^{-1} of salmon testis DNA, 50% formamide, 10%

dextran sulphate, and 2× SSC) was denatured at 85-90°C for 5min and immediately put on ice for at least 15min. The denatured probe was applied to the slide and DNA-DNA in situ hybridization was carried out at 42°C for 14h. Following hybridization, the slides were placed in 50% formamide in 2× SSC at 60°C for 10min, 1× SSC at 60°C for 10min, 0.5× SSC at 55°C for 10min, 0.19× SSC at 50°C for 10min, and 2× SSC at room temperature for 10min. Biotinylated probes were visualized with Rhodamine or fluorescein isothiocyanate (FITC) conjugated with avidin DCS for 1h at 37°C. Chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI) or vectashield mounting medium with propidium iodide (PI) for 10min at room temperature. Hybridization signals were detected using Nikon epifluorescence microscope E-600 equipped with filter sets for Rhodamine/DAPI or FITC/PI. Digital images were recorded using a CCD camera and analyzed with the Lucia-FISH Image System.

Results and discussion

The fragment of ITS-1 containing partial 5.8S and 18S rDNA sequences was consistently amplified for each of the two scallop species. Sequence data showed that the ITS-1 fragment has a length 340bp with 47.1% GC content in *C. farreri*, and 337bp with 50.1% GC content in *C. nobilis*. By alignment comparison, two ITS-1 regions display a total of 87 variable sites, among them 21 are gaps, and 66 are transitions or transversions. The similarity between the ITS-1 amplified from *C. farreri* and *C. nobilis* is 75.4%. Owing to the sequence variation of ITS-1 between *C. farreri* and *C. nobilis*, a species-specific probe was developed.

Using the optimized hybridization and washing conditions, FISH of biotin-labeled ITS-1 probe from genomic DNA of *C. farreri* can reliably distinguish *C. farreri*, *C. nobilis*, and their hybrids, according to the number of signal loci detected in interphase nuclei or metaphase chromosomes. More than 20 metaphases and 100 interphase nuclei were examined for each species and their hybrids. Results showed that two signal loci were observed in the metaphase spreads and interphase nuclei of *C. farreri* (Figs. 1A, 1B, and 1C). No obvious signal locus was detected in *C. nobilis*. Only one weak signal was observed in the interspecific hybrids between *C. nobilis*♀ and *C. farreri*♂ (Figs. 1D, 1E, and 1F).

In this study, we present a method of developing species-specific probe for FISH in scallops and the optimization of FISH conditions. One of the advantages of this approach is that construction of probes is based on PCR-labelling, which can be performed easily. Another merit is that the time-consuming preparation of metaphase chromosomes is not required. We can identify the hybrid by detecting of the signal located in interphase nuclei.

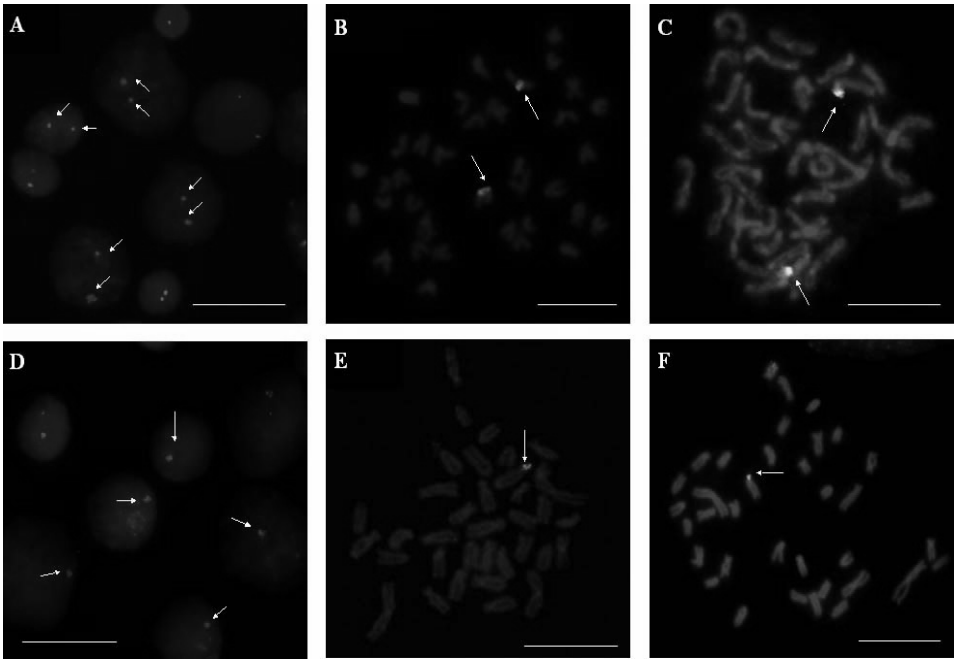

Fig. 1. Chromosomal location of ITS-1 in *C. farreri* and the hybrids (*C. nobilis*♀×*C. farreri*♂) by FISH. A: By rhodamine/DAPI detection system, two ITS-1 signals were detected in interphase nuclei of *C. farreri*; B and C: By rhodamine/DAPI and FITC/PI detection systems respectively, two ITS-1 signals were detected in metaphase chromosomes of *C. farreri*; D: By rhodamine/DAPI detection system, one ITS-1 signal was detected in interphase nuclei in hybrids; E and F: By Rhodamine/DAPI and FITC/PI detection systems respectively, one ITS-1 signal was detected in metaphase chromosomes of hybrids by FISH. Bars=5μm

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UNDERSTANDING HOW MARINE VIBRIOS CAN CAUSE MORTALITIES IN LARVAL TURBOT

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Heavy mortalities can often occur during early rearing of flatfish larvae whilst they are fed on live prey organisms such as rotifers or *Artemia*. Bacteria are acknowledged to be a major cause of such losses and *Vibrio splendidus* has been implicated as a cause of some larval rearing crashes. In turbot larvae from a commercial hatchery in Northern Spain the bacterial flora of larvae fed on *Artemia* was dominated by *V. splendidus* even though the *Artemia* bacterial flora was comprised almost solely of *V. alginolyticus*. Sixteen bacteria from turbot larvae, mainly from batches of larvae experiencing poor survival, were tested for virulence against turbot larvae and 4 isolates, from 3 separate batches of larvae and at three different times of year, were found to be highly virulent. These were all *V. splendidus* biotype 1 but, as previously found, many other *V. splendidus* isolates from the same larval rearing system were avirulent.

The principal isolate studied, *V. splendidus* DMC-1, was representative of the dominant organisms isolated from a batch of larvae experiencing very poor survival, but a similar, highly virulent organism was isolated as a minor component of the gut bacterial flora of a parallel batch of larvae experiencing high survival. *V. splendidus* DMC-1 did not cause significant mortalities when added to the water in which first-feeding turbot larvae were held. However, when rotifers were pre-incubated with *V. splendidus* DMC-1 and fed to larvae, mortalities increased after 2d suggesting that larvae must be delivered to the digestive tract in order to cause larval mortalities. Histological examination and immunohistochemistry with antiserum to *V. splendidus* DMC-1 showed that bacteria adhered to the intestinal tract of larvae; damage to the intestinal epithelium was evident with signs of enteritis.

To further investigate the reasons for damage to the intestinal epithelium a bank of Tn10 transposon mutants of *V. splendidus* was prepared. *V. splendidus* was strongly haemolytic on blood agar and when approximately 10 000 transposon mutants were screened on blood agar, 4 were identified that had lost haemolytic

activity. Two of the haemolysin-negative (hly-) mutants were selected for further study. Apart from being non-haemolytic, the mutants had also lost cytotoxicity to turbot tissue culture cells and had lost virulence for turbot larvae. Mortalities in turbot larvae fed on rotifers colonised with hly- *V. splendidus* were similar to those in control larvae and there was no damage to the intestinal tract observed on immunohistochemistry even though the intestinal tract was heavily colonised with the hly-bacteria.

Determination of the site of transposon insertion in the hly-bacteria revealed two types of insertion that caused the hly-phenotype and loss of virulence for larvae.

The first group of mutants are deficient in production of a toxin closely related to a well-recognised bacterial cytotoxic enterotoxin. The second group had a mutation on an adjacent gene that showed clear homology to a bacterial virulence regulating gene. The only difference found between the hly-mutants and the wild type *V. splendidus* DMC-1 was in haemolysin production, and no other factor was identified as being under the control of the regulatory gene. Comparison of approximately 8kb of the *V. splendidus* genome with the genomes of *V. cholerae, V. vulnificus*, and *V. parahaemolyticus* indicated that the haemolysin and regulatory genes appeared to be an insertion in the *V. splendidus* genome as they showed low homology with *Vibrio* genes.

These results allow us to understand how bacteria can kill flatfish larvae and open the way for rational strategies to be devised for identifying probiotic bacteria that might prevent such bacteria from causing damage to the larval digestive tract and subsequent death.

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EFFECT OF *ARTEMIA* ENRICHMENT ON MASS CULTURE OF PACIFIC BLUEFIN TUNA *THUNNUS ORIENTALIS* LARVAE

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Introduction

The enrichment of *Artemia* with docosahexaenoic acid (DHA) and choline have been demonstrated effective in different species (Craig and Gatlin, 1996; Gapasin and Duray, 2001) though, in some cases, the effect was not conclusive (Wilson and Poe, 1988; Hanley et al., 1998). However, there is no information on the effect of *Artemia* enrichment with DHA and choline on the growth performance of the Pacific bluefin tuna (PBT; *Thunnus orientalis*) larvae, where the need for a suitable larval diet is still unresolved problem. This area therefore needs to be clarified.

Materials and methods

One thousand PBT larvae (19 days old) were randomly divided into five groups in duplicate 500-l tanks and fed with either *Artemia* (INVE Aquaculture, Belgium) enriched with oleic acid (Diet 1), DHA (Diet 2), DHA + choline 1000ppm (Diet 3), DHA + choline 2000ppm (Diet 4), or stripped knifejaw larvae (Diet 5, reference diet) for 12 days.

Lipids were analyzed according to Folch et al. (1957). The fatty acid methyl esters were analyzed with a gas chromatograph (G-3000; Hitachi, Tokyo, Japan). Peak quantification was done with an integrator (D-2500; Hitachi, Tokyo, Japan). Choline content was determined by kit (Wako, Osaka, Japan).

Data were normalized and significant differences were determined by a Tukey multiple comparison test, using the SPSS for Windows (v. 10.0).

Results and discussion

The DHA enrichment of *Artemia* significantly elevated the DHA levels to 13.9, 13.8, and 12.5mg.g^{-1} DW in Diets 2, 3, and 4, respectively; however, the levels were much lower than the reference diet (26.9mg.g^{-1} DW; Diet 5). The levels of total choline and phosphatidylcholine (PC)-binding choline in enriched *Artemia* and tuna larvae are shown in Figs. 1 and 2, respectively. The results of rearing experiments are shown in Table I.

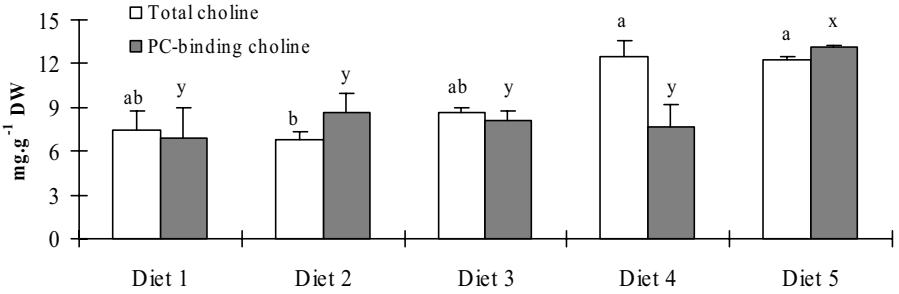

Fig. 1. Total choline and PC-binding choline content in different diets.

Although the growth and survival rate was improved in DHA-enriched groups compared to the fish fed with DHA-deficient diet, it was negligible compared to the enhanced growth of the fish larvae-fed group (Diet 5). Therefore, it suggests that the enrichment of *Artemia* with DHA or choline would not be sufficient to resolve the dietary problem of PBT larvae. This may be attributed to the lower levels of PC-binding choline in Diets 2, 3, and 4 than those in Diet 5. This assumption is supported by the role of PC observed by Kanazawa (1993). It may also be attributed, in part, to the lower DHA/EPA ratio in Diets 2 (1.0), 3, (1.0) and 4 (0.9) than those in Diet 5 (3.5) (Copeman et al., 2002).

Table I. Rearing results of tuna larvae fed either on enriched *Artemia* or fish larvae (*n=22).

	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**
Initial length (mm, n=20)	12.2±1.0	12.2±1.0	12.2±1.0	12.2±1.0	12.2±1.0
Final length (mm, n=30)	17.7±1.8*^{3}	18.5±1.4	19.1±1.3	19.1±0.8	43.3±3.4
Initial weight (mg, n=20)	28.5±7.4	28.5±7.4	28.5±7.4	28.5±7.4	28.5±7.4
Final weight (mg, n=30)	*65.1±17^{c}	88.2±16.9^{b}	96.9±18.9^{b}	93.8±12.9^{b}	1175.5±244.3^{a}
Survival rate (%)	6.6±1.4^{c}	32±3.5^{b}	30.2±12.7^{b}	30.5±14.8^{b}	61.7±10.6^{a}

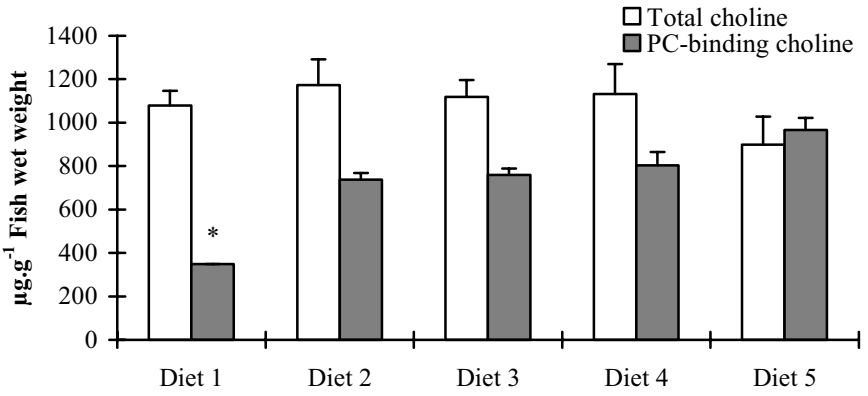

Fig. 2. Total choline and PC-binding choline content in tuna larvae fed with different diets. *no replicates.

Conclusions

The simple enrichment protocol of *Artemia* with DHA and choline is not effective in PBT larvae. Further studies on the elevation of PC-binding choline or the elevation of the DHA/EPA ratio in *Artemia* are therefore required.

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SKELETAL DEVELOPMENT AND ANOMALIES IN REARED DUSKY GROUPER (*EPINEPHELUS MARGINATUS*)

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Introduction

Dusky grouper is a new candidate species for aquaculture, whose reproduction and larval rearing has only recently been achieved (Marino et al., 2003). In order to optimize the larval rearing of this species, its skeletal development was studied. Furthermore, the skeletal anomalies observed in different rearing trials were followed up in order to get some indication of the best performing larval rearing method. The assumption underlying this approach is that skeletal elements are genetically fixed, and so any alteration in their number or shape can be considered as probably being a consequence of altered environmental conditions during embryonic (number of vertebrae) or larval (number of fin rays) development.

Materials and methods

Some 85 individuals (from 0 to 75 days post-hatching (dph)) were subjected to skeletal development study, all belonging to the same egg batch. All the samples (n=85) were anaesthetized (ethylene glycol-monophenyl ether, Merck, 0.2-0.5ml.l^{-1}), fixed in buffered formalin (4% in phosphate buffer 0.1M pH 7.2), and stained in toto with alizarin red for calcified structures and alcian blue for cartilage (Taylor and Van Dike, 1985). Observations were performed on the left side of the fish under stereomicroscope.

To study skeletal anomalies, two different larval rearing cycles of dusky grouper (*Epinephelus marginatus)* were performed (2001 and 2002), and the eggs from each split into two groups, one reared following Large Volume methodology (Cataudella et al., 2003) (lots BS), and the other in 'green-water' tanks (Saroglia and Ingle, 1992) (lots ROS). All the individuals (576 individuals, see Table I) were analyzed according to the protocol set up by Boglione et al. (2001) for morphological quality assessment.

Table I. Characteristics of sampling used for morphological quality assessment

Origin	code	age	n	$SL_{average}$	LS_{range}
reproduction event: 2001 Large Volume reared larvae in SMEG (Borgo Sabotino, LT), 5 larvae.l^{-1}	BS01	50	47	5.2	3.7-6.2
Same egg batch as BS01 but reared with green water methodology in Maricoltura Farm, Rosignano Solvay (LI), 8.5 larvae.l^{-1}	ROS01V-78	78	48	3.4	2.1-5.6
Same egg batch as BS01 but reared with green water methodology in Maricoltura Farm (Rosignano Solvay, LI), 21 larvae.l^{-1}	ROS01VI-50	50	18	1.8	1.3-2
	ROS01VI-54	54	20	1.8	1.3-2.3
	ROS01VI-82	82	48	4.5	3.9-5.2
reproduction event: 2002, Large Volume reared larvae in SMEG (Borgo Sabotino, LT), 7 larvae.l^{-1}	BSO2-46	46	53	1.9	1.3-3.7
	BS02-70	70	122	2.8	1.9-5.2
Same egg batch as BS21 but reared with green water methodology in Maricoltura Farm (Rosignano Solvay, LI), 8 larvae.l^{-1}	ROS02-v1	60	20	1.83	1.4-2.4
as above but 28 larvae.l^{-1}	ROS02-v5	60	50	1.9	1.2-2.5
as above but 16 larvae.l^{-1}	ROS02-v6	60	150	2	1.3-2.9

Results

Four developmental phases were identified. Phase I (from hatching to 3-4mm TL): swim bladder activates and eyes become pigmented in each larva. Differentiation occurs in skeletal elements of mouth (Meckel cartilage, maxillar, articular, angular, dental, quadrate, and palatine) and opercular plate (preopercular and opercular). This consists of the transition from eleuteroembryonic to larval phases, through the acquisition of protopterygiolarva characteristics (according to Balon, 1975). A continuous embryonic finfold is the only fin, and the dorsal spiny rays (the future 1st and 2nd dorsal ray), the cleithrum, the 1st spiny ray, and the basypterygium of the pelvic fin start their differentiation. The notochord is at the preflexion stage. Phase II (4-6mm TL): premaxillar and ectopterygoid are discernible in the cranium, while in the opercular plate the subopercular and interopercular complete their differentiation. The caudal complex starts its development: parahypural, epuralia, hypuralia, and lepidotrichia are evident while notochord flexion takes place, giving the larvae the capability of faster movements. The 2nd dorsal and the two pelvic spiny rays augment their dimension (assuming a triangular section), are considerably lengthened and with differentiated spines lining each margin. This consists of the transition from protopterygiolarva to pterygiolarva. Phase III (6-11mm TL): the hyoid arch completes its differentiation. Pectoral, pelvic, anal and caudal fins reach their final number, while the dorsal and anal fin pterygiophores are differentiating; the scapulocoracoidea car-

tilage begin to ossify. All the vertebrae (24) are ossified. Phase IV (11-35mm TL): all the larvae longer than 35mm complete the skeletogenetic process. At the end of this step, the dusky grouper is a juvenile, with scales and adult pigmentation.

The 2001 BS01 lot showed the best morphological quality (only 8.5% of the observed individuals showed at least one deformation, only one of them showing a severe anomaly; i.e., one deformed vertebra) and the other 2001 lots (ROS01) the worst ones. In 2002 substantial ameliorations were observed in the ROS02 lots, but without reaching the same quality as that shown by the BS01 lot. Among the ROS02 individuals, one lot reared at an intermediate stocking density (ROS02v6, 16 larvae.l^{-1}) shows a better quality than the others 2002 lots (BS included).

Discussion and conclusions

The analysis of fin differentiation patterns may provide some indication of the locomotory capabilities of grouper larvae and juveniles. The first fin to differentiate is the caudal (6mm TL, 20dph), the fin most involved in transmitting muscular force for propulsive tail strokes in C-start type of fast-start locomotor behavior, commonly used by fish in escape or attack responses (Hale, 1999). Consequently, at this stage, dusky grouper pterygiolarvae are capable not only of feeding on passively captured prey but also actively capturing (and selecting) faster-moving preys. The presence of the three spiny rays actually gives the pterygiolarvae the capability of quickly changing direction (up-down by moving the 2nd dorsal rays; left-right by the pelvic rays). Starting from 14mm TL, the resorption of larval spiny rays begins and the last little spines disappear in all the pterygiolarvae longer than 19mm. At this stage adult pigmentation covers 2/3 of the body and the scales are differentiated. At this stage the dusky grouper probably begins its settlement in the benthic habitat. The skeletal development of the vertebral axis is similar to many other teleosts: the ossification of neural and haemal arches precedes that of vertebral bodies in both cephalic and caudal directions. The final number of pleural ribs is acquired after the final number of vertebrae is attained, as in *Sardinops melanostictus* (Matsuoka, 1987) and in *P. major* (Matsuoka, 1987).

The presence of the elongated spiny rays (never previously described in reared marine species) makes the dusky grouper larvae and post-larvae extremely sensitive to handling activities (such tank cleaning, transfers, and sizing) owing to their fragility which makes them highly susceptible to breaking and also to pathogens. LV larval rearing, which does not involve any of the above-mentioned activities, unlike intensive rearing, could thus be considered as the most promising larval rearing methodology for the dusky grouper.

'2001' LV rearing gave the best quality juveniles, and the 'green-water' reared larvae of the same year the worst, although considerable qualitative amelioration was observed in the 2002 green water reared lots.

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EFFECT OF PHOTOPERIOD MANIPULATION ON RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) EGG QUALITY: A GENOMIC STUDY

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Egg quality can be defined as the ability to allow the development of a normal embryo. In fish, like in other lower vertebrates, the egg contains a large amount of yolk. Because yolk plays an important role during embryo development, many studies have tried to link yolk composition and developmental success observed after fertilization. In contrast, the possible role of non-yolk cytoplasmic components accumulated during oogenesis – such as structure and regulatory proteins, cortical alveoli content, and messenger RNAs – has received far less attention.

However, components such as messenger RNAs are known to be essential for early embryonic development. In bovine two-cell embryos, a relationship between embryonic developmental competence, assessed in terms of time of first cleavage, and the expression of IGF1 mRNA was reported.

Whereas external factors such as food quality and availability during vitellogenesis can be expected to influence egg quality through yolk components, other extrinsic factors may act through the alteration of non-yolk cytoplasmic components. Indeed, the possibility that specific messenger RNAs may also be affected by rearing conditions such as egg post-ovulatory ageing has been suggested by preliminary work in rainbow trout concerning a small number of genes.

Among external factors or rearing conditions suspected to affect egg quality, photoperiod manipulation of spawning date is probably of great interest because of its wide use in aquaculture.

The present work intends to 1) characterize the effect photoperiod manipulation of spawning date on rainbow trout (*Oncorhynchus mykiss*) egg quality and 2) analyze any possible link between egg transcriptome and egg developmental capacities.

For this purpose, female rainbow trout of an autumn-spawning strain were exposed to a photoperiod treatment used to obtain an early summer spawning in fisheries. Early embryonic development was monitored with special interest for survival at eyed-stage (ES) and at yolk-sac resorption stage (YSR). Noticeable morphological abnormalities observed at YSR were also carefully monitored. In addition, for each female egg transcriptome was analysed by microarray analysis.

In the present study, large differences in terms of egg quality were observed. Indeed, embryonic survival at eyed-stage ranged from 0% to 99%. Those differences were further increased at YSR, where the percentage of live alevins exhibiting no noticeable malformations (ANA, live, and normal alevins) allowed the identification of 3 groups. A first group of 7 females presented a proportion of ANA ranging from 0% to 8%. A second group of 6 females exhibited a proportion of ANA ranging from 29% to 44%. A last group of 5 females exhibited a proportion of ANA over 71%.

At YSR, a high proportion (55%) of observed abnormalities was characterized by an incomplete yolk-sac resorption. This malformation seemed more frequent than what was previously reported after fertilization of rainbow trout eggs collected after post-ovulatory ageing.

Statistical analysis of microarray data showed that after photoperiod manipulation of spawning date, the mRNA level of a significant number of genes was reflective of egg developmental competence. While these results remain to be confirmed by real-time PCR, the identity of involved genes might point out some cellular or molecular mechanisms associated with photoperiod-induced egg quality defects.

In conclusion, photoperiod manipulation of spawning date can induce egg quality defects. However, egg quality observed after fertilization is extremely variable between females. In addition, egg quality defects induced by this experimental condition seem associated with yolk-sac resorption problems. Finally, a large number of mRNAs exhibit a differential abundance between low and high quality egg samples. This suggests that the oocyte transcriptome before fertilization can be reflective of egg developmental capacities after fertilization.

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EARLY WEANING OF BARRAMUNDI, *LATES CALCARIFER* (BLOCH), IN A COMMERCIAL, INTENSIVE, SEMI-AUTOMATED, RECIRCULATED LARVAL REARING SYSTEM

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Introduction

The production of live food for larvae of barramundi (Asian sea bass), *Lates calcarifer*, like for most species of marine fish, constitutes a major operational cost and bottleneck in a commercial hatchery.

We present a commercial system that tackles these issues by using two of the latest products available in larval culture methods. We have developed an intensive, green-water, recirculated larval system and a continuous, high density, recirculated rotifer system using the HUFA enriched chlorella paste (DHA-enriched Super Fresh V12 Chlorella) produced by Pacific Trading Co. Ltd, Japan. The other major improvement to the system is the use of the micro-diet, Gemma Micro (GM), from Skretting for early weaning. *Artemia* have effectively been replaced in the larval diet by GM making the larval culture process more efficient and economic and producing higher quality weaned juveniles.

Materials and methods

The rotifer system has two 1000-l tanks each with a foam fractionator, floc traps (sediment filter), fluidised-bed biological filter, and one UV unit for new water disinfection. The chlorella paste is fed automatically and continuously to the two rotifer tanks 24h a day from a standard refrigerator via two peristaltic pumps.

The algal paste offers tremendous advantages, particularly because the rotifers can be produced at high density in small volume cultures. A base rotifer density of less than 100.ml^{-1} is maintained between larval batches. When rotifers are required, the systems are run at a density of 1000-1500.ml^{-1} with a lead time of 7d. The algal paste is already enriched with essential fatty acids and no further en-

richment is required. The unconsumed rotifers are kept in optimal nutritional value in the larval tanks by maintaining a low algal paste cell density.

The culture system is clean, with low levels of ciliates (free-swimming and attached; e.g., *Vorticella*) and based on simple technology. Bacteriological studies showed that the recirculating system carries a more stable bacterial population than batch systems and there is an almost total absence of harmful *Vibrio* spp. The rotifers are then continuously and automatically pumped directly (no rinsing) from the rotifer tanks to the larval tanks. Forty to sixty percent of the rotifer cultures are harvested per day (over 1 billion rotifers). Both rotifer systems have been run continuously, simultaneously or alternately, for over two years without the need of master cultures.

The use of the chlorella paste has been priced at AUD$ 0.17 (€ 0.10) per million rotifers produced. The daily labour requirement to maintain the system is less than 1h and the construction of the system only requires small tanks, standard pumps, and PVC and plastic welding.

The larval system consists of two 6000-l tanks in parallel, with a classic recirculated water treatment system. The larval tanks are stocked at 100 larvae per litre (a total of 1.2 million larvae) and the larval rearing regime is described in Fig. 1. As per the rotifer system the chlorella is continuously and automatically fed to the larval tanks to keep a constant low algal density. The rotifer density is adjusted by changing the size of the outlet self-cleaning screens (63-500µm) and/or by adjusting the pumping rate to the larval tanks.

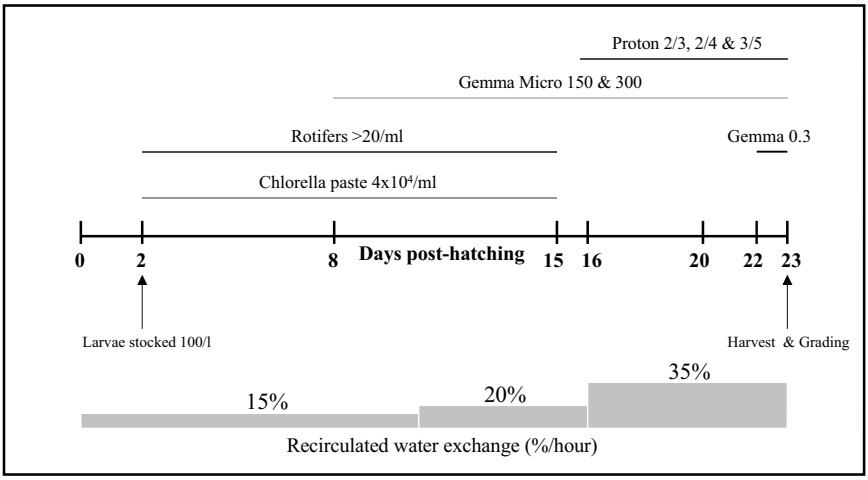

Fig. 1. Larval rearing regime for barramundi, *Lates calcarifer*, in the intensive recirculated system from first feeding larvae to metamorphosis. Artificial diets: Gemma Micro (Skretting); Proton (INVE); Gemma (Skretting). Temperature: 30-31ºC. Salinity: 30-32ppt.

The other major improvement to the system was the use of the microdiet, Gemma Micro, from Skretting (Nutreco) for early weaning. The latest batch of barramundi was produced without the use of any *Artemia*. The larvae were fed only on rotifers for 6 days and then co-fed with GM for 7 days and were weaned by D16 with only 5% weaning mortality. Over 700 000 fry were produced from this batch with a survival of 58%. In the previous batch less than 2kg of *Artemia* per million weaned fry (Day 18-20) were used with less than 0.5% weaning mortality. This represented a 95% reduction in *Artemia* use compared to previous batches (Table I). For economic reasons, once the larvae are weaned, Gemma Micro is slowly replaced by the microdiet, Proton (INVE), before moving on to the nursery feed, Gemma (Skretting) (Fig. 1).

Table I. History and production parameters of the intensive larval system and cost of weaning and post-weaning feeds (including labour) per million of barramundi weaned fry produced (15mm). AUD$1 = € 0.6.

	Batch Number (month-year)					
	03-03	**08-03**	**10-03**	**12-03**	**02-04**	**04-04**
Larval stocking density (per l)	54	70	84	100	103	106
Fry produced at D25 (15mm) × 10^3	278	450	448	612	577	734
Survival (%)	44	54	45	51	47	58
End of weaning (dph)	25-35	20-26	22-24	22-24	18-20	16-18
Weaning mortality (%)	1-2	1-2	0	1-2	0	5
Deformity rate (% assessed @ 100mm)	< 0.1	<0.8	< 0.1	< 0.2	< 1.0	< 0.5
Artemia (kg) per 10^6 weaned fry	20.5	6.6	12.5	10.1	1.2	0
Cost of enriched *Artemia* (including labour) (AUD$)	5700	2,500	3900	3300	700	0
Gemma Micro (kg) per 10^6 weaned fry	0	4.6	20.5	14.2	12.1	13.2
Cost of Gemma Micro (AUD$)	0	1400	6500	4500	3800	4200
Proton (kg) per 10^6 weaned fry	11.5	6.2	5.8	10.9	10.2	11.8
Cost of Proton (AUD$)	500	200	200	400	400	500
Total cost in AUD$ per 10^6 weaned fry	**6200**	**4100**	**10 600**	**8200**	**4900**	**4700**

Results and discussion

Using this system we have routinely achieved a survival rate of 44-58% from 2 day old larvae through to 23-day-old weaned fry (15mm). The deformity rate of the fingerlings assessed at 100mm was less than 1% and was not greater than previous batches reared using *Artemia* (Table I). The latest batches of larvae weaned quickly with Gemma Micro and grew to 100mm 15-25% faster than previous batches (9-10 weeks compared to 11-12 weeks from hatching). There is also less size variation, a great advantage since barramundi are very cannibalistic

and need frequent grading on reaching 3-4 weeks old. Once transferred to the nursery, the fish are much healthier and less susceptible to stress (handling, grading, etc.).

The total cost of weaning and post-weaning feeds (including labor) has been summarized in Table I and is 20% cheaper, using GM and minimal *Artemia,* than the standard method. The labour requirement (and operational cost) has been significantly reduced to one person dealing with both the larval system and the live feed system. The capital cost of constructing the system was also minimal as the system was mainly built in-house using PVC and assembled using plastic welding.

More than six million barramundi fry have been produced using this system at the Darwin Aquaculture Centre over the last 24 months. The rotifer system is currently experimented, with great results, by several European commercial hatcheries. We will be producing technical and scientific papers late 2005 describing the rotifer and the larval systems.

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A GNOTOBIOTIC *ARTEMIA* TEST SYSTEM FOR HOST-MICROBIAL INTERACTIONS

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A model system was developed to evaluate the effect of the microbial community composition offered to *Artemia*. Using axenically-hatched *Artemia* fed axenic food particles (algae, yeast, or bacteria), a gnotobiotic environment is created in which survival and development of this crustacean can be related to the microbes present. In a first set of experiments, two isogenic strains of baker's yeast (*Saccharomyces cerevisiae*) were used to feed *Artemia*: a wild type strain and its isogenic mnn9 mutant, defective in the extension of the mannose chain of cell wall-bound mannoproteins. The genetic background, yeast growth phase, and growth medium appeared to be important parameters determining the quality of yeast cells as feed for *Artemia*. A very strong positive correlation between *Artemia* performance and yeast cell wall chitin and glucan content was obtained, while mannose content was negatively correlated. Mnn9 yeast cells grown until the exponential phase in minimal medium proved to be excellent feed for *Artemia*, yielding an average 95% survival and 4-mm-sized animals after 6 days at 28°C, which is comparable to the best results obtained with algal feed. This set of experiments also demonstrated that the standard growth test yields highly reproducible results. Furthermore, yeast cell viability and the method used to kill/sterilize the cells are important parameters influencing nauplii performance in these gnotobiotic conditions.

In a second series of experiments, a larger set of isogenic yeast strains and two algal species were tested. Yeast cell wall mutants were always better feed for *Artemia* than their respective wild type. Yeast cells harbouring null mutants for enzymes involved early in the biochemical pathway for mannose extension of cell wall-bound mannoproteins performed best as feed for *Artemia.* Yeast cells defective in chitin or β-glucan production or with reduced mannoprotein phosphorylation scored second. These results suggest that any mutation affecting the yeast cell wall composition and/or scaffolding is sufficient to improve the digestibility for *Artemia.* The results with algae indicated that within a species, strains can have different nutritional value under gnotobiotic conditions. The

growth phase was another parameter influencing feed quality, although here it was not possible to reveal the exact cause.

The use of probiotics is receiving considerable attention as alternative approach to control microbiota in aquaculture farms, especially in hatching facilities. However, application with consistent results is hampered by insufficient information of their mode of action. In a third set of experiments, the effect of bacteria on *Artemia* performance in the gnotobiotic test was investigated. Dead (allowing to investigate their nutritional effect) or live bacteria (allowing to evaluate their probiotic effect) were offered as additional feed items. Therefore, nauplii were cultured in the presence of ten bacterial strains combined with four different major axenic live feeds (two strains of *Saccharomyces cerevisiae* [wild type and mnn9] and two strains of *Dunaliella tertiolecta*) differing in their nutritional value. In combination with poor- and medium-quality live feeds, dead bacteria (constituting maximal only 5.9% of the total ash-free dry weight supplied) exerted a strong effect on *Artemia* survival but a rather weak or no effect on individual length. These effects were reduced or even disappeared when medium- to good-quality major feed sources were used, possibly due to improvements in the health status of *Artemia*. Some live bacteria, such as GR 8 (*Cytophaga* spp.) improved (not always significantly) nauplii performance beyond the effect observed with dead bacteria, independently of the feed supplied.

In a final series of experiment, *Artemia* was fed mainly bacteria (90 or 99% of the total ash-free dry weight offered) in combination with a small amount of yeast cells (for instance 1% of the total ash-free dry weight offered). It was found that wild type yeast cells had no significant effect on the performance of *Artemia*, while mnn9 cells had a synergistic effect on *Artemia*.

Transcriptome analysis on *Artemia* indicated that mnn9-fed *Artemia* display, in relation to WT-fed *Artemia,* a considerable amount of differential expressed genes within 4 to 8 hours after the initial contact to this feed types.

In conclusion, the gnotobiotic *Artemia* test system has proven to be an elegant system to start understanding the effect of various feed items. It is hoped that this test will allow development of better feed particles for, in particular, crustacean larvae.

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THE MICROBIOLOGY OF PHYLLOSOMA REARING OF THE ORNATE ROCK LOBSTER *PANULIRUS ORNATUS*

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Rock lobsters, of the Family Palinuridae, are the most valuable single species wild fisheries sector in Australia. Wild fisheries harvest yield has plateaued at sustainable limits and there is considerable interest in the development of a rock lobster aquaculture sector. However, a major challenge is the successful rearing of the larvae (phyllosomas) of Palinuroidea rock lobsters that have a larval phase extending between 6 to 20 months, depending on the species. The only commercially successful crustacean aquaculture sectors include species that have a larval phase of only a few days or weeks. With a larval phase of many months it is necessary to reduce phyllosoma mortality to an absolute minimum.

A major cause of larval mortality is believed to be due to opportunistic pathogenic bacteria. To improve phyllosoma survival, an effective strategy is required for microbial management. We have therefore employed a holistic approach to understand the microbial dynamics within the larval rearing system including scanning electron microscopy, traditional culture based and molecular analyses (DGGE; clone libraries) as well as fluorescent in situ hybridisation (FISH) and quorum sensing analysis. This effort is directed at 4 identified microbiological compartments in the rearing system; 1) the water column, 2) the biofilm, 3) the live larval feed (*Artemia*) and 4) the phyllosomas themselves.

Larval rearing runs are consistently characterised by a high rate of phyllosoma mortality. After 30 days of larval rearing survival rates are typically only 5-10% with increased mortalities being observed around the time of moult. In the early stages (of 11 stages) molting occurs approximately every 7-9 days. Mass mortalities often occur after 14-21 days of larval rearing and are indicative of bacterial infection. Histopathological examination reveals extensive proliferation of bacteria in the hepatopancreas and gut. Lesions are observed, including granulomas in the thoracic connective tissue. FISH analysis revealed bacteria within and through the hepatopancreas tubule lumen and intestine with the majority of the bacterial community being represented by vibrios.

The culturable microbial community within the system is diverse with representative members from the Alphaproteobacteria, Actinobacteridae, Firmicutes, Cytophaga-Flavobacetri/Bacteroides and Gammaproteobacteria identified from each compartment of the larval rearing system. Dominant strains isolated from the water, biofilm and phyllosoma environments included *Vibrio parahaemolyticus, V. alginolyticus*, and *V. proteolyticus.* DGGE fingerprints revealed a dynamic bacterial community in the various compartments. Surveys of the bacterial community for the thermolabile hemolysin (tlh), thermostable direct hemolysin (tdh) and toxR genes revealed only the presence of the tlh gene. However, colonies exhibiting hemolysin activity on blood agar plates were not positive for tlh. The identification of the protease is presently unknown. Acyl-homoserine lactone (AHL)-dependent quorum-sensing (QS) systems are detectable from day 3 of larval rearing. Peak AHL activity was found to occur at the time of mass mortalities of phyllosomas. AHLs were found in the biofilm and phyllosomas.

Phyllosomas are readily colonised by a diverse consortia of marine bacteria. Scanning electron microscopy (SEM) analysis demonstrated adhesion of filamentous bacteria to the carapace within 2-3 days post-molt. By 7 days post-molt the maxillipeds and basal and coxal endites are extensively fouled and entangled. The fine setae of the second maxillipeds are highly fouled. Coccoid, but not filamentous, bacteria are observed in the region of the anus. FISH and clone library analysis have confirmed that external fouling is primarily due to *Thiothrix*-like filamentous bacteria and is initially restricted to the mouthparts. The bacteria clearly interfere with the ability of the phyllosoma to process and masticate feed and likely leads to a progressive decline in nutritional status of the phyllosoma and susceptibility to opportunistic pathogenic bacteria. Both histopathology and FISH evidence indicates infection and invasion of the digestive tract and hepatopancreas of putative pathogenic strains of *V. alginolyticus*. A significant proportion of isolated *V. alginolyticus* posses at least one hemolysin gene. However, active exo-protease production is observed in strains that test negative for hemolysin genes. Virulent factors may be controlled by AHL-dependent quorum-sensing systems.

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INVESTIGATING THE FATTY ACID REQUIREMENTS OF LARVAL STRIPED TRUMPETER *LATRIS LINEATA* DURING THE LIVE FEEDING PERIOD AND USING DOSE-RESPONSE TECHNIQUES

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Determining fatty acid requirements in larval fish feeding on live prey is difficult. Many early investigations used enriched live foods with a variety of commercial preparations, fed them to larvae, and then based the dietary fatty acid requirement on the diet supporting maximum growth. However, commercial preparations contain differing amounts of essential nutrients and it is not always clear why performance is enhanced. Specialty oils are now available that contain large proportions of the essential fatty acids for marine fish, namely ARA (20:4n-6), EPA (20:5n-3), or DHA (22:6n-3). Using combinations of these speciality oils and other components we have developed oil-based emulsions with graded amounts of the target nutrient while keeping other essential nutrients constant. These emulsions facilitate nutrient requirement studies using dose-response designs similar to those more easily undertaken in juvenile and adult fish using formulated feeds.

Striped trumpeter is an Australian, marine finfish with aquaculture potential. Our research has recently focused on determining fatty acid requirements during the larval phase. In three separate dose-response experiments we determined the dietary DHA and ARA requirement of striped trumpeter larvae during rotifer and *Artemia* feeding in replicated, 300-l tanks. The first experiment used seven experimental emulsions with graduated n-3 polyunsaturated fatty acid (PUFA) and constant ARA to enrich rotifers. Larvae were fed from 5-18 days post-hatch (dph). Neither survival nor growth was affected by the range (10-31 mg n-3 PUFA·g dry matter^{-1}, DM) of n-3 PUFA in the diet. An inverse relationship between larval and dietary concentrations of DPA-3 (22:5n-3) was recorded, and

the presence of DPA-3 in elevated amounts in larvae when dietary DHA was suppressed suggested elongation of EPA in an attempt to raise tissue DHA concentrations. This trend between tissue DPA-3 and dietary DHA was used to calculate a dietary requirement of 12.7 mg DHA·g DM^{-1}. During this experiment an inverse relationship between dietary n-3 PUFA and the incidence of vacuolation (lipid droplets) identified histologically in the gut enterocytes and liver suggested problems with lipid transport when the diet was lacking in n-3 PUFA. Further, larvae fed low n-3 PUFA were startled by light and had reduced feeding compared with their counterparts on higher dietary n-3 PUFA.

A second experiment determined the dietary DHA requirement of larvae during the *Artemia* feeding period (16-36 dph). Five experimental emulsions were used to enrich *Artemia* to produce a diet with graduated DHA (0.1-20.8 mg·g DM^{-1}) and constant ARA. At 36 dph, a significant, positive relationship was identified between larval growth (dry weight and standard length) with dietary DHA, indicating 20.8 mg DHA·g DM^{-1} was optimal for near-maximum growth in striped trumpeter larvae fed *Artemia*.

In the last experiment larval growth and survival was unaffected when they were fed rotifers with graduated ARA (1.3-11.2 mg·g DM^{-1}) but constant n-3 PUFA and 18:2n-6. Larvae receiving higher dietary ARA had elevated total prostaglandin E and F concentrations and were less resistant to a hypersaline challenge. The experiment demonstrated that increasing dietary ARA above 1.3 mg·g DM^{-1} had no benefit for striped trumpeter larvae.

Dose-response designs using live feeds are technically challenging and labour demanding. However growth and survival curves generated from such intensive studies are an excellent means for quantifying nutrient requirements. Further, the use of additional techniques to assess larval performance, such as physiological challenges, biochemistry, determination of feed intake, tissue histological examination, and behavioural observations, complement more conventional performance indices, and are now routinely used in our larval experiments. Finally, we are attempting to develop greater consistency with larval nutrient requirement studies within and between species to allow better comparisons of nutrient requirement data. To this end the dose-response formulations and methods discussed here have since been applied to studies investigating the fatty acid requirements of Atlantic cod and Senegal sole larvae in a collaborative project involving researchers in Australia, Scotland, and Spain.

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EFFECTS OF BROWN TIDE ON HARD CLAM LARVAE AND IMPLICATIONS FOR POPULATION RECRUITMENT

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Introduction

Brown tides of the picoplanktonic alga, *Aureococcus anophagefferens* (Pelagophyceae, ~2µm equivalent spherical diameter), have occurred for nearly two decades in shallow, temperate bays in the mid-Atlantic USA, where they can attain maximum densities of up to 1000-2000 cells.µl^{-1} (Bricelj and Lonsdale, 1997). They are known to adversely affect juvenile and adult bivalves, including the hard clam, *Mercenaria mercenaria*, an important commercial and recreational fishery and aquaculture species, via arrestment of gill ciliary currents and thus feeding inhibition (Bricelj et al., 2001). The toxin produced by *A. anophagefferens* has not been chemically characterized but its action has been described as analogous to that of the neurotransmitter dopamine (Gainey and Shumway, 1991). Brown tide is most commonly a summer phenomenon and therefore coincides with the period of spawning and larval development of hard clams in this region, yet limited information is available on the effects of brown tide on larval stages of bivalves. It is expected that larvae will be more vulnerable to the adverse effects of brown tide than post-metamorphic stages. The only larval study conducted to date was on bay scallops, *Argopecten irradians* (Gallager et al., 1989), yet effects of harmful algae are known to be highly species-specific.

The main objectives of this laboratory study are thus to investigate the density-dependent effects of a toxic isolate of *Aureococcus anophagefferens* on growth and survival of critical larval stages of *M. mercenaria* (from D-stage to pediveligers). Experimental results are being incorporated in a larval biochemically-based numerical simulation model (Hofmann et al., 2003) to assess the possible influence of brown tide on recruitment success of natural clam populations in US Atlantic bays and thus evaluate the potential for rehabilitation of this valuable fishery via restocking efforts.

Materials and methods

Hard clam broodstock were conditioned and induced to spawn at IMB's Marine Research Station. First-feeding veligers (D-stage larvae) were reared in aerated 17-l conicals at 22°C and 30ppt salinity (three replicate tanks per treatment) and subsampled every 3 days to determine shell lengths and cumulative mortalities using video microscopy. Larvae were offered a baseline, optimum diet [50 *Isochrysis galbana* (t-Iso) cells.µl^{-1}] supplemented with increasing concentrations of a toxic isolate of *A. anophagefferens* (CCMP 1708) from West Neck Bay, New York, ranging from 100-800 cells.µl^{-1}, for 15 days. Controls consisted of optimum, and high (150 t-Iso cells.µl^{-1}) concentrations of clone t-Iso to account for concentration-dependent effects. The high-density control was equivalent in biovolume to the highest brown tide treatment. A non-fed treatment held in 0.22-µm-filtered seawater was also included to determine whether brown tide mimicked the effects of starvation.

The relative ability to graze upon (ingest) algal cells was determined in a separate experiment in which larvae were exposed for 2-4d to the following treatments: 1) non-fed, 2) unialgal *A. anophagefferens* at 400 cells.µl^{-1}, and 3) unialgal t-Iso at a volume equivalent cell density of 50 cells.µl^{-1}, and gut contents of individual larvae were observed qualitatively by epifluorescence microscopy. All experiments were conducted by adding non-axenically cultured microalgae harvested in late exponential growth phase, as previously described by Bricelj et al. (2001).

Conclusions

Larvae experienced significantly higher mortalities and lower growth rates in all treatments supplemented with *A. anophagefferens* than in the two t-Iso controls. Cumulative mortalities at 15d were comparable at brown tide levels ranging from 200-800 cells.µl^{-1}, and comparable to those in the non-fed treatment. Adverse effects on growth and survival were time-dependent (detected earlier at higher *A. anophagefferens* densities). Growth rates were inversely related to the concentration of brown tide. It is noteworthy that the lowest *A. anophagefferens* concentration tested in the present study (100 cells.µl^{-1}) was sufficient to elicit significant reduction in growth relative to controls (~66% at 15d of exposure), whereas hard clam juveniles (~7mm in initial shell length) were able to acclimate and fully recover after ~2wks of exposure to a comparable concentration (80 *A. anophagefferens* cells.µl^{-1}) offered in a mixed suspension with t-Iso (Bricelj et al., 2004).

Observations of gut chlorophyll *a* autofluorescence revealed that early veligers had empty guts (comparable to the starved treatment) when fed the unialgal suspension of *A. anophagefferens* at bloom levels for 4 days but not at 2 days, sug-

gesting that feeding incapacitation is time-dependent and requires a few days of exposure to take effect. Deleterious effects on larval ingestion, growth and survival are not attributable to the effects of high cell density or small algal size, but the mechanism of action remains uncertain.

The preliminary experiments reported in this study were unable to discriminate between the toxic effects of *A. anophagefferens* cells and the associated algal filtrate. However, previous studies have shown that the toxic effects in adult bivalves are only elicited by contact with cells and not by dissolved metabolites present in cell-free filtrates (reviewed by Bricelj and Lonsdale, 1997). Additional research is required to determine whether this is also true for larvae.

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INFLUENCE OF TANK VOLUME ON VITELLOGENESIS AND SPAWNING PERFORMANCES IN SEABASS (*DICENTRARCHUS LABRAX*)

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Introduction

Fish sexual maturation and reproduction in captivity are influenced by environmental factors such as photoperiod, temperature, salinity, oxygenation, and water quality, but also by rearing factors such as density, confinement (during the manipulations), and rearing volume. All these factors can have a limiting or an activating role on the reproduction and can have an influence on the spawn quality (Bromage and Roberts, 1995).

Studies concerning the effects of breeding density on fish growth and welfare showed that this factor influences the social behavior (Ellis et al., 2002). It can be suggested that the breeding volume could influence fish welfare and its spawning capacities. In *Epinephelus akaara*, Okumura et al. (2002) showed that water height and pond volume could limit the implementation of the reproductive behavior preceding the spawn.

We tried to estimate the influence of tank volume during gametogenesis on individual spawning performances and identify a minimum breeding volume which would block fish reproduction. The present study was conducted in sea bass *Dicentrarchus labrax* which spawns spontaneously in captivity. For this species, the most important factors (temperature and photoperiod) required for the good maturation are identified and controlled; nevertheless aquaculturists encounter problems due to the wide variation of gamete quantity and quality (Fornies et al., 2001).

Materials and methods

Sea bass (initial mean weight: 748±13g, mean±SD) were reared until the end of vitellogenesis (from the end March to the end of December) in 1, 3, 8, 16, and 32-m^3 tanks and were subjected to natural variations of photoperiod and tem-

perature of Brest harbor. The rearing density (equal for all sizes of tanks) was 3 fish.m^{-3} and sex ratios were 2 males for 1 female. At the end of vitellogenesis, breeders were transferred in 2-m^3 spawning tanks. In each of the 15 spawning tank, 1 female was placed with 2 males (n=3 females for each rearing volume of the first period). Individual spawning performances were assessed (e.g., number of spawn, fecundity, viability rate, hatching rate, larval deformation rate).

Results and discussion

During the first two months of the experimentation, growth rate was significantly (Newman-Keuls test, P>0.05) lower or nil in smaller volume tanks (1m^3) compared to larger volume tanks (8, 16, and 32m^3). In August, oocyte diameters were significantly (Newman-Keuls test, P>0.05) lower in the smallest ones (1, 3, and 8m^3) compared to the largest ones (16 and 32m^3).

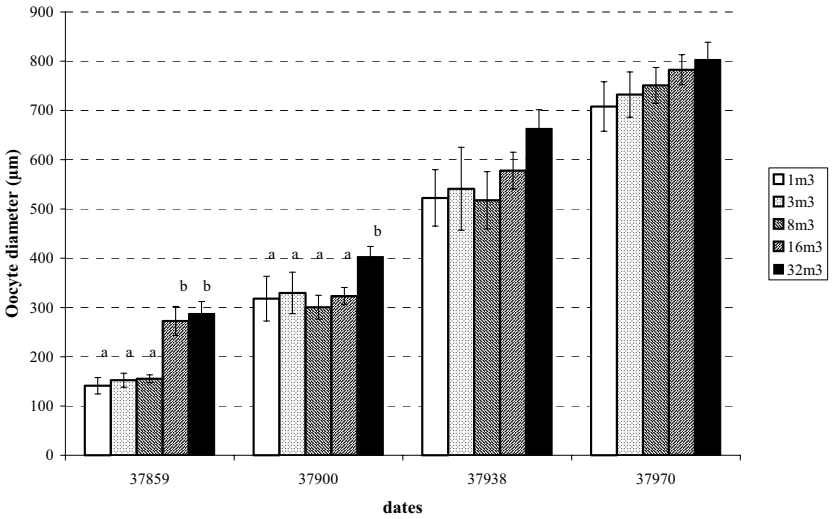

Fig. 1. Effects of tank volume on changes in oocyte diameter from the end of March to the end of December (same letter denotes homogeneous groups (P>0.05))

At the end of the experiment, fish mean weight in the 1-m^3 tank was significantly lower than in the 3-m^3 tank, but oocyte diameters were not significantly different between tank volumes (Fig. 1). This suggests a longer acclimatization process in smaller rearing volumes. Changes in levels of plasma oestradiol in females and levels of plasma 11-ketotestosterone in males were not significantly different between the different tanks. A peak of plasma oestradiol was observed four months before the first spawn collection. Some females did not spawn (7 of 15) but at least one spawn was collected for each rearing volume. For the smallest volumes, the more difficult period of acclimatization (the first three months, until the end of June), results in a bad specific growth rate. But it does not seem

to have an influence on the sexual maturation. There is no significant difference of oocyte diameters at the end of vitellogenesis. A delayed growth was only seen for the smallest volume ($1m^3$), resulting in a lower specific growth rate (Table I).

Table I. Female characteristics at the end of vitellogenesis. The values for oocyte diameter correspond at mean±SD.

♀	Rearing volume	Pre spawning weight	SGR	Oestradiol	Oocyte diameter 15/12/03	N° of spawns
14	3	2237	0.182	1.715	666.0±8. 1	2
11	8	1680	0.187	0.928	835.3±12.1	2
13	8	1400	0.223	0.467	848.0±12.4	2
3	16	1562	0.234	0.847	790.0±11.7	2
7	16	1583	0.213	1.687	830.0±11.9	2
15	1	1469	0.140	0.450	660.0±9.3	1
8	16	1656	0.178	1.123	788.0±10.3	1
6	32	940	0.173	1.484	797.3±6.3	1
2	1	1256	0.108	0.958	770.7±12.6	0
5	1	936	0.033	0.315	693.3±18.0	0
4	3	1291	0.209	2.057	816.0±10.1	0
9	3	1674	0.158	1.599	714.7±9.3	0
1	8	1177	0.191	0.433	795.3±12.3	0
10	32	919	0.315	0.477	825.3±9.7	0
12	32	1465	0.238	0.696	828.0±8.9	0

Table II. Female spawning performances. Two numbers for the same female and the same parameter, corresponds to the values of two spawns.

♀	Rearing volume	No. of spawns	Fecundity (eggs. $spawn^{-1}$)	Relative fecundity ($eggs.kg^{-1}$)	Viability rate (%)	Hatching rate (%)	Larval malformation rate (%)
14	3	2	387 260	379 868	88.9	68.3	8.3
			462 500		94.5	70.7	19.7
11	8	2	414 690	469 943	77.6	76.8	13.6
			374 820		72.9	78.3	5.3
13	8	2	207 610	276 258	64.7	31.3	62.0
			179 150		80.1	51.6	19.7
3	16	2	173 450	309 458	52.0	38.3	13.5
			309 920		88.7	75.8	17.6
7	16	2	288 980	215 352	83.5	89.8	11.6
			51 920		83.5	45.0	16.3
15	1	1	35 240	23 988	63.6	21.9	18.2
8	16	1	133 930	80 878	0.0	0.0	0.0
6	32	1	95 640	101 748	98.2	92.3	29.6
2	1	0	-	-	-	-	-
5	1	0	-	-	-	-	-
4	3	0	-	-	-	-	-
9	3	0	-	-	-	-	-
1	8	0	-	-	-	-	-
10	32	0	-	-	-	-	-
12	32	0	-	-	-	-	-

The spawning quality indicators (fecundity, viability, hatching, and larval malformation rates) do not show significant differences between females (Table II). A high individual variation of spawning performances was observed. The lack of spawning observed in a few females cannot be attributed to fish age since it was equivalent to seabass used by Fornies et al. (2001).

Conclusions

Spontaneous spawning was not hindered in seabass kept in 1-m^3 tanks during vitellogenesis. High individual variations of spawning performances were recorded. It has not been possible to demonstrate a significant effect of the tank volume ranging from 1-32m^3, on spawning quality. However, a 1-m^3 tank is supposedly limiting for sea bass reproduction due to influence on the welfare of the fish. Further work should be carried out on the effect of rearing density on spawning performances.

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SPERM QUALITY EVALUATION IN FISH

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Introduction

Sperm characteristics have been investigated in order to determine a group of parameters that allows the evaluation of sperm quality. The choice of the appropriate spermatic parameters depends on sperm application (e.g., laboratory fertility studies, sperm cryopreservation, sperm bank or production strategies).

In the present work we have determined some sperm characteristics in Senegalese sole (*Solea senegalensis*). There is no information on sperm quality in this species, and all reproductive problems have been attributed to the bad quality of the eggs.

Other studies, such as sperm cryopreservation, require the evaluation of milt prior and after cryopreservation. Specific biomarkers that allow the evaluation of the spermatozoon are used to check sperm quality. In sperm banks, also genome must be conserved and DNA should be evaluated. In the present study, several markers of sperm quality were checked in fresh and cryopreserved sperm from normal and sex-reversed rainbow trout (*Oncorhynchus mykiss*) and from gilthead seabream (*Sparus aurata*).

Materials and methods

Sperm extraction and preparation

Rainbow trout sperm was collected from normal males (n=10) using a catheter introduced in the urogenital pore. Sex-reversed males (n=10) were sacrificed to extract testicular sperm using a bisturi and sperm was incubated in a maturating medium (Maturfish, IMV) during 2 h. For cryopreservation, sperm was diluted 1:3 (v/v) in the extender medium (#6 Erdhal and Graham medium + 7% DMSO and egg yolk), loaded into 5 ml straws and frozen in liquid N_2 (10min exposed

horizontally at 1cm from N_2 surface). Sperm thawing was performed in a 60ºC water bath for 30s. Gilthead seabream sperm was extracted (n=10) using abdominal massage, collecting the sperm with a syringe without needle. Half of each sample was cryopreserved diluting the sperm 1:6 (v/v) in the extender medium (1% NaCl + 5% DMSO + 10mg.ml^{-1} BSA). Sperm was loaded into 5 ml straws and frozen in a styrofoam box as describe before. Sperm thawing was performed in a 60ºC water bath for 30s. Senegalese sole sperm was extract (n= 20) pressing the testicles and collecting the sperm with a 1-ml syringe. Sperm was introduced in Eppendorf tubes and maintained refrigerated (10ºC) until analysis.

Fresh and frozen/thawed sperm analysis

Seminal plasma osmolarity, pH, cell concentration, and sperm volume were measured in fresh samples using the same procedure for all species.

Sperm motility was determined activating 1µl of sperm with 200µl of seawater for marine species or DIA532 activating solution for freshwater species (Billard, 1977). Cell viability was determined using IP fluorescent dye in a flow cytometer. The percentage of non-fluorescent cells was identified as viable cells. Fertility was determined in rainbow trout using batches of 10 000 eggs and in seabream using batches of 2000 eggs. ATP content was determined by bioluminescence (ATP assay KIT CLS II, Boeringer). Mitochondrial status was measured by flow cytometry using the fluorescence probe JC1 (Molecular Probes). Cells with mitochondria membrane low potential (green cells-MMLP) were recorded as damaged cells. Sperm resistance to osmotic solutions was determined using flow cytometry and IP dye. Rainbow trout spermatozoa were exposed to 10 and 300mOsm.kg^{-1} solutions for 2min and the viability of cells was measured under these conditions. For seabream sperm, exposure was performed in a 300 and 1100 mOsm.kg^{-1} solutions and the resistance of spermatozoa for 5min was recorded. Nucleus DNA was assed using the comet assay method. DNA fragmentation was measured by single-cell gel electrophoresis using BrI to visualize the comets. Images were recorded and the percentage of DNA fragmentation was determined using Komet 5.0 software.

Results and discussion

Results showed in Table I demonstrated that the volume of sperm collected is very variable within and between the analysed species. Senegalese sole produces very small quantities of milt which could be responsible for the problems encountered in the reproduction of this species. Also, cell concentration was lower compared with the values registered for rainbow trout or seabream, but is in accordance with the values reported for other flatfish species, such as turbot (Chereguini et al., 2001).

Table I. Sperm characteristics from rainbow trout, gilthead seabream, and Senegal sole.

	Volume collected	Cell concentration	Osmolarity	pH
Rainbow trout	2-25ml	$17.1 \times 10^9.ml^{-1}$	305	8.0
Seabream	1-7ml	$11.2 \times 10^9.ml^{-1}$	389	8.3
Senegalese sole	10-60µl	$1.48 \times 10^9.ml^{-1}$	344	-

Other specific characteristics of milt, such as motility, could be a good indicator of sperm quality. After sperm activation by contact with fresh or seawater, spermatozoa movement from several species decrease in few seconds (Rurangwa et al., 2004). In *S. senegalensis*, sperm motility last no more than 1.30min after activation with seawater (data not show), and we have detected a variable range of motility within males (Table II). Sperm motility in fish is sometimes correlated with fertility (Billard, 1988). According to these data, sperm quality and production in *S. senegalensis* could be one of the principal causes of the appearance of a high number of non-fertilized eggs.

Normal rainbow trout, sex-reversed trout, and seabream males produced high motility sperm (Table II).

Table II. Biomarkers of sperm quality determined in fresh and frozen sperm from normal and sex-reversed rainbow trout males, gilthead seabream, and sole.

	Trout	Sex-reversed trout	Seabream	Sole
Fresh Sperm				
Sperm motility (%)	91.1	90.8	95	40-75
Sperm viability (%)	98.9	91.6	-	
Fertility (%)	89.5	82.7	77	
ATP (%)	-	4.52×10^{-8}	3.17×10^{-5}	-
MMLP (%)	-	-	11	-
Sperm resistance (%)	94.5 / 96.6	68 / 90.5	85 / 78	-
DNA fragmentation (%)	11	-	28.2	-
Frozen Sperm				**-**
Sperm motility (%)	45	24.6	70	-
Sperm viability (%)	58.6	48	71.5	-
Fertility (%)	72	57.7	75.6	-
ATP (%)	-	7.37×10^{-8}	0.18×10^{-5}	-
MMLP (%)	-	-	22.7	-
Sperm resistance (%)	23.1 / 34.5	17.8 / 35.5	60 / -	-
DNA fragmentation (%)	30.3	-	31.4	-

The analysis of specific biomarkers of sperm quality is very important to check spermatozoa status. The determination of these parameters allows the detection of changes in sperm quality and is relevant in cryopreservation studies. Cryopreservation allows the storage of sperm material and can be used either in laboratory investigation or in production facilities. The determination of cryopreserved sperm quality is vital to assure the same fertility rates as in fresh sperm. A

slightly decrease in sperm quality after cryopreservation as been reported for several species. In the present study, sperm motility and viability slightly decreased in cryopreserved sperm when compared with fresh analysis. However, with the exception of sex-reversed rainbow trout sperm, fertility rates using cryopreserved sperm were as higher as in fresh.

The energetic metabolism of spermatozoa was analysed determining ATP content and the status of mitochondria. After cryopreservation, there was a decrease in ATP content in seabream sperm which could be related with the damage observed in mitochondria (Table II). Plasma membrane resistance is also a good indicator of cell status, because spermatozoa should be capable of maintain the osmotic equilibrium in different osmolarities. This test simulates the effect of motility activation during the process of fertilization, and as been correlated with cryopreservation ability (Cabrita et al., 2001). Sperm resistance to osmotic solutions decreased after cryopreservation both for rainbow trout and seabream, being this test a good indicator of sperm quality after cryopreservation. DNA was also affected after cryopreservation, especially in rainbow trout were the percentage of DNA fragmentation reach the 30% (Table II). This test could be very important if we intent to create a sperm bank from specific males in selection programs.

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EARLY LARVAL CULTURE OF MARINE ORNAMENTAL DECAPODS: GETTING IT RIGHT FROM THE START

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Introduction

The culture of marine ornamental species is regarded as a sustainable alternative to the harvest of wild specimens for the aquarium trade industry. Although in recent years the culture of ornamental decapod crustaceans has deserved special attention, feasible protocols that allow commercial scale culture of the most valuable and/or heavily traded species are still missing (Calado et al. 2003a). One of the bottlenecks impairing the captive culture of ornamental decapods is early larval feeding, with most species displaying heavy larval mortalities in the first zoeal stages. Additionally, the few surviving larvae can delay their larval development for days or even weeks, and in most cases die before metamorphosing. Due to the extended larval development of most cultured species, it is important to evaluate larval quality at hatching in order to avoid wasting time and money in the culture of poor-quality larvae. The present work evaluates the importance of larval batch size and first zoeal stage starvation on larval survival of the fire shrimp *Lysmata debelius*, the cleaner shrimp *L. amboinensis* and *L. grabhami*, the emerald crab *Mithraculus sculptus*, the ruby crab *M. forceps*, and the arrow crab *Stenorhynchus seticornis*.

Materials and methods

Three mated pairs of *L. debelius*, *L. amboinensis*, and *L. grabhami* and six mated pairs of *M. sculptus*, *M. forceps*, and *S. seticornis* were kept separately in the laboratory during approximately 4 months (temperature 25±1ºC, salinity 34±1‰, photoperiod 12h light:12h dark) and were fed ad libitum 4 times a day a mix of frozen foods (adult *Artemia* biomass, adult *Artemia* enriched in *n*-3 fatty acids, adult *Artemia* enriched in *Spirulina*, newly hatched *Artemia*, mysids, shrimp, squid, and mussels). Total numbers of hatching larvae were counted per pair and classed as good- or poor-quality batches according to the number of hatched larvae being close to the number of expected larvae estimated by existing relationship between log adult size and log number of larvae. One hundred

larvae pooled from good- or poor-quality larval batch were randomly selected and placed in 10-l cylindro-conical fibreglass tanks (as described by Calado et al., 2003b) (temperature 25±1ºC, salinity 34±1‰, photoperiod 12h light:12h dark) and either supplied newly hatched *Artemia* nauplii at a density of 5 nauplii.l^{-1} immediately after hatching, or left under starvation for the first day after hatching and later supplied the larval diet previously described. Six replicates per treatment were used. Larvae of *L. debelius*, *L. amboinensis*, and *L. grabhami* were cultured until the fifth zoeal stage, while larvae of *M. sculptus*, *M. forceps*, and *S. seticornis* were cultured to megalopa.

Average number of surviving larvae of each species fed immediately after hatching, or starved during the first day after hatching, from good- or poor-quality larval batches, was compared using a one-way analysis of variance (ANOVA) using the software Statistica™ (version 6), after checking the assumptions. When ANOVA results were significant ($p<0.05$), the Tukey's multiple comparisons test was used (Zar, 1996).

Results and discussion

The average number of larvae produced per batch by the ornamental decapods in the present work varied through the study period (Table I).

Table I. Average (± standard deviation) number of hatched larvae of good- (GB) and poor-quality (PB) larval batches. n = number of larval batches.

	Average number of larvae	
	GB	**PB**
L. debelius	1243 ± 252 (n = 17)	182 ± 51 (n = 8)
L. amboinensis	2234 ± 361 (n = 15)	302 ± 124 (n = 7)
L. grabhami	1823 ± 182 (n = 12)	132 ± 31 (n = 10)
M. sculptus	551 ± 102 (n = 10)	103 ± 21 (n = 7)
M. forceps	493 ± 124 (n = 11)	92 ± 44 (n = 8)
S. seticornis	378 ± 92 (n = 8)	108 ± 32 (n = 7)

Poor-quality larval batches were generally observed during the first month of captivity, when most organisms were still acclimatising to laboratory conditions and diets. Among ornamental shrimp (*L. debelius*, *L. amboinensis*, and *L. grabhami*), poor-quality larval batches were recorded when shrimp moulted and several embryos could still be seen attached to the old exoskeleton. This type of event is generally a consequence of poor water quality, namely pH fluctuations and low Ca^{2+} concentrations, and could also be detected through the presence of "curled" larvae which were unable to inflate and assume the typical appearance of the first zoeal stage. Additionally, newly moulted adults also failed to assume their "normal" look, displaying twisted, curled antennas, rather than smooth and straight appendages. Concerning the studied marine ornamental crabs (*M. sculp-*

tus, *M. forceps*, and *S. seticornis*), poor-quality larval batches coincided with the presence of several embryos in the bottom of the maturation tanks. As for ornamental shrimp, the cause of this type of larval batches among crabs can also be a direct consequence of poor water quality. Since the studied crabs can become ovigerous without moulting, no exterior sign of "osmotic stress" could be detected on broodstock. After the stabilization of water quality parameters (ammonia, nitrite, and nitrate kept bellow detectable levels, pH between 8.0 and 8.2, and Ca^{2+} concentration at 400-420mg.l^{-1}), no poor-quality larval batches were ever recorded.

The effect of early larval starvation on good and poor-quality larval batches survival is resumed in Table II.

Table II. Average (%) (± standard deviation) survival of larvae fed immediately after hatching (F), or starved during the first day after hatching (ST), from good- (GB) or poor-quality (PB) larval batches. Different superscript letters in the same row represent significant differences ($P<0.05$).

	Average larval survival			
	F-GB	**ST-GB**	**F-PB**	**ST-PB**
L. debelius	87.2 ± 6.2^{a}	79.1 ± 4.2^{a}	59.3 ± 4.1^{b}	24.0 ± 5.7^{c}
L. amboinensis	82.6 ± 8.3^{a}	76.5 ± 2.3^{a}	52.2 ± 8.3^{b}	15.6 ± 7.4^{c}
L. grabhami	80.0 ± 5.2^{a}	73.8 ± 3.5^{a}	48.7 ± 6.3^{b}	11.1 ± 5.3^{c}
M. sculptus	92.5 ± 3.4^{a}	81.5 ± 5.2^{a}	62.6 ± 4.9^{b}	29.3 ± 2.4^{c}
M. forceps	96.4 ± 3.0^{a}	83.8 ± 6.7^{a}	68.0 ± 6.7^{b}	33.9 ± 5.6^{c}
S. seticornis	88.1 ± 1.3^{a}	72.2 ± 5.2^{a}	55.4 ± 9.3^{b}	18.1 ± 3.0^{c}

Starvation during the first day after hatching of good-quality batch larvae did not induce any significant deleterious effect on survival, when compared with larvae from the same batches fed immediately after hatching. However, since the larval development of none of the studied species was followed to metamorphosis, results should be regarded with caution, given that starved larvae may show higher mortality in later larval stages. In fact, Calado et al. (2005) reported that the negative effect of starvation of the first zoeal stage of Monaco shrimp *L. seticaudata* is more pronounced in late-stage larvae and during settlement. The effect of early starvation was significantly amplified when larvae from poor-quality batches were used. This result probably reflects the poor physiological condition of larvae exposed to osmotic stress. The fact that even larvae from poor quality batches fed immediately after hatching displayed lower survival than starved larvae from good quality larval batches reinforces the importance of the deleterious effects of precocious exposure to osmotic stress during the incubation period and/or after hatching. It is also possible that broodstock exposure to inappropriate water condition could have affected food consumption, inducing lower yolk reserves on hatched larvae. This possibility was visually confirmed through the absence of the typical bright coloration of yolk reserves (accumu-

lated on the posterio-dorsal end of the carapace) on larvae from poor-quality batches.

Conclusions

The present work shows that larval batch size can be a good indicator to assess the larval quality of marine ornamental decapods, and that only after fully stabilizing broodstock in captivity should larval culture be attempted. This procedure assures that only factors affecting larval culture (e.g., larval diets, temperature, rearing density) are truly being evaluated and that possible negative effects recorded are a consequence of unsuitable culture conditions, rather than promoted by poor larval quality. This study also reinforces the importance of providing suitable larval diets immediately after hatching to decapod larvae, since their plasticity can allow them to delay larval development for extended periods, leading to higher production costs. Additionally, the lower probability of successfully metamorphosing decreases larval production feasibility and increases the chances of poor juvenile shrimp production.

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IN VITRO EVALUATION OF THREE DIFFERENT CASEIN-BASED MICROCAPSULES FOR FIRST-FEEDING OF FISH LARVAE

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Introduction

Formulation of artificial feeds for early stages of marine fish is a highly desirable objective in order to reduce the dependency of cultures on live food. Despite recent developments in this field, complete replacement of live food still do not result in acceptable growth and survival rates during the early larval stages of most species (Yúfera et al., 2002). Several problems, related to the stability of particles in water, the inclusion of macro and micronutrients as well as attraction to ensure adequate intake, must be solved. Within this context, it was considered of interest to evaluate the potential use of a new type of protein microparticle developed using the technique of complex coacervation (CC), which is characterized by not using aggressive chemicals. The present work was aimed to evaluate the physical properties of the CC particles prepared using casein and protamine, as well as perform a preliminary in vitro assay of their digestibility. This approach has proved to be highly valuable in testing micro feeds prior to their in vivo evaluation (Alarcón et al., 1999). Experiments were designed to compare CC particles with *Artemia* nauplii and other two different types of microparticles previously developed by Yúfera et al. (1996) and Fernández-Díaz and Yúfera (1997).

Materials and methods

CC microparticles were prepared by addition of 1% pH 10 protamine solution drop wise in 100ml of 1% pH 11 casein solution maintained under agitation by a Polytron. Casein was selected as the main ingredient because of its high nutritional value and good digestibility, while protamine was used since is a peptide containing a high number of arginine residues, a target amino acid for protein hydrolysis carried out by trypsin present in the gut of larvae. When the coacervate suspension flocculated, 12ml of 1N NaOH were added and the precipitated resuspended. After stirring, particles were collected, washed with a saline solu-

tion (3.5% NaCl), and stored at 4°C until used. Size distribution of the particles was measured by laser light scattering (Mastersizer) and morphology observed by scanning electron microscopy. Floatability of particles in saline water (3.5% NaCl) was measured gravimetrically using a Soerensen cylinder. Casein-based microcapsules prepared by interfacial polymerization (IP) or ionic gelification (IG), as well as freeze-dried *Artemia* nauplii, were prepared as described in Yúfera et al. (1996) and Fernández-Díaz and Yúfera (1997). In vitro assays were carried out by incubating enzyme extracts obtained from 15-day-old sea bream *Sparus aurata* larvae (2.4 unit protease activity.ml^{-1}) with the different microparticles or *Artemia* nauplii in carbonate buffer (pH 8.5). Assays were adjusted to a ratio enzyme:protein of 0.3U.mg^{-1}. Mixtures were continuously agitated in a rtary digestion vessel (T^a=24.3±0.8ºC). Samples were taken at different moments (0, 0.5, 2.5, 4.5, 6.5, and 8h) and centrifuged immediately (16 000g, 10min, 4ºC). The supernatant was used for free amino acids (Chu et al., 1983) and soluble protein (Bradford, 1976) determination. A duplicate set of tubes without the enzyme extract was used to evaluate auto-hydrolysis and leaching of samples.

Results and discussion

An example of the internal and external morphology of the CC microparticles is shown in Fig 1. It seems they are formed by the aggregation of a number of smaller units but the final result presents a good spherical shape with a slightly porous surface. Previous analysis has shown that this structure enables entrapping of lipid-walled microparticles and liposomes and results in greater stability. Under the conditions used in the present experiment a significant proportion of the obtained particles (40.43±7.02%) were in the range from 50-200µm. This is an interesting result since intake of particles exceeding 200-250µm may be severely limited by mouth size of most larvae, while those lower than 50-75µm could be less attractive.

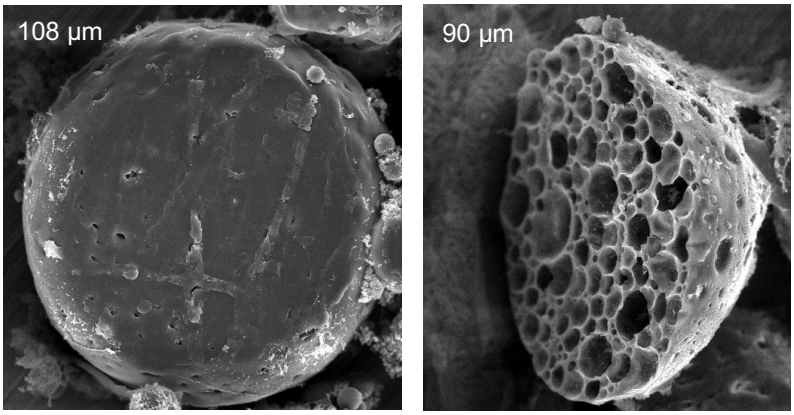

Fig. 1. Photomicrograph of casein microparticles coacervated with protamine. The upper numbers indicate the total width of the picture.

Regarding the floatability of particles within the indicated size range, 60% sedimented in the first 10min, but after this time a slow sink took place, with 20% of them remaining in the water column after 30min. This is also an interesting feature, considering that measures were carried out in a static water column which lacks of the aeration usually provided in larval rearing tanks that could provide extra buoyancy to the microparticles.

Some of the main problems to be solved when developing a microparticle suitable for feeding of fish larvae are related to their stability in water (what greatly determines both their nutritive value and water quality) and the ability of being digested by the scarcely developed gut of the larvae (Yúfera et al., 2000; Langdon 2003). Results obtained in the in vitro assays showed that, in the absence of protease activity, IP and especially IG particles were characterized by a high level of leaching, evidenced by the release of soluble protein to water. In contrast, both *Artemia* nauplii and CC particles were characterized by a low liberation of protein to water (Fig. 2). When enzyme extracts were added to the mixtures, all the particles were progressively hydrolyzed, this being measured by the increase of free amino acids in the medium (Fig. 2). In this case, similar digestion rates were observed for the three assayed particles, while a high release of free amino acids, even without the addition of proteases (control), was measured in *Artemia* nauplii. This result could be explained considering the high contents in those molecules existing in this live food.

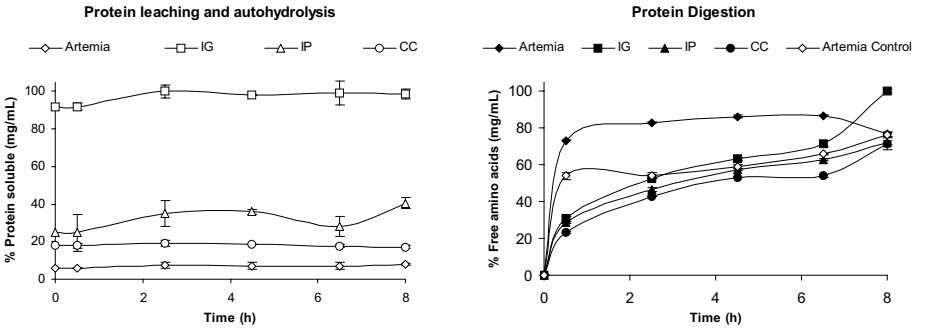

Fig. 2. Protein leaching and digestibility of *Artemia* nauplii, CC, IP, and IG microparticles under in vitro conditions. Values are the mean of two duplicates.

Conclusion

A preliminary evaluation of the microparticles developed using the technique of complex coacervation show that their stability in water and degradability by larval digestive enzymes could make them interesting candidates for further developments in the field of artificial feeds for larvae of marine fish.

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FASTING INDUCES HEAT SHOCK PROTEINS EXPRESSION IN LARVAL FISH

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Introduction

Quantifiable indicators of stress have been sought by aquaculturists to monitor the effect of husbandry conditions and practices on larval rearing. Within this context, the expression of heat shock proteins holds promise. As the protein synthetic machinery is up-regulated in the early life stages and feeding is crucial during this phase, we hypothesized that nutrient deficiency will induce hsps in fasting larvae. To this end, we investigated fasting influences on hsp70 and hsp90 expression in sea bream (*Sparus aurata*) larvae.

Materials and methods

Sea bream larvae were reared in the facilities of the ICMAN (Puerto Real, Cadiz, Spain). At 22 days after hatching, a number of larvae were separated into four experimental groups in different tanks. One group was deprived of food for 12h, another one was placed in a warm (25ºC) water tank for 1h, and a third one was held under reduced oxygen (3.5mg.l^{-1} O_2) for 1h. A control group was held under the normal rearing conditions (regularly fed on *Artemia* nauplii, 5.3mg.l^{-1} O_2, and 20ºC). In each case, a group of 10-12 larvae were sampled 1h after stressor exposure and also after a 24-h recovery period. The larvae were killed by submersion in ice-cold water (4ºC), washed quickly with tap water, deep-frozen, and freeze-dried for later determination of HSP70 and HSP90 expression.

Freeze-dried sea bream larvae were powdered using a ceramic mortar maintained at low temperature by continuous addition of liquid nitrogen. The samples were diluted (250mg.ml^{-1}) in Tris 50mM, pH 7.5 buffer containing a commercial cocktail of protease inhibitors (Sigma). The homogenate was sonicated (couple of pulses), centrifuged (14 000rpm, room temperature, 3min), and the supernatant was diluted 1:1 using 2× Laemmli's buffer, boiled (95ºC for 5-8min), and

kept frozen at -20°C. The protein concentration of the homogenate was determined using the bicinchoninic acid with bovine serum albumin as standards.

Western blot analysis was carried out to assess HSP70 and HSP90 expression in samples according to established protocols (Vijayan et al., 2003). The primary antibody for HSP70 was a mouse monoclonal antibody while HSP90 was probed using a rat polyclonal antibody (StressGen, Vancouver). The band intensities were scanned and quantified Chemi imager^{TM} using the AlphaEase software (Alpha Innotech, CA) and expressed as percent of fed control. The data were compared using either one-way analysis of variance (ANOVA) or two-way ANOVA (fasting and heat shock as independent variables), respectively. The data were log-transformed to meet the criterion of homogeneity of variance, while non-transformed data are shown in the figures.

Results and discussion

In sea bream larvae, the HSPs were induced very quickly (1h of stressor exposure).The complete recovery of HSPs in sea bream after stressor exposure within 24h suggests a faster turnover of HSP70 and HSP90. The 22-d-old sea bream larvae had significantly higher HSP70 protein expression in response to a 12-h food deprivation compared to the fed control larvae (Fig. 1). The HSP70 response to food-deprivation was similar in magnitude to that seen with 1h heat shock (+5^{o}C) in sea bream larvae. The HSP70 was undetectable in the fed control, and both food-deprivation- and heat shock-induced HSP70 expression returned to undetectable levels after 24h recovery. Exposure of sea bream larvae to 1h reduced oxygen (3.5mg.l^{-1}) appeared to induce HSP70 expression, but the changes were not statistically significant (Fig. 1).

HSP90 protein expression was significantly induced and similar in magnitude, by food-deprivation, heat shock and reduced oxygen levels in sea bream larvae (Fig. 2). The enhanced HSP90 response to food-deprivation and heat shock was similar to that seen with HSP70, except that the magnitude of induction was greater for HSP90 (~150) compared to HSP70 (~75%). HSP90 appeared as a doublet in sea bream larvae, whereas only a single band was apparent in rainbow trout larvae. Recovery of larvae from all three stressors resulted in the HSP90 content returning to pre-stress control levels (Fig. 2).

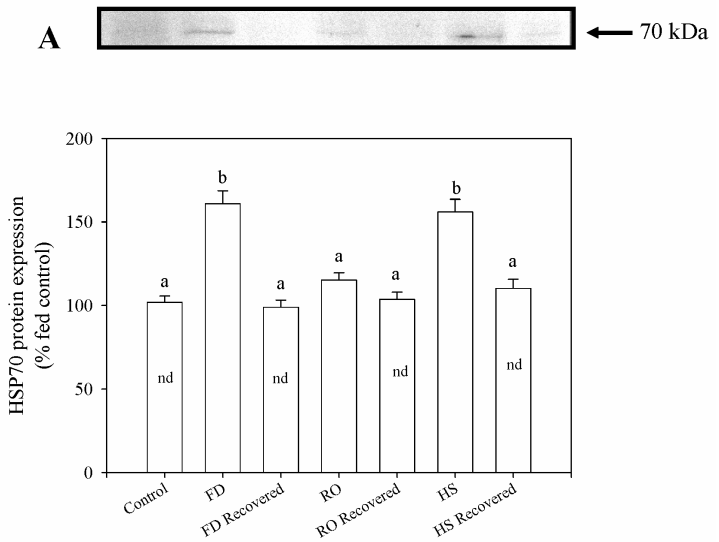

Fig. 1. HSP70 protein expression in sea bream larvae. Twenty-two-day-old sea bream larvae were either food deprived (FD), exposed to reduced oxygen (RO), or heat-shocked (HS; +10°C) and were allowed to recover for a 20-h period before determining HSP70 protein expression. (A) Representative western blot of HSP70 protein expression and the lanes on the blot correspond with the bars on the histogram. (B) Densitometric values of HSP70 protein were quantified and expressed as percent fed control. Values represent means + SEM (n = 4; each sample consisted of 3-4 pooled larvae); different letters denotes significant differences (One-way ANOVA, $p<0.05$).

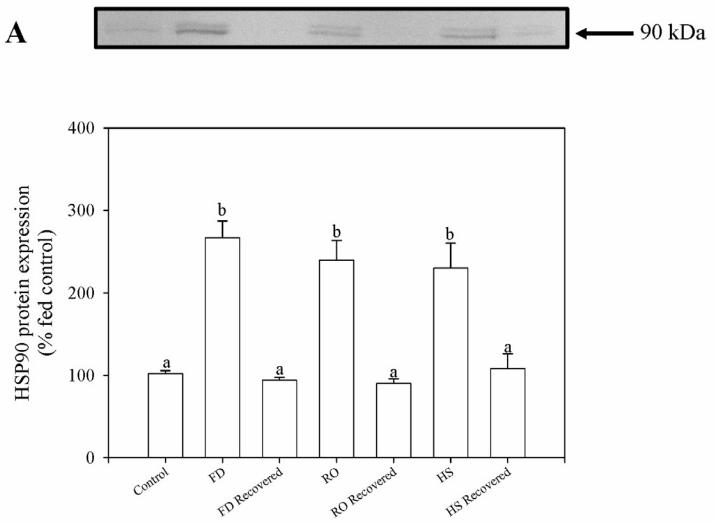

Fig. 2. HSP90 protein expression in sea bream larvae. All details as in Fig 1.

Our results demonstrate for the first time that food deprivation stimulates HSP70 and HSP90 protein expression in early life stages of fish. The induction of heat-shock proteins are commonly used as indicators of cellular stress and increases in response to stressors impacting protein homeostasis (Parsell and Lindquist, 1994). Although the mechanism for heat-shock induction with food deprivation was not explored in this study, it is abundantly clear that the early life stage is a period of rapid growth involving both protein synthesis and breakdown (Mommsen, 1997). This high protein turnover is sustained by influx of free amino acids from food (Ronnestad et al., 1999) and, consequently, amino acid restriction associated with food deprivation may lead to enhanced proteolysis in larval fish (Conceicao et al., 1997). This enhanced endogenous tissue breakdown may affect cellular protein homeostasis leading to poor growth and survival, as seen in larval fish deprived of food (Cunha et al., 2003).

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FATTY ACID COMPOSITION OF SPOTTED SAND BASS *PARALABRAX MACULATOFASCIATUS* DURING EARLY ONTOGENY

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Introduction

In marine fish with indirect ontogeny, such as spotted sand bass, the digestive capacity is limited since the digestive tract is undifferentiated in early stages (Peña et al., 2003). Studies of fatty acids (FAs) during early ontogeny in this species can help improve survival and growth. FAs have an important role in larval nutrition, as energy and essential fatty acid (EFA) sources for their development (Fernández-Borras, 1993). The objective of the present study was to evaluate the FA composition of spotted sand bass during the early ontogeny under laboratory culture conditions.

Materials and methods

Eggs from 10 spawns were reared in cylindro-conical tanks within a closed system (SC-12), described by Alvarez-González et al. (2001). Fertilized eggs were maintained at 23°C, 37ppt salinity, 6mg $O_2.l^{-1}$, and 7.4µg NH_4-$N.l^{-1}$. Hatching was designated d0 and the experiment lasted 30d after hatching (DAH). Total lipids and fatty acid contents were recorded for different stages and specimens: embryos (d0), eleutheroembryos (1dah); fed and starved larvae (2, 3, 4dah); fed larvae (7, 10, 15, 20, 25, 30dah). Larvae were fed with rotifers (*Brachionus plicatilis*) and *Artemia franciscana* (nauplii, juvenile, and adult). The samples were rinsed with formic acid 0.4M, freeze-dried, and stored at -80°C until biochemical analysis. Analysis was also made of total lipids by extraction with chloroform:methanol:water (2:1:1.8) (Bligh and Dyer, 1959); FAs prior to transesterification and methylation (AOAC, 1995) were analysed by gas chromatography.

One-way ANOVA was applied to fed and starved larvae (2, 3, and 4dah), followed by PLSD test and by non-parametric variance analysis of Kruskal-Wallis and Mann-Whitney U-test.

Results and discussion

Four phases of the lipid and fatty acid metabolism were identified in *P. maculatofasciatus*, during early development same than *Dicentrarchus labra*x, *Sparus aurata* and *Stizostedion lucioperc*a (Diaz et al., 2002): The first phase corresponds to the endotrophic period, beginning at oocyte activation and its completion just after mouth-opening at 0 to 2dah (Peña et al., 2003). It is characterized by an intense use of all the fatty acids, and a mean docosahexaenoic to eicosapentaenoic and arachidonic acids (DHA/EPA/ARA) ratio of 3:1:1. The second phase (endo-exotrophic) followed the first feeding and is completed with total exhaustion of yolk reserves at 2-3dah. It is also marked by weak hepatic and pancreatic activity at 4dah (Peña et al., 2003). Most of the fatty acids reached their minimum values at 2dah (14:0>18:1n-9>20:5n-3>16:1n-5>16:1n-7) and at 3dah (16:0>18:0>22:6n-3>20:4n-6>17:0>15:0>18:1n-7>16:2). Same pattern was observed on *Dentex dentex* (Mourente et al., 1999) and *Solea sengalensis* (Mourente and Vázquez, 1996). Some fatty acids appeared for the first time at 2dah (18:3n-3>18:2n-6) and at 3dah (20:1n-7). The mean DHA/EPA/ARA ratio was 4:1:1.5. All FA decrease at minimum values at 2 or 3dah. A marked preference on the saturated 16:0, the monoenoic 18:1n-9, and the PUFA 22:6n-3 (223 to 33, 15 to 3, and 27 to 4 respectively). Same pattern was observed on *Perca fluviatilis* (Abi-Ayad et al., 2000) and *Dentex dentex* (Mourente et al., 1999). The third phase (exotrophic) started with important glycogen accumulation in the liver at 4dah and concluded with the formation of the complete digestive tract at 12-16dah (Peña et al., 2003). All FAs increased exponentially by using rotifers to feed the larvae at 2-15dah. Also, a dramatic decline in the DHA/EPA/ARA ratio was observed 2:1:2 at 4dah to 0.3:1:0.7 at 15dah. Alvarez-González (2003) confirm this observation by the maximum protease activity found at 12-16dah. And the fourth phase (exotrophic) started when the hepatic, pancreatic, and intestinal activities of the larvae were functioning as in adults at 16-24dah (Peña et al., 2003). All the fatty acids increased by adding *Artemia* at 15-30dah, reaching their minimum proportion from in the DHA/EPA/ARA ratio observed 0.1:1:0.5 (25dah). Alvarez-González (2003) confirms these observation since the acid protease activity was surpassed by the alkaline protease activity at 25dah.

Table I. Fatty acids (mean ± SD, n = 3) dynamic in starved (s; 0-3dah) and fed (f; 2-30dah) *Paralabrax maculatofasciatus* larvae.

FA	Day										
ng ind^{-1}	**0 *s***	**1 *s***	**2 *s***	**3 *s***	**2 *f***	**3 *f***	**4 *f***	**10 *f***	**15 *f***	**25 *f***	**30 *f***
14:0	**28** ± 0.4	**15** ± 8.2	**3** ± 0.4	**4** ± 1.2	**3** ± 0.2	**4** ± 1.4	**12** ± 4.7	**61** ± 16	**164** ± 27	**2639** ± 380	**10230** ± 2404
15:0	**4** ± 0.1	**2** ± 1.0	**1** ± 0.0	**2** ± 0.0	**1** ± 0.0	**1** ± 0.4	**6** ± 0.2	**20** ± 3.8	**64** ± 12	**807** ± 57	**2169** ± 425
16:0	**223** ± 6.7	**100** ± 40	**41** ± 3.1	**115** ± 3.2	**48** ± 4.1	**33** ± 11	**183** ± 6.3	**678** ± 112	**2367** ± 301	**34034** ± 83	**86610** ± 15656
17:0	**4** ± 0.1	**2** ± 1.0	**1** ± 0.1	**3** ± 0.3	**2** ± 0.1	**1** ± 0.3	7 ± 0.1	**26** ± 3.6	**124** ± 11	**1940** ± 35	**4951** ± 530
18:0	**18** ± 0.5	**9** ± 3.5	**5** ± 0.3	**19** ± 5.7	7 ± 0.4	**5** ± 1.2	**30** ± 0.8	**89** ± 11	**354** ± 20	**6257** ± 424	**13929** ± 1451
19:0	nd	nd	nd	nd	nd	nd	nd	nd	**23** ± 2	**213** ± 9	**732** ± 121
24:0	**2** ± 0.1	**1** ± 0.3	tr	tr	tr	tr	tr	tr	tr	tr	tr
Σsat.	**279** ± 8	**128** ± 54	**51**± 3.8	**142** ± 10	**61** ± 4.8	**43** ± 14	**239** ± 12	**875** ± 146	**3095** ± 373	**46073** ± 997	**118623** ± 20586
16:1n-5	**3** ± 0.0	**8** ± 0.1	**1** ± 0.6	**3** ± 1.5	**1** ± 0.1	**1** ± 0.4	**5** ± 0.1	**10** ± 2.3	**66** ± 8	**1438** ± 260	**7908** ± 1474
16:1n-7	**1** ± 0.0	**1** ± 0.2	tr	**1** ± 0.0	tr	tr	**1** ± 0.0	**6** ± 1.5	**24** ± 3	**268** ± 7	**693** ± 136
18:1n-7	**3** ± 0.1	**1** ± 0.6	**1** ± 0.0	**1** ± 0.1	**1** ± 0.0	**1** ± 0.2	**3** ± 0.1	**9** ± 1.6	**111** ± 3	**2229** ± 57	**6442** ± 835
18:1n-9	**15** ± 0.3	**5** ± 2.4	**2** ± 0.1	7 ± 1.3	**3** ± 0.2	**3** ± 0.9	**12** ± 0.2	**37** ± 7.4	**361** ± 21	**5513** ± 261	**13183** ± 1527
20:1n-7	tr	tr	tr	tr	tr	**1** ± 0.0	**1** ± 0.0	**3** ± 0.4	**10** ± 1	**165** ± 18	**306** ± 28
Σmono	**21** ± 0.4	**15** ± 3.3	**4** ± 0.7	**11** ± 2.9	**4** ± 0.8	**5** ± 1.6	**22** ± 0.5	**64** ± 13	**572** ± 36	**9613** ± 603	**28533** ± 4000
16:2n	**2** ± 0.1	**1** ± 0.0	tr	**1** ± 0.0	**1** ± 0.1	tr	**2** ± 0.4	**4** ± 0.1	**52** ± 8	**539** ± 93	**1597** ± 234
18:2n-6	tr	tr	nd	nd	**1** ± 0.1	**4** ± 1.1	**29** ± 0.9	**132** ± 37	**358** ± 30	**3372** ± 156	**8840** ± 1314
18:3n-3	tr	tr	nd	nd	**1** ± 0.1	**2** ± 0.7	**16** ± 1.1	**99** ± 23	**681** ± 38	**7935** ± 360	**19637** ± 2667
18:4n-3	tr	tr	tr	nd	tr	tr	tr	tr	**83** ± 5	**725** ± 45	**1859** ± 229
20:4n-6	**9** ± 0.6	**3** ± 1.4	**1** ± 0.0	**6** ± 0.3	**2** ± 0.1	**1** ± 0.4	**9** ± 1.8	**41** ± 5.0	**153** ± 2	**2921** ± 197	**11040** ± 821
20:5n-3	**10** ± 0.8	**5** ± 1.4	**1** ± 0.1	**3** ± 0.2	**1** ± 0.1	**2** ± 0.6	**4** ± 0.8	**24** ± 1.2	**212** ± 5	**5855** ± 5798	**14373** ± 1165
22:6n-3	**27** ± 3.5	**14** ± 4.1	**4** ± 0.2	**17** ± 0.6	**5** ± 0.1	**4** ± 0.1	**9** ± 3.4	**31** ± 3.9	**73** ± 6	**609** ± 9	**1040** ± 28
ΣPUFA	**47** ± 4.9	**23** ± 6.9	**6** ± 0.3	**26** ± 1.1	**11** ± 0.4	**13** ± 3.8	**70** ± 8.4	**332** ± 71	**1611** ± 94	**21957** ± 1659	**58386** ± 6458
ΣFAME	**347** ± 13.	**167** ± 65	**61** ± 1	**180** ± 14	**76** ± 6	**62** ± 20	**332** ± 21	**1271** ± 231	**5279** ± 504	**77643** ± 3258	**205542** ± 31044
DHA/EPA/ARA	**3:1:1**	**3:1:1**	**3:1:1**	**6:1:2**	**4:1:2**	**3:1:1**	**2:1:2**	**1:1:2**	**0.3:1:0.7**	**0.1:1:0.5**	**0.1:1:0.8**

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ACHYRANTHES ASPERA ENHANCES THE EFFICIENCY OF ANTIGEN CLEARANCE FROM THE HOST *LABEO ROHITA*

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Introduction

As soon as any foreign particle invades, the immune system of the host detects it, mounts immune response against it, and eliminates it from the host body as quickly as possible. This is called antigen clearance. Antigen clearance is associated with a downregulation of the immune response and apoptosis of most antigen-specific T-cells (Buckland, 2004). Though antigen clearance has been studied in several mammals by various techniques, no such study has been conducted with fishes. *Achyranthes aspera*, L. (Family: Amaranthaceae) is an herb that stimulates the non-specific immunity of carps (Vasudeva et al., 2004; Vasudeva and Chakrabarti, 2005). This investigation aims to study the effects of *A. aspera* in antigen clearance from the spleen of Indian major carp *Labeo rohita*, rohu.

Materials and methods

Rohu (200±27g) were cultured under two feeding conditions: test diet and control diet. Test diet was prepared using 0.5% *A. aspera* seed along with other feed ingredients like fish meal, wheat flour, cod liver oil, vitamins, and mineral premix. Control diet contained all ingredients, except the seed. After 28d of feeding, fish were injected intraperitoneally with Bovine serum albumin, BSA (500µl). The diffused small pieces of spleen of fish were collected on d14 of immunization. Tissues were fixed in 1% glutaraldehide and 2% paraformaldehyde in 0.1M phosphate buffer and standard procedures were followed for further tissue processing. Ultrathin sections (70nm) were cut using glass knives in a ultramicrotome. The sections were slowly lifted onto nickel grids and were blocked with 2% fish gelatin. Then the sections were labelled by incubating with rabbit anti-BSA antibodies for 12h and were washed with 1% fish gelatin in phosphate buffer. After treating with primary antibody, the grids were labelled with secondary antibody; goat anti-rabbit-IgG conjugated with 15-nm gold particles (TAAB), washed, and stained with uranyl acetate. Stained sections were viewed for labelled particles under transmission electron microscope.

Results and discussion

Immuno-labelling showed the presence of gold particles in spleen sections of *L. rohita*. In control group, the particles were heavily crowded and more numerous (Fig. 1a), whereas in the spleen (Fig. 1b) of fish fed with *A. aspera* seed incorporated diet, the number of gold particles was less than the control group. This indicated that the injected antigen was efficiently cleared in fish fed with plant ingredient incorporated diet compared to the fish fed with control diet.

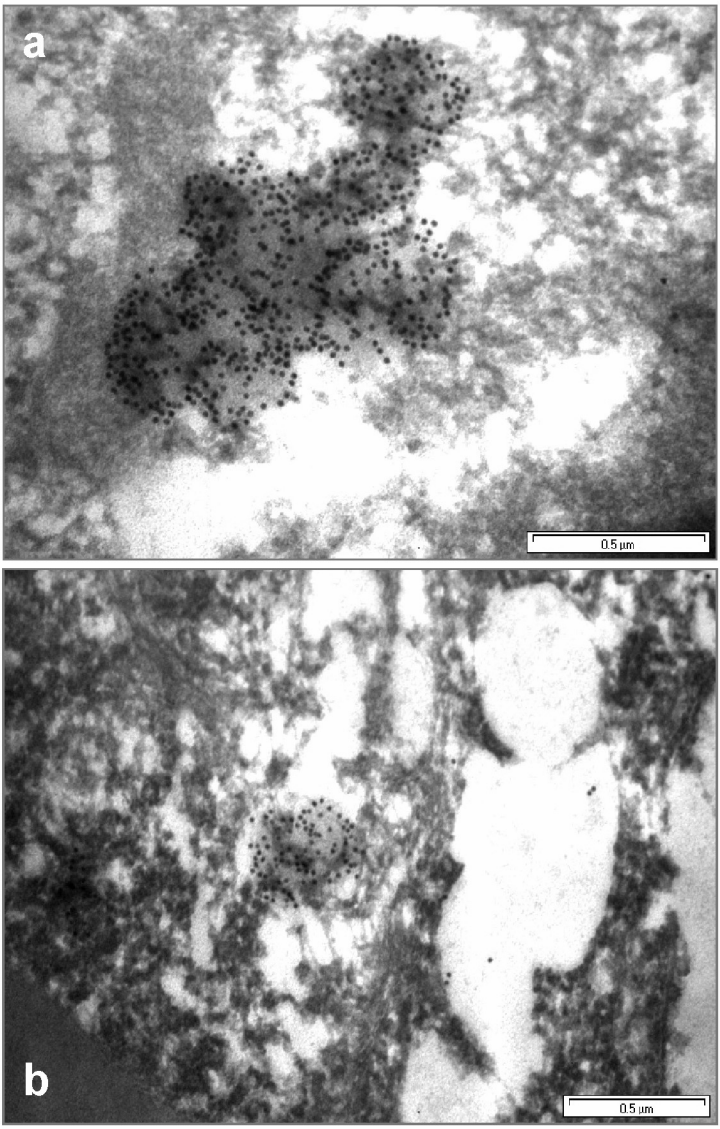

Fig. 1. Electron micrographs of spleen (a) control (b) *Achyranthes*-treated rohu.

The host's immune system recognized the invading antigen and tried to clear it as quickly as possible to confer safety of the host and to protect the host from the onset of infection. The treatment with itraconazole reduces the concentration of histoplasma antigen in blood and urine, suggesting the rapid clearance of fungemia (Joseph et al., 2002). In the present study, high amount of BSA was found in the spleen of control fish even 14d after immunization. The immune system of this carp is therefore poor in clearing the invaded antigen; hence high mortality is recorded in this species during disease outbreaks. The feeding of plant-incorporated diet may boost the immune system of this fish.

In conclusion, treatment with *A. aspera* has enhanced the immunity of *L. rohita* that has efficiently eliminated the BSA from the system compared to the control.

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FACTORS AFFECTING EARLY GROWTH OF NILE TILAPIA FRY IN HAPA-IN-POND ENVIRONMENTS

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Introduction

Hapas, usually suspended in fertilized ponds, have long been used for tilapia fry production (Santiago et al., 1985; Bhujel, 1997) and recently for fry rearing (Little et al., 2003). In fish breeding programs, members of a full-sib family usually share a common tank or hapa prior to tagging and communal testing. This tends to increase resemblance between family members which may reduce the efficiency of breeding programs. For convenience of monitoring and identification of families, hapas are often arranged in rows over the pond. If ponds are heterogeneous, for example with respect to nutrient availability, the spatial arrangement of hapas may create an environmental correlation among neighbouring units. The aim of this study was to quantify the common environmental and genetic effects on early rearing stages of tilapia in hapa-in-pond nursing conditions.

Materials and methods

25 full-sib families of Nile tilapia, *Oreochromis niloticus,* were produced by single-pair mating. Fry were produced and reared from hatching to swim-up in separate 6-m^2 hapas suspended in fertilized ponds. At swim-up, four groups of 30 fry each were obtained from each family. A total of 54 hapas (2m × 1m × 1m) were installed in each of two 4500-m^2 ponds, in two columns of 27 hapas. Ponds were fertilized with chicken manure at a rate of 50kg dry matter $ha.day^{-1}$. The fry groups were randomly stocked into the inner 25 hapas at a stocking density of 15 $fry.m^{-2}$. The remaining two hapas at either end of each row were controls (not stocked). Two treatments were assigned to either column: 40% protein

commercial formulated feed twice daily or no supplementary feed. Temperature, dissolved oxygen (DO), and pH were measured twice weekly with a portable DO meter (WTW® model multi 340i meter at a depth of 30cm inside each hapa. On days 14, 21, 28, and 35 fry were counted, bulk weighed, and average weight recorded. On day 42 fish were removed from the hapas, and individual body weight (BW) and standard length measurements taken.

Survival rate (S_t %) was calculated as $S_t = (N_t/N_0) \times 100$, where N_t is the number of fry at day t and N_0 is the number of fry at stocking. Due to heterogeneity of variances of fish among ponds in the main experiment, BW data was log-transformed. Genetic, environmental, and spatial variability effects were analysed with the following model (ASReml; Gilmour et al., 2002):

$$Y_{ijkl} = \mu + p_i + t_j + \beta_l \log(INWT_{ijkl}) + \beta_2 d_{ijkl} + u_{ijkl} + h_k + e_{ijkl} \qquad \text{(Model 1)}$$

where Y_{ijkl} = logarithm of the 42-day body weight of an individual; μ is overall mean; p_i is fixed effect of pond (i = 1, 2); t_j is fixed effect of dietary treatment (j = 1,2); β_1 is regression coefficient of logarithm of initial body weight; $log(INWT_{ijkl})$ is a co-variable of the logarithm of initial body weight of an individual; β_2 is regression coefficient of number of fry in the kth hapa at the end of the experiment; d_{ijkl} is the effect of number of fry on individual l; u_{ijkl} is random additive genetic effect of the lth individual; h_k is a random effect of the kth hapa; and e_{ijkl} is a random residual effect associated with an individual.

Heritabilities (h^2) and common environmental/hapa effects (c^2) for BW were obtained from the complete data set. A bivariate setting of Model 1 in which BW in pond A and BW in pond B were considered separate traits was used to obtain h^2, c^2 in each pond, and genetic correlation (r_g) between traits. The r_g was used to evaluate the presence of genotype by environment (GXE) interaction. The mean performance of few full-sib families in each pond was plotted to illustrate the GXE interactions. The effects of water quality, pond, treatment and week of sampling on BW were determined by the GLM procedure of SAS (1989).

Results and discussion

The h^2 estimates for 42-day BW from the whole data set was 0.01 with high c^2 effects (Table II). The h^2 estimate for BW in pond B was 0.05. The h^2 estimates in pond B were consistent with those obtained for 45-day BW (Tave and Smitherman, 1980). Heritability in pond A was higher (0.59) but there were lower c^2 effects for pond A than pond B. This indicates that h^2 estimates in Nile tilapia are environmentally dependent. Common environmental/hapa effects should be reduced to improve heritability of BW in Nile tilapia. The r_g estimates for the two traits (BW in pond A and pond B) was -0.27, which is well below

unity, suggesting GXE interaction. GXE interactions of the crossover type are also implied by Fig. 1. However, given the high standard errors, the existence of GXE interaction is not conclusive. We found significant spatial autocorrelations ($\chi_2^2 = 7.6$; $p = 0.0224$) across rows and hapa columns (Table I), indicating that ponds were heterogeneous with respect to environmental factors affecting fry growth. Lower h^2 estimates were associated with higher pond heterogeneity (Table I). Since patterns of spatial variability are not known before hand, spatial autocorrelation should be included in breeding programs using hapa-in-ponds systems, to determine underlying environmental patterns. However, spatial autocorrelations are more important in environments with poor water quality.

Table I. Heritability (h^2), common environmental effect (c^2) and genetic correlation (r_g) estimates for body weights in pond A and B and the spatial autocorrelations across rows (ρ_r) and columns (ρ_c) within ponds. Body weight in each pond was considered as a distinct trait and was used for estimation of r_g.

Trait	h^2	c^2	r_G	ρ_r	ρ_c
BW in both ponds	0.01 (0.06)	0.36 (0.05)	-	0.29	0.22
BW pond A	0.59 (0.19)	0.14 (0.06)	-0.27(0.69)	0.16	-0.06
BW pond B	0.05 (0.11)	0.29 (0.07)		0.26	0.33

Morning and afternoon DO and afternoon pH significantly affected fry BW (Table II). This indicates that differences in BW had to do with the amount of DO available in the hapas. Extended periods of hypoxia may reduce growth (Chervinski, 1982) and cause mortality (Coche, 1982) in Nile tilapia. We did not find any differences in survival between ponds or treatments in this study. The observed GXE interaction may be a response to the differences in DO levels in the two ponds.

Table II. Marginal (Type III) mean square values of water quality, pond, fish survival, sampling week and treatment effects on body weight of Nile tilapia fry reared in a hapa-in pond system

Source	df	Type III SS	F value	P-value
Pond	1	31.17	167.08	<0.0001
Week	5	544.91	584.13	<0.0001
Treatment	1	0.19	1.03	0.3108
Fish survival	1	1.17	6.29	0.0124
Morning DO (mg l^{-1})	1	1.11	5.95	0.0151
Afternoon DO (mg l^{-1})	1	1.00	5.38	0.0207
Morning Temperature (˚C)	1	0.04	0.23	0.6307
Afternoon Temperature (˚C)	1	0.16	0.88	0.3478
Morning pH	1	0.13	0.69	0.4061
Afternoon pH	1	0.85	4.57	0.0330
R-Square	0.88			

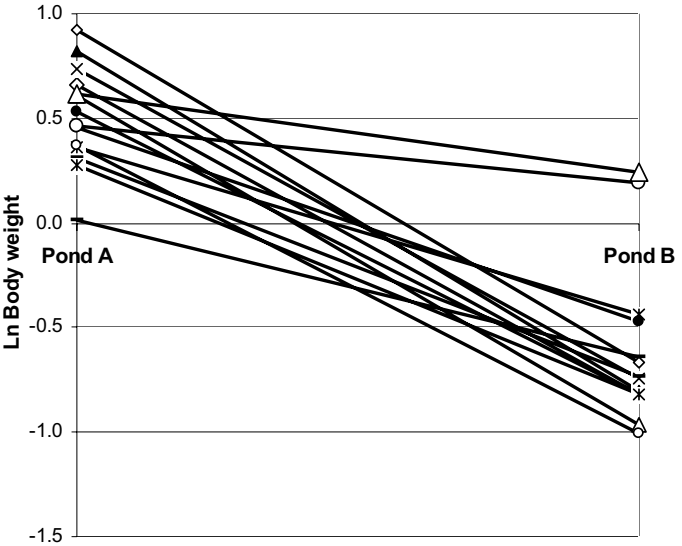

Fig.1. Mean body weights of full-sib families of Nile tilapia fry reared under two different ponds and dietary environments. Fry were given supplementary artificial protein diet or fed naturally on pond food.

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ARTEMIA: AN ANIMAL EXTREMOPHILE

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The word extremophile is usually applied to certain microbes that inhabit, and often require, environments that we consider extreme in terms of their physical and chemical characteristics. If one chooses to apply this term to plant and animal taxa, the brine shrimp *Artemia* definitely qualifies as an animal extremophile. Thus, the motile stages are among the best (probably *the* best) of all animal osmoregulators, enabling success in severely hypersaline environments; these astonishing capabilities will be summarized. In addition, the encysted embryos, or cysts, appear to be without equal in the animal kingdom when it comes to tolerating high doses of UV and ionizing radiation, years of continuous anoxia while hydrated at physiological temperatures, thermal extremes, and desiccation-hydration cycles. Such abilities enable *Artemia* to survive and even thrive in incredibly harsh environments – their evolutionary strategy has been to live where few others can, and their ecological success rests firmly on an ensemble of biochemical, biophysical, and physiological adaptations that will be summarized. Indeed, the massive numbers of *Artemia* that are found in certain locations (the Great Salt Lake in Utah, USA, being a good example) are only possible because of those adaptations. What emerges from the considerable amount of research on these embryos, carried out by a number of scientists around the world, is their uniqueness as a model system for the study of resistance to severe stress by animal cells. In addition, some of the information gained from the study of this amazing animal has been used to produce some rather unexpected and valuable applications, and these will be considered briefly.

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MANAGEMENT OF LARVAL HEALTH IN CULTURED STRIPED TRUMPETER *LATRIS LINEATA*

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Introduction

In a hatchery situation, the effective management of factors that affect the health of fish larvae is vital to ensure high survival, growth and larval quality. Striped trumpeter *Latris lineata* is a temperate marine finfish being investigated in Tasmania for its aquaculture potential. The specific health issues affecting striped trumpeter larvae, with the potential to constrain juvenile production, are betanodavirus (Munday et al., 2002), bacterial enteritis and intestinal dysfunction (Cobcroft et al., 2004a; Bransden et al., in press), a myxozoan parasite (Grossel et al., 2003), and skeletal malformations (Cobcroft et al., 2001). We have adopted several techniques to successfully manage viral and bacterial pathogens and myxozoan parasites in larval production.

Materials and methods

For production trials and experiments from 1997-2005, gametes were hand-stripped from captive striped trumpeter broodstock. Following detection of betanodavirus, fertilised eggs were surface-disinfected with ozone, at 1mg.l^{-1} for 1min, prior to incubation. An ozonation system was installed in 2003 to disinfect all incoming hatchery water. Larvae were cultured in tanks ranging from 300-3000 l, using either a clearwater or a greenwater regime. Larvae were fed rotifers *Brachionus plicatilis* at 10.ml^{-1} daily from 5 days post-hatching (dph) and enriched *Artemia* at 1-2.ml^{-1} from 15dph. Larval quality was monitored routinely using gross morphometric observations for growth and condition indices, histological examination of tissues (especially the intestine and liver), and survival to specific developmental stages. Experiments were conducted to assess bacterial flora in larvae, and wet smears of brain and central nervous system tissue of

post-larvae were examined for the presence of myxozoan parasites. Histopathology and in situ hybridisation (ISH) were used to study the myxozoan disease.

Results and discussion

Survival of striped trumpeter larvae has often been low and the time of mortality variable between batches. Since adopting the practice of ozone disinfection of eggs, and other control measures including disinfection of equipment between trials, there has been no detection of betanodavirus in cultured *L. lineata* (Battaglene and Morehead, in review). There was increased survival to later stages in batches of larvae stocked with ozonated eggs, and this was further improved by reducing larval stocking density (Table I). Subsequently, other factors were identified with a greater impact on larval survival.

Table I. Comparison of larval rearing trials in 300-l tanks, which received eggs with and without ozone disinfection at 1mg.l^{-1} for 1min. Larvae reared at 16 ± 1^{o}C on rotifers fed twice daily.

Ozonation of eggs	Antibiotic OTC	No. of tanks	Stocking density (larvae.l^{-1})	Age at finish (dph)	Survival (%)	
					Mean	SE
No	No	3	30	13	31.2	2.8
No	No	4	30	12	16.7	2.2
No	No	3	30	12	30.6	1.5
No	No	3	30	12	33.7	1.2
Mean (no ozonation)					28.1	7.7
Day 3	Yes	24	15	18	61.3	6.4
Day 1 & 3	Yes	4	15	14	50.3	2.6
Day 1 & 3	No	4	15	14	20.3	4.2
Day 1 & 3	No	4	10	15	69.2	4.1
Mean (with ozonation)					50.3	21.4

Changes in the intestine of larvae have been identified in some batches undergoing mortality events. Pathology included decay of the posterior intestine, grossly associated with swelling of the anterior region of the mid gut and blockage of the hind gut. Histological examination of larvae identified bacterial enteritis characterised by sloughing of gut epithelial cells associated with bacteria. At early stages of this infection, larvae had an opaque grey colouration of the mid gut, referred to as 'grey gut' syndrome. Experiments using antibiotics demonstrated that high bacterial loads were an important factor in larval mortality. Treating larvae with antibiotics improved survival, increased digestion, and reduced the incidence of grey gut syndrome and the presence of urinary calculi, resulting in more viable larvae. However, the use of antibiotics is not an option for hatcheries and a new water filtration and ozonation system was implemented. The resultant improvement in water quality allowed greater microbial control,

without antibiotics, and enabled better interpretation of experimental results by limiting confounding influences associated with a highly variable supply of seawater from the Derwent Estuary. Grey gut syndrome persisted in larvae reared in ozone-disinfected water and was no longer associated with severe degeneration of the gut or high mortality. Recent investigations suggest it was related to larval diet, apparently reflecting a fatty acid imbalance that interfered with lipid transport (Bransden et al., in press).

A high proportion of the post-larvae produced to date (>85%) exhibit jaw malformation. A comparative study with kingfish reared in New Zealand showed a high degree of similarity in malformation (Cobcroft et al., 2004b). No definitive cause of malformations in *L. lineata* was established despite considerable research into the vitamin and lipid enrichment of live feeds (Bransden et al., in press). The onset and severity of jaw malformation was delayed from around 30dph to after 50dph through a combination of better husbandry practices, the use of ozonated seawater, higher feed rates, and lower larval densities and temperatures.

Whilst early larval mortality has been reduced, fish surviving over 30dph frequently developed nervous conditions, including walling, flashing, loss of swimming equilibrium and inability to feed. Post-larvae have succumbed to sudden episodes of irreversible scoliosis and lordosis of the spine from 60dph. Examination of the brains and spinal cords of these fish showed severe meningoencephalomyelitis caused by a myxozoan parasite, *Kudoa neurophila* (Grossel et al., 2003). A single-round polymerase chain reaction (PCR) diagnostic assay has been developed to detect *K. neurophila* in striped trumpeter larvae (Grossel et al., 2005). The infective stage of the parasite entered the fish host via epithelial cells as early as 25dph. The presporogonic stages enter the spinal cord via the connecting peripheral nerves where terminal stage sporogonic plasmodia develop throughout the brain and spinal cord between 105-130dph. Fish cultured to 100dph in ozonated seawater, at >700mV ORP for 10min, tested negative to the PCR assay and showed no signs of *K. neurophila,* demonstrating the effectiveness of ozonation as a barrier to the parasite.

Conclusions

Larval survival and growth have increased and the incidence of disease has decreased following the introduction of ozonation of eggs and culture water. Investigations are continuing to determine the potential role of nutrition and environmental factors on jaw malformation.

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INGESTION OF LIVE FOOD BY *LITOPENAEUS VANNAMEI* LARVAE AT DIFFERENT STOCKING DENSITIES

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NUTRITIONAL PHYSIOLOGY DURING DEVELOPMENT OF SENEGALESE SOLE (*SOLEA SENEGALENSIS*)

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Qualitative and quantitative dietary imbalances are one of the main causes of sub-optimal performance and quality often observed in larviculture. The Senegalese sole, a species with a complex metamorphosis, difficulties in weaning, and with occasional problems of malpigmentation and skeletal deformities, is a good model species to study larval nutritional physiology. In addition, the early metamorphosis and acquisition of a peculiar non-proactive bottom-feeding behaviour make early weaning an important issue in sole hatcheries. In fact, problems in reproducibility of weaning success in sole may be largely due to extended feeding on a diet (*Artemia* metanauplii) with inadequate characteristics for bottom-feeding. The present work reviews recent findings in different aspects of nutritional physiology during the development of Senegalese sole, in an attempt to optimize the composition of sole diets and to understand what the limiting factors for weaning sole are.

Both digestive enzyme activity and tracer studies using ^{14}C-*Artemia* show that sole larvae, even at young stages, have a high capacity for digesting live prey. This is reflected in a high growth potential and low mortality rates for this species during the larval stage compared to other marine fish species.

Early introduction of inert microdiets in co-feeding with *Artemia* does not seem to affect intestinal processes, based on the observation of the digestive enzymes profile. However, when co-feeding is not provided, intestinal activity may be depressed. Furthermore, early introduction of microdiets in co-feeding with *Artemia* may have a positive effect on survival rates, but at the expense of lower growth rates and higher size dispersal. This may reflect variation in the adaptation capacity of individual larvae to inert microdiets.

High dietary neutral lipid (soybean oil) content results in reduced growth, accumulation of lipid droplets in the enterocytes and affects the capacity of Senegalese sole larvae to absorb and metabolise dietary fatty acids (FA) and amino acids

(AA). Through tube-feeding of different ^{14}C-lipids and free FA it has been shown that FA absorption efficiency increases with unsaturation and that sole larvae spare DHA from catabolism. In addition, it was demonstrated that absorption efficiency varies according to molecular form, being highest for free FA, lowest for triacylglycerols, and intermediate for phospholipids.

Live preys commonly used in larviculture do not seem to have a balanced AA profile for sole larvae. Furthermore, the ideal dietary AA composition probably changes during development. Rotifers and *Artemia* metanauplii are apparently deficient in one or more of the following AA depending on the larval development stage: histidine, sulphur AA, lysine, aromatic AA, threonine, and arginine. It has also been demonstrated that supplementation of *Artemia*-fed larvae with AA in deficiency, increases AA retention and reduces AA catabolism. When supplementing larval diets with limiting AA it should also be considered that sole larvae have different absorption and retention efficiencies for individual AA and that they have the capacity to spare indispensable AA. In addition, the absorption of free AA is faster and more efficient than that of complex proteins.

In summary, sole larvae appear to have a good digestive capacity from the onset of exogenous feeding and a good capacity to spare essential nutrients such as DHA and indispensable AA. Still, sole larvae may face difficulties in digesting diets with high levels of dietary neutral lipid and/or complex proteins, and this is likely to be reflected by a lower performance. A balanced dietary AA composition may improve growth and nitrogen utilization in sole larvae. Improvements in biochemical composition of inert microdiets for sole are likely to contribute to the reproducible weaning success of Senegalese sole.

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DEVELOPMENT OF METHOD FOR DETERMINING CONSUMPTION IN MARINE FISH LARVAE

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Introduction

Determining the relative consumption rates of different feeds is a key step in their evaluation. Environmental conditions, different feeding systems and schedules, the use of attractants, and feed types may impact the amount of feed consumed by fish larvae. Optimization of diets, feeding, and rearing conditions for high feed consumption rates can lead to improved survival and growth of fish larvae fed both live and formulated diets.

Materials and methods

Artemia nauplii were marked with the inert metal oxides of yttrium, ytterbium, lanthanum, or dysprosium. *Artemia* nauplii were added to a solution containing 1 l of seawater and 250mg of marker and aerated vigorously in an Imhoff cone (n=3 for each marker). Samples were taken at intervals, rinsed copiously, and analyzed to determine uptake of markers. The marked organisms were added to clean seawater and sampled over time to determine marker depletion.

A trial was run to quantify the number of live *Artemia* nauplii consumed by an individual fish larvae based on the yttrium concentration in the nauplii. *Artemia* nauplii were marked as above and counted into groups (n=5) of 1, 5, 10, 20, and 40. Yttrium concentration was analyzed using inductively coupled plasma-optical emission spectroscopy (ICP-OES) and plotted to generate a standard equation that could then be used to determine the number of nauplii consumed by larvae. Three-day-old lingcod (*Ophiodon elongatus*) were allowed to feed on marked nauplii for 60 minutes. Full larvae were removed and dissected to visually enumerate consumed nauplii. The dissected larvae and gut contents were analyzed for yttrium. *Artemia* nauplii numbers obtained with the ICP-OES were compared to numbers obtained visually by regression.

Results and discussion

Artemia quickly took up markers to concentrations required for consumption studies (~2.0% dry weight) (Fig. 1). Upon being placed into clean seawater, the markers decreased over time (Fig. 2). This could affect the accuracy of consumption calculations. To adjust for depletion, a sample of marked *Artemia* may be taken at time zero and averaged with a sample taken at the end of the feeding period, if the feeding period is more than a few minutes.

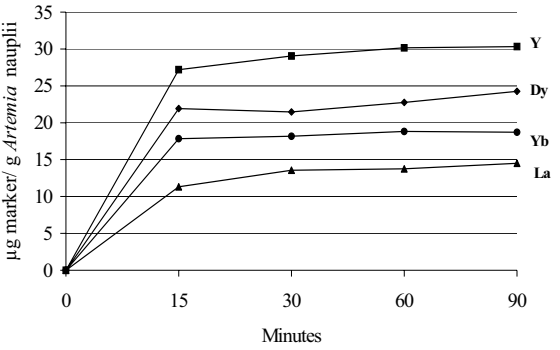

Fig. 1. Mean concentration of yttrium (y), dysprosium (dy), ytterbium (yb), and lanthanum (la) over time by *Artemia* nauplii.

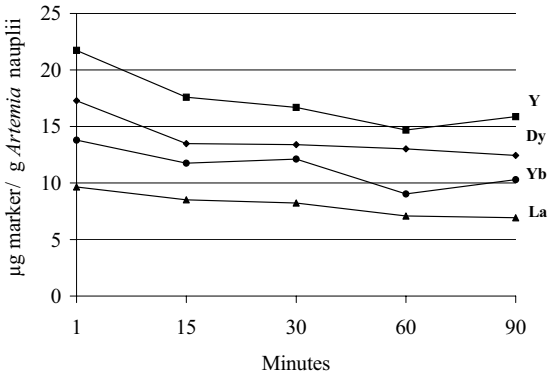

Fig. 2. Mean concentration of markers over time in *Artemia* nauplii in clean (10°C, 30ppt) seawater.

Although the ICP-OES method may not quantify the exact number of organisms consumed by a fish, it was accurate within the specified range of organisms consumed by larvae in this trial. There was a strong correlation (r^2=.99) between yttrium concentration and number of nauplii (Fig. 3). For nauplii in the gut of a larval lingcod, there was also a strong correlation (r^2=.90) between nauplii enumerated visually and nauplii enumerated with the ICP-OES method (Fig. 4). The

ICP-OES has the potential to be more accurate than visual enumeration because digestion can make individual organisms difficult to distinguish. The ICP-OES was also used to quantify ingestion of marked microparticulate diets and rotifers (data not shown).

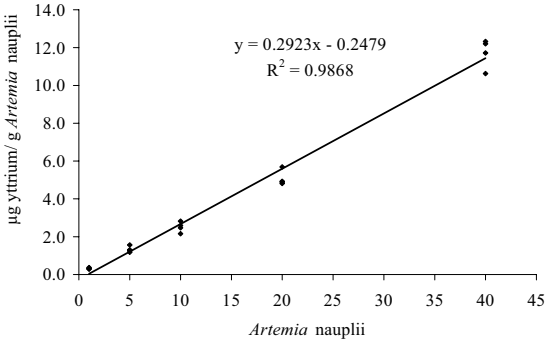

Fig. 3. Yttrium concentration in individual *Artemia* nauplii and replicates of 5, 10, 20, and 40 *Artemia*.

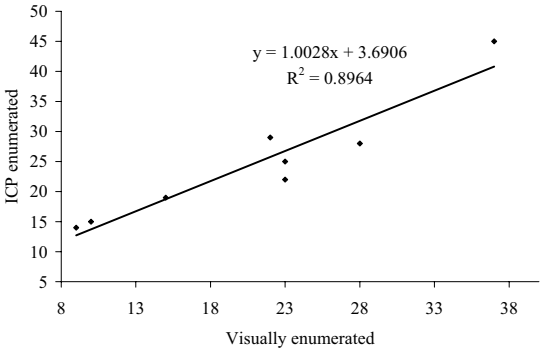

Fig. 4. Correlation of visually and ICP enumerated *Artemia* nauplii consumed by individual lingcod larvae (n=8).

Conclusion

Artemia nauplii and rotifers can be marked with yttrium, ytterbium, lanthanum, or dysprosium to concentrations required for consumption studies. All the markers depleted over time, however, we do not feel depletion would significantly influence consumption values as long as the feeding period is short or the depletion rate is accounted for during the feeding portion of a consumption trial. Enumerating live feeds consumed using markers may be more accurate than visual counting because digestion can make live feeds difficult to distinguish. Enumeration of microparticulate diet consumption is very difficult to do by dissection. The method presented in this paper allows for the direct comparison of live and microparticulate feeds in small fish larvae, with accurate quantification of the amount of material ingested.

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AMINO ACID IMBALANCES IN FISH - A NEW STRATEGY FOR AN OLD PROBLEM

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Most studies on amino acid imbalance and dietary selectivity have been conducted with mammals and rats. In rats for instance, dietary amino acid deficiencies cause a rapid (within minutes) decrease in food intake. Teleost fish, particularly juveniles, show no apparent aversion to indispensable amino acid-devoid diets, although possible taste aversion has not been distinguished from neurological feedback control. Therefore, it remains to be answered if amino acid attractant(s) can override neurological responses. Indispensable amino acid (IAAs) requirements in fish are frequently determined with diets of different palatability and feed intake is often neglected or superimposed on changes in diet utilization efficiencies. Some studies simply do not provide information on diet intake or feed utilization or do not include a comparison of growth between diets composed of free amino acids (FAA-based) and corresponding protein-based diets. It is therefore imperative to better understand how dietary amino acid imbalance, taste and dietary intake interact in fish, and how this can impact amino acid requirement estimates. Attempts have been made to unify the recognition of IAAs through neural signalling in all animals although the evidence in fish of the same basic mechanism of nutritional stress is missing.

As a first step in resolving the dilemma of neurological feedback response in larval/juvenile fish, we employed an amino acid balanced, casein-gelatin-based diet (protein-based) as control, in addition to a diet covering estimated amino acid requirements, but based on synthetic amino acids (FAA-based). The AA-based diet formulation used in the present study has already been tested in cichlids. Two additional "amino acid imbalanced" diets – each devoid of five indispensable amino acids – were also included: a diet devoid of Lys, His, Ile, Phe, and Trp, called (-)Lys; and a diet devoid of Arg, Thr, Val, Leu, and Met, called (-)Arg. In treatment 1, fish were fed the FAA-based balanced diet in four meals each day throughout the experiment. In treatment 2 [(-)Lys(-)Arg group], fish

were fed the (-)Lys and (-)Arg diets as alternating meals; i.e., (-)Lys, (-)Arg, (-)Lys, (-)Arg), each day throughout the experiment. In treatment 3 [(-)Lys(-)Lys group], fish were fed the (-)Lys diet as first two meals in the morning and then the (-)Arg diet as two meals in the afternoon. In treatment 4 [(-)Lys/(-)Arg group], fish were fed four meals of the (-)Lys and (-)Arg diets on alternate days. We report here the effects of dietary amino acid imbalances on the diet intake, fish growth and body free amino acid concentrations in a juvenile cichlid, midas (*Amphilophus citrinellum*).

The first feeding experiment showed that feed intake of midas fed to satiation did not differ in naïve fish (day 1) or on day 2 among a protein-based, FAA, (-)Lys or (-)Arg groups. The second experiment showed that the feed intake of midas on day 1 and day 2 in the FAA diet-fed group was significantly higher than that of (-)Lys(-)Arg, (-)Lys(-)Lys, and (-)Lys/(-)Arg groups. On day 31 of feeding experiment, the feed intake of midas in the FAA treatment group was significantly lower than (-)Lys(-)Arg, (-)Lys(-)Lys and (-)Lys/(-)Arg treatment groups. In summary, our results indicated that midas did not decrease feed intake when fed an amino acid imbalanced diet compared with an amino acid balanced diet. Secondly, fish increased their feed intake of amino acid imbalanced diets although they had initially lower weight gain and lower feed conversion rates. It appears that the midas' response to (-)Lys or (-)Arg diets is dissimilar to that in mammals or birds, and that the neurological signals involved may be processed or interpreted differently. It is hypothesized that due to its high tolerance of amino acid imbalances, the fish is the ideal model organism to re-examine metabolic responses to dietary disproportions of amino acids. Furthermore, this study suggests that fish fed IAAs-imbalanced diets in an appropriate sequence may benefit fish growth through hyperphagia. The sequential use of disproportionate AA diets may also be used as a tool to eliminate the negative effect of IAAs deficient diets (plant protein-based) on fish growth.

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REGULATION OF ASYMMETRY IN ATLANTIC HALIBUT (*HIPPOGLOSSUS HIPPOGLOSSUS*) – GENE EXPRESSION OF MyoD AND PITX2

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Introduction

Most vertebrates appear bilaterally symmetrical, but many of their internal organs are asymmetric. Flatfish hatch as symmetrical larvae with asymmetric internal organs like other vertebrates. During metamorphosis they become an externally asymmetric and right-sided flatfish. Although halibut are right-sided, up to 19% in commercial hatcheries turn out to be left-sided. It is not known why this phenomenon occurs, but it is likely to be due to a combination of genetic and some environmental factors. Pitx2 and MyoD have been studied in relation to asymmetry (Hashimoto et al., 2002). The transcription factor Pitx2 is asymmetric expressed in the left lateral plate mesoderm (LPM) in all vertebrates studied, and has important roles in vertebrate pituitary, eye, branchial arch, hindlimb, brain development, as well as having a key function in regulating left-right asymmetry of internal organs (Ryan et al., 1998). Another transcriptional factor, MyoD, is involved in control and regulation of muscular development (Weintraub et al., 1993), and has been observed asymmetrically expressed in the earliest somites of halibut embryos. In symmetric fish, like rainbow trout, this factor is expressed symmetrically in the somites (Delalande and Rescan, 1999). By screening these asymmetric gene expressions patterns in embryos and later relating these expression patterns to the proportion of left- and right-sided halibut juveniles, we tried to determine whether these factors are involved in the regulation of asymmetry in the halibut.

Materials and methods

Halibut embryos were sampled in a commercial halibut hatchery, from ½-gastrulation to hatching and fixed as described by Hall et al. (2003). Whole-mount in situ hybridisation (WM-ISH) was performed on dechorionised em-

bryos as described by Hall et al. (2003), but with some modifications. Gene expression was studied in ten different egg groups and eight different developmental stages with DIG-RNA antisense probes for Pitx2, MyoD1, and MyoD2. Sense probes were also used as negative controls. Paraffin sections were made of selected WM-ISH embryos to identify the expression more precisely. In addition, a screening of asymmetric expression at two different stages for both MyoD2 and Pitx2 was performed. This was done in five different egg groups. Left-sided juvenile halibut were registered at the end of metamorphosis in the different egg groups and related to the asymmetric gene expression.

Results and discussion

Pitx2 was first detected at ½-gastrulation in the anterior part of the head (Fig. 1a). At later stages the expression was detected by histology in the jaw, brain, and the developing teeth and eye precursors. Pitx2 was also found on the left side of LPM (Fig. 1b) from the 30-somite stage until close to hatching in 97% of the embryos. This is consistent with expression patterns in other vertebrates, where this left-sided expression regulates left-sidedness of several internal organs (Ryan et al., 1998).

Two isoforms of MyoD were identified in halibut: MyoD1 and MyoD2. In rainbow trout these two genes are probably the result of an early genome duplication event about 100 million years ago (Delalande and Rescan, 1999). The expression patterns of the two isoforms were distinct from one another. MyoD1 was first expressed in the adaxial cells at ¾-gastrulation, which are located close to the notochord (Fig. 1c). MyoD2 was detected in the developing somites (Fig. 1d). From the 20 somite stage the two isoforms were both expressed in the whole of the somites where expression occurred. However while MyoD1 was expressed symmetrically, MyoD2 was expressed asymmetrically in the developing somites (Fig. 1e) at several early development stages (¾-gastrulation, blastopore closure, 20-somite stage, and 30-somite stage).

There was no link between the proportion of embryos showing asymmetric MyoD2/Pitx2 expression and the proportion of left-sided metamorphosed halibut juveniles. This indicates that MyoD2 and Pitx2 are probably not involved in the regulation of external asymmetry in halibut. The functional significance of the asymmetric MyoD2 expression is not known. Pitx2 is probably an important factor in the regulation of left-right asymmetry in internal organs. A few of the embryos displayed symmetrical expression of Pitx2 in the LPM, and these embryos were also highly deformed in the head and tail. This indicates that Pitx2 can be a possible candidate responsible for the deformities observed in farmed halibut and other fish. The symmetrical expression in brain, eyes, and lower jaw is consistent with the critical role of this factor for normal development of this tissues in both humans and fish (Ryan et al., 1998). The different expression patterns

and sequences indicate different roles for MyoD1 and MyoD2. MyoD1 is probably not a muscle regulation factor as in other fish, because of a lacking conserved motif (bHLH). Halibut have another intact MyoD1 protein that can perform the regulation of muscle, which is the original function of the ancestral MyoD. MyoD2 might have become redundant in halibut, which is a common feature in duplication processes. It is also possible that MyoD2 has acquired a new function, which is observed for MyoD2 in amphioxus (Urano et al., 2003).

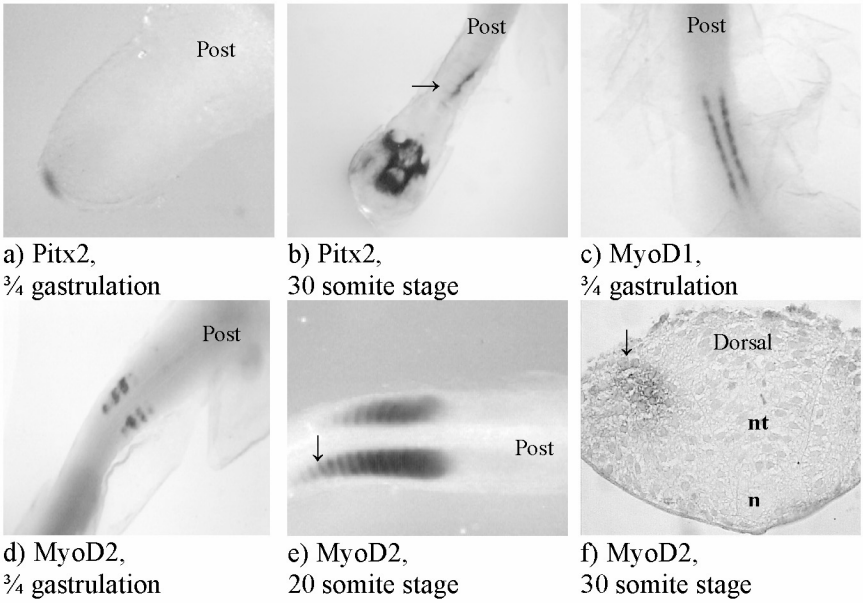

Fig. 1. Gene expression by in situ hybridisation of Pitx2, MyoD1, and MyoD2 in halibut embryos. a) Pitx2 expression in the anterior part of the head at ¾-gastrulation; b) Asymmetric Pitx2 expression in the left LPM at 30 somite stage; c) MyoD1 expression in the adaxial cells at ¾-gastrulation; d) MyoD2 expression in the developing somites ¾-gastrulation; e) Asymmetric MyoD2 expression in the somites at the 20 somite stage; f) Paraffin section of MyoD2 at the 30 somite stage which shows only expression on the left side of the embryo. Arrow indicates asymmetry, post=posterior part of the embryo, n=notochord, nt=neural tube.

Conclusion

The asymmetrically expressed Pitx2 in the LPM most likely has a key function in regulating left-right asymmetry of internal organs in halibut. MyoD1 is involved in the regulation of muscle differentiation in halibut. MyoD2 may have become redundant, or has another and new, still unknown function in halibut.

Acknowledgements

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CHANGE OF LIPID AND FATTY ACID COMPOSITION DURING DEVELOPMENT OF *GALAXIAS MACULATUS* (*OSMERIFORMES: GALAXIIDAE*) EGGS AND LARVAE

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Introduction

It is recognized that environmental factors such as temperature, salinity, light, or available food type affect the lipid contents and fatty acid composition of aquatic organisms. For instance, successive changes in salinity during the migrations between fresh and saltwater in diadromous fish have been shown to cause significant changes in their metabolism (Sheridan, 1989), obviously affecting their biochemical composition mainly as a preparation or response to the osmoregulation process originated by the change of environment.

Since *Galaxias maculatus* can indiscriminately live in lacustrine and estuarine environments (freshwater and brackishwater, respectively), the aim of the present study was to compare the evolution of the fatty acid composition of eggs and larvae from estuarine, freshwater, or cultured populations in order to understand the effect of their broodstock origin.

Materials and methods

Sexually mature freshwater and estuarine broodstock were captured wild, and cultured broodstock were taken from F1 adults reared in captivity from wild parents. Eggs were obtained by artificial fertilization and incubated at 13±1°C and 0ppt for freshwater and cultured broodstock, and 10ppt for estuarine broodstock.

After fertilization, eggs from various females at 3-4 hours post-fertilization (1st cleavage), and embryons in the epibolic stage, embryons in the organogenesis stage, embryons in the ocular pigmentation stage, and embryos close to hatching were sampled. Once the larvae were hatched, they were kept in starvation in 2-l tanks at room temperature (12±1°C) at the same water salinity condition as in

incubation. Samples were then taken from newly hatched larvae, larvae with half of the yolk-sac reabsorbed, and larvae with the yolk-sac fully reabsorbed.

Each analysis was performed in duplicate from two samples from each pool and expressed as a percentage of dry weight. Total lipid was extracted from 0.1g of samples (eggs or larvae) and homogenizing in chloroform:methanol (2:1, v:v), according to Folch et al. (1957) and stored at -80°C prior to analysis. Fatty acid methyl esters were prepared from the extracted lipids according to Morrison and Smith (1964). Fatty acids were identified by separation in a gas chromatograph (Hewlett Packard 5890 series II Plus, Wilmington, USA) using a 30m × 0.25mm i.d. × 0.25μm capillary column HP-225 (Hewlett Packard, Wilmington, USA). Nitrogen was used as a carrier gas. Fatty acids were identified by comparison to a well characterized standard such as GLC 462 (Nu-Chek Prep, Elysian, USA).

Results and discussion

The results indicate that the level of lipids in embryos and larvae from estuarine broodstock is lower than those from freshwater. This is probably a product of changes in salinity, not changes in diet (Table I).

The ratio n-3/n-6 polyunsaturated fatty acids (PUFA) increased considerably after hatching in larvae from estuarine fish, but not in those from freshwater or cultured conditions. The ratio of n-3/n-6 PUFA in eggs and larvae from estuarine fish corresponds to a marine pattern, while those from freshwater clearly correspond to a freshwater pattern. This agrees with Tocher et al. (1995), where changes in the composition of fatty acids resulted from a change in salinity rather than diet in juveniles of some salmonid species. In three cases, DHA was more abundant than EPA. It was also observed that both PUFAs were primarily conserved during embryogenesis and then consumed during larval development, independent of the original environment of the breeder (Table I).

Conclusion

Both environment and diet of broodstock fish affected lipid and fatty acid composition of *Galaxias maculatus* embryos and larvae as well as their development, suggesting that differences in the requirements of first-feeding fish may be predicted for larvae coming from different environments or reared in different water salinity. Further experiments are conducted to confirm this hypothesis.

Table I. Change of lipids, ratio n-3/n-6 PUFA , DHA (22:6n-3), and EPA (20:5n-3) during embryonic and larval development of *Galaxias maculatus*: eggs at 3-4 hours post-fertilization (A), embryons in the epibolic stage (B), embryons in the organogenesis stage (C), embryons in the ocular pigmentation stage (D) and embryo close to hatching (E), newly hatched larvae (F); larvae with half yolk-sac reabsorbed (G); and larvae with yolk-sac fully reabsorbed (H).

Populations	Fatty acids	A	B	C	D	E	F	G	H
Estuarine	% lipids	17.8±1.45	12.6±0.38	12.4±0.23	13.0±1.18	24.3±1.13	20.8±2.62	24.1±1.72	20.3±1.12
	n-3/n-6	7.3±0.46	5.6±0.33	4.4±0.08	6.4±0.33	4.7±0.72	16.7±1.31	15.1±0.24	11.0±0.86
	EPA	8.9±0.13	6.9±0.18	6.7±0.15	7.4±0.15	3.6±0.61	9.3±0.21	6.6±0.20	4.2±0.06
	DHA	19.5±0.62	15.7±0.3	15.2±0.91	17.9±0.54	10.7±1.97	24.3±0.8	21.7±1.72	14.2±0.43
Captivity	% lipids	26.4±0.37	22.9±0.97	19.3±1.95	12.6±0.33	18.5±0.09	29.8±1.58	28.9±0.50	25.6±1.83
	n-3/n-6	5.6±0.40	6.2±0.14	6.3±0.20	7.2±0.39	6.7±0.67	7.2±0.30	7.0±0.20	4.3±0.36
	EPA	4.9±0.06	4.7±0.03	4.6±0.10	5.1±0.16	5.2±0.01	5.0±0.51	5.0±0.06	2.1±0.01
	DHA	17.0±0.47	16.0±0.19	18.7±0.89	22.2±3.39	20.5±0.00	23.2±2.33	21.0±2.03	10.6±0.1
Freshwater	% lipids	24.4±1.49	21.8±0.58	24.9±0.00	26.3±1.07	24.2±2.97	23.1±0.03	24.7±2.24	27.1±1.57
	n-3/n-6	2.5±0.08	3.2±0.00	3.2±0.35	1.1±0.01	2.7±0.09	2.2±0.00	2.4±0.13	1.9±0.00
	EPA	4.6±0.06	5.8±0.01	5.3±0.45	2.2±0.12	4.1±0.29	3.2±0.00	2.2±0.16	1.4±0.04
	DHA	8.4±0.14	9.9±0.02	9.0±1.38	6.3±0.35	9.4±0.7	8.6±0.01	5.8±0.02	4.7±0.22

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DIFFERENTIAL EXPRESSION OF DIGESTIVE ENZYMES PRECURSORS IN YOLK-SAC AND UNFED LARVAE OF *PAGRUS PAGRUS*

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Introduction

The knowledge of digestive physiology of fish larvae is crucial to formulate adequate inert diets to their nutritional needs. One of the most critical steps of larval development is the transition from endogenous to exogenous feeding. Yolk sac exhaustion, mouth opening and food availability should be synchronized to avoid prolonged periods of starvation that debility the larval physiological status. The aim of the present work is to evaluate the effects of fasting at gene expression level of three digestive precursors (amylase, trypsinogen and bile salt-activated lipase) in red porgy larvae (*Pagrus pagrus*) in order to understand the functioning of physiological mechanisms implied under such situation.

Materials and methods

Eggs of red porgy were obtained by natural spawning from captive brood stock. Newly hatched larvae were transferred to 300 L tanks with flow-through water supplied at a constant temperature of 19.5±1 ºC and salinity of 33 ‰. Constant illumination was provided during the first two weeks switching to a photoperiod of 12L:12D afterwards. Larvae were fed with *Brachionus rotundiformis* at 10.ml^{-1}. Fed and unfed larvae were sampled at 0, 3, 5 and 7 days after hatching (dah).

Total RNA was extracted from pooled larvae and reverse transcribed using the RETROscript kit (Ambion). RT-PCR experiments were carried out to study the expression of α-amylase, trypsinogen and bile salt-activated lipase (BAL) mRNAs. Specific primers for the red porgy precursor sequences were designed based upon the cDNA obtained in previous studies (data not shown) (Table I). Amplification of the housekeeping gene β-actin using primers from winter

flounder (Douglas et al., 2000), was performed to provide an internal control of gene expression. Controls were performed using single primers (to check for single primers artifacts) and both primers without template (to check for external contamination).

Table I. Oligonucleotide primers used in RT-PCR reactions. Tm: melting temperature; MW: molecular weight.

Primer	Bases	Sequence 5'→3'	Tm	MW
Amylase 5'	20	GAT CTG TCT GCT GTG TAC GG	55.2	6155
Amylase 3'	20	ATC CGT CTC CAC AAG TCT GG	56.5	6053
Trypsinogen 5'	20	GAG AGC ATG ATA TCT ACC GC	52.4	6126
Trypsinogen 3'	20	CGC AGA ACA TGG CAT CGG TG	59.3	6167
BAL 5'	20	GGT GAC GTT GGG ATA CCG TG	58	6229.1
BAL 3'	20	GGC CAG GTT GTT TAC AAC AG	54.2	6157

The amplification conditions were 2min at 94ºC; 31 cycles of 29s at 94ºC, 30s at 61ºC, 1min at 72ºC; 2min at 72ºC. Products were resolved on a 1.8% agarose gel and compared to a 100-bp ladder (Amersham Pharmacia Biotech AB). Expression was quantified using a GelDoc 1000 video gel documentation system (Bio-Rad) with Multianalyst software. The amount of precursor's transcript relative to the amount of actin transcript was calculated once the background was subtracted.

Results and discussion

Red porgy larvae hatch with elevated levels of expression of the three precursors studied, although amylase showed the highest amount of transcript (5.26) (Fig. 1). During the endogenous feeding period, amylase and BAL expression decreased reaching same transcripts values at mouth opening (around 0.53), while trypsinogen expression declined slightly and showed approximately 6 times higher value of transcript than the precursors mentioned above. During the exogenous feeding stage there was a similar pattern of expression of the three precursors: an initial increase until 5dah and a posterior decrease until 8dah, being this decline more pronounced in trypsinogen. The three precursors showed an increase of their expression under starvation conditions until 5dah. Afterwards, trypsinogen synthesis was not influenced by fasting, while BAL and amylase showed a continuous increase of their expression until 1.25 and 1.50, respectively (Fig. 2).

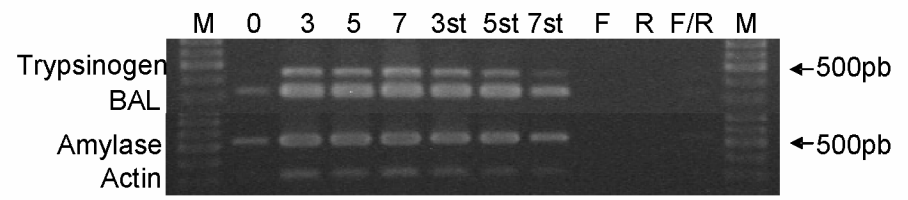

Fig. 1. RT-PCR analysis of trypsinogen, BAL and amylase expression during larval development of the red porgy (*Pagrus pagrus*). The age of the larvae (dah) are indicated above each lane. Total larval cDNA was used as a template for PCR. Amplification products (330, 442, and 549pb, respectively) were resolved on an 1.8% agarose gel using low range ladder (Amersham Pharmacia Biotech AB) as markers (M). Actin mRNA was amplified (310pb) as a housekeeping gene. The three controls did not show any expression signal (F, forward primer; R, reverse primer; F/R, both primers without template).

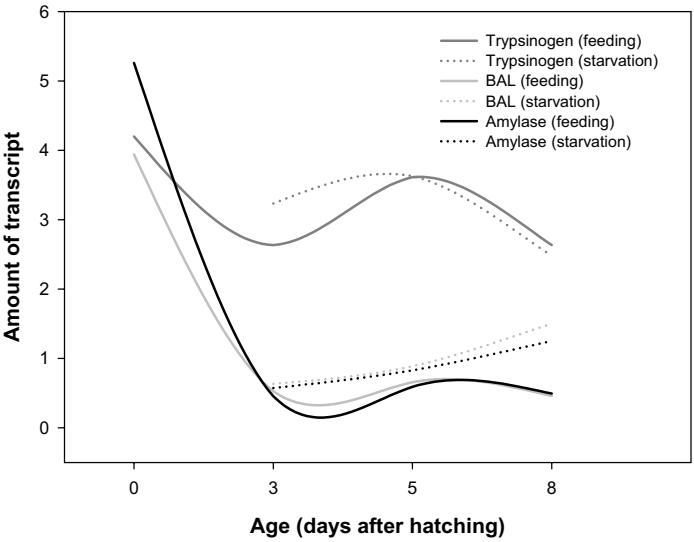

Fig. 2. Amount of trypsinogen, BAL, and amylase transcripts relative to amount of actin in *P. pagrus* larvae under feeding and starvation conditions. Striped fragment indicates mouth opening.

Conclusions

Red porgy larvae are preparing their enzymatic equipment from hatching in order to be ready for external food digestion at first feeding. According to the carnivorous nature of this species, trypsinogen is the main precursor synthesized, being BAL and amylase expression reduced 6 times from mouth opening. Day 5 after hatching is a critical point from which starvation conditions generate dif-

ferences in expression of precursors. There is an increase of expression of the three precursors before 5dah probably as a strategy of survival against the new environmental situation. In this sense, food absence also resulted in an increase of expression over that of fed larvae. After that date, trypsinogen was not modulated by feeding conditions, whereas BAL and amylase experienced an increase. A possible explanation of these results could be that, due to an easer digestion of carbohydrates and lipids than proteins, larvae synthesize BAL and amylase to maximize the energy procurement.

Acknowledgements

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ZOOTECHNICAL IMPROVEMENTS IN THE LARVICULTURE OF EUROPEAN MARINE FISH

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In recent years marine fish hatcheries in Europe have been forced to intensify their production and introduce more automation in order to reduce larviculture production costs. The produced fry quantity increased while the fry quality was maintained, reducing at the same time the production costs in order to increase the profitability.

This process has also been addressed to the production of sea bream fry, considered from many producers' point of view as one of the most complicated among the Mediterranean species. This is mainly due to the fact that important phyto- and zooplankton production is needed in order to obtain successful larviculture.

Comparing the historical figures of fry production from 2000 to 2005, the main production indices have drastically changed, but this time without a substantial growth of investments or hatchery volumes and areas.

An example of observable improvements is the number of fry produced per employee per production season. This number more than doubled from 200 000 up to over 500 000 fry per person. Another visible change is the production of fry per production volume, that has risen in the last five years from 20 000 up to an average of 50 000 fry from 0.5-1g.

On the contrary to what happened in the previous decade, these important changes were mainly obtained by improving the zoo-techniques, substantially helped by new, simple, and affordable technical adaptations and implementations. The most important changes have been introduced in the live food production units, as well as in the larval and weaning sectors.

In live food production, the mass culture of algae evolved towards high-density and continuous culture systems instead of the traditional batch culture systems. Using these systems, the production of the two most important algae species used in marine fish larviculture – *Nannochloropsis* and *Isochrysis* spp. – per-

formed in 150-l culture volumes for example results in a daily yield of 50 l algae suspension at 150 resp. 25 million cells per ml. These cultures can proceed for several weeks and result in a strongly reduced manpower need, consumables, and space requirements.

Traditional low-density rotifer batch cultures evolved towards high-density batch culture systems and low-maintenance recirculation or flow-through systems. The new techniques give a daily yield of around 1.5 billion rotifers per metric tonne and the predictability of these systems is very high. Also for the hatching and enrichment of *Artemia*, automatic hatching, separation and harvesting systems are being developed, resulting in a strongly reduced manpower production system.

Adoption of the new live food techniques at production scale resulted in, next to a considerable reduction in manpower and space requirements, a levelling out of the variations in the live food quality.

In the larval rearing sectors, stocking densities of larvae increased to more than 150 000 larvae per metric tonne, in the most produced species, sea bream and sea bass. These high stocking densities are made possible by an early transfer of fish larvae (day 20-25) into the weaning unit and an early onset of weaning with high valuable early weaning diets. In this way, the need for phyto- and zooplankton is strongly reduced. Automation in the larval rearing sector, such as constant monitoring of dissolved oxygen levels, temperature, the introduction of self-cleaning surface skimmers, etc. greatly reduced manpower requirements.

In the weaning sector, the increased efficiency of oxygen transfer into the circulating water has considerably reduced the production cost. The use of recirculation systems or semi-closed systems stabilizes the water quality and reduces the cost for warming up the water in the cold season, levelling out the variations in the composition of used seawater or borehole water. Automation of the food distribution and size grading of the fry resulted again in reduced manpower needs.

Furthermore, some zoo-technical adaptations were applied successfully to reduce the incidence of the most important deformities (skeletal malformations) in sea bream fry to a very low incidence.

Overall, the adoption of the zoo-technical improvements is resulting in a better survival rate, while the quality standards for the fry are scoring higher than before. The final result is a higher profitability for the fry producers.

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PROBIOTICS FOR SHRIMP LARVICULTURE – REVIEW OF FIELD DATA FROM ASIA AND LATIN AMERICA

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Introduction

Major losses in penaeid hatcheries are due to *Vibrio* spp., which are opportunistic bacteria. They are normally present in the culture facilities as well as in the larval gut flora and in the live feed and they usually cause disease in sub-optimal culture conditions. The application of antibiotics is expensive and detrimental; i.e., selection of bacteria that are drug-resistant or more virulent, prevalence of drug residues in reared animals. Probiotics, which compete with bacterial pathogens for nutrients and/or inhibit the growth of pathogens, can be a valid alternative to the prophylactic application of chemicals (antibiotics and biocides). Here, we report on the performance of a commercially available mixture of *Bacillus* strains (SANOLIFE® MIC), using data from an Asian hatchery (*Penaeus monodon*) and a Latin-American hatchery (*Litopenaeus vannamei*) as examples.

Materials and methods

A mixture of specific *Bacillus* strains was designed following a research program on the ability of numerous *Bacillus* strains to inhibit a range of pathogenic *Vibrio* strains, to grow under conditions prevailing in shrimp hatcheries, and to degrade waste products. These strains were then included in bioassays and challenge tests in order to confirm the lack of toxin production and pathogenicity to target organisms. Various mixtures of *Bacillus* strains were evaluated in laboratory-scale and pilot-scale conditions. Finally, the best-performing mixture (SANOLIFE® MIC) was assessed in commercial or pre-commercial conditions in various Asian and Latin-American countries.

The commercial product includes *Bacillus* spores and nutrients. These spores were germinated prior to application in the rearing tanks by suspending the product in sterile water (drinking water or disinfected seawater) for up to 8h. The appropriate amount of germinated *Bacillus* was immediately applied to the

tanks in order to reach a final concentration of 1-5×10^4cfu.ml^{-1}. The probiotics were applied daily.

In three separate tests run over a period of 1 year, the performance of the *Bacillus* mixture was compared to antibiotic prophylactic and negative control in a Thai hatchery. *P. monodon* nauplii were reared (4 × 175-l tanks per treatments in each test) until PL10, and fed a combination of *Chaetoceros*, *Artemia* nauplii, and Lansy Shrimp (40% live food substitution) larval diet (INVE).

The performance of the *Bacillus* mixture was compared to prophylactic antibiotics (in combination with formaldehyde treatment in early PL stages) and negative control (without the addition of any chemicals) in a Brazilian hatchery. Each 20-MT tank was stocked with 5 million *L. vannamei* N3.$tank^{-1}$. Water disinfection included chlorine addition followed by neutralization with thiosulphate. Shrimp larvae were fed microalgae, *Artemia*, and commercial larval diets.

Results and discussion

In replicate tests run in Thailand, daily application of SANOLIFE® MIC gave results similar to those observed with prophylactic antibiotic (Fig. 1).

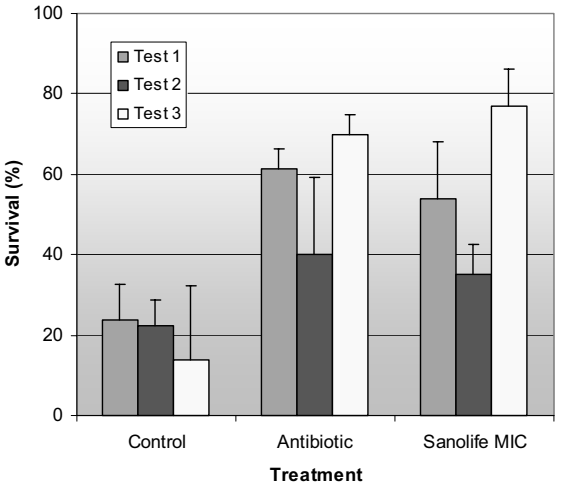

Fig. 1. Survival of *Penaeus monodon* larvae at PL10 in 3 tests run in Thailand.

In 2 of these 3 tests, tank biomass was also recorded at harvest. The tank biomass of the negative control (18.6±15.6g.$tank^{-1}$), was significantly lower than recorded for the antibiotic (48.3±22.3g.$tank^{-1}$) and probiotics tanks (58.3±28.7g.$tank^{-1}$). There were no significant differences between antibiotic and probiotic treatments.

Similarly, the daily application of SANOLIFE® MIC to 20-MT tanks stocked with *L. vannamei* led to marked improvement in survival compared to the negative control (Fig. 2). Unfortunately, the lack of replicates inherent to field evaluations prevented statistical analyses.

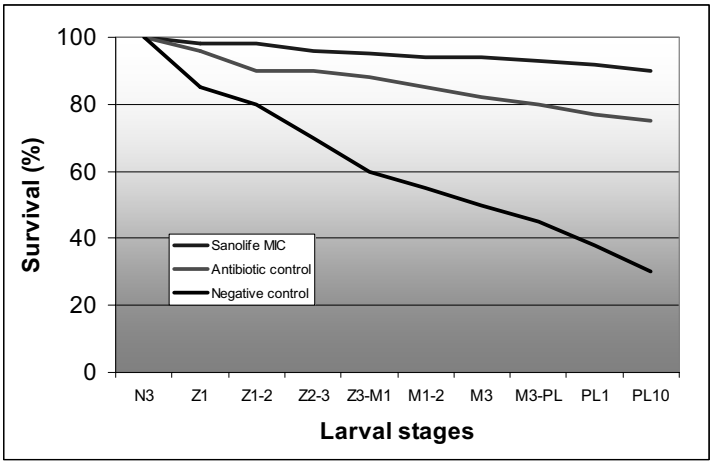

Fig. 2. Survival of *Litopenaeus vannamei* larvae in a commercial Brazilian hatchery. The performance of SANOLIFE® MIC was compared to prophylactic antibiotic (in combination with formaldehyde treatment in early PL stages) and negative control (no chemicals added).

Conclusions

- The performance of SANOLIFE® MIC was evaluated in numerous hatcheries in Asia and Latin America with *P. monodon* and *L. vannamei*.
- As shown in the two examples, probiotics have the potential to produce results similar to those obtained with antimicrobials and, importantly, in a cost-effective manner.
- Obviously, minimizing the risk of vibriosis demands a multi-disciplinary approach, including good hygiene and sanitation measures to reduce the input of potential pathogens, as well as a suitable farm management. Products such as probiotics are only effective and cost-beneficial when they are properly applied together with a suitable farm management.
- Interestingly, this probiotic mixture also gave very promising results in mitten crab larviculture with increased survival and dry megalopa body weight.

Acknowledgements

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THE IMPACT OF MUTATIONS IN THE QUORUM SENSING SYSTEMS OF *AEROMONAS HYDROPHILA, VIBRIO ANGUILLARUM*, AND *V. HARVEYI* ON THEIR VIRULENCE TOWARDS GNOTOBIOTICALLY CULTURED *ARTEMIA FRANCISCANA*

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Quorum sensing, bacterial cell-to-cell communication by means of small signal molecules has been previously shown to regulate the expression of several virulence-associated phenotypes, including the production of proteases, haemolysin, and toxin. Therefore, disruption of quorum sensing has been suggested as a new anti-infective strategy for aquaculture. So far, two major quorum sensing systems have been found in Gram-negative aquatic pathogens. Like many other Gram-negative bacteria, *Aeromonas hydrophila* and *Vibrio anguillarum* were found to contain an acylated homoserine lactone (AHL)-mediated quorum sensing system. A totally different multichannel system was described in the luminescent bacterium *V. harveyi*. The first channel of this system is mediated by an AHL. The second channel is mediated by the so-called autoinducer 2 (AI-2), which is a furanosyl borate diester. The chemical structure of the third autoinducer is still unknown. All three autoinducers are detected at the cell surface and activate or inactivate target gene expression by a phosphorylation/dephosphorylation cascade. A system similar to the *V. harveyi* one was recently discovered in *V. anguillarum*, in addition to its previously mentioned AHL-mediated system.

Quorum sensing mutants of *A. hydrophila* AH-1N, *V. anguillarum* NB10, and *V. harveyi* BB120 have been constructed before and therefore, these strains were chosen as representatives to study the impact of mutations in the quorum sensing systems of aquatic pathogens on their virulence. To this end, a model system using gnotobiotically grown *Artemia franciscana* was developed. Working in the absence of other bacteria is important for this type of experiments since they might cause bias. Indeed, AI-2-mediated signalling was found to be present in many different species. As far as we know, it is the first time that this type of experiments was done in a well defined gnotobiotic environment. Sterile *Ar-*

temia nauplii, obtained by hatching of decapsulated cysts, were cultured in filtered and autoclaved artificial seawater and fed dead LVS3 bacteria. In a first experiment, we found that a single addition of 10^7 CFU·ml^{-1} of LVS3 was sufficient to obtain a survival of *Artemia* of more than 80% after 48h. This feed level was used in all further experiments.

A first challenge test showed that the wildtype *Aeromonas hydrophila*, *V. anguillarum*, and *V. harveyi* strains were pathogenic to *Artemia*, with the *V. harveyi* strain likely being an opportunistic pathogen. A further series of challenge tests revealed that mutations in the AI-2-mediated channel of the *V. harveyi* quorum sensing system abolished virulence of strain BB120, whereas mutations in the AHL-mediated channel had no effect on its virulence. Furthermore, we investigated whether an exogenous source of AI-2 could restore the virulence of an AI-2 non-producing mutant. Filter-sterilised washwater of an AI-2-producing *V. harveyi* culture was chosen as an exogenous source of AI-2 because it is not yet possible to obtain the molecule in a purified form. The washwater could indeed restore the virulence of the AI-2 non-producing mutant.

It has been shown before that blocking the AHL-mediated quorum sensing system of *A. hydrophila* AH-1N with signal molecule analogs decreased exoprotease production by the bacterium. However, we found that mortality of *Artemia* caused by the same *A. hydrophila* strain was not different from that caused by its quorum sensing mutants. Also for *V. anguillarum* NB10, we found that neither mutations in its AHL-mediated system nor in its dual channel system had any effect on its virulence towards *Artemia*. Our data are in accordance with results obtained before with rainbow trout (*Oncorhynchus mykiss*) challenged to the same *V. anguillarum* wildtype and mutant strains.

All together, our results indicate that disruption of quorum sensing could be a good alternative strategy to combat infections caused by *V. harveyi*. Several pathogenic *V. harveyi* strains are found to be resistant against antibiotics and disinfectants. Therefore, disruption of the AI-2-mediated channel of the *V. harveyi* quorum sensing system might prove to be a good additional tool to combat infections caused by this pathogen.

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OPTIMISATION OF LIVE FEED ENRICHMENT WITH INNOVATIVE PRODUCTS: FIELD RESULTS

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Introduction

A major concern in intensive marine hatchery operations is the production of high quality fish. Still better results are obtained in extensive systems, where natural zooplankton is the main source of food. Juveniles produced in this type of system grow much faster and are of better quality than the ones originating from intensive rearing operations. This study looks into the nutritional aspects during the first weeks of feeding. The effect of an appropriate enrichment on quality of turbot and halibut fry was also evaluated.

Materials and methods

Rotifers were cultured in standard conditions (yeast and algae), harvested and transferred to an enrichment tank. The first experiment used a density of 3000 rotifers.ml^{-1}. Temperature was kept at 23°C, salinity at 22ppt, and the enrichment product MultigainlowARA (DANA FEED A/S, Denmark) was dosed at 0.2g.million^{-1} rotifers. Samples were taken before enrichment and after 2 and 8 hours of enrichment. Samples were analysed for lipid, protein and HUFA composition. In the second experiment, rotifers were kept at 1000.ml^{-1} at 24°C in 30ppt seawater and 0.2g MultigainlowARA was added per million rotifers. Samples were taken for vitamin A, astaxanthin, thiamine, and iodine analyses after 2-4h of enrichment.

Turbot were evaluated at first grading (140 000 fry), *Artemia* feeding (day 15-32) being the only variable (Multigain versus control). *Artemia* enrichment was done in standard conditions (0.5g.l^{-1} for 24h at 28°C and density of 300.ml^{-1}). Fry were graded at an average weight of about 1g over different mesh sizes and evaluated for deformities (sum of operculum, malpigmentation, and eye migration). Fish were defined as 'big' when their weight was more than 1.5g, 'medium' between 0.9 and 1.3g, and 'small' less than 0.7g.

A production batch of halibut was carried through metamorphosis and grown up to 1-4g when deformities (eye migration and pigmentation) were assessed on a total of 298 juveniles. *Artemia* feeding was exclusively on Multigain-enriched *Artemia* (standard procedure, see above).

Results and discussion

For the first rotifer enrichment experiment, rotifers had a lipid content of 9.1% (on dry matter) before enrichment. The lipid increased to 14.9 and 15.2% after 2 and 8 hours of enrichment, respectively, and protein decreased from 60.1% to 54.4 and 56.3%, respectively. Some selected fatty acids are presented in Table I. The results show that enrichment causes an increase in lipids and a corresponding decrease in protein. This is to be expected as the main part of the enrichment product is lipid based. Comparing these values with natural zooplankton (copepods), these levels are in the upper range (11-14% lipids in natural zooplankton, calculated from van der Meeren, 2003). Protein levels in natural zooplankton are reported within a greater span; 38% on dry weight to 72% (Hamre et al., 2002). Enriched rotifers have a total lipid DHA value of 2% (dry matter), a bit lower than reported for natural zooplankton (e.g., 1.9-2.9%, calculated from van der Meeren (2003)).

Interesting from this experiment is that the difference between 2 and 8 hours enrichment is not reflected in the enrichment levels. This has a practical consequence for the hatcheries that could benefit from a shorter enrichment procedure.

Table I. Results of the analysis of some fatty acids methyl esters (FAME) in rotifers before enrichment and after 2 and 8 hours of enrichment with MultigainlowARA. Values are expressed in $mg.g^{-1}$ dry weight.

	ARA	**EPA**	**DHA**	**n-3 HUFA**	**n-6 HUFA**
Non-enriched	0.27	3.33	2.39	8.9	3.6
2h enriched	1.2	4.7	16.62	25.44	11.87
8h enriched	1.89	5.93	20.3	31.05	14.1

For the second rotifer experiment, the following values were obtained after 2-4 hours enrichment on MultigainlowARA (in ppm on DM basis): vitamin A: 2.45, astaxanthin: 143.2, vitamin C: 1191, thiamine: 6.1, and iodine: 34.4.

Comparing these values to wild zooplankton levels, the vitamin A level is within the same range (0.2-2.7ppm; Moren et al. (2001)); there is a clear increase in astaxanthin levels after enrichment (<10ppm in non-enriched rotifers, not published data) up to more than 100ppm, though this is still below levels found in the wild (244ppm; own sampling, unpublished data) to 748ppm (van der Meeren, 2003). A significant increase in vitamin C to more than 1000ppm was observed, levels known to have an effect on stress resistance in larvae. Thiamine

levels are on the lower side (requirement at least 10ppm for halibut), but also iodine could be increased by further enrichment, as levels in zooplankton are mostly more than 50ppm (K. Hamre, pers. comm.).

These results show that by enrichment with a product formulated with all these necessary nutrients, the rotifer composition can be altered to mimic more closely the natural prey.

For turbot, the results of size distribution and deformities are presented in Fig. 1. Turbot that were fed multigain-enriched *Artemia* were bigger than the control, reflected in the size distribution. Also, mortality was lower in this group as well as the deformed fish, which resulted in a significant higher output of good fry in this particular hatchery. Better results can be explained by the improved composition of the *Artemia* (results not shown here), mainly in terms of DHA, vitamin and mineral content.

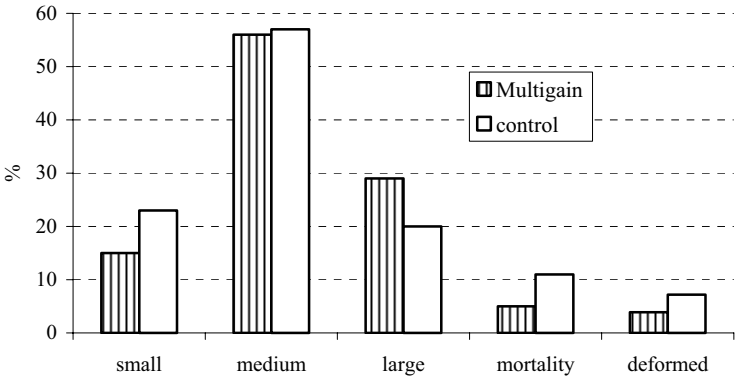

Fig. 1. Effect of *Artemia* enrichment on size distribution, mortality and deformities in turbot fry

Table II. Number and percentage of halibut juveniles with no deformities (pigmentation and eye migration) within sampling groups. Juveniles expressing perfect pigmentation are also presented. n = 298.

Sampling	perfect juveniles		perfect pigmentation	
group (n)	number	%	number	%
A (36)	23	64	28	78
B (61)	22	36	38	62
C (45)	25	56	34	76
D (78)	49	63	63	81
E (78)	36	46	50	64
TOTAL (298)	155	52	213	71

Results of the quality assessment of 298 halibut juveniles from one production batch are presented in Table II. Of the juveniles looked at, 52% expressed perfect pigmentation and eye migration. Looking exclusively at pigmentation, 71% of the sampling group showed perfect pigmentation, meaning that the limiting factor in this case was the eye migration. From a practical point, the outcome was lest stringent and more than 70% of this group was accepted for further on-growing. The variations among groups could be explained by several factors like *Artemia* quality and enrichment conditions (e.g., oxygen, temperature), conditions during first feeding of the larvae, and impact of employees.

Conclusions

Young marine larvae that are still depending on live food are depending on the quality of this prey for their proper development. Natural zooplankton is hereby the reference for optimum nutrition. It is possible to manipulate the composition of rotifers and *Artemia* to a certain extent to come closer to the preferred composition. Still some nutritional factors are unknown and should be investigated. Enrichment products reflecting these needs can be formulated in order to provide the fry with the best nutritional balance. These studies have shown that it is possible to improve growth and quality of marine fish larvae by using an adapted enrichment procedure.

Acknowledgements

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CHANGES IN THE NUTRITIONAL APPROACH FOR CULTURING AND ENRICHING ROTIFERS AND *ARTEMIA*: IMPACTS ON PRODUCTION EFFICIENCY AND ECONOMICS

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In marine fish hatcheries rotifer mass production is still a very labor-intensive activity because of the needs for regular harvesting, cleaning, and concentrating of the rotifers. Furthermore rotifers might suffer from all these manipulations and their quality might be affected. Recent improvements in rotifer diet formulation have considerably improved the industrial production and enrichment of rotifers.

Inclusion in the new rotifer diet (Culture Selco Plus®) of fertility enhancers as well as the use of higher quality ingredients has improved culture performances – i.e., 25% shorter culture periods, 50% higher rotifer outputs, reduced bacterial counts (1-2 log units), and lower floccule loads than with earlier culture diets. These improvements have immediate consequences on live food quality, harvesting efficiency, and the overall economics in the hatchery. Culture crashes are considerably reduced as well as less frequent upscaling from stock cultures.

The use of formulated diets is not restricted anymore to application in high density cultures but can also be practiced under typical Asian hatchery conditions in large culture tanks set up with low rotifer densities (> 20-m^3 tanks, < 50 rotifers.ml^{-1}); since rotifers display a better condition and water quality can be better controlled, direct in-tank enrichment can now be practiced (e.g., with Protein Selco Plus®).

New formulations of concentrated pastes combine the properties of liquid and dry enrichment products and can be used without previous harvesting and rinsing of the rotifers, reducing manipulation stress in the rotifers and handling time for the workers. Since rotifers are not exposed to handling stress, the enrichment kinetics are far superior, e.g., more than 45mg.g^{-1} n-3 HUFA (consisting of more than 50% DHA) can be achieved within 12h enrichment with Protein Selco Plus®. Besides the lipid enrichment, rotifers are boosted with immuno-stimulants and contain up to 2000ppm vitamin C. The enrichment of rotifers in the culture

tank is not restricted to short rearing cycles. It has also been performed under Asian hatchery conditions in large flat-bottomed tanks ($12m^3$) using the combination Culture Selco Plus® for 8 days followed by a Protein Selco Plus® enrichment on day 9 without intermediate rinsing of the rotifers. After this 9-day culture-enrichment experiment, rotifers reached $25mg.g^{-1}$ DW n-3 HUFA and 1500ppm Vit C in less than 4h while dissolved oxygen levels could be kept at 3.3ppm without using pure oxygen.

Artemia enrichment is no longer focusing exclusively on lipid enrichment but inclusion of algal concentrates, specific peptides, amino acids, minerals, and other micro-elements have allowed to develop species-specific and larval-stage specific enrichment products (e.g., Plani Selco® for turbot, sole, halibut, etc.; Gadi Selco® for cod, haddock, etc.; Serrani Selco® for seabass, mullet, grouper, etc.; and Spari Selco® for seabream, dentex, etc.). Rational use of the species-specific boosters allows reduction of enrichment periods and working at lower temperature, securing lower DHA catabolism as well as improved phospholipid retention. By the time *Artemia* has reached its most digestible stage and would start to use its own reserves, it has been nutritionally modified with freshly ingested concentrates. The short enrichment at colder temperature considerably reduces the energy cost for the hatchery but also reduces the production of *Artemia* faeces and molting wastes which could result in secondary bacterial contaminations.

Experiments with *Artemia* quality enhancers (e.g., protein enhancers) have shown that minor changes in enrichment strategy can result in a drastic change in the protein/lipid ratio and HUFA balance of enriched *Artemia* nauplii. Starting with a protein booster followed by a species-specific lipid enrichment, a protein sparing effect has been noticed. During enrichment the relative protein content in *Artemia* is typically decreasing as a result of the boosting effect of the lipid emulsions. Depending on the enrichment strategy and using the combination of protein boosters and species-specific emulsions the protein content could be corrected or even increased to 58%. These protein enhancers combine the properties of liquid and dry Selco®'s and will be important tools in further improving larval quality for species in which the protein/lipid ratio plays an important role.

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THE EFFECT OF THE DUSK PHOTOPERIOD CHANGE FROM LIGHT TO DARK ON THE TIMING OF HATCHING OF EGGS OF THE SPOTTED ROSE SNAPPER (*LUTJANUS GUTTATUS*)

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Introduction

The eggs of rosy spotted snapper (*Lutjanus guttatus*) have been observed to hatch soon after the dusk change in photoperiod from light to dark. Hatching of sergeant major and triggerfish eggs has also been observed to normally occur after dusk and can be delayed with continual illumination (Tucker 1998), and it has been suggested that in these species hatching enzyme is controlled by photo-receptors. The present work aimed to determine the effects of photoperiod on hatching of rosy spotted snapper eggs.

Materials and methods

Mature rosy spotted snapper were caught and brought to the experimental facilities of the Centro Regional de Investigación Pesquera (CRIP), Bahia de Banders, Cruz de Huanacaxtle, Nayarit, Mexico. Female fish with oocytes greater than 400µm were selected and induced to ovulate with LHRHa EVAc slow-release implants (Prof. Y. Zohar, University of Maryland Biotechnology Institute, Baltimore, USA). The females were placed with males in the spawning tanks that received a natural photoperiod from windows in the laboratory. The fish spawned the day after being implanted between 8pm and 1am, and eggs were collected the following morning. Two studies were made on the hatching of the eggs. In the studies, approximately 100 eggs were pipetted into 200-ml glasses previously filled with filtered and UV-treated water. The incubation glasses were placed in flow-through water baths that maintained the same temperatures as the spawning tanks (28.5-30°C).

In the first study, eggs were collected close to 6am (sunrise). Glasses were placed for incubation in a darkened room using black plastic and a similar room which had a single 15W neon white light approximately 50cm above the incubators. Eggs placed in the dark room were collected in the dark before sunrise, and

eggs placed in the light room were collected soon after as the sun rose. The photoperiod treatments were applied by moving glasses from the light room to the dark room in a lightproof box. The following photoperiods were applied to the eggs: a continuous dark group, a light group which received light continuously from 6am, and 3 different groups that received light from 6 am followed by light to dark changes 14, 18, and 22 hours after spawning. Each hour from 11am until hatching was completed, the glasses were checked for hatched larvae (for the dark treatment glasses were removed, checked, and not returned) if hatched larvae were observed a glass was removed from the incubator, treated with formalin, and the larvae and unhatched eggs counted. The study was performed twice with eggs from 2 different females, study 1a and 1b.

In the second study eggs were collected from a total of 7 different females that spawned at different times from 8pm to 1am. The eggs were incubated as above but under a natural photoperiod. Hatching rate was assessed as above.

Results and discussion

Generally, hatching was observed to start 18h after spawning and to be complete by 22h after spawning. Little variation was observed in hatching under different photoperiods. In study 1a the eggs were observed under all photoperiods to initiate hatching between 19-20h after spawning and finish by 22h after spawn (Fig. 1).

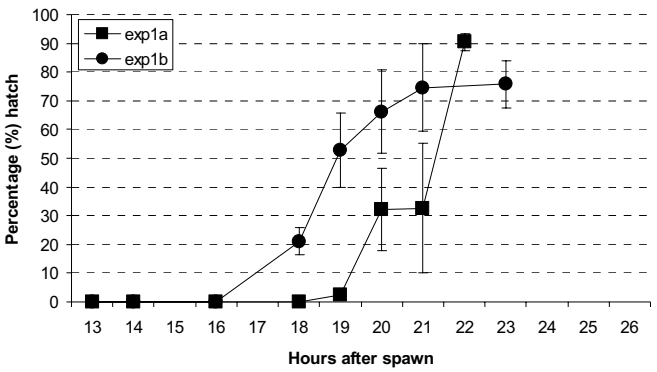

Fig. 1. Mean percentage hatched (±1 standard error mean) at each hour after spawning for all photoperiod groups in exp 1a and 1b.

In study 1b all eggs in photoperiod treatments started hatching 18-20h after spawning and finished 21h after spawning (Fig. 1). There was inconclusive evidence that hatching was affected by continuous light from 6am. In study 1b, final hatch rates ranged from 75.8-91.8% after 21h in all groups except the continuous light group that exhibited hatch rates between 45.7-83.4% (n=4). How-

ever in study 1a hatch rates after 22h from all groups ranged from 62.8-95.1% and hatch rates for the continuous light group ranged from 75.7-95% (n=4). Photoperiod did not appear to affect hatching time.

There was more variation in hatching times between individuals in study 2. Hatching was observed to be initiated from 16-18h and finish 19-22h. However, the majority of the eggs hatched between 18-20h. A maximum of 10% hatch was observed before 18h. Although there was more variation in hatching time between individuals, there did not appear to be an effect of spawning time on duration of incubation.

It has been observed in gilthead sea bream (Barbaro et al., 1997), turbot (Mugnier et al., 2000), and bullseye puffer (Duncan et al., 2003) that LHRHa-induced spawning treatments can accelerate oocyte development and advancing the normal time of spawning, suggesting that LHRHa interferes with diurnal ovulation patterns. Alterations in spawning time in relation to a fixed hatching time, for example hatching at the dusk, suggests light to dark change in photoperiod could result in insufficient time for correct larval development and cause poor larval quality. However, this study suggests that in the rosy spotted snapper incubation time is fixed at 18-22h and spawning time or dusk photoperiod changes do not affect the time of embryonic development.

Conclusions

The dusk photoperiod change from light to dark and different spawning times (8pm-1am) did not affect duration of incubation. Generally eggs hatched 18-22h after spawning. There was a suggestion that constant light should be avoided for the incubation period as it can result in variable hatch rates.

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DOES LARVAL SIZE LIMIT EARLY WEANING IN SENEGALESE SOLE, *SOLEA SENEGALENSIS* (KAUP, 1858)?

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Introduction

Weaning has traditionally been the bottleneck in sole farming. In recent years some progress has been made in Senegalese sole weaning. Cañavate and Fernández-Díaz (1999) were able to wean 8-mg sole using co-feeding to obtain 39% survival while Engrola et al. (2005) using sudden-weaning achieved 69% with 14-mg sole. According to Ribeiro et al. (1999), post-larvae at 3mg are able to ingest, digest, and absorb nutrients, having a morphologically complete tract and equipped with the necessary digestive enzymes.

This experiment was designed to verify whether early sudden weaning of sole is possible and to understand how digestive capacity of post-larvae is affected.

Materials and methods

Post-larvae ~1mg dry weight (DW) were transferred to 4-l white flat bottom plastic tanks and acclimatised for four days until the beginning of the experiment. Enriched frozen *Artemia* metanauplii were supplied to the post-larvae until the weaning started. The experiment consisted of three treatments done in triplicate. In all treatments, post-larvae were sudden-weaned after one day of fasting. Treatments consisted in sudden-weaning post-larvae with different weights: SW1 - 1mg; SW2 - 2mg, and SW4 - 4mg. The experiment lasted 21 days where growth, growth dispersion, and enzymatic capacity were evaluated. *Artemia* metanauplii were offered to the post-larvae two times per day: one in the morning (10:00 am) and other late in the afternoon (6:00 pm). The inert diet used in this experiment was AgloNorse no. 2 (0.6-1.0mm) (EWOS, Norway) and was continuously supplied by automatic feeders for 22 hours a day. Each tank was stocked with 220 sole post-larvae. At the beginning of each treatment, 15 post-larvae were randomly sampled from each tank for weight and length evaluation, a total of 45 fish per each treatment. Sampling for weight and length were done

once a week and throughout the experimental period. Pools of 30 post-larvae per replicate were sampled for enzymes weekly, one week after the beginning of treatment. During the experiment, post-larvae were reared at 19.79±1.20°C of water temperature, with a salinity of 36.00±0.00‰. Dissolved oxygen saturation in the water was at 94.36±2.89%.

For enzymatic determination, the assays were performed in whole-body post-larvae given the small size of the fish. All enzymatic determinations were done as described by Ribeiro et al. (2002).

In the experiment, coefficient of variation (CV) was calculated using the formula CV = (treatment standard deviation/treatment mean) × 100 and was used to determine the inter-individual length variation among fish in the same treatment. Data are presented as arithmetic means with their standard deviations. One-way ANOVA was used to test differences between treatments. Differences were considered significant when $P<0.05$. When differences were found ($P<0.05$), Tukey´s Honest Significant Difference (HSD) test was used to determine which specific treatments differed significantly. All statistical analysis was carried out using the Statistica 5.1 package software (StatSoft, Tulsa, USA).

Results

Initial weight at weaning had a significant impact on post-larval final weight, with sole from SW1 (3.18±3.16mg) being significantly smaller than sole from SW4 (5.12±2.77mg). Total length was significantly lower in the SW1 treatment (11.03±3.75mm) than fish from the SW2 and SW4 treatments (13.45± 3.31 and 14.88± 2.43mm, respectively). The distribution of length was high, with post-larvae from SW4 treatment (16.34%) being significantly more homogeneous than post-larvae from SW1 (34.03%) or SW2 (24.64%). At the end of the experiment, the survival rate of sole post-larvae from SW4 treatment was significantly higher (90.06±10.01%) than the other two treatments SW2 (39.65±7.03%) and SW1 (38.46±3.75%).

No significant differences in post-larval trypsin specific activity were determined during the experiment between treatments (Fig. 1A). An almost twofold decrease of amylase specific activity in the post-larvae was determined between the first two samplings from treatment SW1 (Fig. 1B). In the first sampling, amylase activity was significantly higher ($P<0.05$) in the post-larvae from treatment SW1 than for SW2 and SW4. No significant differences for the specific activity were found between the treatments in the two remaining samplings ($P>0.05$). A twofold and threefold decrease of alkaline phosphatase specific activity were observed in SW2 and SW4, respectively, between the first and second sampling (Fig. 1C). The activity was always significantly lower ($P<0.05$) in post-larvae from SW2 compared to post-larvae from the SW4 and SW1 treat-

ments. Concerning the specific activity of leucine-alanine peptidase (Fig. 1D) in the first sampling, the activity was significantly higher ($P<0.05$) in the group that was eating inert diet for a week (SW1) than in the post-larvae that were still eating *Artemia* metanauplii (SW2 and SW4). In the second sampling, post-larvae from both treatments that were fed with inert diet (SW1 and SW2) presented similar results compared to the post-larvae that were eating live food (SW4). In the last sampling, SW1 and SW4 had very similar results.

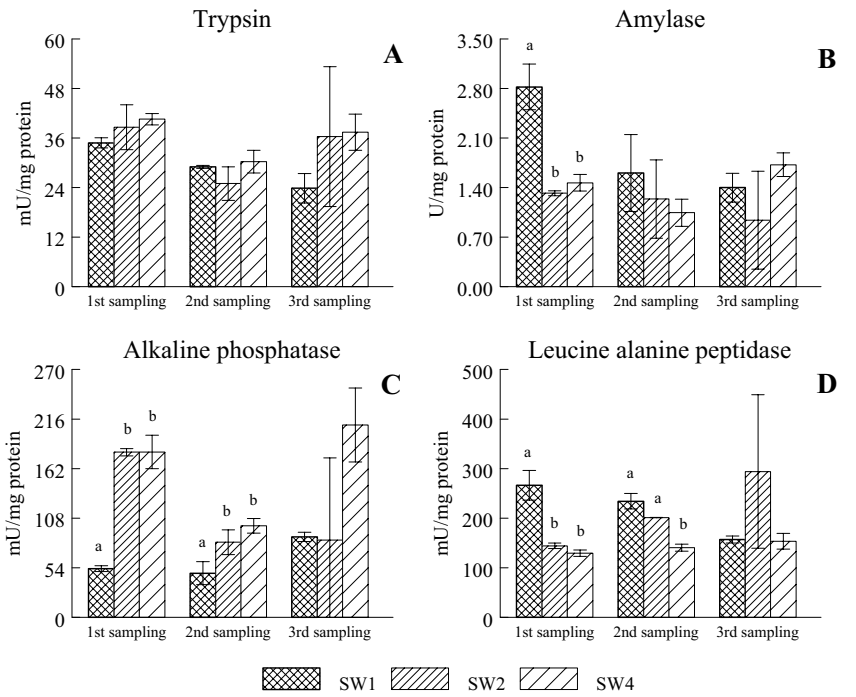

Fig. 1. Specific activity of trypsin (A), amylase (B), alkaline phosphatase (C), and leucine alanine peptidase (D) in whole sole post-larvae during the experiment. Mean ± SD. (n = 3) with the different superscript letter are significantly different ($P<0.05$).

Discussion

Trypsin activity values suggest that sole post-larvae have an adaptation period to inert diets, with reduced feed intake. According to Zambonino-Infante and Cahu (2001), trypsin activity is directly related to dietary protein intake. In the present study it was possible to observe a constant decrease in trypsin specific activity in the post-larvae from SW1, suggesting that they were eating inert diet at a maintenance ration but could not support growth and survival. In treatment SW2, sole post-larvae seemed to need about a week to adapt to the inert diet. After that,

trypsin values were similar to SW4, and some compensatory growth was observed. Still, adaptation to inert diet in SW1 and SW2 was at the expense of a higher mortality. It is clear from this work that it is difficult to successfully wean 1- or 2-mg DW post-larvae with a sudden-weaning strategy. Nevertheless, it is possible to wean 4.4-mg DW post-larvae with a survival rate of 90.06% (SW4 treatment). A higher alkaline phosphatase activity as found in post-larvae from the SW4 treatment also suggests a better developmental and nutritional status of the fish and this can explain the higher survival rate in this group as also observed in Ribeiro et al. (2002).

In this study it was observed that larval size does affect early weaning in Senegalese sole. This may be related with reduced feed intake or insufficient stimulation of digestion by inert diets in sole smaller than 4mg DW.

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PATHOGENESIS OF A THAI STRAIN OF WHITE-SPOT SYNDROME VIRUS (WSSV) IN SPF *LITOPENAEUS VANNAMEI*

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Introduction

White spot syndrome virus (WSSV) is a serious threat to shrimp aquaculture. Strategies to control WSSV have been experimentally tested but still no treatment is available. Effective control measures can only be achieved by understanding the pathogenesis of WSSV. In this study, a standardized oral inoculation procedure (Escobedo-Bonilla et al., 2004) was used to unravel the pathogenesis of WSSV with special emphasis on the determination of the primary site(s) of replication (portal of entry) and the search for the cause of death in specific pathogen-free *Litopenaeus vannamei*.

Materials and methods

Specific pathogen-free (SPF) *L. vannamei* Kona-strain were acclimatized (salinity of 15g.l^{-1} and 27°C) and housed in 50-l glass aquaria equipped with water heater, mechanical filter and continuous aeration.

A Thai WSSV stock was used. The virus titer was $10^{5.6}$ shrimp infectious dose 50% endpoint (SID_{50}.ml^{-1}) by oral route (Escobedo-Bonilla et al., 2005). A dose of 10^4 SID_{50} in 50µl was made in phosphate-buffered saline (PBS) pH 7.4 and it was orally inoculated to shrimp with a fine pipette tip.

Six shrimp were collected at 0, 6, 12, 18, 24, 36, 48, and 60h post-inoculation (hpi). Shrimp were fixed in Davidson's (24-48h), changed to 50% ethanol (24h), and embedded in paraffin. Per time point, three shrimp were sectioned longitudinally and three were cross-sectioned at three levels: 1st, at the level of the mouthparts; 2nd, at the middle part of the pereon; and 3rd, at the posterior part

of the pereon. Organs analyzed were foregut (esophagus, stomach) and midgut (hepatopancreas, anterior midgut cecum) in the digestive system, respiratory system (gills), excretory system (antennal gland), integument, and internal organs (heart, hematopoietic tissue, lymphoid organ, nervous tissue, gonads).

For immunohistochemistry (IHC), tissue sections (4μm) were incubated (1h at 37°C) with a monoclonal antibody against VP28 (2mg.ml^{-1}), washed and incubated (1h at 37°C) with 1:200 dilution of sheep anti-mouse biotinylated antibody, washed and incubated (30min) with 1:200 dilution of streptavidine-biotinylated horseradish peroxidase complex, developed with 0.01% of 3,3'-diaminobenzidine (DAB), counter-stained with Gill's hemaluin, washed, dehydrated and mounted. WSSV-infected cells were counted in five fields (400×) selected at random. Infected epithelial cells were expressed as percentage and the infected non-epithelial cells were expressed as cells.mm^{-2}. WSSV DNA was detected in cell-free hemolymph (0, 6, 12, and 18hpi) by one-step PCR and WSSV-infected hemocytes were determined by indirect immunofluorescence (IIF) (Escobedo-Bonilla et al., 2005).

Results and discussion

The evolution of WSSV infection in different target organs is shown in Figs. 1-6. Arrowheads show the first time point with positive cells. At 12hpi, four out of five (80%) shrimp were WSSV-positive. The primary sites of WSSV replication were foregut (0.3%), gills (0.4 cells.mm^{-2}) and antennal gland (0.4 cells.mm^{-2}). At 18hpi, five out of five (100%) shrimp were infected. WSSV spread to midgut, integument and internal organs (hematopoietic tissue, lymphoid organ and gonads). Epithelial cells in midgut, cardiac, and neuronal cells were not affected. From 24hpi until the end of the experiment (60hpi) the most susceptible organs were foregut, gills, antennal gland, integument, lymphoid organ, and hematopoietic tissue. These organs showed areas of necrosis due to WSSV infection starting from 36hpi. WSSV DNA was first detected at 12hpi in cell-free hemolymph from the five shrimp collected, but infected hemocytes were only found from 36hpi onwards.

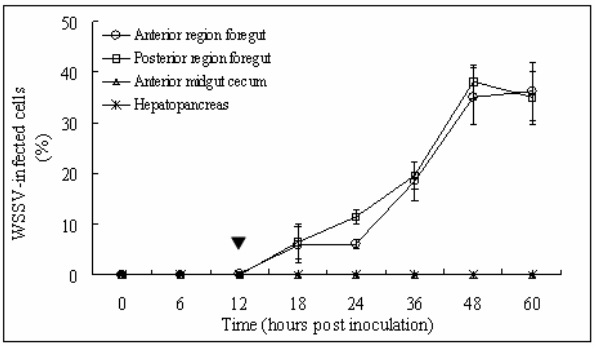

Fig. 1. WSSV infection in epithelial cells of the foregut and midgut in the digestive system.

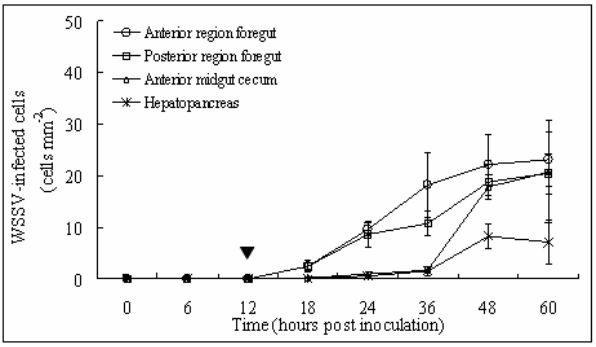

Fig. 2. WSSV infection in cells of connective tissues of the foregut and midgut in the digestive system.

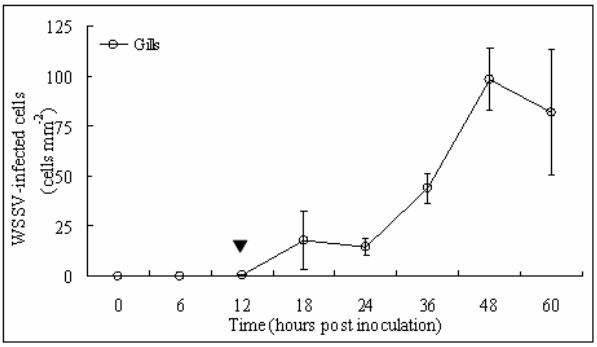

Fig. 3. WSSV infection in cells of gills.

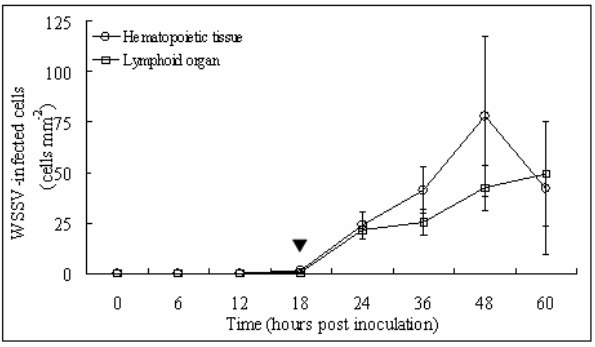

Fig. 4. WSSV infection in hematopoietic tissues and lymphoid organ.

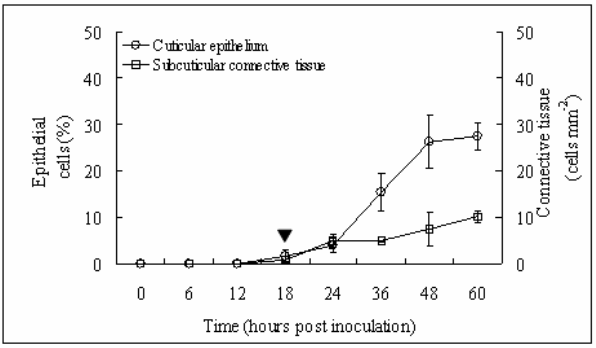

Fig. 5. WSSV infection in the integument.

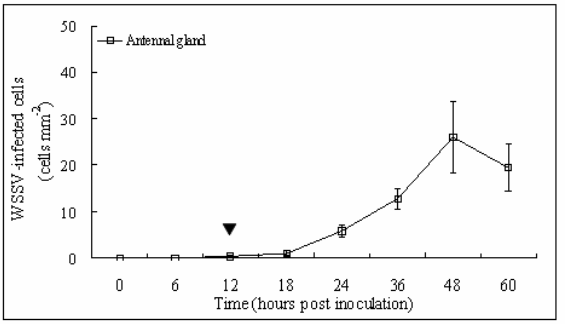

Fig. 6. WSSV infection in cells of antennal gland.

The oral intubation of a high dose of WSSV in *L. vannamei* revealed primary replication in foregut, gills, and antennal gland. In the digestive system, WSSV withstood chemical and physical barriers in order to replicate in epithelial cells of foregut. Afterwards, WSSV crossed the basal lamina and spread through the connective tissues of the midgut (18hpi). It is more difficult to understand how the gills and antennal gland got infected early after inoculation (12hpi). It is possible that inoculum spilled over the mouthparts and the base of the antenna may have reached the gills through the scaphognatite and the antennal gland through the excretory pores. Once WSSV crossed the basal lamina at the sites of primary replication, it was carried away by hemolymph circulation to the heart and reached lymphoid organ, hematopoietic tissue, and gonads at 18hpi. The integument of the gill chamber was probably infected with virus shed from the gills. PCR results suggest that WSSV spreads in hemolymph in cell-free form. Hemolymph circulation fostered the systemic infection of WSSV. By the end of the experiment (60hpi) organs such as gills, antennal gland, integument, and foregut showed areas of necrosis, indicating loss of structure and function. These organs perform gas exchange, transport and excretion of CO_2 and ammonia, salt, water, and acid-base balance that are critical for the homeostasis of shrimp. Thus, multi-organ dysfunction is probably the cause of death in WSSV-infected shrimp.

Acknowledgements

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EFFECTS OF ASCORBIC ACID ENRICHMENT BY IMMERSION OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) EGGS AND EMBRYOS

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Introduction

Ascorbic acid (AA) is an essential micronutrient in the diet of teleost fish, including salmonids, which do not have gulonolactone oxidase activity (Dabrowski, 1990). AA requirements for different teleost fish have been well-documented (Dabrowski, 2001) and vary within fish ontogeny; e.g., larval metamorphosis and gonad maturation. The concentrations of AA vary among tissues, but gonads (ovaries and seminal plasma) usually contain the highest levels, several-fold higher than in blood plasma (Blom and Dabrowski, 1995; Ciereszko and Dabrowski, 1995). This would suggest that in scurvy-prone teleosts, AA is of particular relevance to completion of reproduction and quality of gametes. Tissues such as ovaries depend upon delivery and cellular uptake of AA to meet localized requirements of this vital antioxidant (Guarnaccia et al., 2000). The objectives of this investigation were to evaluate the effect of an immersion enrichment of different forms of AA (free or ester) on the concentration of total AA and dehydroascorbic acid (DHA), their retention time at different developmental stages, and the survival of rainbow trout offspring.

Materials and methods

Sperm and eggs of rainbow trout were obtained from Troutlodge, Inc. (Sumner, WA, USA). They were shipped overnight on ice to Columbus (OH, USA) and used within 24h after stripping. Sperm motility was checked before experimentation and semen samples with >90% initial motility (n = 4) were pooled and used for fertilization. Sperm concentration was determined using spectrophotometry as described by Ciereszko and Dabrowski (1993).

To standardize fertilization, 24 subsamples of ova (4g each) were fertilized with 10µl of sperm in 10ml of dechlorinated city water. One minute after fertilization, each subsample of eggs was drained of water and fertilized eggs were immersed for 3h in one of four solution of the vitamin: 0 (control), 100, and 1000mg.l^{-1} L-ascorbic acid (Sigma, St. Louis, MO, USA) and 2000mg.l^{-1} L-ascorbyl-2-polyphosphate (AP) (Phospitan C, Showa Denko K.K., NY, USA) at 7.9°C with six replicates per treatment. After 3h, vitamin solutions were removed and each subsample of eggs was transferred into separate PVC baskets with a nylon screen bottom and incubated in California-type hatching trays (Flex-a-Lite Consolidated, Inc., Tacoma, WA, USA). Three replicates of eggs were used to evaluate embryo survival at the eyed stage and hatching**,** whereas the three other replicates were sampled at different stages – e.g., 3h and 24h after fertilization, at the eyed stage (13d), and at hatching (33d) – for determination of total TAA and DHA concentrations. TAA and DHA were measured following Dabrowski and Hinterleitner (1989).

Significant differences among treatments were determined by ANOVA and the differences between means were tested with Duncan's multiple-range test using STATISTICA (Tulsa, OK, USA). Differences were considered significant at $P<0.05$. Normality and homogeneity of variance were confirmed for all data and percentage data were arcsin-transformed prior to statistical analysis.

Results and discussion

The average of TAA and DHA of non-fertilized eggs were 183.1±27µg g^{-1} and 21.8±4.3µg g^{-1}, respectively. The results show that the highest amount of TAA was observed 3h after fertilization in all treatments but no significant differences were obtained at any time and treatment ($P>0.05$). TAA was reduced during incubation (embryonic development) but, after hatching, the amount of TAA in alevins increased in wet weight in AA groups (Fig. 1, TAA). Also, the mean of DHA decreased in all of the groups from 3h after fertilization until the eyed stage and then increased at hatching (Fig. 1, DHA).

Survival in the control group at hatching was 84.4±7.8 %, and was significantly ($P<0.05$) higher than in fish treated with 100, 1000mg AA l^{-1}, and 2000mg AP l^{-1} (68.9±17.6, 20.6±12.5 and 50.7±17.5%, respectively).

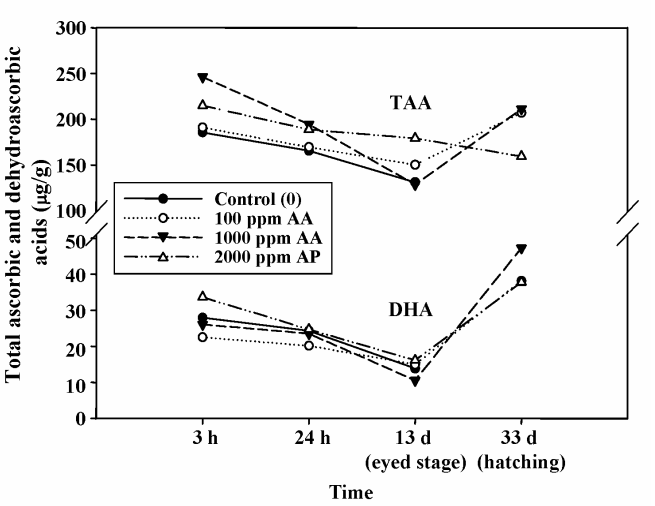

Fig. 1. The effect of different levels of ascorbic acid solution on TAA and DHA in eggs (n=6), embryos (n=6), and alevins (n=9) at different developmental stages in rainbow trout.

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ENERGETICS OF LARVAE AND POSTLARVAE OF THE PACIFIC OYSTER *CRASSOSTREA GIGAS*

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Introduction

Increase in the protein content of microalgal food increases the survival and/or growth of bivalve larvae and postlarvae, depending on the species of microalgae employed and on the stage of bivalve development (Uriarte and Farías, 1999). Interesting results have been obtained when conditioning Pacific oyster broodstock by feeding with microalgae containing unusually high protein levels. Larvae, and subsequently the postlarvae, obtained from this broodstock had significantly higher survival than those obtained from control broodstock, although no effect was noted in the larval-postlarval growth rates (Uriarte et al., 2004). The objective of the present study was to determine how the quality of the microalgae affected the physiology of Pacific oyster larvae and postlarvae.

Materials and methods

D larvae were obtained after the first 48h of incubation and separated into six 500-l tanks at a concentration of 5 larvae.ml^{-1}. Two tanks were randomly selected for feeding larvae with a normal diet consisting of a 50:50 mixture by cell count of *Isochrysis* aff. *galbana* (clon T-Iso) and *Chaetoceros neogracile* (I+G); another two tanks were used for feeding larvae with high-protein *I.* aff. *galbana* (IH), and a final pair of tanks for feeding with a mixed diet (IH+L) made of 60% IH and 40% of a lipid emulsion containing highly unsaturated n-3 fatty acids (DHA+EPA) following Uriarte et al (2003).

The larvae were cultivated for 23d at 25°C until reaching the pediveliger stage capable of metamorphosis. Metamorphosed larvae were then cultivated in tanks wth fibreglass plates as settlement substrates and were presented with diets the same respective diets as given prior to settlement. Postlarval culture was carried out until the juvenile oysters reached an average length of 5.1mm (± 0.05).

The growth rate (G) was transformed into its energetic equivalents using biochemical analysis on each developmental stage. Ingestion rate (I), respiration

rate (R), and excretion rate (U) were measured for the D larvae, umbonate larvae, pediveliger larvae, and postlarvae measuring 2 and 5mm in length. (I) was determined using a Coulter particle counter based on the rate of reduction in numbers of microalgal cells from 200-ml volumes of seawater and applying the method of Bayne et al. (1985), (R) was measured using 500-µl chambers for larvae and 1500-µl chambers for postlarvae using Strathkelvin polarographic electrodes, and (U) was measured using the Solorzano method with 200-ml seawater volumes and 6-h incubations. The gross growth efficiencies (K1 and K2) and the O/N index were estimated for each developmental stage (MacDonald, 1988; Bayne et al., 1985, respectively).

The data were analyzed using parametric ANOVA when they were normal, and when non-normal, using non-parametric Kruskal Wallis ANOVA.

Results

The rate of food ingestion was affected significantly by the interaction among developmental stages of the oyster and the diet ($P=0.025$). The lowest ingestion rate of 177µJ.h^{-1} occurred in D larvae fed with the IH and I+G diets, umbonate and eyed larvae, and 2-mm postlarvae showed intermediate values, and higher values were observed in umbonate larvae fed with the I+G diet . The highest ingestion rates were in 5-mm postlarvae, at 2752µJ.h^{-1}.

The respiration rate was affected by the stage of development ($P<0.00001$) but not by the diets. The lowest respiration rate was observed in umbonate and eyed larvae (20µJ.h^{-1}), while intermediate values were observed in D larvae and 2-mm postlarvae (190µJ.h^{-1}), with the highest values in 5-mm postlarvae (1120µJ.h^{-1}).

Preliminary observations were made on excretion by umbonate larvae over a period of 48h, finding that the highest concentration of ammonium in the medium occurred at 12h. Based on this result, all further measurements of excretion rate were confined to 12-h periods. The ammonium excretion rate varied significantly between developmental stages ($P<0.00001$), observing the lowest excretion rate in D larvae (0.04µJ.h^{-1}), intermediate in eyed larvae and 2-and 5-mm postlarvae (1.59µJ.h^{-1}), and the highest in umbonate larvae (3.01µJ.h^{-1}). The diets used had no apparent effects on the excretion rates of the oyster.

The growth rate was significantly affected by the interaction between developmental stage and diet ($P<0.007$). D larvae showed the lowest growth rate among the different developmental stages, with the I+G diet producing the lowest growth of this stage. Umbonate and eyed larvae had a growth rate similar to that of the D larvae when fed the I+G diet ; their growth was, however, significantly higher on the IH and IH+L diets (Fig. 1A). The 5-mm seed had the best growth rate, independent of diet. In the 2 mm seed the growth was higher with the I+G

and IH+L mixtures than with IH (Fig. 1B). In relation to the organic weight gain with respect to the initial organic weight, the best growth during the experimental period was experienced by the D larvae and umbonate larvae with 36% and 41%.day^{-1}, respectively. This value dropped to 28%.day^{-1} in the eyed larval phase, dropping rapidly after metamorphosis to values of less than 1%.day^{-1}.

The gross growth efficiency (K1) was affected by the interaction between the stage of development and the diet ($F=5.156$; $df=8,15$; $P=0.003$). The K1 values were greater in 2-mm seed fed on I+G and IH+L mixtures, intermediate in 2-mm postlarvae fed on IH and 5 mm seed (32%), and significantly less in larval stage (1.4%) independent of diet. The values for K2 were greater in postlarvae of 2 and 5mm (52.8%), intermediate in D larvae, and lowest in umbonate and eyed larvae (1.6%), independent of diet. Similarly, the O/N index was affected only by the stage of development ($F=38.202$, $df=4,15$; $P<0.00001$), with all the larvae showing a value significantly lower (<5.7) than both postlarval groups (>14.6).

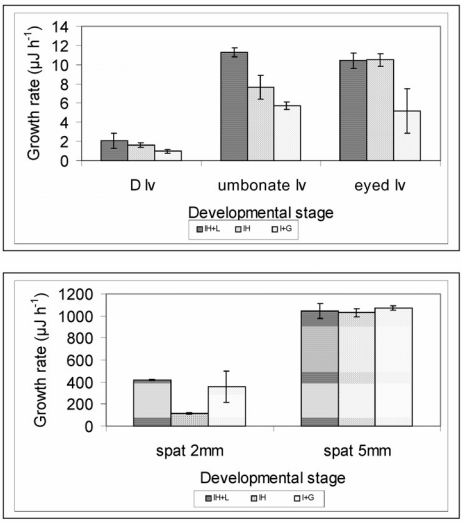

Fig. 1. Growth rate transformed into its energetic equivalents on each developmental stage. 1A: larval stages of development, 1B: postlarval stages of development. Each value is a mean of two replicate tanks.

Discussion

The best growth and highest K1 value, seen in the 2-mm postlarvae with the I+G and IH+L diets, may be an indication that in this developmental stage, eicosapentanoic (EPA) and/or arachidonic (AA) acids are of key importance. These nutrients are not found in the IH diet according to analyses made by Farías et al. (2003). The significantly lower growth of pediveliger larvae with the I+G diet may indicate that the IH diet, and to a lesser extent the IH+L diet, provides the best amount of amino acids and essential fatty acids for the morphometric and physiological processes which occur at the end of this stage.

The extremely low value for K2 in umbonate and eyed larvae could be related to characteristics of the period of metamorphosis, namely active morphogenesis. In fact the ingestion rate in umbonate larvae is less than in the preceding larval phase. Tendency toward negative energy balances have been observed in the pectinid *Argopecten purpuratus*, in the premetamorphic period (Marín et al., 2002). The low value of the O/N index prior to metamorphosis shows that the protein catabolism for production of energy is highly relevant in larval stages, while the O/N index rises significantly in the postlarval stage. This is probably the result of an increase in importance in carbohydrates as *Crassostrea gigas* assumes its benthic and intertidal existence.

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ENRICHMENT OF *BRACHIONUS PLICATILIS* WITH PREMIUM MICROALGAE: *ISOCHRYSIS GALBANA* VAR. T-ISO

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Introduction

The biochemical composition of microalgae for aquaculture purposes can be dramatically modified using semicontinuous culture techniques. Since the nutritive value of live feed is a crucial factor affecting growth and survival of fish larvae and the composition of filter feeders depends strongly on the diet (Ben-Amotz et al., 1987; Whyte and Nagata, 1990; Frolov et al., 1991; Fábregas et al. 2001), high quality microalgae can be bioencapsulated through the filter feeders in order to deliver the required nutrients to the larvae. In this experiment, the rotifer *Brachionus plicatilis* was enriched for 24h with *Isochrysis galbana* var. T-ISO (CCMP1324) cultured in semicontinuous regime with five renewal rates (RR). The different renewal rates modified nutrient and light availability in the microalgal cultures, and consequently the biochemical composition of the cells, as previously tested in the species *I. galbana* (Otero et al., 1997).

Materials and methods

Cultures were carried out using tubular glass units (diameter 30mm) containing 80ml of culture. Culture medium consisted on autoclaved seawater (salinity 3.5%) enriched with nutrients: KNO_3, 4mM; NaH_2PO_4, 0.22mM; $ZnCl_2$, 2.29µM; $MnCl_2$, 3.37µM; Na_2MoO_4, 2.72µM; $CoCl_3$, 0.38µM; $CuSO_4$, 0.465µM; ferric citrate, 21.2µM; thiamine, 1mg.l^{-1}; biotine, 13.4µg.l^{-1}; vitamin B_{12}, 9.4µg.l^{-1}. Tubes were subjected to a 12h:12h light:dark regime with an irradiance of 162µmol of photons.m^{-2}.s^{-1}, at a temperature of 21°C. Aeration was supplemented with CO_2 in order to keep pH below 7.8. Tubes were inoculated with 7.2×10^6 cells.ml^{-1} and cultures were allowed to grow until they reached early stationary phase, with an average cell density of $60.40\pm8.6.10^6$ cells.ml^{-1}. Once cultures reached stationary phase, the semi-continuous regime was started with a daily renewal of 10, 20, 30, 40, and 50% of the culture volume. Three replicates were maintained for each renewal rate. When steady-state was reached, biomass was collected for biochemical analyses and feeding of rotifers.

Five groups of rotifer cultures with three replicates each were starved for 12h and fed afterwards microalgae from different renewal rates, diets being established on a cell-number basis (27 000 cells per rotifer). After 24h of feeding, rotifers were collected on a sieve and freeze-dried for protein, lipid, carbohydrate, and C/N/H analysis or frozen for fatty acid analysis.

Results and discussion

The different RR modified nutrient and light availability in the microalgal cultures, and consequently the biochemical composition of the cells. Protein content increased from 3.42pg.cell^{-1} in the RR of 10% to 5.12pg.cell^{-1} in the RR of 40%, as nitrogen availability became higher, and decreased again in the RR of 50%. The evolution of the protein suggests that cultures were nitrogen-limited up to the RR of 40%. Carbohydrate and lipid contents were also strongly affected, and decreased continuously with increasing RR in conditions of nitrogen limitation. Carbohydrates decreased from 9.65-2.50pg.cell^{-1}, while lipid content decreased from 6.11-3.88pg.cell^{-1} (Fig. 1).

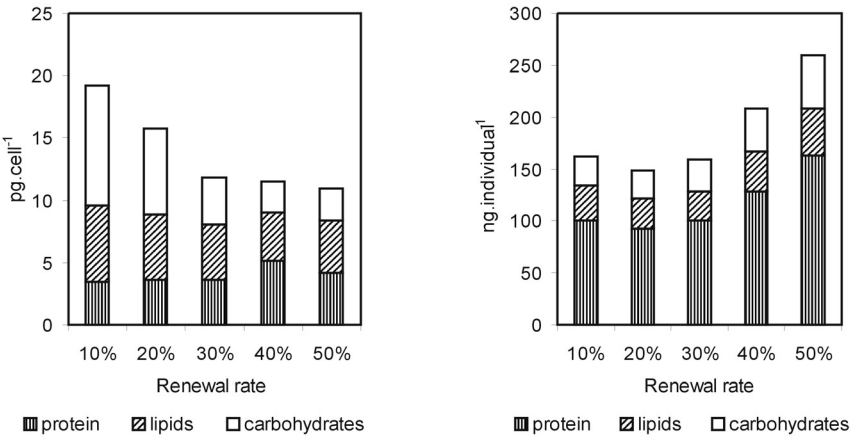

Fig. 1. Composition of the organic fraction of *Isochrysis galbana* var. T-ISO (left) and *Brachionus plicatilis* (right), expressed as pg.cell^{-1} and ng.rotifer^{-1}, respectively.

In nutrient-sufficient cultures both fractions remained constant. The changes in the composition of the microalga were reflected on the rotifers. Protein content remained almost constant from the RR of 10-30%, approximately 100ng per individual, and then increased up to 163ng of protein per individual in the highest RR, despite of the drop in the protein content of the microalgae cultured with a RR of 50%. Lipids decreased slightly from 34ng per rotifer at the RR of 10% to 28ng per rotifer at the RR of 30% and then increased up to 45ng per rotifer in the individuals fed the highest RR. Carbohydrate content was negatively correlated to microalgal content, and increased from 28-50ng per individual with increasing

RR (Fig. 1). Lipid and carbohydrate contents both in microalgae and rotifers were slightly and negatively correlated, while the correlation between respective protein contents was high and positive, being r^2=0.9214 up to the RR of 40% and r^2=0.3500 if the values for the RR of 50% were included.

The fatty acid profile of both microalgae and rotifers was also affected by the renewal rate. The percentages of saturated and monounsaturated fatty acids decreased with increasing renewal rate, while polyunsaturated fatty acids experimented an increase. 18:3ω-3, 18:4ω-3 and 22:6ω-3 are the most abundant PUFAs of T-ISO and their percentages over the total fatty acids increased by 50% in the cultures with a renewal rate of 40% with regard to the lowest renewal rate of 10%. Particularly, DHA increased from 6.33% to 9.51% of the total of fatty acids (Fig. 2).

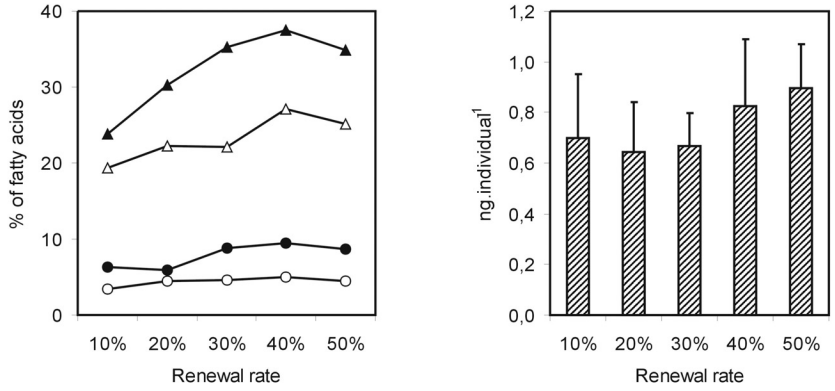

Fig. 2. Left: percentages of ω-3 PUFAs and DHA over total fatty acids in T-ISO and *B. plicatilis*. -▲-, ω-3 PUFAs in T-ISO; -△-, ω-3 PUFAs in *B. plicatilis*; -●-, DHA in T-ISO; -○-, DHA in *B. plicatilis*. Right: evolution of DHA content in *B. plicatilis* fed T-ISO from different renewal rates.

The evolution of the fatty acids in the rotifers was similar to the microalgae, but the sum of ω-3 PUFAs represented a maximum of 27% of the total fatty acids compared to the 37% reached in T-ISO. The highest DHA percentage was 5%, and the lower percentage of DHA in rotifers in comparison to microalgae was compensated by an increase of EPA. Despite of this, the DHA content per individual increased with increasing renewal rates, reaching a value of 0.90ng per individual in the rotifers fed microalgae obtained with a renewal rate of 50% (Fig. 2). The total content of ω-3 PUFAs in the rotifers also increased with increasing RR, from 3.31ng.individual^{-1} to a maximum of 4.90ng.individual^{-1} in the RR of 40% (data not shown).

Our results suggest that microalgae from the lowest renewal rates were poorly digestible due to the high levels of storage products accumulated under deffi-

cient nutritional conditions. The increase of nitrogen availability in the microalgal cultures maintained with higher renewal rates led to a dramatic decrease in the cellular carbohydrate and lipid contents and higher levels of protein. The reduction in the amount of lipids was nevertheless accompanied of an increase in PUFAs content, which results on an improvement in the nutritional quality of T-ISO.

Thus, semicontinuous culture techniques are efficient tools to optimize the composition of microalgae for rotifer enrichment, and rotifers are capable of assimilate the high amounts of protein and polyunsaturated fatty acids provided by these high-quality microalgae, increasing their nutritional value for fish larvae culture.

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EVALUATION OF POTENTIAL PROBIOTICS FOR COD LARVAE: IMPACT OF INITIAL SCREENING STRATEGY

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Two different isolation strategies were used to gather a pool of candidate probiotics for cod (*Gadus morhua* L.) larvae. The dominant intestinal bacterial flora of individual larvae and isolates showing antagonistic activity against *Listonella anguillarum* HI610 – using the replica-plating method – were brought to pure culture and phenotypically characterized. Larvae used in the study were sampled from well performing groups between 10 and 23 days post-hatch. The larvae originated from 4 different Norwegian hatcheries. Two groups were reared using a flow-through system (I1 and I2), while two other groups were reared using a recirculation system (R1 and R2). These four groups were fed intensively reared rotifers. The fifth group was reared using a flow-through system, but fed mainly natural plankton (E). Only antagonistic isolates were collected from groups I1 and R1. From a pool of 413 dominant and 98 antagonists, 27 dominant and 28 antagonistic isolates were chosen for evaluation of probiotic potential.

All isolates were initially characterized using 16 tests. The antagonistic activity of the potential probiotic strains against 4 pathogens (*Listonella anguillarum* HI610, *L. ang*. F99, *Marinomonas*, and *Vibrio logei*) and against 3 dominant isolates (the most dominant isolate from groups I2, R2, and E) was tested in a well diffusion agar assay. Haemolytic activity was tested by growing the isolates in Marine Broth for 24 hours and then spotting 10µl onto blood agar plates. Tolerance for fish bile was tested by exposing the isolates to 10% fish bile for 1.5h, and then checking for growth. API ZYM was used to examine for production of 19 constitutive extracellular enzymes. The ability to adhere to cod intestinal mucus was determined by adding probiotic candidates to 96-well Maxisorb plates pre-incubated with mucus. After incubation and washing, the quantity of bacteria still remaining was determined fluorometric using PicoGreen. Maximum

growth rate in Marine Broth and in cod intestinal mucus will be measured for the potential probiotic strains within May this year.

The initial phenotypic characterization showed that all isolates were gram-negative and that 95% were rods. The dominant microflora showed high variability on individual larval level, both regarding microflora composition and number of CFU in each larva. The percentage antagonistic isolates of total CFU was found to constitute 2.0, 1.5, 1.1, 43.0, and 2.8 for the rearing conditions I1, I2, R1, R2, and E, respectively. Among the dominant and the antagonistic isolates, 9 and 15 isolates, respectively, displayed antagonistic activity against one or more of the pathogen bacteria, while 3 and 13 isolates, respectively, were antagonistic to three or four pathogens. 4 dominant and 10 antagonistic isolates displayed antagonistic activity against one or more of the dominant isolates. Haemolytic activity was found with 63% of the dominant isolates and 46% of the antagonistic isolates. All isolates were able to grow after exposure to fish bile. The production of extracellular enzymes varied between isolates within the two groups, both regarding which enzymes that were produced and the magnitude of production. The API ZYM test showed that nearly all 55 isolates had high alkaline and acid phosphatase activity and high leucine arylamidase activity. Varying activity was recorded for esterase (C4), esterase lipase (C8), valine arylamidase, phosphohydrolase, β-galactosidase, α-glucosidase, β-glucosidase, and glucosaminidase. Low activity was found for the remainder of the tested enzymes. The abilities to adhere to mucus were quite similar for the dominant and the antagonistic isolates. Up to 2% binding to intestinal mucus was recorded for one third of the isolates, 44% of the isolates displayed between 2 and 5% binding, 10% of the isolates had between 5 and 10% binding, and 15% of the isolates displayed more than 10% binding to intestinal mucus.

The antagonistic isolates showed more extensive antagonism than the dominant isolates, although some of the original antagonistic isolates did not express antagonistic activity after freezing. High proportions of haemolytic isolates were found in both groups and we consider all haemolytic isolates unsuited for probiotics. All isolates indicated ability to survive in intestinal conditions by tolerating exposure to fish bile, enabling oral administration of the probionts. Bacterial production of extracellular enzymes may be expected to contribute to the host´s digestive processes and the API ZYM test showed that isolates within both groups displayed different enzymatic profiles that can be evaluated for their relevance to the host. Adherence to intestinal mucus has been used as a selection criterion, and is considered to be a vital characteristic for probiotic candidates. It was possible to separate isolates with good in vitro binding capacity from isolates showing less binding capacity. It needs to be stressed that these are all in vitro tests and in vivo trials will be carried out to confirm eventual probiotic potential. But these tests will help choose which candidates to use in in vivo trails.

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DEVELOPMENTAL STAGE AND MORPHOGENESIS OF FINFISH LARVAE, WITH SPECIAL REFERENCE TO IMPROVEMENT OF LARVAL HEALTH

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Introduction

To a greater or less extent, there are deformities in hatchery-raised juveniles. It is the key factor for survival in the field after release and for stocking effectiveness. It is also key issue of severe mortality and low market price in aquaculture industry. Fish quality, or ecological robustness, is defined as the ability to adapt to natural conditions at release site. The objective of the production of the hatchery-raised seed is to supply high quality seeds for stock enhancement and aquaculture. One of the main causes of deformity could be some nutritional condition of live feed, such as Vitamin A, D, and DHA concentration. We are now conducting the research and development on determination of suitable nutritional condition of live food, such as rotifer and *Artemia*, for finfish larviculture.

Materials and methods

First, we conducted experimental larviculture of *Paralichthys olivaceus*, *Inimicus japonicus*, and *Takifugu rubripes* with gradient of stocking density of newly hatched embryo, to determine the suitable stocking density of newly hatched embryos.
Second, we examined the relative growth of *P. olivaceus*, *I. japonicus*, *T. rubripes*, *Seriola quinqueradiata*, *S. dumererili*, and *Pagrus major*. Third, we characterized developmental stages of larvae using the combination of relative growth, external and skeletal morphogenesis. Finally, we conducted experimental larval rearing using live food with different Vitamin A and HUFA concentration, determined the safety level of Vitamin A concentration level of live feed, such as rotifer, *Artemia*, and their combination.

Results and discussion

There were remarkable density dependent growth and survival performance in the experimental larviculture on *Paralichthys olivaceus* and *I. japonicus*. Outbreak of biting behavior of larval *T. rubripes* that should be one of the causes of severe mortality was also density dependent. We clarified the presence of the suitable stocking density defined as resulted highest growth and survival for finfish larviculture. The stocking densities of hatched-out embryos were 20 000 ind.m^{-3} for *P. olivaceus*, 15 000 ind.m^{-3} for *I. japonicus*, and 5000 ind.m^{-3} for *T. rubripes*.

Usually, relative growth of finfish had multiple allometric regression curves. We defined the developmental stage according to the characteristics of relative growth in the course of larval development, and distinguished the special features of morphological and skeletal development. The course of development of finfish larvae divided 7-8 stages. The definition of developmental stage differs from the morphological classification of development such as Okiyama (1967) and Minami (1982). Cartilages of skull and shoulder girdle appeared at the stage of free embryo in common. These components developed using endogenous nutrients. Another skeletal component appeared and developed using exogenous nutrients. The causes of skeletal deformities should classify by endogenous/maternal effects and exogenous one.

We conducted the experimental larviculture of *P. olivaceus*, *I. japonicus* and *Pagrus major* to clarify the effect of vitamin A concentration on occurrence of deformity. To evaluate the effect of rotifer feeding period, we used L-rotifer cultured by *Chlorella* enriched by *Nannochrolopsis* and *Artemia* enriched by Marine omega® as control. For treatment, we used L-rotifer enriched by experimental agents with vitamin A concentration of 150IU.g^{-1}, 750IU.g^{-1}, 1500IU.g^{-1}, and 7500IU.g^{-1}, respectively, and *Artemia* enriched by Marine omega®. In the case of *P. olivaceus*, there were no significant differences of the performance of larviculture in the course of rotifer feeding period. In the case of using rotifer with 212IU.g^{-1} vitamin A concentration reflected 750IU/g vitamin A concentration of enrichment agent, lowest skeletal abnormality occurrence rate was achieved at 38-46dah, just after metamorphosis. Specific skeletal abnormality appeared at the case of excess and deficiency of vitamin A concentration, respectively. We also conducted the experimental larviculture using L-rotifer enriched by *Nannochrolopsis* and *Artemia* enriched by experimental enrichment agents with gradient of vitamin A concentration, such as 150IU.g^{-1}, 750IU.g^{-1}, 1500IU.g^{-1}, and 7500IU.g^{-1}, respectively. There were no significant differences of the performance of larviculture in the course of rotifer feeding period. In the course of *Artemia* feeding period, highest growth rate and lowest malpigmentation achieved in the case of feeding *Artemia* with 325IU.g^{-1} vitamin A concentration reflected 750IU.g^{-1} vitamin A concentration of enrichment agent. Skeletal abnormality

occurrence rate was lowest in the same case, also. Excess and deficiency of vitamin A of live feed affected the health of hatchery-raised juveniles of *Paralichthys olivaceus*, *I. japonicus*, and *Pagrus major*. In the case of *Paralichthys olivaceus*, vitamin A concentration of rotifer should be most important key issue for occurrence of skeletal deformity. Rotifer with Vitamin A concentration of 212IU.g^{-1} reflected 750IU.g^{-1} of enrichment agent was recommended for improvement of the health of hatchery-raised juveniles. Vitamin A concentration of *Artemia* (50-2831IU.g^{-1}) did not cause skeletal deformity of hatchery-raised *P. olivaceus* juveniles raised by rotifer with Vitamin A of 212IU.g^{-1}. Safety level of Vitamin A concentration of *Artemia* for *P. olivaceus* should be higher than reported level of 50IU.g^{-1} *Artemia* on a dry matter basis (Dedi at al., 1995).

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EFFECT OF TEMPERATURE ON THE SEXUAL MATURATION OF PIKE-PERCH (*SANDER LUCIOPERCA* L.)

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Introduction

The pike-perch (*Sander lucioperca*, L.) has been a highly valued additional fish of traditional pond fish farming for many years. In the last decade, the increased demand for valuable freshwater fish led to attempts to raise pike-perch under industrial conditions to a size suitable for human consumption. Several research groups have initiated feeding and rearing experiments on pike-perch using artificial diets under controlled conditions in indoor systems (Zakes, 1996; Rónyai et al., 2003; Molnár et al., 2004).

The intensive tank-rearing and induced spawning of pike-perch has been elaborated, although the fry and fingerling production of pike-perch is still based on pond-nursing under natural conditions and the spawners still have been reared under pond conditions (Hilge and Steffens, 1996). The success of pike-perch fingerling production strongly depends on the environmental conditions and the natural food abundance of nursing pond. The gonad development of spawners kept in ponds depends on the seasonal variation. The spawners' pairs are formed when the water temperature reaches 10-14°C after the winter. However there is no information about the possibilities of the reproduction of the artificially reared pike-perch where the water temperature is constantly high.

The aim of the experiment was the evaluation of the temperature effect preparatory to reproduction on gonad development of artificially reared pike-perch.

Materials and methods

The experimental fish originated from the offspring of broodstock artificially spawned in tanks. The fish were reared in an indoor recirculation system and fed with artificial fish diets only. The fish were kept in 2-m^3 tanks at a water temperature between 18-25°C in an indoor system.

Two groups were formed in November from fish 1.5 years old; each group consisted of 20 pairs of broodstock fish. The first group was cooled gradually and subsequently water temperature was maintained at level below 10°C, and the other group was kept at 19±2°C. The fish was not fed during the trial. The gonad samples were taken monthly from 3 females and 3 males per group during 4 months. Ovaries and testes were removed from each fish; gonadosomatic index (GSI) was determined as follows: GSI (%) = gonad weight × (body weight - gonad weight)$^{-1}$ ×100.

Histological examination of the gonads was carried out on sections. A 10-mm transverse of the ovary and the whole testis was fixed in Bouin's fluid and stored in 70% alcohol solution until processing. Tissues were dehydrated with a series of alcohol solutions. Tissue samples were then embedded in paraffin and were cut at 5-µm sections. The sections were stained with haematoxylin and eosin. Ovarian development was examined by a histomorphometric analysis by Rinchard and Kestemont (1996), while development stages in testis were examined by the method of Billard (1990).

Results and discussion

The GSI was 0.40% for females and 0.02% for males on 28 of November, when the groups were formed. The gonad weight and the GSI of the group kept at low temperature started to increase. After 4 months (March 30), the GSI reached 3.04% for females and 0.37% for males on average. But the fish kept at 19±2°C the GSI had not increased (Table I). The values of the GSI at each sampling date under different temperature conditions were significantly different ($P<0.05$).

Table I. Changing of the gonad weight (GW) and GSI of the pike-perch kept under cold (<10°C) and warm (19±2°C) conditions

Date	Temp.	Female			Male		
		FW (g)	GW (g)	GSI (%)	FW (g)	GW (g)	GSI (%)
November 28	-	989±51	3.91±0.12	0.40±0.02	1003±77	0.22±0.06	0.02±0.01
January 6	cold	978±66	9.83±0.21	1.02±0.05^{a}	997±81	0.37±0.08	0.04±0.01^{a}
	warm	990±36	4.17±0.29	0.42±0.01^{b}	973±110	0.25±0.05	0.03±0.01^{a}
February 11	cold	1057±115	27.33±10.21	2.61±0.70^{a}	1009±53	3.40±0.52	0.34±0.04^{a}
	warm	1073±87	4.37±0.38	0.41±0.03^{b}	1023±182	0.27±0.08	0.03±0.01^{b}
March 30	cold	980±70	29.40±16.04	3.04±1.50^{a}	903±210	3.30±1.30	0.37±0.14^{a}
	warm	915±203	4.03±0.15	0.46±0.11^{b}	904±115	0.47±0.12	0.05±0.01^{b}

Temp.: treatment under different temperature, FW: fish weight, GW: gonad weight, GSI: gonadosomatic index; the a and b values are significantly different ($P<0.05$).

The results of the histological examination are summarised in Table II. The ovary developmental stage was I-II and only spermatids were observed in the

testis at the beginning of the trial (Fig. 1). The female and male gonad development of the fish kept at low temperature was more pronounced, than at 19±2°C. The ovary stage III-IV was reached after 4 months and sperm cells were observed in the testis after 1-2 months for fish kept at low temperature. On the contrary, the ovary stage of the fish kept at warmer temperature stagnated at I-II and only several spermatid cells were observed in the testis (Fig. 2).

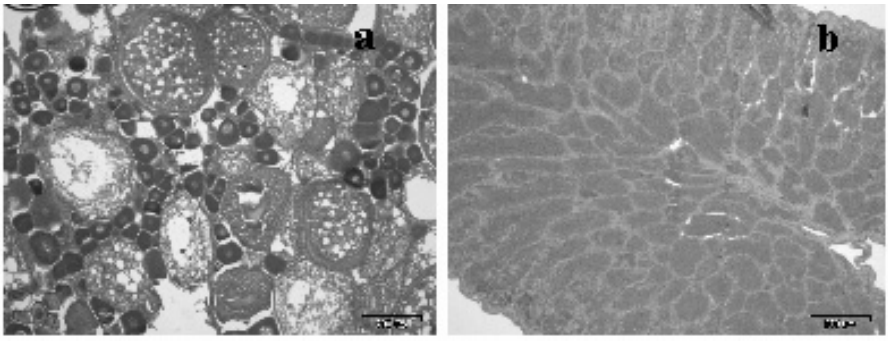

Fig. 1. Histological sections of the ovary 100× (a) and testis 100× (b) of pike-perch at the beginning of the trials (28 November). The ovary development stage (ODS) was I-II and the testis was undeveloped with spermatids (stage I-II).

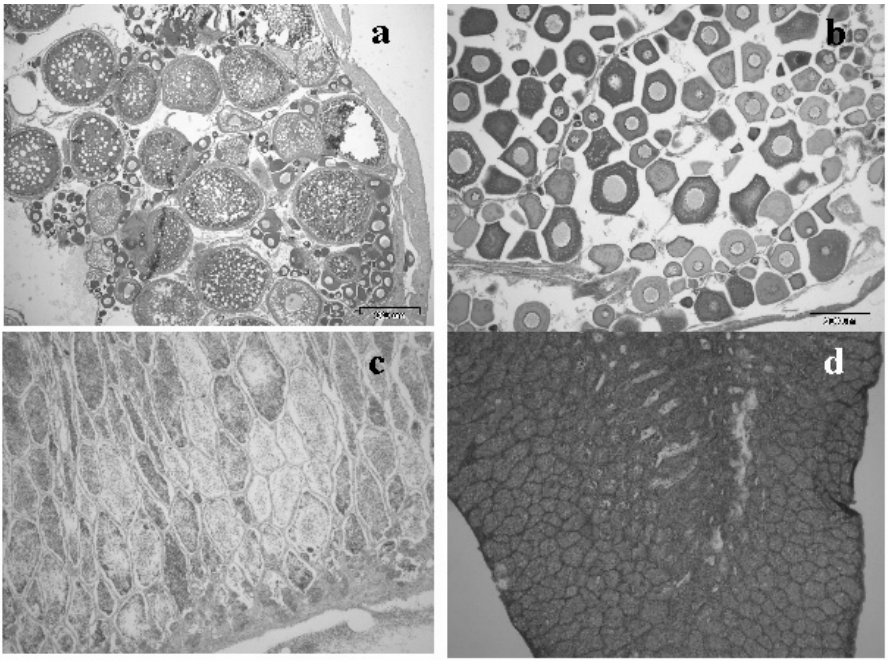

Fig. 2. Histological sections of the ovary at cold 40× ODS: early IV (a) and warm 100× ODS: I, few II (b) and of the testis at cold 100× spermatozoa fill the lumen at stage IV (c) and warm 100× spermatogonia with few primary spermatocytes at stage I-II (d) treatment of pike-perch on March 30.

After the trial 3 pairs from each group were induced for spawning with injections of carp pituitary extract. However, the females didn't ovulate, only sperm was stripped from the males kept at low temperature. The reason of unsuccessful propagation probably was the poor conditions of brooders. The histological examinations of fish from this trial demonstrated that the gonad maturation of the artificial reared pike-perch could be induced by temperature regulation. However, for the proper gonad development of pike-perch reared in an indoor system a cold period of several months required.

Table II. Development stages of the gonads at the start (November 28) and at each sampling date under different temperature conditions (cold: <10°C, warm: 19±2°C).

Date	**Ovary development stage**		**Testis development stage**	
November 28	I-II		I-II	
treatment	Cold	Warm	Cold	Warm
January 6	II	I-II	III	I-II
February 11	III	I-II	IV	I-II
March 30	IV	I-II	IV	I-II

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EFFECT OF DIFFERENT LIVE FOOD ENRICHMENTS ON EARLY GROWTH AND LIPID COMPOSITION OF ATLANTIC COD LARVAE (*GADUS MORHUA*)

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Introduction

Interest in the intensive production of Atlantic cod (*Gadus morhua*) in Canada began in the early 1990s, driven by a reduced supply from the wild fisheries and, consequently, high market prices. In addition to this, there was a perceived need to develop alternate species to salmon culture, and Atlantic cod has been shown to be a suitable candidate. The current production protocol used in Newfoundland uses rotifers (*Brachionus* sp.) and *Artemia* during the larviculture. Only a few recent studies compared and evaluated optimal live food enrichments procedures for cod (O'Brien-MacDonald et al., in press; Park et al., in press). The objectives of this study were to evaluate the effects of different enrichment protocols – three during the rotifer phase, and four during the *Artemia* phase – in the early growth, survival, and lipid composition of Atlantic cod larvae.

Materials and methods

Newly hatched cod larvae were stocked in 3000-l tanks at a density of 50 larvae.l^{-1} (2 replicates), and reared to 60dph (721°d) on rotifers and *Artemia* enriched differently. Water management and feeding schemes followed previous protocols outlined in Brown et al. (2003). Three different treatments were tested during the rotifer phase, using commercially available products as a single enrichment or in combination with algae paste. The products tested differed in their proximate composition (Table I) and fatty acid profiles. The treatments were: 1) Spray-dried *Schizochytrium* sp., 2) Spray-dried *Crypthecodinium* sp., and 3) a combination of dried *Schizochytrium* sp. and *Pavlova* sp. paste. Rotifers were enriched in 300-l conical tanks at 22°C for a period of 24h. Enrichments were added to the rotifer tanks twice in a twelve hour period at 9:00 am and 3:00 pm, with the amounts added each time as per the manufacturers' directions. Enriched rotifers were supplied to the larvae from 1-43dph (11-482°d) at a density of 4000.l^{-1}. During the *Artemia* phase, the same treatments were tested in addition

to a combination of a lipid-rich emulsion and dried *Schizochytrium* sp. as a fourth treatment. *Artemia* cysts were hydrated, descapsulated, and hatched according to standard practices and subsequently enriched for 24h using protocols similar to those described above for rotifers. *Artemia* were supplied to the larvae from 37-60dph (443.3-721°d) three times daily as necessary to sustain a prey density of 1000.l^{-1}.

Table I. Proximate composition of the enrichments tested. Values are according to the manufacturers' information.

Proximate composition (%)	**Enrichments**			
	***Schizochytrium* sp.**	***Crypthecodinium* sp.**	***Pavlova* sp. paste**	**Emulsion**
Protein	20	10	52	*
Lipids	38	23	20	65
Carbohydrate	17	9	22-24	*
Ash	20	44	*	3
Moisture	4	4	91	30

Pooled samples of larvae were collected weekly for growth measurements (dry weight) and biweekly for lipid analysis (total lipids, lipid classes and fatty acid profiles). Samples of the experimental enrichments, algae paste and enriched live-food were collected in three replicates for lipid analysis. In addition, samples of enriched live-food were collected for analysis of total protein and amino acid composition.

Differences in growth between treatments were analyzed using an ANCOVA (analysis of covariance). The General Linear Model (Minitab Version 13.1) was employed and the effect of tanks was not significant. Lipid profiles between treatments are being analyzed using one-way ANOVAs with Tukey's multiple comparison test to compare bi-weekly differences. Significance was set at $\alpha = 0.05$ for all analyses.

Results and discussion

Survival measurements were based on visual observations. In order to reduce stress on the larvae, there is no systematic assessment of survival rates before weaning. Therefore, a qualitative scale (0 to 5) was used to measure the weekly survival rates. At 39dph (468.5°d), before the start of the *Artemia* phase, larval densities in the tanks were evened. The combination of dried *Schizochytrium* sp. and *Pavlova* sp. paste resulted in best survival rates during the experimental period (Table II).

Table II. Survival of Atlantic cod larvae fed different enrichments, based on a qualitative scale (0 to 5).

	Treatment 1	Treatment 2	Treatment 3	Treatment 4
Start experiment	5	5	5	
End rotifer phase	2	1	3	
Start *Artemia* phase	5	5	5	5
End experiment	2	2	3	3

Following the same trend, this treatment also resulted in faster growth ($p=0.006$) during the rotifer phase (10.15%.d^{-1} specific growth rate) when compared to treatment 2 (8.14%.d^{-1} SGR). On the other hand, during the *Artemia* phase, the larvae from treatment 1 grew at a faster rate (13.9%.d^{-1}, $p<0.001$) than those from treatment 3 (7.5%.d^{-1}) and those from treatment 4 (5.44%d^{-1}). After 630°d the fastest growth rates for all treatments were observed.

In the present study, each enrichment was tested with both types of live food – rotifers and *Artemia* – supplied during the larval rearing of Atlantic cod. However, when comparing different treatments, the survival and growth produced by enriched rotifers or *Artemia* did not follow the same trend. Overall, the fastest growth and best survival rates were not produced by the same treatment.

McEvoy et al. (1996) showed that *Artemia* nauplii enriched for 18h with a diet rich in ethyl esters did not contain ethyl esters but this diet resulted in a remarkable increase in triacylglycerol. The authors suggested that the nauplii can actively modify the dietary fatty acid profile. Similarly, rotifers enriched with fatty acids as ethyl esters rapidly incorporated these fatty acids in other lipid classes (Rainuzzo et al., 1994). Lipid nutrition of larvae is recognized as one of the bottlenecks for mass production of many species, and the effects of essential fatty acids on the growth, survival and stress resistance of marine larvae has received increasing attention in the past few years (Sargent et al., 1999; Izquierdo et al., 2000; Tocher, 2003).

The findings reported here are the first stage of a study that will also analyze the lipid composition of enriched live-food and larvae. Lipid analysis will be fundamental for interpretation of results. The lipid classes are being determined by Iatroscan TLC/FID and extracts are being fractionated for determination of fatty acid composition in neutral and polar lipids.

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EFFECT OF MICROENCAPSULATED DIETS ON GROWTH AND DIGESTIVE TRACT DEVELOPMENT OF THE SPOTTED SAND BASS (*PARALABRAX MACULATOFASCIATUS*) LARVAE

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Introduction

Among the inert feeds used for the substitution of live prey for larviculture, the microencapsulated feeds have the highest stability in water. In addition, the microcapsules membrane can be made from the same ingredients used for the formulation. The present work attempts to determine the effects on growth and survival of spotted sand bass larvae fed with microcapsules diets.

Materials and methods

The larvae were reared in a closed circuit. Greenwater culture was maintained during 12 days after hatching (dah) with microalgae *Nannochloropsis oculata* (300 000 cells.ml^{-1}). Larvae were fed with rotifers *Brachionus plicatilis* (1-10 rotifers.ml^{-1}) from 2-15dah. From 15dah, larvae were fed with *Artemia* nauplii and juveniles (2-6 nauplii.ml^{-1}) as a control treatment. Two types of microcapsules were supplied as experimental treatments at 15dah. Larvae were co-fed with live prey and microcapsules during 5d. Microcapsules were made by interfacial polymerization as described by Yúfera et al. (1999) modified for the present experiment.

Digestive tract development was determined by external (alkaline phosphatase and N-aminopeptidase) and internal (leucine-alanine peptidase) enzyme activities. The expression of *P. maculatofasciatus* α-amylase was studied by real-time

PCR using Assays-by-Design^{SM} service TaqMan$^{®}$ MGB probes and a mix of unlabeled PCR primers (FAM™ dye labelled). The Eukaryotic 18S rRNA (Applied Biosystems) was used as endogenous control for normalising mRNA levels of the target gene.

Ten individuals per tank were sampled every 5d for growth evaluation. Survival was determined at the end of the experiment. The treatments were tested in three replicates and the results were tested with a Kruskal-Wallis ANOVA by ranks. Significant treatments were determined by a Siegel and Castellan multiple comparison of mean ranks. All analyses were made using the statistical package STATISTICA version 6.1 (Statsoft, Inc., Tulsa, Oklahoma, USA).

Results and discussion

Size growth of larvae fed with live preys and MCC were significantly higher ($p<0.005$) at 15dah. This may not be due the experimental treatments, because the microcapsule supply at that day had just started. Larvae fed with live preys shown the highest growth at 20dah as an adaptation of larvae to the microcapsule feeding, because larvae fed with MCH and live prey were significant ($p<0.05$) larger than the larvae fed with MCC (Fig. 1). Weight was only significantly different ($p<0.05$) at 5dah on larvae fed with MCC.

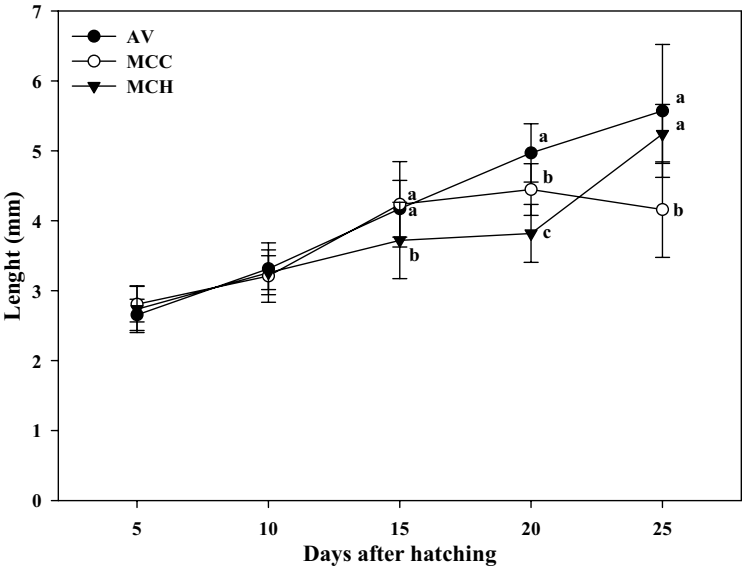

Fig. 1. Mean growth and standard deviation of larvae fed with live prey and microcapsules. AV= Live prey treatment; MCC = Squid meal microcapsules; MCH = Fish protein hydrolysate microcapsules. Different literals show significant differences ($p<0.05$).

Survival from larvae fed with live preys was significantly ($p<0.05$) higher than the others treatments. No significant differences ($p<0.05$) were found between microcapsules treatments.

Table I. Survival percent of larvae fed with the different treatments at 25dah. Distinct literals show significant differences ($p>0.05$).

Treatment	Survival (% ± SD)
AV	6.42 ± 2.26^{a}
MCC	0.28 ± 0.14^{b}
MCH	0.68 ± 0.32^{b}

Internal digestion enzymes displayed a higher activity at 22dah, whereas the external digestion enzymes activities were higher at 25dah. This suggests that between both 20 and 25dah, adult digestion in spotted sand bass begins, since according to Peña et al. (2003) the spotted sand bass has a juvenile-like digestive tract at 22dah. Also, Cahu and Zambonino-Infante (2001) mentioned that the internal digestion enzymes activities are substituted by the external digestion enzyme activities as the fish grows. Enzymatic activities of larvae fed with live prey were higher than larvae fed with microcapsules but only the live prey treatment displayed a higher activity of internal digestion enzymes at 25dah than 22dah.

Table II. Enzymatic activities (total mU) of 20dah larvae and 25dah spotted sand bass juveniles.

Treatment	Alkaline phosphatase (total mU)		N-aminopeptidase (total mU)		Lecine-Alanine peptidase (total mU)	
	22 dah	25 dah	22 dah	25 dah	22 dah	25 dah
AV	0.2915	0.619	0.01583	0.09168	125 243.50	308 226.45
MCC	0.0001	-	0.00003	-	101 854.86	23 290.90
MCH	0.0043	0.0307	0.00088	0.00353	132 312.73	26 939.30

The mRNA coding for amylase levels at 20dah was lower than the mRNA levels at 25dah. Both MCH and MCC treatments displayed a significant ($p<0.05$) higher mRNA levels than the live prey treatment at 20dah. All of the treatments displayed a significant mRNA level ($p<0.05$) at 25dah, whereas the MCH treatment showed the higher mRNA level and the MCC treatment showed the lowest. Increments of α-amylase expression could be due to a high demand of amylase for the transformation of glycogen to lipids from the low fatty acids content of the feed used on this work (data not shown). The reduction on the α-amylase expression from the MCC treatment could be due a post transcriptional negative effect (Table III).

Table III. mRNA levels (ΔΔCt) of larvae fed with live preys and microcapsules. Different superscripts show significant differences ($p<0.05$).

Treatment	Amylase expression (ΔΔCt)	
	20dah	25dah
AV	17.56 ± 0.70^{b}	22.91 ± 0.58^{c}
MCC	25.66 ± 0.33^{a}	17.18 ± 3.05^{d}
MCH	27.08 ± 0.82^{a}	29.03 ± 0.35^{e}

Conclusion

Growth results in the present work show that the MCH treatment can be selected as a substitute for the live prey supply during the spotted sand bass larviculture. Survival obtained in both live prey and microcapsule treatments were below than the obtained by previous works on spotted sand bass larviculture.

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LARVAL REARING OF SPOTTED ROSE SNAPPER *LUTJANUS GUTTATUS* UNDER EXPERIMENTAL CONDITIONS

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Introduction

The spotted rose snapper (*Lutjanus guttatus*) is a marine fish species with high potential for aquaculture in Mexico and other Latin American countries. This is one of the red snapper species that are highly appreciated in local markets. At present, in several East Pacific countries juveniles of *L. guttatus* are being captured and stocked in floating cages for on-growing until they reach the market size. Because supply of larvae and juveniles cannot be dependant on availability of wild fish, efforts are currently done to develop larviculture techniques for their controlled production in fish hatcheries.

In the development of larviculture techniques for *L. guttatus*, is necessary to test adequate rearing protocols with live food and artificial microdiets. To date there is no detailed feeding protocols for larval rearing of the species. Therefore, in the present study we tested water volume exchange and feeding protocols to cultivate *L. guttatus* larvae at experimental scale.

Materials and methods

Fertilized eggs of spotted rose snapper with an average diameter of 0.71±0.02mm were incubated in 600-l circular black fiberglass tanks with sea water filtered with sand, 20-µm cartridges, and UV light. During egg incubation the water temperature oscillated between 28-30°C with a constant salinity of 35ppt. No water exchange was done at this time and constant illumination and aeration were maintained. After 20 hours of incubation the eggs started to hatch. Three days post hatching (dph), the water flow was started and a water volume exchange of 50% was done daily from that moment and was increased to 200% at 10dph and up to 600% from 31dph onwards.

Feeding of spotted rose snapper larvae started at 1dph by adding to the larval tanks a mix of microalgae (*Nannochloropsis oculata*, *Isochrysis* sp., and *Tetraselmis* sp.) with a density of 100 000 cells.ml^{-1} which was maintained daily until 14dph. Rotifers (*Brachionus rotundiformis*) were added to the larval tanks in 1dph at a density of 10.ml^{-1} and were reduced to 6.ml^{-1} in 24dph. Both microalgae and rotifers density was verified four times a day and new live food was added as required. A mix of live instar I nauplii of *Artemia* and HUFA (A1 Selco)-enriched *Artemia* (above instar II nauplii) were offered to the fish larvae twice each day starting on 24dph. Due to significant differences in fish larvae size at this time, the mix of *Artemia* consisted of 50% instar I nauplii and 50% enriched *Artemia* which are bigger in size. Enrichment of *Artemia* was done for 24h in two steps, one of 16h, and a second of 8h. Initial density of *Artemia* was 0.5 nauplii.ml^{-1} and was gradually increased to 1.ml^{-1} until 31dph. From this mix, the amount of instar I nauplii was reduced by 20% every day staring 24dph and the amount of enriched *Artemia* was increased accordingly. Weaning started at 31dph using a microbound diet made of decapsulated cysts of *Artemia* and fish meal as protein sources (García-Ortega et al., 2003). During weaning, a reduction of 10% per day in the amount of *Artemia* was done and the amount of artificial microdiet was increased as the larvae were gradually adapting to it. After ten days of weaning, all the larvae were actively feeding exclusively on the microdiet. The microdiet initial particle size was 150-300µm and it was increased to 300-500µm and 500-700µm as the larvae grew in size. Fish larvae weight and total length was recorded at 0, 5, 10, 15, and 58dph. A summary of the water volume exchange and feeding protocol is shown in Fig. 1.

In a second feeding trial, a similar feeding protocol was tested with the addition of the tropical copepod *Tisbe monozota*, which is currently produced in closed systems (Puello-Cruz et al., 2004). A mix of nauplii, juveniles, and adult of copepods were offered to the larvae at a density of 0.5.ml^{-1} from 3-14dph. The rotifer density was increased to 15.ml^{-1} at 10dph and then reduced again to 10.ml^{-1} at 14dph, when feeding of snapper larvae with the mix of *Artemia* was initiated.

Results and discussion

Fertilized eggs of this species are pelagic and present one oil drop. Fertilization rate was 77.6±10.2% and hatching initiated 20 hours after fertilization with an average hatching rate of 92.3±2.6%. The newly-hatched larvae measured 2.7±0.2mm in total length. The mouth and anus were open at 3dph. Snapper larvae relied on yolk reserves until it was absorbed and initiated exogenous feeding by 3dph. Growth of larvae and post larvae of hatchery-reared spotted rose snapper is shown in Fig. 1. High mortalities were observed in the first days after the start of exogenous feeding and during the rotifer-feeding phase from 4 to 24dph. Similarly, growth during this phase was poor, probably due to inadequate prey size and nutritional quality of live food, especially the poor content of HUFA in

rotifers. Further mortality was also observed during weaning. An estimated 0.5% survival was obtained from hatched to post-weaned larvae. Poor growth and survival during the rotifer phase at hatchery conditions have been observed in other Lutjanidae species. However, growth of snapper larvae improves with the use of small strain rotifers and after *Artemia* feeding and weaning onto artificial microdiets (Duray et al., 1996; Watanabe et al., 1998). After the start of *Artemia* feeding, a fast and constant increase in growth of *L. guttatus* was observed from a total length of 4.86mm at 15dph to 48.8mm at 58dph (Fig. 1). The growth of *L. guttatus* larvae in the first trial was higher than the previously reported for hatchery-reared Pacific red snapper (*L. peru*) of the same age (Duncan et al., 2002). Similarly, growth in mutton snapper (*L. analis*) larvae presents a steady increase after 21dph, reaching a length of 22.2mm at 38dph when feeding on *Artemia* and artificial microdiets (Watanabe et al., 1998).

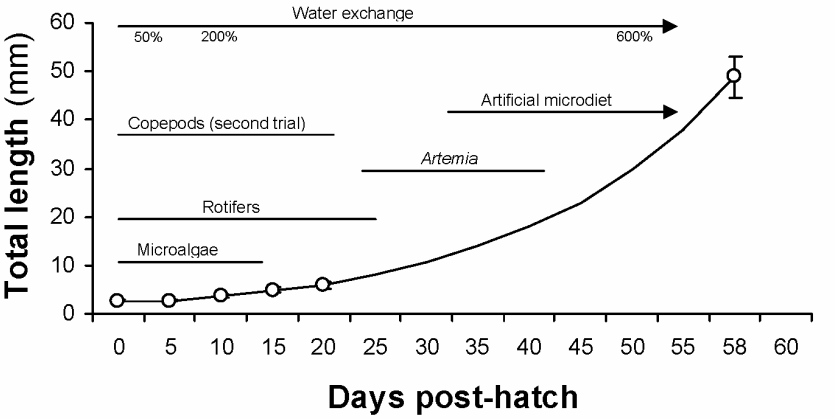

Fig. 1. Feeding and water exchange summaries and growth of *Lutjanus guttatus* larvae during culture at experimental conditions.

Better results in terms of growth and survival in snapper larviculture are expected with the use of tropical copepods as first food (Singhagraiwan and Doi, 1993). In our second trial, the use of copepods improved survival compared to the first trial until 19dph when it ended. Thanks to its small size (from 68µm at nauplii stage) and high content of highly unsaturated fatty acids, *T. monozota* offers a promising alternative to improve growth and survival in feeding trials with larvae of snapper species.

Conclusions

Larvae of spotted rose snapper *L. guttatus* can be cultivated under experimental conditions with microalgae, rotifers, copepods, *Artemia*, and artificial microdiets. Modifications in the present feeding protocol are required to include prey

food of adequate size at first feeding and the use of enriched rotifers to improve survival and growth during this phase in the larviculture of *L. guttatus*.

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ESTIMATION OF DAILY FOOD INTAKE AND GUT EVACUATION UNDER DIFFERENT FEEDING CONDITIONS IN AFRICAN CATFISH (*CLARIAS GARIEPINUS*) LARVAE

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Introduction

Different methods have been used to estimate food intake and evacuation in fish larvae and juveniles. The type of diet used in these investigations (e.g., zooplankton organisms or inert artificial microdiets) can affect the accuracy of the estimates of food consumption. For example, zooplankton organisms are easily broken into pieces inside the larval gut and thereby complicate their individual count. On the other hand, some artificial microdiets may lack natural attractants that are important for food acceptability.

Decapsulated cysts of *Artemia* were used as test diet to estimate food intake and gut evacuation in larvae of the African catfish (*Clarias gariepinus*). The estimations were based on the temporary accumulation of ascorbic acid 2-sulfate (AAS) measured in fish larvae after feeding on decapsulated cysts. AAS is a stable derivative of ascorbic acid that occurs naturally in the encysted embryos of *Artemia* and is converted into ascorbic acid few hours before the nauplii start hatching (Mead and Finamore, 1969). The first signs of liberated ascorbate from developing embryos of *Artemia* have been detected after 16h of cyst hydration (Dabrowski, 1991). The larval and juvenile stages of some teleost fish are unable to utilize AAS as a precursor to the synthesis of ascorbic acid, and no natural occurrence of AAS has been detected in fish (Dabrowski et al., 1990). Thus, the AAS in decapsulated cysts of *Artemia* with less than 16 h of hydration do not convert into ascorbic acid and can be used as natural marker in food consumption studies with African catfish larvae.

Materials and methods

Hatchery-produced catfish larvae were reared at a constant temperature of 28°C. In the first experiment, the gut contents of fish larvae fed ad libitum were calculated as they changed during the first five days after the start of exogenous feeding. In addition, it was verified whether the ingested AAS was retained or broken down in the fish. Catfish larvae were kept at an initial density of 1200 larvae per 17-l aquaria. Four diet treatments were tested in triplicate. Feeding with live *Artemia* nauplii or decapsulated cysts started at the beginning of exogenous feeding (two days after hatching) and was repeated every four hours, from 08:00 to 24:00. In treatment one catfish larvae were fed exclusively decapsulated cysts of *Artemia* during the entire experiment to measure the AAS content in fish derived from cysts (gut contents). In treatment two a combined feeding of *Artemia* nauplii and decapsulated cysts was carried out to estimate the retention of AAS in fish. In this treatment nauplii were offered to the fish larvae in non-sampling days and decapsulated cysts were fed exclusively during the sampling days. In treatment 3 (control) only live nauplii were fed to the larvae during the entire experiment. Comparison between treatment 1 and 3 should give an indication how daily food consumption changed during the first days of exogenous feeding. To reassure that AAS biosynthesis in fish larvae did not occur, even when other sources of ascorbic acid are provided, a fourth diet treatment was included using a dry artificial diet with a source of ascorbic acid different from AAS. Fish samples were taken every four hours on days 1, 3, and 5 after the start of exogenous feeding. Samples were taken 15 min before the first daily meal and three hours after each meal. In the control treatment, samples were taken only three hours after the fourth daily meal.

In a second experiment, the gut evacuation rate of larvae was determined under different feeding conditions. Because the gut evacuation rate might be affected by meal frequency, two feeding strategies were applied during the sampling days. In the first treatment, a continuous feeding regime was applied: after the first meal with decapsulated cysts, subsequent feeding was done with live instar I nauplii of *Artemia* which contained no AAS. In the second treatment, a discontinuous feeding strategy was followed: after the first feeding with decapsulated cysts, no additional food supply was offered during the rest of the day. As control diet, *Artemia* nauplii were offered to the fish in the third treatment. In all diet treatments the fish were fed with nauplii in between sampling days. Fish larvae samples were taken at days 1, 3, and 5 after the start of exogenous feeding. In the continuous feeding treatment, samples of fish were taken 15min before the first daily meal and three hours after each meal during the sampling days. For the discontinuous feeding treatment, fish samples were taken before the daily first meal and at 3, 7, 11, and 15h after that first meal. In the control treatment, fish samples were taken only three hours after the fourth daily meal.

In both experiments fish samples were shock frozen in liquid nitrogen and stored at minus 80°C until AAS determination. The number of fish larvae in each sample for AAS analysis ranged from 80-120 on day 1, 40-50 on day 3, and 15-20 on day 5 depending on the fish weight in both experiments. The fish weight during the sampling days was determined before first feeding and after every feeding with exception of the last feeding. The dry matter of *Artemia* cysts, artificial diet and fish during the sampling days in the experiments were determined by drying the samples in an oven at 60°C for 24h. Samples of decapsulated cysts of *Artemia*, hydrated for 1 and 3h after brine storage, and instar I nauplii were also prepared for determination of AAS content. Extracts of *Artemia* cysts and catfish larvae were prepared as described by Nelis et al. (1994). Reversed-phase, ion-paired, high-performance liquid chromatography (HPLC) was used for quantification of AAS. The HPLC system consisted of a LKB 2249 pump, an N-60 valve injector fitted with a 100-µl loop, a Philips PYE Unicam PU 4025 UV detector and a Merck Hitachi D-7500 integrator. A 3-µm Adsorbosphere HS C18 (150 × 4.6mm) column was used with a mixture of acetonitrile and ammonium phosphate buffer (60mmol, pH 5.0) containing 32mmol TBAP (1.5:8.5 ($v.v^{-1}$)) as mobile phase. Flow rate was $1ml.min^{-1}$ and detection was performed at 254nm.

The number of cyst present in the gut or gut content was estimated considering the amount of AAS measured in the fish samples divided by the AAS content of cysts, divided by the number of fish in the sample. GC was also calculated based on body dry weight (BDW) of larvae. Gut evacuation was calculated from data of experiment 2 as the remaining percentage after 7, 11, or 15h of deprivation of food with AAS from the initial number of cysts after the first feeding. Food consumption was calculated by subtracting the gut evacuation from the gut contents and was expressed as percentage of fish body weight.

Results and discussion

Decapsulated *Artemia* cysts were highly accepted by fish larvae in both experiments. Fish were actively feeding on decapsulated cysts every time feeding with cysts was done. From experiment 1, results show that the number of food particles ingested during the first meal and during the rest of the day was close to the total number of cysts measured at the end of the day. This indicates a tendency of the larvae to fill completely the gut with food particles in each meal. As fish increment its size, the volume available to store food also increases. In this way the fish is able to ingest an increasing amount of food in each meal. Once the gut is filled, no additional ingestion of food occurs until more space in the gut is available.

Food evacuation differed significantly ($P<0.05$) between continuous and discontinuous feeding treatments in experiment 2. After 7h of ingestion of the first

meal at day 1 after the start of exogenous feeding, the fish larvae evacuated approximately 87% of the amount of food ingested in that meal compared to 43% when discontinuous feeding was applied. Hence, 3 h after the second meal ingestion, the newly ingested food has a significant effect on the evacuation rate of the previous meal. During the continuous feeding regime, food evacuation occurred faster than when a discontinuous feeding was applied. On days 1 and 3, more than 90% of the food ingested in the first meal was evacuated after 15h. During the first days after the start of exogenous feeding (i.e., from day 1 to 3) the gut evacuation rate of *C. gariepinus* appears to be independent of body weight. Gut evacuation rate presented a similar pattern as food consumption, increasing during the first days of exogenous feeding and decreasing as fish growth continued.

Food consumption in catfish larvae increased from an average of 46.5% of their body dry weight (BDW) at day 1 after the start of exogenous feeding to 53.8% (BDW) at day 3. Thereafter, food consumption decreased to 27.8% (BDW) at day 5. Within the same day and from one meal to the next the fish is growing fast, consequently the gut size increases and more capacity to store food becomes available. Evidently, the amount of food ingested by the fish larvae increases with their body weight. However, if we express food consumption as percentage of body weight after the start of exogenous feeding, in *C. gariepinus* larvae the amount of ingested food decreases with age from day 3 onwards.

Conclusion

This method provided a useful tool for the determination of food consumption and gastric evacuation in fish larvae and permits adequate standardization. It avoids the individual count of food particles or marker substance by the direct analysis of the total amount of AAS in fish and in diet samples.

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OFIMER PROBIOTIC STUDY ON RAINBOW TROUT. IV. THE SETTLEMENT OF INTESTINAL MICROBIOTA IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) FRY SUBMITTED TO PROBIOTIC TREATMENT

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Introduction

Probiotic treatments may be particularly useful to secure the settlement of fish intestinal microbiota. In a previous study, a dietary supplementation with *Pediococcus acidilactici* protected trout fry against the vertebral column compression syndrome, suspected to be caused by a pathogenic bacterium (Aubin et al., 2005). However, intestinal microbiota appeared to be highly variable in time, and between rearing locations (Huber et al., 2004; Gatesoupe et al., 2005). The present paper is an attempt to characterize the microbes associated with intestinal mucus in trout of two nurseries, where they were fed with a probiotic yeast from start-feeding onwards.

Materials and methods

The experiments were conducted in two different experimental nurseries located in Brittany, one in Plouzané (AFSSA) and the other in Sizun (SEMII). In both sites, trout fry were fed with a commercial diet, either supplemented or not with the yeast *Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079 (10^6cfu.g^{-1} in the dry diet). In Plouzané, trout from one spawn were allocated in duplicates of 3000 fish each, for the control and the experimental group. Another spawn was used in Sizun, with triplicates of 800 fish each. The trout were reared in river water in Plouzané, and in spring water in Sizun. The water inlet was UV-treated and the mean temperature was 11°C in both sites, but with two different varia-

tion ranges (2 and 0.1°C of standard deviation in Plouzané and Sizun, respectively). Three fish were sampled in each tank at regular intervals for microbial examination, after 20h starvation (at 0, 7, 14, 21, 28, 49, and 112 days post start-feeding [dpsf] in Plouzané, and at 10, 20, and 30dpsf in Sizun). Aubin et al. (2005) described the microbiological methods.

Results

In both sites, there was no significant difference in total aerobic bacteria between the control and the experimental groups, nor were different the counts of autochthonous yeast. However, there were significant differences among sampling dates in Plouzané (Fig. 1). The bacterial counts were maximum at 7dpsf in Plouzané, and at 10dpsf in Sizun, with the same level (105 and 10^7cfu.g^{-1}, respectively). Then the bacterial load decreased in both sites from the second fortnight onwards (Fig. 1; Gatesoupe et al., 2005). Autochthonous yeast were detected during the first month in Plouzané, where the maximum was reached at 28dpsf (10^4cfu.g^{-1}). The same level of autochthonous yeast was observed in Sizun at 10-20dpsf, but it decreased afterwards (10^3cfu.g^{-1} at 30dpsf). In Plouzané, the autochthonous yeast were dominated at 14dpsf by one strain of *Leucosporidiales* (89% of the isolates), then the dominance turned to *Candida* sp. (99.8 and 85% of the isolates at 21 and 28dpsf, respectively). In Sizun, *Debaryomyces hansenii* YB3A3 was dominant at the three dates of sampling (97-100% of the isolates), as already observed previously (Aubin et al., 2005). Meanwhile, the probiotic yeast was retrieved at 10^3cfu.g^{-1}, without significant difference between sites and sampling dates.

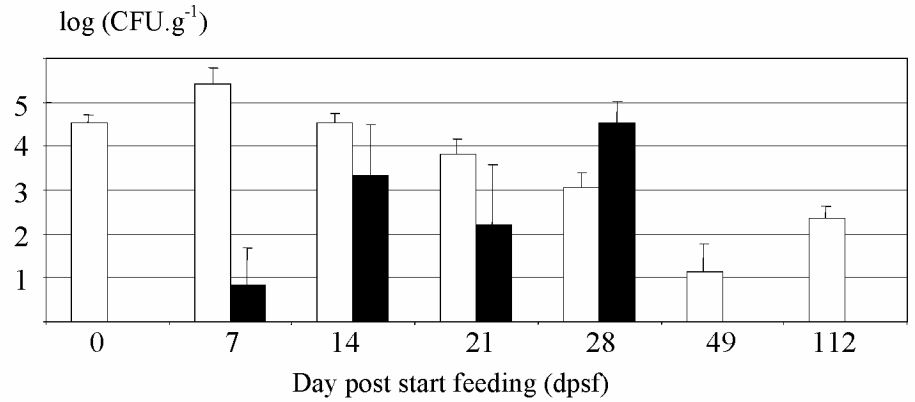

Fig. 1. Microbial counts in the intestine of trout fry in Plouzané in cfu.g^{-1} ($\log_{10}$ transformed, ± standard error, n = 4). The grey and the black bars correspond to cultivable aerobic bacteria and autochthonous yeast, respectively.

The dominant bacteria were also highly variable in time, while depending on the probiotic treatment, in Plouzané (Fig. 2). *Pseudomonas* spp. were strongly dominant at 0dpsf, and at 7dpsf in the control group. The dominant strains were different in the two groups till 21dpsf, but not afterwards. *Bacillus* sp., then again *Pseudomonas* spp. were dominant in both groups at 28 and 112dpsf, respectively. In Sizun, the dominance of *Pseudomonas* spp. was observed in both groups at 10 and 20dpsf (50% of the isolates). Then at 30dpsf, *Providencia* sp. became slightly dominant (23% of the isolates) in highly diversified microbiota. In a previous experiment in Sizun, the dominance of *Pseudomonas* spp. was also observed at 20dpsf in the group fed with the probiotic, but not in the control group (Aubin et al., 2005).

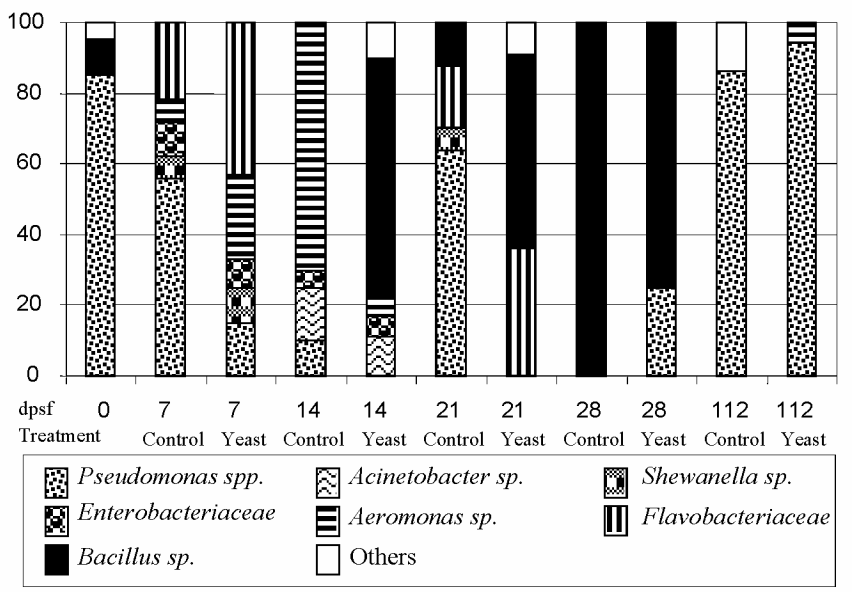

Fig. 2. Proportion of the dominant strains of bacteria isolated in the intestine of trout fry in Plouzané (% of the isolates), according to the sampling date (dpsf), and to the probiotic treatment (dietary yeast or control).

Discussion

The high variability of microbiota observed between fish farms seemed to apply to nurseries too. However, some trends may be worth further confirming. It seemed that *Pseudomonas* spp. were hosted in large amounts in the intestinal mucus during the first days of feeding. Then the bacterial load decreased, while diversity and variability increased. There was no consistent effect of the probiotic yeast on microbiota, but the treatment might be useful directly for the host. So far, the probiotic effects of *S. cerevisiae* var. *boulardii* have not been observed in trout fry, but the treatment increased the resistance of trout against bac-

terial infection at a later stage (Quentel et al., 2005). Some effects initiate early though they can be observed only several months after; e.g., the prevention of vertebral compression (Aubin et al., 2005). The supplementation of a dry diet with live *D. hansenii* stimulated gut maturation in sea bass larvae (Tovar et al. 2004), whereas autochthonous yeast are seldom isolated in marine fish. The presence of numerous CFU of autochthonous yeast during the first month may be thus important for trout development. *D. hansenii* was present in large numbers in Sizun from 10 to 30dpsf, but yeast were scarce before 14dpsf in Plouzané, and *Leucosporidiales* or *Candida* sp. have not been documented for any probiotic effect. Moreover, yeast were not detected in trout fry in another nursery (Viviers de France, Lévignacq; data not shown), though autochthonous *S. cerevisiae* were found there after five months of feeding (Gatesoupe et al., 2005). In front of this haphazard colonization of trout intestine by yeast, it may be safe to introduce a probiotic strain from start feeding onwards.

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EFFECTS OF TWO CULTURING TECHNIQUES ON THE GROWTH, SURVIVAL, AND LARVAL QUALITY OF *DENTEX DENTEX*

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Introduction

Dentex dentex is one of the Sparidae of interest for Mediterranean finfish aquaculture diversification. Although it is a fast growing species, survival during the larval stage is the main bottleneck for its study and final adaptation to commercial farming conditions (Sweetman, 1992).

In the present study, larvae hatched from the same batch of eggs were reared under two culturing techniques: "mesocosmos", used in studies of morphological description and in commercial fish farms in Greece (Divanach and Kentouri, 2000), and intensive rearing, mainly used in Spanish finfish farming industry. The aim of this comparison is to point out the principal culture parameters involved in the differences observed in growth, skeletal deformations and larval survival between the two rearing techniques.

Materials and methods

Three females and six males of *Dentex dentex* were kept under controlled and constant temperature (19 ± 1 ºC) and photoperiod (14L:10D). A single batch of eggs was incubated, and newly hatched larvae (0dph) were randomly transferred to four tanks: two black bottom 500-l tanks for intensive rearing, and two white-bottomed 1500-l tanks for mesocosmos (Divanach and Kentouri, 2000). Culture conditions are shown in Table I.

Feeding sequences of both culture techniques only differed during rotifer (*Brachionus* sp.) feeding phase. Intensive reared larvae were fed daily with 10 rotifers ml^{-1} in green water (*Tetraselmis chuii, Isochrysis galbana* var. Tahiti and *Nannochloropsis gaditana*). The rotifers were enriched at 250 rot ml^{-1} with 0.3 gr l^{-1} DHA Selco (INVE). In mesocosmos, no rotifer was added to the population established beforehand by a phytoplancton bloom (*Tetraselmis chuii,*

Isochrysis galbana var. Tahiti and *Nannochloropsis gaditana*) produced before the introduction of larvae to the tank. From 15dph to the end of the experiment, all larvae were fed with enriched *Artemia* nauplii (500 nauplii.ml^{-1}, 0.6g.l^{-1}, DHA Selco, INVE). Weaning started at 25dph.

Table I. Initial larval density and culture conditions.

Culture parameters	Intensive	Mesocosmos
Initial larval density (larvae.l^{-1})	47.5	3.5
Temperature (ºC)	18.9-21.5	19.2-22.2
Oxygen (mg.l^{-1})	4.7-8.4	6.8-8.9
Salinity (‰)	31.7-36.1	32.8-35.6
pH	7.7-8	7.7-8.1

Twenty larvae per tank were sampled on days 0, 7, 15, 20, 31, and 40dph for growth determination (total length and dry weight). On day 31 dph, 20 larvae per tank were sampled to determine skeletal deformations using the double staining technique (Dingerkus and Uhler, 1977; Potthoff, 1984), descriptions of normal morphology can be found in Koumoundouros et al. (1999; 2000; 2001). At the end of the experiment, on 40dph, surviving larvae from each tank were counted. Results were analysed by a t-test (StatgraphicsPlus 4.1, Microsoft Inc.).

Results and discussion

Total length, dry weight, and skeletal deformations results are shown in Figures 1, 2, and 3, respectively. Survival averaged 6.58% in mesocosmos and 1.58% in intensive rearing.

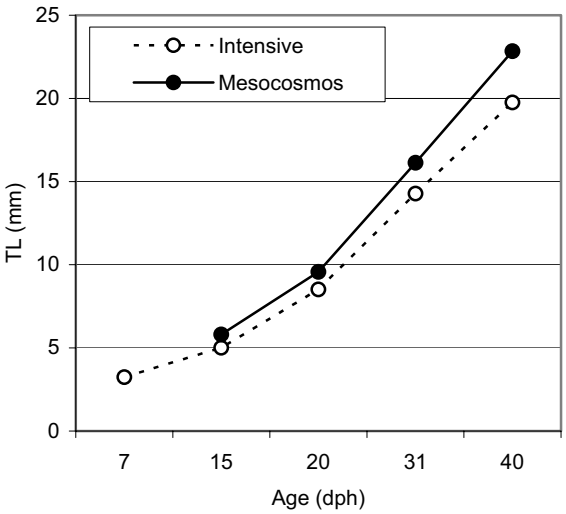

Fig. 1. Changes in total length of *Dentex dentex* larvae under different culture parameters.

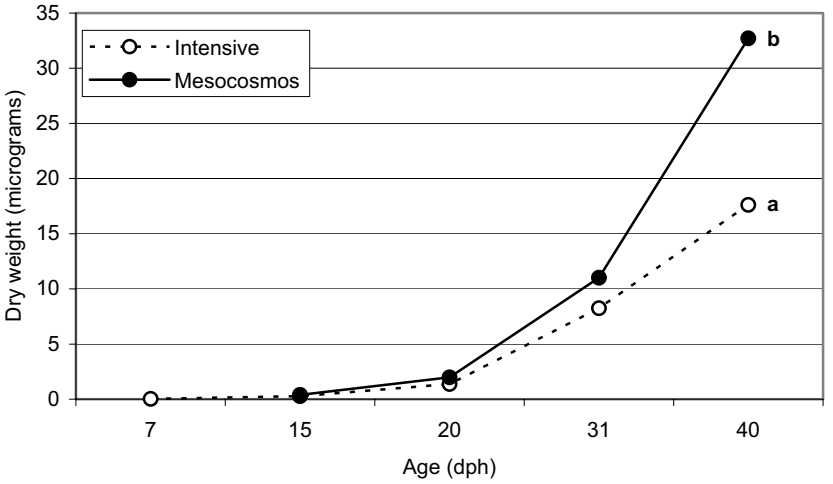

Fig. 2. Changes in dry weight of *Dentex dentex* larvae under different culture parameters.

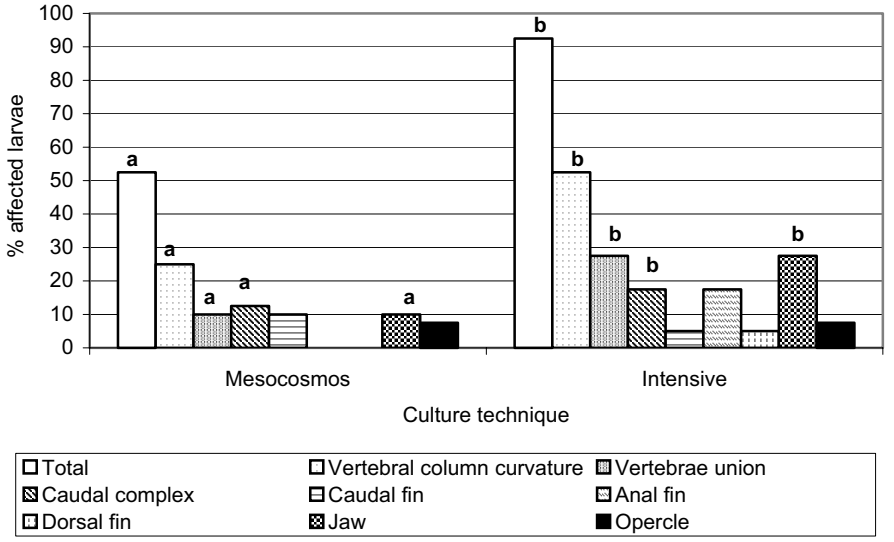

Fig. 3. Percentage of *Dentex dentex* larvae with skeletal deformations under different culture parameters.

Although larvae reared in mesocosmos showed a higher total length and dry weight, only dry weight at 40dph was statistically different than the dry weight of intensively reared larvae at the same age. Significant differences were observed in survival at 40dph and in the incidence of skeletal deformations at

31dph, both for percentage of deformed larvae and for some deformation types such as those related to vertebral column and to the caudal complex.

Larvae used in the present study came from the same batch of eggs in order to minimise genetic variability, hence differences observed can mainly be attributed to the rearing conditions. Over all the culture parameters, initial larval density and initial prey density and quality are the factors suspected to affect growth and survival performance, while skeletal deformities might also be affected by hydrodynamics.

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NATURE AND LEVEL OF DIETARY PHOSPHOLIPIDS AFFECTS THE DIGESTIVE PHYSIOLOGY AND LARVAL QUALITY OF EUROPEAN SEA BASS LARVAE

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Introduction

Lipids are the main energy source in developing larvae and their requirements in marine fish larvae have been extensively studied. Particular attention has been paid to phospholipids (PL) and highly unsaturated fatty acids (HUFA). It has been reported that a diet with 19% lipids with ca. 9% PL induced a good growth in European sea bass larvae, since larvae might utilize PL more efficiently than neutral lipids (NL) (Cahu et al., 2003). Like most marine fish, European sea bass has an absolute requirement for HUFA. A deficiency in HUFA delays growth, induces mortality, reduces resistance to stress and results in malformations associated to nutritional disorders. The essential fatty acid requirements are known to vary both qualitatively and quantitatively. Their optimal levels for marine fish are around 3% dry matter for EPA+DHA (Sargent et al., 2002).

The present study aims to evaluate the effect of the nature (PL vs. NL) and level of *n*-3 HUFA of dietary lipid supply on growth, digestive enzymatic activities, incidence of skeletal malformations and histological organization of the intestine and liver in European sea bass larvae from first feeding up to metamorphosis.

Materials and methods

European sea bass larvae (7dph) were fed until 37dph with five isonitrogenous and isolipidic microdiets (4 replicates) with different levels of HUFA, which differed in their lipid class composition, PL vs. NL, and level of EPA and DHA. Fishmeal, containing 17% lipids, was previously defatted to control dietary lipid composition by only the addition of oils (marine lecithin, Phosphomins©, and soybean lecithin). Diet names indicated the percentage of EPA and DHA contained in the PL and NL fractions: PL5 (4.8%), PL3 (2.3%), PL1 (1.1%), NL1

(1.3%) and NL3 (2.6%). Marine phospholipid fraction in PL diets varied in inverse relation to soybean lecithin, whereas NL diets contained soybean lecithin in an inverse proportion to marine TAG (cod liver oil). To evaluate the level of maturation of the digestive system, lipase, and brush border intestinal enzymes (alkaline phosphatase, AP and aminopeptidase N, AMPN) were measured at 30 and 37dph, while trypsin and amylase secretions were measured at 37dph (n = 20-50 larvae). Pancreatic and intestinal enzymes were analysed according to Zambonino and Cahu (1999) and Cahu et al. (2003).

Results and discussion

At 37 dph, significant differences in larval growth and incidence of skeletal malformations were observed between groups (ANOVA, $P<0.05$, Fig. 1). Almost half of the fish fed PL5 diet had deformities. This value was very low (6.6%) in the group fed the diet PL1. Fish fed diets with PL5 and PL3 presented a high prevalence of scoliotic vertebral columns (35.3 and 10.2%, respectively), while head deformities were less frequent. Fish fed with the diet NL1 showed an equal percentage of skeletal malformations affecting the vertebral column and operculum (7.9 and 8.2%, respectively), this value of deformities was higher than in the control group (Fig. 1). An effect of oxidation of HUFA could be evoked (Sargent et al., 2002) but it is unlikely since the formulation of experimental diets felt within the recommended NRC levels for dietary antioxidants (NRC, 1993).

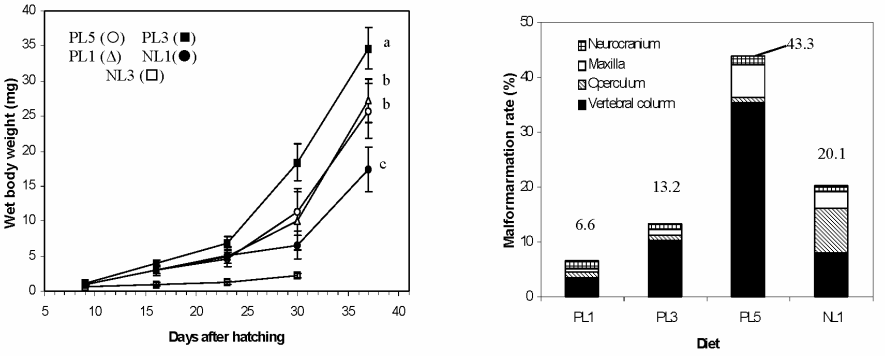

Fig. 1. Growth and skeletal malformations in European sea bass juveniles (age: 71dph) fed diets containing different levels of EPA and DHA. Means ± S.D. (n = 4) with different superscript letters for the same day are significantly different ($P < 0.05$).

At 30, the highest AP specific activity was observed in fish fed PL3, while no differences were observed between the other groups (PL1, PL5, and NL1), which were lower than in fish fed PL3. At day 37, the lowest AP activity was detected in larvae fed PL1, no differences being found between the other groups. No differences were observed in AMPN specific activity of larvae fed different diets at 30dph. At day 37, AMPN specific activity was 30% lower in larvae fed

PL1 (control group) compared to the rest of treatments (Fig. 2). All groups exhibited a good level of trypsin secretion, while the fish fed the highest levels of EPA+DHA in the PL fraction (PL5 diet) showed lower values of amylase secretion than the rest of the groups (ANOVA, $P<0.05$; Fig. 2). It should be noted that although larvae fed PL1 and NL1 diets exhibited the highest values of trypsin and amylase secretion, these values should not be attributed to a major maturation of the digestive system, since the specific activity of intestinal brush border enzymes (particularly AP) was lower in these groups in comparison to fish fed PL3 diet. High secretion of pancreatic enzymes in larvae fed PL1 and NL1 might be correlated to the high dietary content of linoleic acid, since C_{18} fatty acids regulate the pancreatic secretion (Shintani et al., 1995).

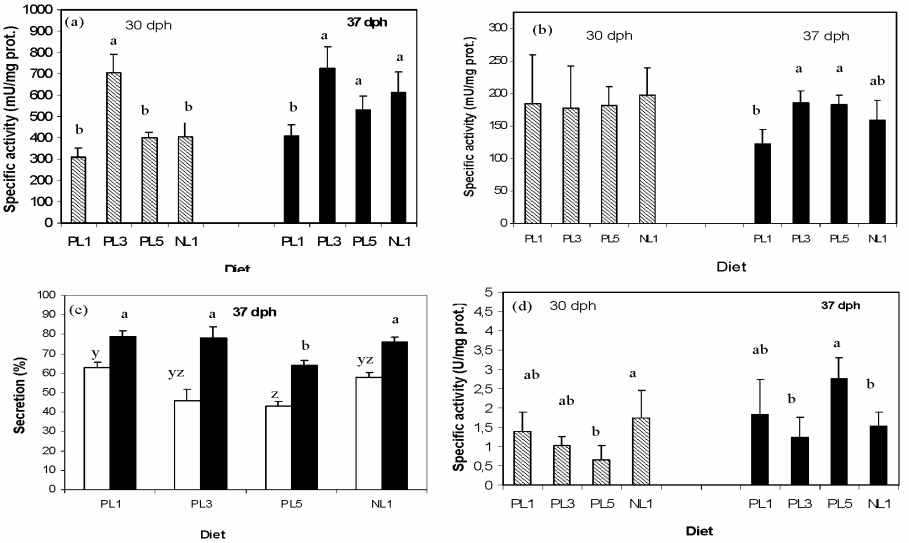

Fig. 2. Specific activity of AP (a), AMPN (b), trypsin (□, c), amylase (■, c) and lipase (d) in 30 and 37dph larvae fed diets containing different levels of EPA and DHA.

Specific activity of lipase was affected by diets (ANOVA, $P<0.05$; Fig. 2). At 30dph, lipase specific activity was two times higher in fish fed NL1 than in PL5 group. At 37dph, the lipase activity in PL5 group was 55% and 45% higher than in fish fed PL3 and NL1, respectively. These changes might be related to the high lipid deposits observed in the intestine rather than to a lower maturation of the digestive tract, since lipids accumulated in the intestine might have disrupted the metabolic capacity to digest, absorb and export lipids into the circulatory system. The change in lipase activity in PL5 group at day 37 compared to day 30 was not attributed to a stimulation of lipase by its substrate, i.e. NL. This change reflected an indirect consequence of intraluminal fatty acid concentration resulting from the hydrolysis of PL, which in turn stimulated a hormonal mechanism involving secretin (Sheele, 1993). Changes in the histology of the prevalvular

intestine were only observed in fish fed NL3 diet, which showed large deposits of small lipid droplets. Large intercellular lipid vacuoles were also noted. Fish fed NL diets exhibited a lower lipid accumulation in this region than in the other groups, indicating different patterns of lipid absorption and accumulation, depending on the level and class of dietary lipids. The form of supply dietary HUFA has a direct effect on fat storage in the liver, which might be due to a higher influx of chylomicrons and/or VLDL and differences in lipid exportation capacities of the intestinal mucosa.

Conclusions

The quantity and form of EPA and DHA (NL or PL) supplied in the diet were determining for European sea bass larvae in terms of digestive physiology and incidence of skeletal deformities. Larvae used EPA and DHA more efficiently when they were present in the PL rather than in the NL fraction of the diet. These results must be taken into account in the formulation of compound diet for larvae. Indeed, lipid and HUFA are generally provided by fish oil, which is a neutral lipid, but marine phospholipid would be a better source of HUFA.

Acknowledgements

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THE USE OF PHYTOPLANKTON AND ZOOPLANKTON IN THE CONDITIONING OF ADULT *MYTILUS EDULIS* (L.)

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Introduction

Previous work has suggested that an omnivorous diet of zoo- and phytoplankton may supply greater energy and nutrients than a single species diet, thereby enhancing the growth of *Mytilus edulis* adults (Wong and Levinton, 2004). This study aims to examine the effectiveness of a mixed diet of live algae and rotifers in free-swimming or bioencapsulated forms on the promotion of gametogenesis. The conditioning of bivalve broodstock with a diet rich in long chain polyunsaturated fatty acids leads to higher egg yields and benefits larval growth and spat production. Gametogenesis in adult *M. edulis* can be opportunistic when food supplies are favourable and can be accelerated in the hatchery environment. Studies have found that the gametogenic cycle and condition indexes are strongly linked to the storage and consumption of reserves, particularly glycogen (Gabbott, 1975). During winter months the flesh weight decreases as lipid and glycogen reserves are utilised, due to limited food availability and the onset of gametogenesis (Okumuş and Stirling, 1998).

This study focuses on the assimilation of nutrients from live algae and rotifers into the gonadal tissue of adult *M. edulis*, and their effect on animal condition. Rotifers will be used as carriers in a mixed-diet system using algae of different biochemical profiles. Mussel condition will be determined by glycogen content, fatty acid profiles and condition indices.

Materials and methods

Adult *M. edulis*, in a resting gonadal state after the summer spawning season, were retrieved from Waterford Bay during October 2004. The mussels were acclimatised for one week, after which condition indices (Walne, 1976; Hickman and Illingworth, 1980) were determined to provide initial dry flesh weights for

use in feeding ration calculations. Mussels from this same population were randomly distributed amongst replicated experimental tanks and daily fed one of seven diet combinations at a ration of 3% of the initial bivalve meat weight as determined at time 0. The different diets were as follows; (1) rotifers only (*Brachionus* sp.), (2) rotifers enriched with *Nannochloropsis oculata*, (3) rotifers enriched with Tahitian *Isochrysis galbana*, (4) rotifers enriched with *N. oculata* and *I. galbana*, (5) *N. oculata* only, (6) *I. galbana* only, and (7) *N. oculata* and *I. galbana*.

This study was carried out for an eight-week period with sampling every two weeks when whole mussel tissue was removed, vacuum-packed, and frozen at -80ºC for later glycogen and fatty acid extractions and analysis. Condition indices were also determined at each sampling interval. Dry flesh and shell weights were determined by oven-drying at 80ºC for 12h. Non-mantle tissues from pooled mussels were used for fatty acid and glycogen extraction to ensure that nutrient data was indicative of that incorporated into gametogenesis, and not stored in the mantle tissue itself. Glycogen content of the pooled mussel samples was determined using the method of Carroll et al. (1956).

Total lipids were extracted with chloroform:methanol (2:1, v/v) as described by Folch et al. (1957), with 20ml of the solvent solution used to extract each g of mussel tissue. The extracted lipids were then concentrated in aliquots using nitrogen in tared vials until a constant weight was achieved. 0.05% BHT was added to each tube to prevent lipid oxidation and samples were stored at -80ºC until saponification. The lipid extracts were saponified using potassium hydroxide in methanol for 1h at 70ºC after which 1.2M hydrochloric acid in methanol was added, followed by heating at 70ºC for a further 15min to ensure complete methylation of the fatty acids. These fatty acid methyl esters were thrice extracted in hexane to a total volume of 1ml before addition to the gas-chromatography column. Each pooled tissue sample was extracted and separated on the column in triplicate.

The content and composition of the fatty acid methyl esters were separated and quantified using Varian CP-3800 gas chromatograph equipped with a flame ionisation detector and CP-Select capillary column (50m × 0.25mm i.d., Varian Inc., California, USA). Helium at a pressure of 30psi was used as carrier gas; oven temperature was maintained at 150ºC for 35min and increased at 4ºC.min^{-1} to 240ºC and held for 2min. The injector (split mode) and detector temperatures were held at 270ºC and 300ºC, respectively. Methyl esters were identified by comparison with the retention times of correspondingly pure standards (Sigma Chemical Company, UK) and were expressed as a percentage of total fatty acids.

Percentage data was arcsine transformed before analysis of variance (ANOVA) statistical analysis, as according to Sokal and Rohlf (1981).

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LIPID REQUIREMENT AND DYNAMICS DURING LARVAL ONTOGENY OF HADDOCK, *MELANOGRAMMUS AEGLEFINUS*, IN RELATION TO NUTRITION AND PROBIOTICS

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Introduction

Marine fish species such as haddock, *Melanogrammus aeglefinus*, are currently being investigated in Atlantic Canada as an alternative to salmonid aquaculture. Therefore, mastering hatchery techniques to reliably produce sufficient quantities of juveniles to allow genetic stock manipulation has become desirable. Essential fatty acids have been linked to nutrition and immune response to pathogens (see Sargent et al., 1999; Bell and Sargent, 2003 for reviews) and the analysis of the quantities of reserve fatty acids (triacylglycerols [TAG]) and structural fatty acids (phospholipids [PL]) in an organism permits the identification of selectively retained fatty acids and therefore, the detection of any fatty acid deficiencies. The objectives of this study were (1) to determine the fatty acid requirements of haddock larvae during larval ontogeny by comparing larvae fed with two diets commonly used from weaning to the juvenile stage and (2) to investigate the effect on lipid dynamics and bacterial profile of haddock larvae exposed to live cultures of the bacterium *Arthrobacter davidanieli* (RSXII).

Materials and methods

Haddock larvae were reared at the Aquarium and Marine Center of Shippagan, NB, as previously described by Griffiths et al. (2001). In the first experiment, following the rotifer feeding stage (31 days post-hatch [dph]) duplicate groups of haddock larvae were fed by either *Artemia* enriched with Algamac 2000 (Bio-Marine) or the micro-encapsulated diet ArteMac (Bio-Marine) until they reached the juvenile stage. In the second experiment, three groups of haddock larvae were exposed to the candidate probiotic bacterium RSXII and three equivalent control groups were left unexposed. The probiotic was added to the tanks 18h before the first-feeding (1dph) at a final concentration of 10^6 viable cells.ml^{-1} of

tank water. A second dose of probiotic was administered at 7dph, with the bacteria added to the rotifer meal at a concentration of 10^6 cells.ml^{-1}. In both experiments, haddock larvae were sampled at -3, 1, 15, 31, 41, 45, and 54-62dph for dry mass and lipid content and 20 larvae were sample for bacterial community profiling. Lipid samples were stored in pre-cleaned amber glass vials with Teflon liner caps under N_2 in 2ml chloroform at -80°C before extraction following a modified Folch procedure (Folch et al., 1957) described by Parrish (1999). Extracts were applied to Chromarods-SIII for separation of lipid classes by thin-layer chromatography and quantification by FID using an Iatroscan MK-VI. Lipids were fractionated into neutral and polar lipids using column chromatography and fatty acid profiles were determined by gas chromatography of each fraction. Samples of haddock larvae for bacterial profiling were collected in tubes containing 3ml of the nucleic acid fixative RNAlater to allow retrospective characterization of the bacterial communities associated with the larvae. Profiling of the bacterial populations involved PCR amplification of 16S rDNA, followed by separation of amplicons via denaturing gradient gel electrophoresis (DGGE) and subsequent sequencing of major bands as described by Griffiths et al. (2001).

Results and discussion

Haddock larvae fed *Artemia* from weaning until the juvenile stage exhibited both higher growth rates and survival than those fed ArteMac (Fig. 1).

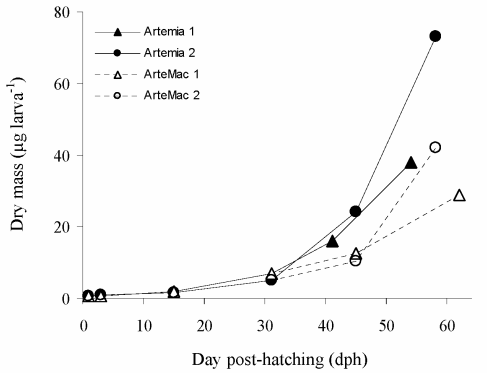

Fig. 1. Dry mass of haddock larvae *M. aeglefinus* fed rotifers enriched with DHA Selco (INVE) during the first 31 days post-hatching (dph) followed by either *Artemia* enriched with Algamac 2000 (Bio-Marine) or the micro-encapsulated diet ArteMac (Bio-Marine) until the juvenile stage. All trials were done in duplicate.

The top-performing larvae fed *Artemia* had relatively low TAG levels (~7%) and trace amounts of sterol esters (SE-WE), while the poorly performing larvae fed ArteMac exhibited elevated TAG levels (13-15%) and significant amounts of SE-WE (Table I). The elevated accumulation of storage lipids by haddock larvae fed ArteMac suggests that this was an inappropriate diet for this species.

Table I. Lipid class composition (mean ± SD) of haddock larvae fed rotifers enriched with DHA Selco (INVE) during the first 31dph followed by either *Artemia* enriched with Algamac 2000 (Bio-Marine) or the micro-encapsulated diet ArteMac (Bio-Marine), until attaining the juvenile stage (duplicate cultures). SE-WE = Sterol esters - Wax esters, KET = Ketone, TAG = triacylglycerols, ST = sterols, AMPL = Acetone mobile polar lipids, PL = phospholipids.

Treatment	Stage	Lipid classes (% of total lipid)					
		SE- WE	KET	TAG	ST	AMPL	PL
Rotifer	Egg	0.0 ± 0.0	0.0 ± 0.0	5.1 ± 0.8	6.8 ± 0.7	2.6 ± 0.4	85.5 ± 1.9
	Hatching	0.6 ± 0.1	0.4 ± 0.5	3.7 ± 1.2	10.6 ± 0.4	0.0 ± 0.0	83.8 ± 2.0
	Larvae 3d	0.2 ± 0.3	0.0 ± 0.0	10.7 ± 4.8	11.4 ± 1.4	3.0 ± 1.7	74.7 ± 7.5
	Larvae 15d	0.0 ± 0.0	1.3 ± 0.8	5.2 ± 0.5	13.0 ± 0.7	1.2 ± 0.5	79.2 ± 0.8
	Larvae 31d	0.3 ± 0.5	1.4 ± 1.1	9.8 ± 4.0	13.0 ± 1.2	1.5 ± 0.6	74.0 ± 1.9
ArteMac	Weaning	2.7 ± 3.0	1.4 ± 0.6	13.0 ± 0.2	16.9 ± 2.2	0.3 ± 0.4	65.4 ± 5.7
	Juvenile	5.2 ± 1.9	1.2 ± 0.6	15.0 ± 1.1	21.1 ± 4.7	0.6 ± 0.2	56.3 ± 8.2
Artemia	Weaning	0.7 ± 1.0	2.8 ± 2.6	7.0 ± 2.7	16.4 ± 8.7	0.4 ± 0.6	71.4 ± 10.9
	Juvenile	0.4 ± 0.6	4.6 ± 1.6	6.9 ± 2.4	17.0 ± 1.3	0.7 ± 0.2	70.1 ± 6.2

Table II. Lipid class composition (mean ± SD) of haddock larvae exposed to the probiotic bacteria *Arthrobacter davidanieli* (RSXII) and control groups. The bacterium RSXII was administered at 1 and 7dph (triplicate cultures). See Table I for lipid class abbreviations.

Treatment	dph	Lipid classes (% of total lipid)			
		TAG	ST	AMPL	PL
	-3	6.9 ± 2.4	7.6 ± 1.5	7.2 ± 9.7	77.9 ± 6.3
Probiotic	1	6.2 ± 0.9	9.5 ± 0.1	2.3 ± 2.9	81.8 ± 3.5
	3	8.1 ± 6.1	10.3 ± 2.4	1.3 ± 0.5	79.4 ± 9.5
	15	7.3 ± 5.8	11.8 ± 0.9	1.1 ± 0.5	79.4 ± 6.0
Control	1	5.3 ± 3.5	8.5 ± 3.3	1.4 ± 1.2	84.4 ± 8.5
	3	6.2 ± 1.9	10.3 ± 1.7	1.5 ± 1.3	81.3 ± 4.4
	15	1.7 ± 1.1	13.8 ± 3.5	1.4 ± 1.9	83.1 ± 6.5

Preliminary data from the second experiment (Table II) indicate that addition of the candidate probiotic *A. davididanieli* (RSXII) enhanced TAG concentrations in haddock larvae by 15dph (Table II) and resulted in higher dry mass values per larvae than those for untreated controls. The high variability in TAG levels recorded for the probiotic treated larvae most likely reflects different levels of colonization between the experimental groups. Bacterial profile are under analysis and will corroborate or infirm this hypothesis. Viral and bacterial infections are known to seriously affect the lipid content of several crustacean species (Stuck et al. 1996; Floreto et al. 2000). A recent study (Pernet, unpublished data) has shown that antibiotic treatment enhanced TAG concentrations in sea scallop larvae which were free of *Vibrio* spp. and resulted in higher post-metamorphic survival and TAG concentration than in non-treated controls, which were con-

taminated with *Vibrio* spp. Thus we propose that application of the probiotic enhanced the general health of haddock larvae by reducing energy allocated to defence.

Future perspectives

The relative proportions of fatty acid as neutral lipids (storage) vs. membrane phospholipids in larvae and in experimental diets are presently process to evaluate (1) the selective incorporation/elimination of specific fatty acids in larval tissues, (2) the fatty acid dynamic during ontogeny of haddock, (3) the nutritional quality of the two tested diets, (4) the specific requirements of haddock larvae for EFAs, and (5) the biochemical response of haddock larvae to the probiotic RSXII. In addition, evaluation of the bacterial communities associated with haddock larvae will provide information concerning the potential use of RSXII as a probiotic for the control of disease outbreaks caused by bacterial pathogens and on the effect of the microbial communities upon larval performance and their physiological response.

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DEVELOPMENT OF ROTIFER STRAINS WITH USEFUL CHARACTERISTICS FOR LARVAL REARING

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Euryhaline rotifer *Brachionus plicatilis* culture has been commonly used for rearing marine fish larvae. Providing cultures with appropriate size of rotifers encourages size-dependent selectivity of the feeding larvae and obtain larvae with higher survival, growth, and stability. This paper reviews the recent progress on the production of rotifer strains with ideal characteristics in terms of size, population growth, tolerance against external conditions, and resting egg formation.

The lorica length of rotifers showed rapid increase during the first 48 hours after hatching. We generally observe 30-40% increase in size during this stage. The lorica length of 2-day-old rotifers ranges from 170 to 320μm from observation using 70 rotifer strains cultured at 25°C. Temperature and salinity optima for population growth vary among strains. It is also found that the tolerance against environment stressors differs among rotifer strains. For example, combined effects of stressors, such as free ammonia, protozoa contamination, and increase of culture water viscosity caused a significant decrease in lifespan, fecundity, and enzyme activity of rotifers. The degree of the decrease, however, greatly varied among rotifer strains. It is therefore important to select a strain with higher stability for mass culture.

Within a strain, rotifer life history parameters can be modified by regulating environmental conditions, especially during resting egg formation, diapause, and hatching. Rotifer clones from resting eggs that experienced diapause have higher population growth. Resting egg formation becomes active when stem females, which hatched from resting eggs, received low quality diet such as baker's yeast or experience starvation, while the succeeding generations received higher quality diets such as *Nannochloropsis oculata*. Biological parameters such as reproductive characteristics and lorica size can also be changed by regulating the culture conditions. Rotifer size generally increases at low temperature and low sa-

linity and fed *Tetraselmis tetrathele*. Under such conditions, the lorica size of some rotifers reached more than 400μm. Four to 10% increase in lorica size was observed when chemicals, such as γ-amino butyric acid, juvenile hormone, and cytochalasin-B were added to the culture medium.

Cross-mating trials between rotifer strains produced hybrids with higher population growth. The lorica size of the progeny strains was intermediate of that of the parental strains. The attempt to produce rotifer hybrids has just started, and this has a great potential to produce strains with desirable characteristics.

Studies on molecular characteristics of rotifers are scarce, even if these studies are important to understand the mechanism involved in the rotifer life cycle, and may be applied to develop further ideal rotifer strains in future. In order to understand the molecular factors involved in cyclical parthenogenesis, sexual reproduction, and resting egg formation, we have been constructing and analyzing *B. plicatilis* EST. So far we cloned 298 unique ESTs. Some of the important genes we found were; protein digesting enzymes, stress responding proteins, signaling receptors, and cell replication relating enzymes.

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EFFECTS OF WEANING AGE AND DIETS ON HISTOLOGICAL ONTOGENY AND DIGESTIVE ACTIVITIES OF PIKE-PERCH LARVAE

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Introduction

Pike perch (*Sander lucioperca*) is one of the percid species who represents a real interest in aquaculture but its culture still presents some difficulties due in part to their small size and fragility (Kestemont and Melard, 2000). Rearing experiments more often concern the juvenile stage than larval one. Studies on larval weaning are rare and lead until now to poor results in terms of growth and survival (Ruuhijärvi et al., 1991). As the other teleostean species, pike perch larvae have much lower digestive enzymatic capacities than adults. This explains at least partially their difficulties to accept a dry diet during their early life stages. Only few authors have studied larval ontogeny of pike perch (Mani Ponset et al., 1994). It is thus required to investigate the effects of different diets and weaning ages on the development of digestive structures and on the activities of digestive enzymes, in order to acquire more knowledge about weaning limits and possibilities, and this is the aim of this work.

Materials and methods

The experiment was conducted with 30 000 pike perch larvae (4 days post-hatching (dph)) produced in a commercial hatchery in the Netherlands (Viskweekcentrum Valkenswaard). At mouth opening, larvae were divided in two groups. In the first group, larvae were fed on artificial diet from mouth opening. In the second, larvae were acclimated during 4 days in a 200-l tank with low water and air flow supply and fed on *Artemia* nauplii (small size) in excess from 8-19h. At 9dph, they were transferred to the experimental unit in a recirculating system of 12 rectangular 20-l grey tanks (50 larvae.l^{-1}). The artificial diet (Lansy, INVE, Belgium) was provided from mouth opening (4dph) and on 9, 15, and 21dph in treatments WD, W9, W15, and W21, respectively, with a period of 6-7d of co-feeding. The control group (A) was fed on *Artemia* nauplii and then enriched metanauplii (Super Selco, INVE, Belgium).

Larvae were collected at 0, 5, 9, 15, 21, 29, and 36dph: 10-15 per treatment for histology and 500, 400, 80, 80, 50, and 30 per tank, respectively, for enzymatic assays. Samples were taken before feeding and immediately stored at -80°C. Trypsin (Try) and amylase (Amy) were assayed on homogenates of entire larvae relieved from head and tail at 0, 5, and 9dph. Enzymes of the cytosol leucine-alanine-peptidase (leuala), and the brush border membrane alkaline phosphatase (AP) and aminopeptidase N (AN) were assayed on homogenates of intestinal segment or entire larvae, as described for pancreatic enzymes. Pepsin (gastric enzyme) was assayed on the pancreatic segment at days 21 and 29. Enzyme activities are expressed as specific activities ($U.mg^{-1}$ protein).

Larvae were fixed in Bouin's fluid, embedded in paraffin, and 6-µm longitudinal sections were stained with Masson Trichrome.

Results and discussion

Pancreatic and intestinal enzymes were detected from hatching. This was also observed for pancreatic enzymes in perch larvae (Cuvier and Kestemont, 2002). Amy, leuala, and AN reached a peak at first feeding and then decreased. Similar pattern of evolution has been described for many other teleostean species. At day 36, no differences were found between enzymatic activities for different treatments, probably because the digestive structures were properly developed and enzymatic activities well regulated.

Specific trypsin activity became almost stable from 15dph, except for control group which was significantly higher than for other treatments, although control and W15 larvae were fed *Artemia* until 21dph. Between days 5 and 9, we observed in WD larvae a sharply increase of trypsin (from 35-90$mU.mg^{-1}$ protein) and amylase activity (from 5-10$mU.mg^{-1}$ protein) (data not figured) and they almost all died at day 11.

Amylase activity sharply increased with mouth opening and then decreased until 36dph. At 29dph, it was significantly higher for W9 larvae than for other treatments. It seems that, as it was seen in sea bass (Zambonino and Cahu, 1994), pike perch larvae have the ability to adapt their enzyme activities in function of the feed ingested.

The specific activity of AP and AN increased between 15 and 29dph concurrently with the decreasing of leuala (Fig. 1). According to Cahu and Zambonino (1994), this evolution reflects the maturation of intestinal enterocytes. This maturation process seems to be impaired and/or delayed for larvae weaned earlier (W15, and especially W9). Indeed, at 21dph, AP reached a peak in larvae weaned at 9 and 15dph significantly higher than in the other treatments (control

and W21). At 29dph, the aminopeptidase activity in W9 larvae was significantly lower than in W21 larvae and leuala activity remained significantly higher for larvae weaned at 9dph. This may reveal a sign of malnutrition (Cahu and Zambonino, 1994).

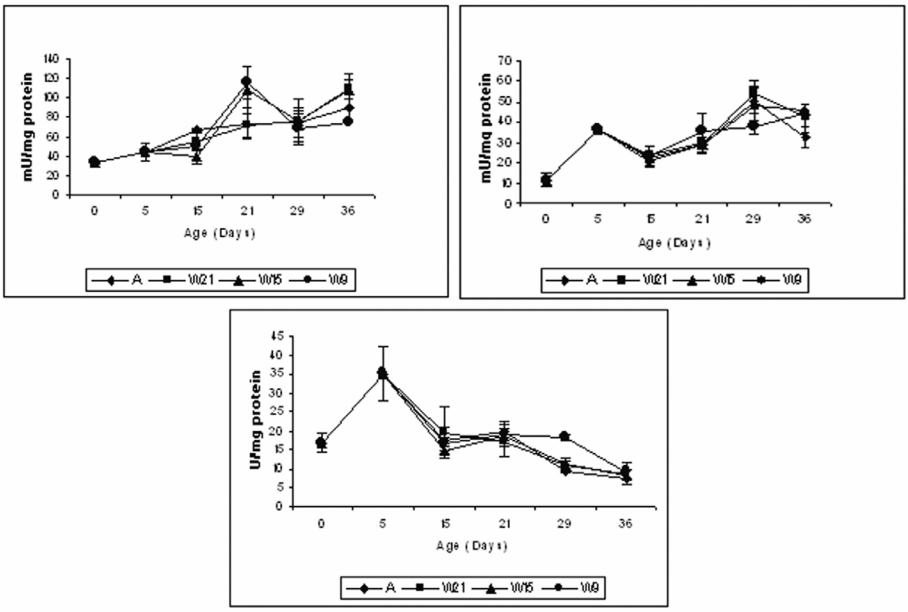

Fig. 1. Top L: Specific alkaline phosphatase activity. Top R: Specific aminopeptidase activity. Bottom: Specific leucine-alanine peptidase activity.

These results are corroborated with histological observations. From mouth opening, the digestive structures, except the stomach, are developed. Intestine is differentiated in two parts anterior and posterior intestine separated by intestinal valvula and the brush border is visible from hatching. At 15dph, a rudimentary stomach is distinguished, becomes recognizable at 21dph, and becomes functional with gastric glands appearing at 29dph, concurrent with pepsin activity. The main effect of the dry diet is observed in W15, and especially in W9 larvae. The intestinal epithelium seems to be atrophied and the height of the enterocytes is clearly lower than in the other treatments (Fig. 2). Indeed, the height of the enterocytes, particularly in the midgut, was cited by Segner et al. (1993) as one of the nutritional indices.

Conclusions

Our results suggest that pike perch larvae can be weaned at 21dph without damages on digestive capacities or development of digestive tract, while earlier weaning impair the onset of maturation processes of the digestive system, both in terms of morphological structures and enzymatic activities.

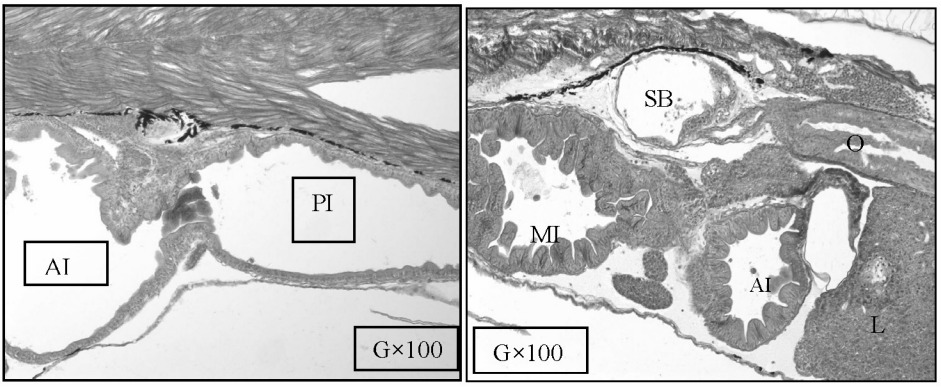

Fig. 2. Intestinal epithelium of W9 and control larvae (Day 15) AI, MI, PI: anterior, median, and posterior intestine, S: swim bladder, O: oesophagus, L: liver.

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HALIBUT FRY PRODUCTION

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The first attempts to grow halibut larvae beyond metamorphosis were successfully carried out in the beginning of the 1970s at Flødevigen Biological Station. However, more than ten years elapsed before the first commercially produced halibut juveniles were seen. In this period, international scientific cooperation brought about trial production and early life stage research in several countries.

Halibut eggs are heavier that most other pelagic fish eggs and are therefore often referred to as mesopelagic. At hatch (~84 daydegrees, dd°) the poorly developed halibut larvae differ from other marine fish by the long yolk-sac stage and relatively large size and by 40d later the larva has resorbed most of its yolk, depending thereafter on exogenous food for further growth and development. In aquaculture, live prey are used for first-feeding, most commonly *Artemia* fed in traditional greenwater cultures, 60d after which the larvae are weaned onto a dry diet.

At present, due to failure of natural spawning in captivity, culture techniques include hand-stripping of eggs from ripe females. Eggs are collected by gently moving a ripe female onto a padded table and lifted above surface level where eggs are stripped and fertilised with milt obtained in a similar manner. One female can ovulate up to 12 batches with an average time interval of 75h. Total egg volume may reach 40% of total body weight.

Fertilized eggs are incubated in upwelling units in total darkness. Dead eggs are separated from live eggs by temporarily introducing a layer of high-saline water in the bottom of the incubator. The amount of removed eggs normally peaks at approximately 24dd° after fertilisation, when the fraction of non-fertilised eggs loses buoyancy. At approximately 70dd° the eggs are colleted by a net, disinfected in a mild solution of seawater and glutaric aldehyde (1:3000), and transferred to yolk-sac tanks. However, some farmers let the eggs hatch in the egg incubators and immediately transfer the larvae.

Large plastic bags floating in lagoons were initially used for larvae during the yolk-sac period, but have later been replaced by 5-15m^3 indoor silos. These

tanks have a conical bottom with a central water inlet, and with an outlet sieve near the top. After introduction of the eggs, hatching is arrested by temporarily exposure to light until the eggs are beyond normal hatching age. By turning off the light the hatching will synchronously be completed within 90min. Unhatched eggs and eggshells are easily removed from the conical bottom. Newly hatched halibut larvae are very fragile, and contact with the outlet sieve has been a major cause of mortality. A continuous salinity gradient preventing the larvae to reach the sieve is made by introducing low salinity water in a vertical tube in the upper part of the silo. The outlet salinity is adjusted to a level lower than the neutral buoyancy of the larvae thus preventing them to reach the surface of the sieve. The main difficulty during the yolk-sac stage is obtaining even larval dispersion in the silo. Immediately after hatching, the larvae will have an adequate distribution as a consequence of the salinity gradient. Later, the larvae become positively rheotactic, and will tend to congregate in the bottom part of the silo at the water inlet. To avoid this, oxygen is gently added at the bottom to create non-directive water currents, an effective method for redistribution of the larvae in the water column.

At an age of approximately 265dd°, the larvae are transferred to first-feeding tanks. At this stage they are positively phototactic and are therefore easily attracted to the surface by use of a dim light. Halibut larvae are first-fed at 12°C in tanks supplied with central aeration to create slow water currents that prevents patching of the prey and also disperse the larvae in the tank, thus leading the prey to the larvae rather then the larvae have to search for the prey. Algae or inorganic particles are added to the water to achieve a turbidity of 1-2NTU, which has been demonstrated to have a profound positive effect on foraging. Use of algae pastes have also been used successfully. Both micro algae and algal pastes has been substituted with clay in a in a recently developed first feeding protocol. This method is more cost efficient compared to application of algae, and also affect water quality positively in that clay aggregate and precipitate organic matter. An automatic tank cleaning system was first made for intensive halibut rearing, and this system has later been widely used also for other species.

Halibut larvae have a nutritional requirement for long chain n-3 fatty acids as EPA and DHA. It is a general relationship between relative content of DHA of the live feed given to the larvae and the content of the halibut larvae. Analysis of larvae offered natural zooplankton as *Temora* sp. or *Calanus finmarchicus,* show contents of DHA above 30 % of the total lipid. When using Artemia nauplii or juveniles as food, it is important to produce Artemia with sufficient nutritional value, and especially the content of DHA of *Artemia* has got strong focus. Recently, studies have shown that the quality (e.g., pigmentation) of the larvae is affected of the content and balance between the DHA, EPA, and ARA. Feeding strategies also affect fry production and quality. By giving meals instead of continuous supply of food significantly improved survival appeared.

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CULTURING EMBRYONIC STEM (ES) CELLS FROM TURBOT, *SCOPHTHALMUS MAXIMUS*

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Introduction

Culturing pluripotent embryonic stem (ES) cells represents a unique model system for in vitro studies of embryo cell growth and differentiation, and represents a connection between in vitro and in vivo manipulation of genes. The establishment of in vitro cultures of turbot ES cells represents a new experimental model for marine flatfish.

Materials and methods

Blastula-stage turbot embryos (Stolt Sea Farm, Øye, Norway) were prepared for cell culture by washing with PBS, disinfected with 70% ethanol, and rewashed with PBS. Batches of embryos (n=100) were squeezed through a cell strainer (0.44μm), releasing most of the cells and without most of the chorions. The cell suspension was centrifuged, pelleting the cells. The cell pellets were added DMEM with 4.5g.l^{-1} glucose and 25mM HEPES, supplemented with 20% foetal calf serum, 50U.ml^{-1} pen.strep^{-1}, 1% 2mM glutamine, 10% 100mM sodium pyruvate solution, 10% 10mM MEM-nonessential amino acids, 2nM sodium selenite, 12.5ng.ml^{-1} fibroblast growth factor basic, 10ng.ml^{-1} human leukaemia inhibitor factor, 50uM 2-mercaptoethanol, 1% salmon serum, and 1% turbot embryo extract. The cells were seeded into cell culture slides, coated with 0.1% gelatine, and incubated in a normal atmosphere incubator at 12°C. The medium was changed every second or third day. For cryopreservation, equal amounts of turbot embryos in medium suspension and complete medium with 50% FCS and 10% DMSO were gently mixed on ice and stored in liquid nitrogen. For alkaline phosphatase staining, cultured cells or fresh embryos were washed with PBS-added complete medium and Innovex brown in equal amounts and incubated overnight. The following day the medium was changed. When positive for alkaline phosphatase, cells or the ICM of embryos had a brownish/purple colour. The stained cells remained viable and could be cultured for a prolonged time. For immunofluorescence staining, ES cells were fixed, permeabilized, and

blocked using standard procedures. Monoclonal mouse anti Oct-4 (Chemikon) was used as primary antibody and goat anti mouse IgG (FITCH conjugated, Chemikon) was used as secondary antibody. Cells were investigated for fluorescence using Olympus B×51. For induction of cell differentiation, complete medium was supplemented with 1UM all trans-retinoic acid.

Results and Discussion

The morphology of ES cells is characterized by their round or polygonal shape with large nuclei and dense cytoplasm. The ES cells were continuously cultured for more than two months. Embryos were cryopreserved and thawed without loss of the potential for proliferation and differentiation, also verified by Oct-4 immunofluorescence staining. Viable ES cells exhibited high alkaline phosphatase activity, a valuable pluripotency marker (Hong and Schartl, 1996).

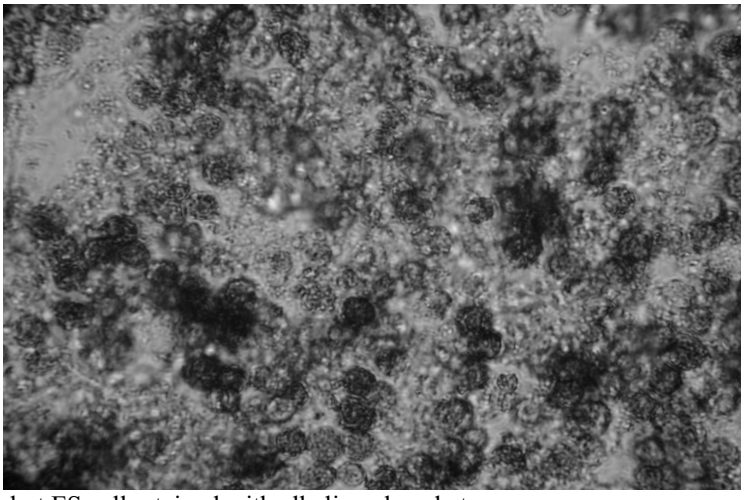

Fig.1. Turbot ES cells stained with alkaline phosphatase.

The pluripotency of turbot ES cells were verified by the presence of Oct-4. Oct-4 is encoded by the POU domain transcription factor and is essential for the development of ICM and required for the maintenance of pluripotency (Saijoh, 1996; Yeom, 1996). The POU genes are highly conserved and probably not stringent specie specific (Kirchhof 2000). In an attempt to detect Oct-4 in marine species, we evaluated an Oct-4 antibody raised in mouse, and successfully confirmed the presence of Oct-4 in turbot ES cells.

No suitable feeder cells has been established for maintaining fish ES cells in an undifferentiated state, so we adopted the feeder-free conditions of zebrafish ES culturing described by Hong and Schartl (1996). Under feeder-free conditions, the cells are dependent on several supplements, like fish embryo extract, bFGF,

and LIF, in combination with Na selenite and mercapthoethanol for self-renewal and for maintaining their pluripotency. In our cultures, bFGF as well as non-identified components present in serum could induce high ES cell proliferation both in the presence or absence of LIF. Reducing or eliminating LIF from the cultures dramatically increased the proportion of dying cells over time, indicating that turbot stem cells could respond to factors derived from other species. For successful long-term culture it was important that the cells attach to the gelatine layer and did not differentiate or die during early days of culture. Only 20% of the plated cells could be kept stable for 60 days, indicating the need for a further optimization of the in vitro culture conditions.

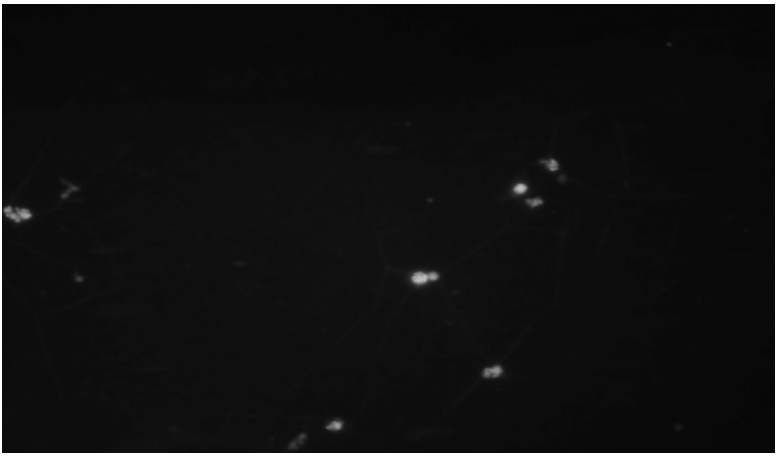

Fig. 2. Oct-4-stained turbot ES cells.

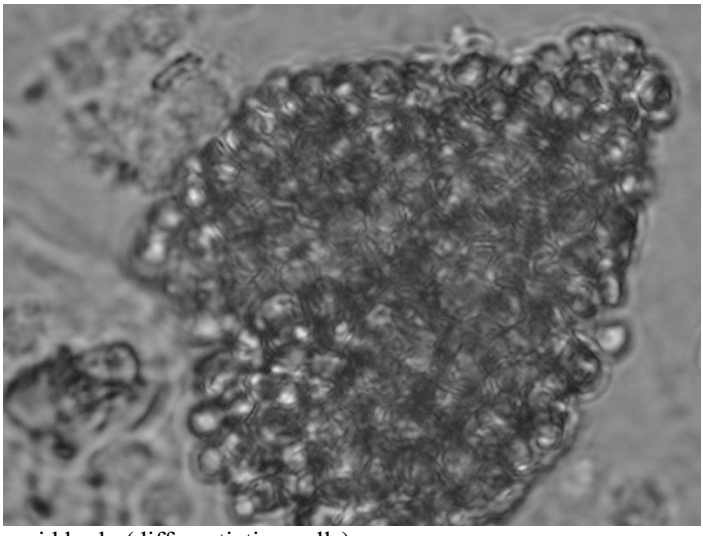

Fig. 3. Embryoid body (differentiating cells).

The turbot ES-like cells were capable of spontaneous differentiation, both in the presence and absence of LIF and formed large embryoid bodies (EB) when culture conditions were changed. The turbot ES cells differentiated more easily when exposed to trans-retinoic acid, but the direction of differentiation was not controllable, although a preference to form EB or neuron-like cells was observed.

Conclusions

The pluripotent nature of turbot ES cells is supported by their round and polygonal cells, high alkaline phosphatase activity, Oct-4 expression, the ability to remain undifferentiated for a prolonged culture period, and the ability to form EBs and undergo differentiation spontaneously but also as a response to changes in the extracellular environment, These characteristics make the turbot ES cells an ideal experimental system for further in vitro studies of flatfish embryo cell growth and differentiation.

Acknowledgements

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LARVICULTURE OF THE WARMWATER MARINE FISH SPECIES COBIA (*RACHYCENTRON CANADUM*)

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Cobia, *Rachycentron canadum*, distributed widely in temperate to subtropical oceans, is a finfish species with emerging global potential for mariculture. Cobia have been cultured in Taiwan since the early 1990s while in the US spawning has been induced using hormone injections and photo-thermal conditioning. Recently research and production has been considered or initiated in the EU, Brazil, and Panama. But cobia production faces several bottlenecks limiting industrial expansion including limitations consistent high quality egg and juvenile production. This presentation will focus on current larviculture research results and ongoing research initiatives for collaborative efforts to accelerate progress in cobia production.

Recent interest in farming of cobia has driven research focused on spawning and raising the young. At the University of Texas Marine Science Institute (UTMSI) we have spawned cobia for the last four years and developed methods for rearing the young in recirculation systems. Spawning of wild-caught and captive-bred cobia takes place in response to photo-thermal conditioning, beginning when day length reaches 13h light and temperature reaches 26°C. Spawning persists through summer and fall conditions at salinities ranging from 28-35ppt. Cobia have been successfully spawned in March through November by staggering the spawning cycle to extend the time of egg availability. Future goals are to achieve year-round spawning.

Cobia larvae exhibit high rates of growth reaching 4-5cm in 30d. They feed on rotifers for the first 3-5d and then *Artemia* until they can be weaned. Their tissues have high lipid and HUFA content; rotifers and *Artemia* enriched with *Isochrysus galbana* or commercial products (Algamac and Aquagrow) in conjunction with greenwater culture provide the best growth and survival of cobia larvae in recirculating aquaculture systems.

In order to broaden the potential for rearing cobia in low salinity or brackish water systems, it is vital to evaluate stage specific salinity tolerances. The egg diameter of cobia spawned at salinities ranging from 28-35ppt decrease with increasing salinity. Eggs are positively buoyant at the spawning salinity but 2-day-old larvae were buoyant at 2ppt below that salinity. Survival of larvae was significantly reduced in 10, 15, and 20ppt treatments compared to controls at 32ppt. Similarly when d7 larvae were reared in 10 and 20ppt their survival was significantly lower than controls, but by d14 larvae survived well in 10 and 15ppt but not in 5-ppt treatments. Young juveniles survive and grow well in 5-ppt water consequently culture at low salinities can be initiated somewhere between 14-30d post-hatch.

Development of weaning diets is crucial to successful commercial production. Several commercial diets are acceptable to late stage cobia larvae but survival is significantly reduced when live prey is eliminated. Promising results were found using a mixture of commercial diet and powdered freeze-dried shrimp. Cobia were completely weaned from rotifers and *Artemia* by 25d post-hatch and survival was not different from live fed controls. Based on this result, future studies are intended to wean larvae at an earlier age using diets based on freeze-dried shrimp meal. Nutrition is paramount to production success and the design of specific diets for all stages, particularly for the broodstock are being planned.

Other issues that are being addressed through a proposed collaborative project between the USA and EU include designing biosecure hatcheries with genetic diversity, development of indicators of egg quality and pathogen detection, optimization of larval and juvenile system design, and growout or production systems. Intense collaborative work on cobia could fast-track this species to a strong position on the global market.

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ADVANCES ON THE GENETICS OF REPRODUCTIVE PERFORMANCE IN PACIFIC WHITE SHRIMP: MULTIPLE SPAWNING CAPABILITY, LATENCY TO SPAWN, AND BIOCHEMICAL TRAITS IN SPAWNED EGGS

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Within captive populations of Pacific white shrimp (*Litopenaeus vannamei*) a small proportion of females spawn up to 25 consecutive times in a 3-4 month production period. Females with such a multiple spawning capacity are highly desirable for larvae production, provided that this trait is inherited and offspring quality is not affected over consecutive spawns or spawning order.

To define if reproductive performance can be selectively improved in production laboratories, in a first instance different biochemical/physiological traits in eggs, as well as other reproductive traits in spawners (vitellogenin in haemolymph, number of spawns, days to first spawn, fecundity) were evaluated for effects on consecutive spawns, and in a second instance for their inheritance in a captive family structured population of shrimp. We found that female condition was not affected by consecutive spawns in terms of biochemical composition of several organs and cytological characteristics of female ovaries. Also, spawning or early larval quality, evaluated by fertilization and hatching rates and by biochemical components such as proteins, acylglycerides, and vitellin levels in eggs, did not deteriorated over consecutive spawns. Finally, consecutive spawns did not negatively affect further larval quality, estimated as survival during culture, resistance to an ammonia stress test at zoea stage, or resistance to a salinity stress test at postlarval stages.

To further define if the first spawn of a female can be used as predictor of its later multiple spawning capability, we evaluated first spawn eggs of all females, including those that produced only one spawn. We found higher levels of vitellogenin, proteins, and acylglycerides for those females that did spawn another three or more times than for those females that spawned only once. An additional trait related to a multiple spawning capacity is the interval of days occur-

ring between eyestalk ablation and first spawn (latency or days to first spawn), which has been found to be considerably shorter for multiple spawning females than for females producing only one spawn. These results confirmed that females with multiple spawning capacity are desirable to improve reproductive performance, and justified the need to further establish if those traits had a significant genetic determination such that upon implementation of a selective breeding program a response to selection would be attained.

We found moderate to large heritabilities for traits related to multiple spawning capacity, as days to first spawn after ablation (h^2 0.41 to 0.54), first spawned eggs acylglycerides (h^2 0.20 to 0.35), and egg vitellin concentration (h^2 0.28 to 0.47), indicating that selection for those traits would result in an improvement of reproductive quality in our population. Finally, we have recently found that number of spawns is also genetically determined, with a heritability of 0.20. Traits such as number of spawns, latency to spawn, egg vitellin, and acylglycerides concentration in first-spawn eggs, indicating a genetic determination is involved in reproductive performance (by a significant heritability value), can be used in a selective breeding program to improve reproductive performance in captive shrimp. Whereas number of spawns and days to first spawn are easy to measure and would allow for selection of females with multiple spawning capacities without manipulation of females or spawns, the use of additional traits in a selection index can increase the precision of the index, and improve further the response to selection. Furthermore, acylglycerides and vitellin are not only predictors of multiple spawning capacities, but large concentration in eggs are associated with high larval quality, and thus their integration into a selection index would result not only in increasing larvae production thorough improvement of reproductive quality, but also in a simultaneous improvement of larvae condition.

The definition of predictor traits in females prior to eyestalk ablation and before spawning are also important, as we have found that vitellogenin levels in haemolymph are higher in females that developed their gonads faster after eyestalk ablation. Furthermore, a significant family means correlation between juvenile female gonad development and adult female spawning performance has also been observed. Further studies on understanding the genetics of those early predictor traits, as well as on the estimation of the genetic correlation between ‘early’ traits and ‘late’ reproductive traits is necessary if further optimization of reproduction in shrimp culture is desired.

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ANTIBODY RESPONSE AFTER IMMUNIZATION WITH *AEROMONAS SALMONICIDA/A. HYDROPHILA* VACCINE AND SURVIVAL RATES AFTER CHALLENGE WITH *A. HYDROPHILA* OF COMMON CARP (C*YPRINUS CARPIO* L.*)* VARIETIES WITH LOW AND HIGH STRESS RESPONSE

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Introduction

The Research Institute for Fisheries, Aquaculture and Irrigation (HAKI) at Szarvas, Hungary has a unique live "gene bank" of common carp, comprising of 17 foreign and 20 Hungarian strains. This "gene bank" was established 40 years ago (Bakos and Gorda, 2001) to maintain a live collection of strains and to produce highly productive hybrids of common carp. Today this unique collection is a good basis for genetic (DNA typing) and stress and disease resistance studies, which are in progress at HAKI. Selective breeding for resistance to infectious diseases would result in improved carp lines for aquaculture, reduce the use of antibiotics and drugs, and ultimately reduce fish losses to disease. The aim of this study was to determine the level of antibodies against *Aeromonas hydrophila* and *A. salmonicida* in four crosses of carp with different genetic background and high or low stress response. Other fish from the same varieties had simultaneously been challenged with *A. hydrophila*. The records of the antibody levels and the survival rate would be used to investigate possible correlation between the genetic background, immune response and survival.

Materials and methods

Males of genetically different Duna wild carp (D) and an inbred parental line Szarvas 22 (22) were selected for high and low stress response. Eggs of one female of Szarvas 22 line were fertilized with the sperms of males with high and low response, so the following combinations of crosses were established: 22×22L (low stress response), 22×22H (high stress response), as well as 22×DL (low stress response) and 22×DH (high stress response). One group of each

crosses was vaccinated (i.p. 0.1 ml.fish^{-1}) against *A. salmonicida/A. hydrophila* (Schering Plough Aquaculture). A boosting dose of vaccine (0.1 ml.fish^{-1}) was applied i.p. 4 weeks following the vaccination. Antibody response was evaluated 1, 3, 5, and 7 weeks after vaccination using Elisa method (Adams, 1992). Other groups of these crosses were experimentally infected with *A. hydrophila* (strain B 2/12) and survival rates had been recorded.

Results and discussion

The antibody response was the highest following 5 weeks after vaccination either against *A. salmonicida* or against *A. hydrophila*. Significantly higher titres of circulatory antibodies against *A. hydrophila* were found in the inbred line 22×22L with low stress response (Fig. 1), while in crosses 22×DL and 22×DH the antibody levels were very low independently of stress response. The same tendency was found in the antibody levels against *A. salmonicida*, where the highest level was measured in crosses 22×22L with low stress response (Fig. 2). Significant genetic variation in antibody response and total level of IgM, measured in Atlantic salmon after immunization, has been previously reported (Stromsheim et al., 1994; Lund et al., 1995).

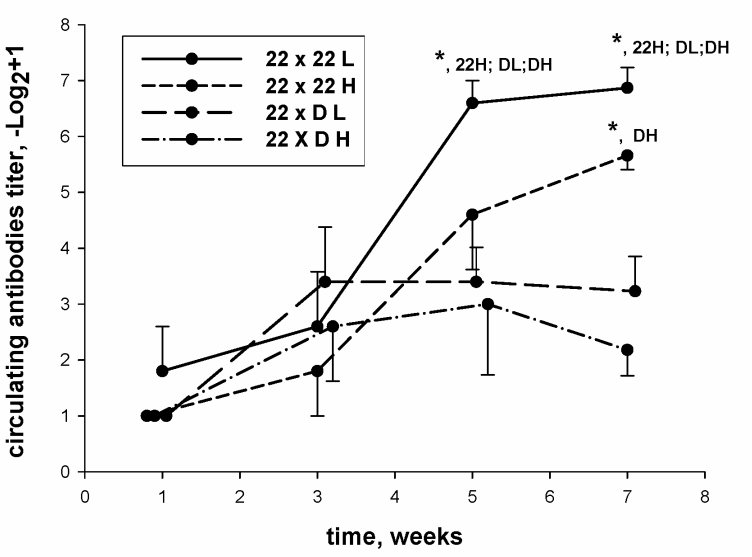

Fig. 1. Circulating antibody levels against *A. hydrophila* in carp crosses with different genetic background and low and high stress response. Data are expressed as means of five fish ±SEM. Significant differences between values obtained at the beginning of experiment (week 1) and following vaccination are indicated by asterisks. Significant differences among crosses are indicated by letters (22×22H: 22H; 22×DL: DL; 22×DH: DH).

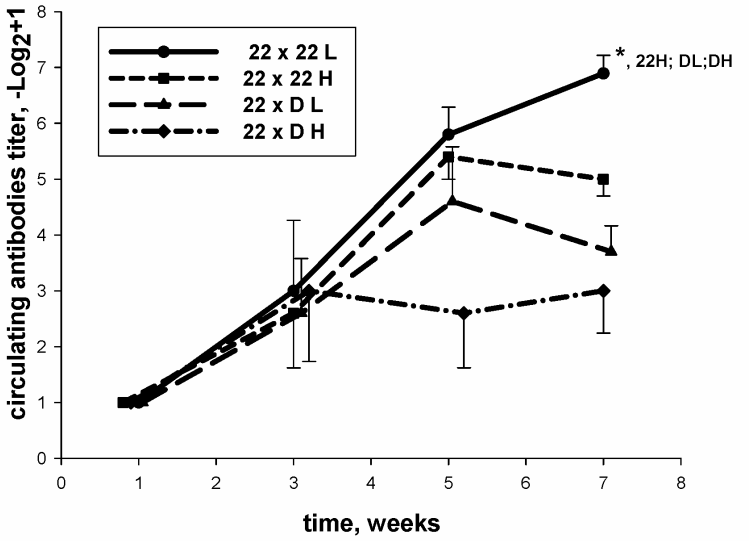

Fig. 2. Circulating antibody levels against *A. salmonicida* in carp crosses with different genetic background and low and high stress response. Data are expressed as means of five fish ±SEM. Significant differences between values obtained at the beginning of experiment (week 1) and following vaccination are indicated by asterisks. Significant differences among crosses are indicated by letters (22×22H: 22H; 22×DL: DL; 22×DH: DH).

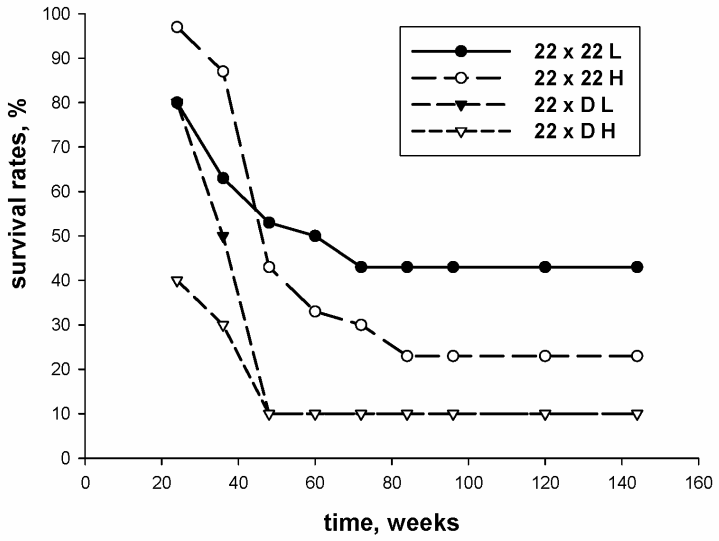

Fig. 3. Survival rates of different crosses of carp with different genetic background and high and low stress response.

Crosses of carp with different genetic background and high and low stress response challenged with *A. hydrophila* had different survival. The best survival was in the case of inbred line 22 with low stress response, while almost 90% of hybrids of 22×D carp died independently of stress response. Survival results correlated very well with the antibody response. Lund et al. (1995) found that in a stepwise multiple regression model, with survival rates in challenge tests with *A. salmonicida* as the dependent variable and the total set of immune parameters as the explanatory variables, the effects of both the total IgM and the level of specific antibodies against A-layer protein after immunization in Atlantic salmon were significant.

Conclusions

Cross of carp 22×22L with low stress response was the most responsive variety to vaccination and had the best survival rate in challenge test with *A. hydrophila*. In our study we used only one parameter of immune system and although survival results correlated very well with the antibody response, further study is needed to evaluate the possible indirect markers when breeding carp for improved resistance against stress and diseases.

Acknowledgements

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A NEW COEFFICIENT FOR EVALUATION OF CONDITION OF FISH LARVAE

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Introduction

Determination of fish condition using Fulton's coefficient requires the length and mass of fish. In the present study, condition of carp larvae was calculated from the length and body perimeter area, using computer photographs of live fish. Measurement of body mass necessary for Fulton's coefficient is very difficult in fish larvae, because drying to obtain accurate weight often results in death. Nowadays, determination of fish growth using photographs is very popular. We propose a new coefficient for fish larval condition evaluation which may be an alternative to Fulton's coefficient.

Materials and methods

One-day-old larvae hatched under laboratory conditions were divided into 2 groups: control and copper. The fish were placed in tanks with dechlorinated clean tap water (temperature 22ºC, dissolved oxygen saturation ~90%, hardness 210mg $CaCO_3.dm^{-3}$, pH 7.7) or with tapwater + 0.2mg.dm^{-3} Cu (as $CuSO_4.5H_2O$), at a density of 300 fish per 180dm^3. Metal concentrations used in the experiment were established based on results of preliminary experiments and literature where they significantly affected the larvae without causing high mortality. Water was exchanged every 3 days to maintain metal concentrations. Fish were fed brine shrimp nauplii ad libitum three times a day. Twenty five fish were randomly sampled daily from each group. Photographic documentation of development was done with a camera and MultiScan computer image analysis. Larval body length and perimeter were measured in the photographs. Fish growth rate over the 30-day period was evaluated, and condition coefficient S was determined using body perimeter area instead of body mass: $S = 2Po.L^{-2}$ where Po=perimeter area (2Po = approximate body area, body thickness was neglected), and L=body length. The S coefficient may be used for evaluation of condition of photographed fish. Growth of larvae reared in polluted water was compared with the control.

Results and discussion

Figs. 1A and 1B show the increase in body length, and particularly in perimeter area, differed between the control and Cu-exposed fish. The differences were statistically significant from 7dph, and probably resulted from the earlier start and higher activity of exogenous feeding by the control fish, while the Cu-exposed fish relied longer on yolk-sac nutrients (Sarnowski 2003) and their feeding activity was reduced (Sarnowski 2005). These results are similar to those obtained by various authors cited in the review by Jezierska and Witeska (2001), and indicate that exposure to heavy metals hinders increase in body length and mass of fish larvae.

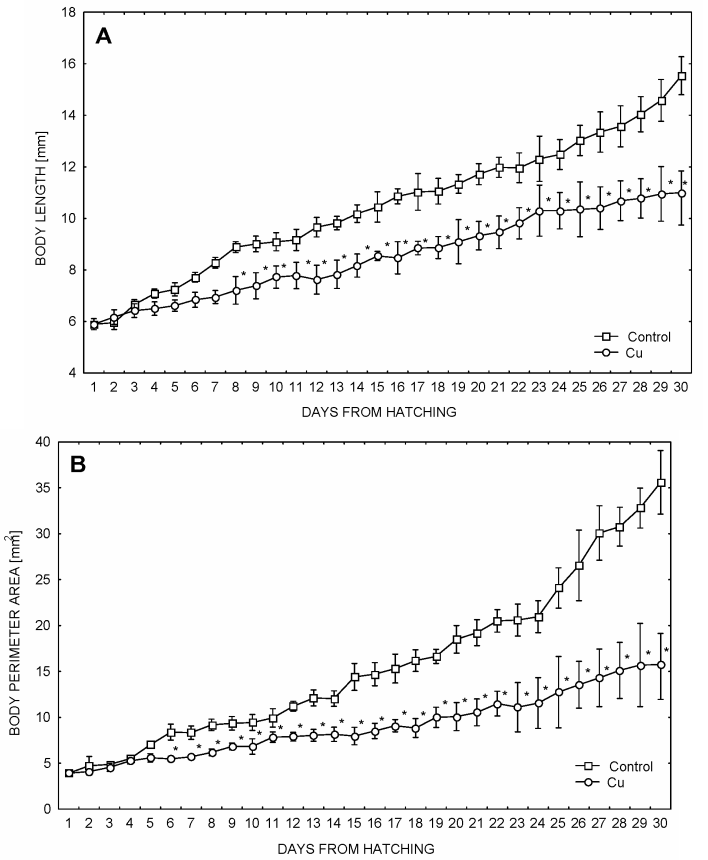

Fig. 1. Growth of common carp larvae in the control, and copper exposure (mean±SD), A - body length, B - body perimeter area.

Figs. 2A and 2B show a distinct correlation between body area (2Po) and fish length (similar to the commonly used mass-to-length ratio). In the control, the

body area: length ratio increased faster (2Po=-36.25+(6.4771×L) than in the Cu-exposed group (2Po=-16.5+(4.0481×L)), which indicates an adverse effect of copper exposure on fish condition.

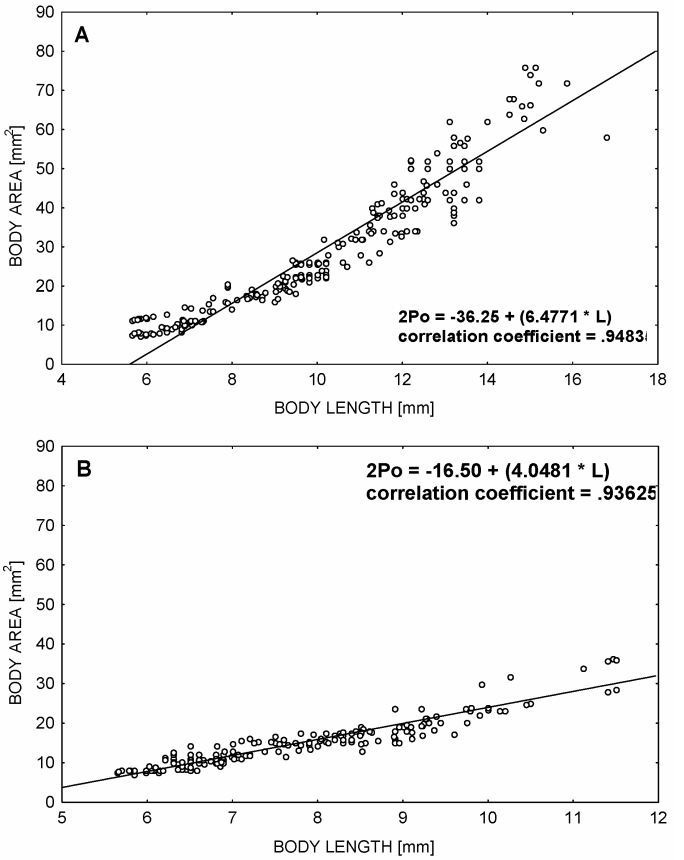

Fig. 2. Relationship between the body length and area of common carp larvae, A – control, B – Cu.

The S coefficient (Fig. 3) shows the changes in fish condition during the experiment. Until 20dph the values of S do not considerably differ between the control and Cu-exposed group. Beginning from 22dph, the Cu-exposed larvae showed significantly lower S values comparing to the control. Under optimum conditions, the condition of growing larvae increased, while in the copper-polluted environment, it decreased. This indicates that long-term copper exposure results in fish slimming. Similar observations were made by Vosylienė and Petrauskienė (1995) for copper-exposed rainbow trout: the fish showed considerably lower condition (calculated as Fulton's coefficient) compared to the control. A reduction in condition of 40-day-old common carp exposed to copper and cadmium was also observed by Sarnowski (2005).

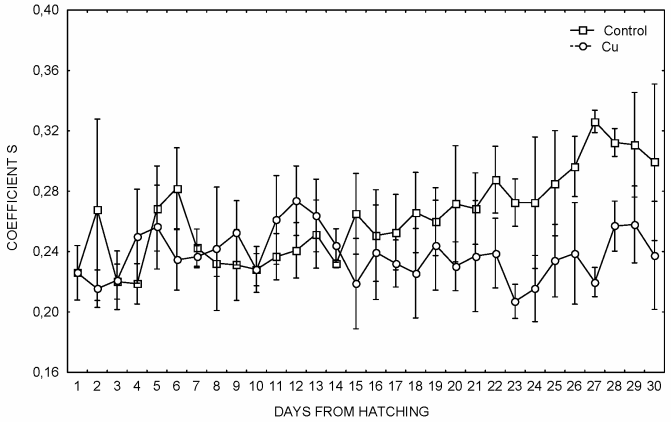

Fig. 3. The changes in S coefficient during experimental rearing of common carp larvae.

It appears that the proposed coefficient S reliably describes condition of larvae and may be used as an alternative to the Fulton's coefficient, especially when fish mortality caused by manipulation during weighing is to be avoided in the first month post-hatching.

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MOUTHPART AND FOREGUT ONTOGENY IN PHYLLOSOMATA OF *PANULIRUS ORNATUS* AND THEIR IMPLICATIONS FOR DEVELOPMENT OF A FORMULATED LARVAL DIET

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Introduction

An inadequate diet has been a major culture impediment of phyllosomata of spiny lobsters, due to a lack of understanding of their larval feeding biology and nutritional requirements (Johnston and Ritar, 2001). This study identifies key structural changes of the mouthparts and foregut of phyllosomata of *Panulirus ornatus* in the early and mid-stages of their ontogenetic development, and infers how these changes may influence the physical characteristics (size, shape, and texture) of a formulated larval diet. A feeding trial using three different textured diets was conducted to determine a preferred diet texture for early stage phyllosomata.

Materials and methods

Ovigerous female broodstock collected from Princess Charlotte Bay (Northern Queensland, Australia) were held in flow-through Nally bins (26°C) until hatch. The newly hatched phyllosomata were transferred to 10-l upwelling recirculating tanks and maintained at 26°C and 36‰ salinity. For morphological examination of the mouthparts and foregut during ontogenetic development, phyllosomata were fed to satiation on a sole diet of enriched *Artemia* spp. For the feeding trial, phyllosomata were reared in 3-l mini-upwelling tanks (50 phyllosomata.l^{-1}). Phyllosomata used in the feeding trial were fed one of three different textured micro-bound diets, and control tanks were fed on-grown *Artemia* as above. At each developmental stage, phyllosomata were removed from the culture vessels for examination and staged after Duggan and McKinnon (2003).

For examination of the mouthparts, phyllosomata (n=10) were fixed for 2-3h at room temperature in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4). Phyllosomata were then prepared for scanning electron microscopy and examined at high vacuum on an LEO VP FEGSEM.

For histological examination of the foregut, phyllosomata were fixed and prepared as above, before being embedded in JB-4 glycol methacrylate resin. The foregut was serially sectioned (transverse) at 2µm on a Sorvall microtome and polychrome-stained. Digital images were observed and captured using an Olympus DP70 camera and Image Pro Plus v.10 software.

The compositions of the 3 micro-bound diet textures used in the feeding trial are shown in Table I. As the nutritional requirements for *P. ornatus* phyllosomata are currently unknown, diet formulations were based on the known requirements of adult *P. ornatus* and larval penaeid prawns.

Table I. Composition of the 3 micro-bound diet textures.

Ingredient	Gelatinous	Paste-like	Hard
Krill meal	56.5	30	30
Squid meal	-	20	20
Fish meal	-	15	15
Krill hydrolysate	2	2	2
Fish oil	5	5	5
Corn oil	1	1	1
Soy lecithin	4	2.5	3
Cholesterol	2.5	2.5	3
Vitamin and mineral premix	3	2	2
Wheat flour	13	13	15
Gelatine	14	5	2

Results and discussion

The mouthparts of *P. ornatus* phyllosomata are well-developed at hatch and the gross mouthpart structure does not change significantly throughout larval development (Fig. 1A), especially the mandibles (Fig. 1B). Density and robustness of setation of the mouthparts does, however, increase during larval development, especially on the ventro-mesial surface of the paired paragnaths, and the number of spinose projections on the maxillules also increases in mid-stage phyllosomata (Instar VIII). Increased setation of the mouthparts in mid-stage phyllosomata suggests that they are able to ingest larger, fleshier prey by generating a stronger feeding current by the presence of extra pappose setae on the maxillules and can maintain fleshy prey items close to their buccal cavity by trapping food particles on the increased setation of the paragnaths.

The foregut of early instar phyllosoma (I-II) is quite simple, with no filter press, but is well-armed with robust lateral and dorso-lateral setae (Fig. 1C). Filter-press development occurs at instar III and is a significant structural development, occurring earlier in larval development than other spiny lobster species. Filter-press complexity increases in mid-stage phyllosomata, with an increase in number of ampullary channels (Fig. 1D). Developmental changes of the foregut after instar III suggests that there may be a shift in dietary regime from soft gelatinous food items to larger, fleshy prey, as the internal triturative and filtration ability of phyllosomata improves throughout larval development.

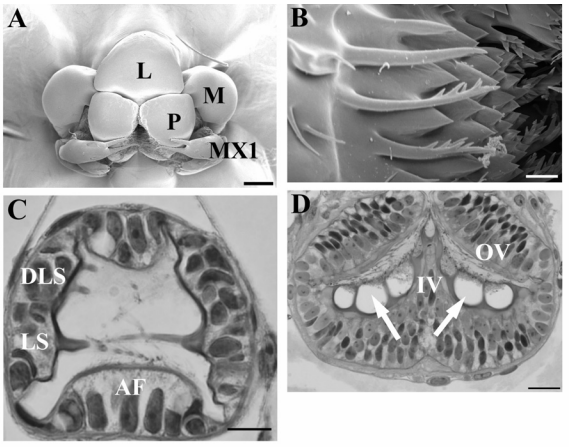

Fig. 1. Scanning electron micrographs of the mouthpart and transverse histological sections of the foregut of phyllosomata of *Panulirus ornatus*. (A) Oral region (instar IV) showing spatial relationship of mouthparts. Scale, 60µm. (B) Molar process (instar I) showing complexity of spinose projections at hatch. Scale, 2µm. (C) Anterior foregut, Instar I. Scale 20µm. (D) Posterior foregut, Instar VIII, showing detail of inner and outer valve setae of the filter press. Scale, 20µm. AF, anterior floor; DLS, dorso-lateral setae; IV, inner valve; L, labrum; LS, lateral setae; M, mandible; MX1, maxillule; OV, outer valve; P, paragnath.

As the early morphological structure of the phyllosomata mouthparts and foregut suggests that their ingestive and digestive capacity is well suited to soft gelatinous dietary items, a feeding trial investigating the effect of different micro-bound diet textures and *Artemia* on survival and growth was conducted (Fig. 2).

Phyllosomata survival when fed only *Artemia* was significantly higher than all three formulated diets. Initially, phyllosomata were attracted to the formulated diets and were observed manipulating the diets with their 1st pereopods and appeared to move diet particles anteriorly toward their mouth. Despite this initial feeding response, phyllosomata appeared to become less interested in the formulated diets as the days of the feeding trial progressed, and were observed feeding more on the smaller dislodged diet particles on the tank floor.

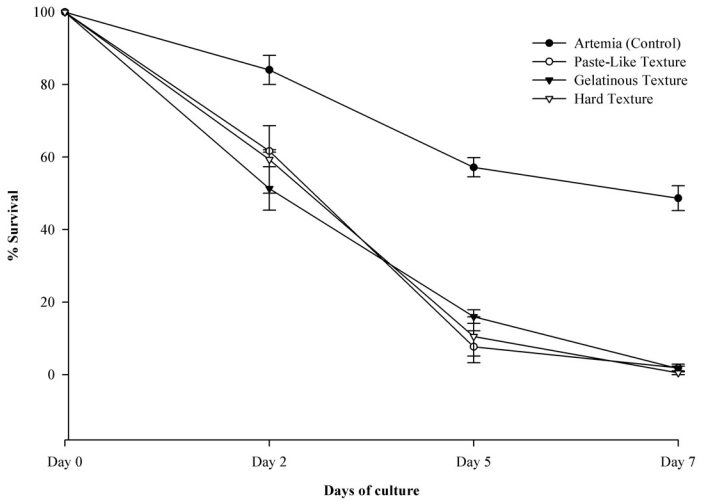

Fig. 2. Survival of instar I *P. ornatus* phyllosomata when fed 3 different textured microbound diets.

Conclusions

Although the mouthpart and foregut morphology of early instar phyllosomata suggests that they may feed on soft gelatinous prey items in the wild, we were unable to determine a preferred diet texture for early stage *P. ornatus* phyllosomata. The formulated diets used in this trial were not palatable over extended time period and were observed to be too large for successful ingestion. Further studies which explore alternate diet sizes and nutritional compositions, using techniques whereby ingestion can be quantified, need to be explored, and will significantly benefit the development of formulated larval diet for *P. ornatus* phyllosomata.

Acknowledgements

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CARBON AND NITROGEN STABLE ISOTOPES ($\delta^{13}C$ AND $\delta^{15}N$) AS NATURAL INDICATORS OF LIVE AND DRY FOOD IN THE PACU *PIARACTUS MESOPOTAMICUS* LARVAL TISSUES

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Introduction

Studies on nutrient assimilation from foods are important information to the understanding of larval feeding and nutrition. The present study proposes the use of the stable isotopes technique to track the individual participation of food sources (live and dry food) on the growth of animals during larval development.

Materials and methods

First-feeding five-day-old pacu *Piaractus mesopotamicus* (total length 5.3±0.2mm and weight 0.43±0.05mg) were fed for a period of 42 days on: (A) *Artemia* nauplii; (D) commercial dry diet (Fry Feed Kyowa B); (A+D) a mixture of both during the entire period; or were weaned to dry food after three (A3D), six (A6D), or 12 days (A12D) on *Artemia* nauplii. In the last treatment, dry food was also offered from the start. The food treatments, as well as the selection of the dry diet and feeding levels, were designed based on preliminary studies.

The samples in each treatment consisted of a pool of larvae (250mg dry weight). Until the 12th day, they consisted of the whole body (without food in its gut); subsequently, due to fast and heterogeneous growth, the animals were separated into size classes, eviscerated, and analyzed separately as head and trunk.

The dried samples were analyzed in a low resolution mass spectrometer (IRMS/EA). The isotopic results for carbon and nitrogen were expressed using the delta per thousand terminology (δ‰), relative to the Peedee Belemnite (PDB) and Atmospheric Nitrogen international standards, respectively, and calculated by the equation: $\delta‰^{13}C$ or $\delta‰^{15}N = [(R\ sample/\ R\ standard)-1] \times 10^3$, where R is the isotopic ratio ($^{13}C/^{12}C$ or $^{15}N/^{14}N$) for the sample and standard,

respectively. δ‰ ^{13}C or δ‰ ^{15}N is the sample enrichment in relation to the standard, with an analytical error in the order of 0.2‰.

Carbon and nitrogen half-life values in the A and D larval tissue were determined according to Ducatti et al. (2002): $y_{(t)} = (q.k^{-1}) + [y_0 - (q.k^{-1})]\ e^{-kt}$, where: $y_{(t)}$ = carbon or nitrogen isotopic concentration in the tissue at a given time t (in ‰); y_0 = initial isotopic concentration in the tissue, and $q.k^{-1}$ = isotopic concentration in the tissue to be attained at the equilibrium threshold (in ‰); k = carbon or nitrogen replacement rate in the tissue (in time units^{-1}); and t = time (days). The half-life value was calculated by: $T = ln2.k^{-1}$, where: T is the half-life in days. The half-life value indicates the time required for 50% of the carbon and nitrogen atoms in the animal tissue to come from the ingested food. Fish growth and survival were also evaluated.

Results and discussion

The isotopic results for both head and trunk fractions showed the same tendency. On the other hand, some differences were expressive between size categories, such as in treatment A+D, where differences of 1.4‰ and 4.9‰ for $\delta^{15}N$ and $\delta^{13}C$, respectively, were observed between the smallest and the biggest classes.

The following results refer to the trunk's fraction of the most representative size class in each sample. The results revealed a larval tissue isotopic profile closely correlated with the corresponding feeding trials (Figs. 1A, 1B). Larvae fed exclusively on nauplii or dry food gradually changed their isotopic composition, reflecting the signals from their respective foods. Starving larvae (S) practically did not change their isotopic composition in relation to the initial larval sample.

Results on the D-larvae $\delta^{15}N$ values were not conclusive, since the initial larvae were richer in ^{15}N than the dry food by amplitude in the order of 2.1‰ (Fig. 1B). The isotopic similarities between foods and the initial biological material have been one of the limiting factors for the application of stable isotopes in studies in this field (Schlechtriem et al., 2004).

The carbon isotope half-life value (T) differences between A-Larvae and D-larvae were quite expressive (Fig. 1A). The isotopic dilution velocity in the tissues of young animals is closely related to addition of new tissue (Hesslein et al., 1993). In fact, the growth results could explain those half-life values, since differences in growth were also high, especially during the first weeks, when D-Larvae showed a much slower growth ($P<0.05$) than A-Larvae.

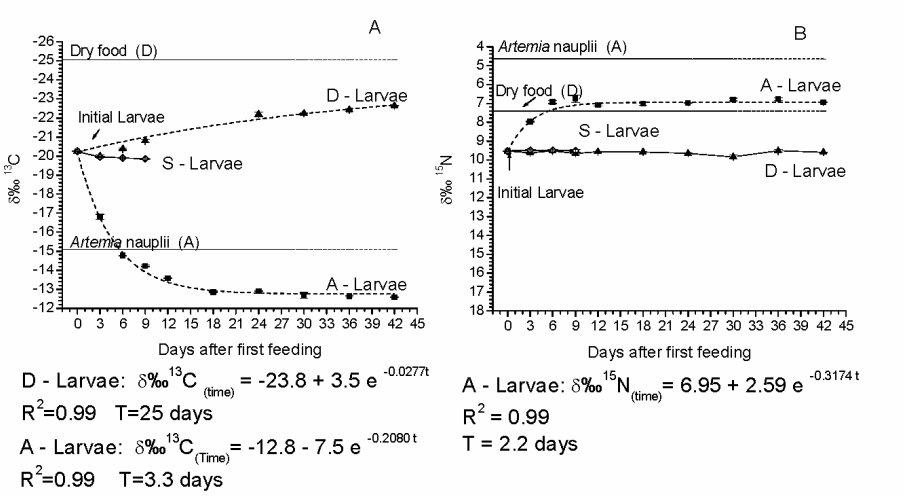

Fig. 1. $\delta^{13}C$ (A) and $\delta^{15}N$ (B) values for starving pacu larvae (S-larvae) and for larvae fed exclusively on *Artemia* nauplii (A-larvae) or on dry food (D-larvae). A dashed line between the observed data represents the exponential curve fitted by the corresponding mathematical expression.

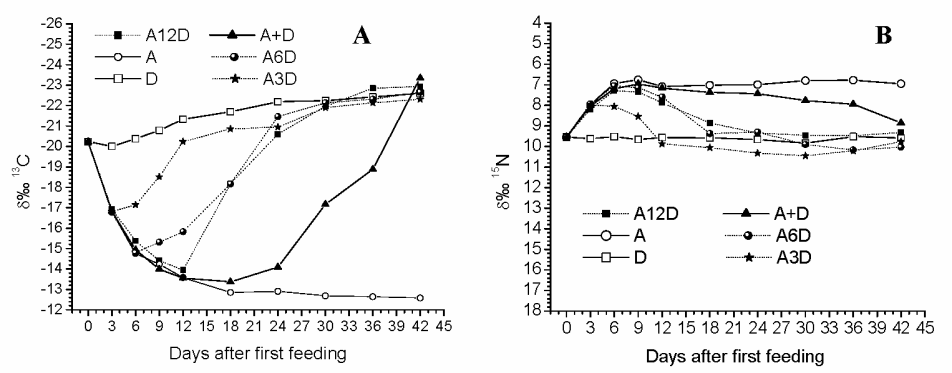

Fig. 2. $\delta^{13}C$ and $\delta^{15}N$ values for pacu larvae weaned from live food to dry food at three (A3D), six (A6D), and 12 days (A12D) after the beginning of exogenous feeding and for larvae fed on nauplii + dry food during the entire period (A+D). Animals fed *Artemia* nauplii alone - A (empty circle) or dry food - D (empty square).

Larvae that had access to both types of food (A3D, A6D, A12D, and A+D) clearly revealed the periods when nutrients from nauplii and later the dry food were assimilated. In this latter case, different moments at the onset of nutrient retention from dry food in the larval tissue were revealed (Figures 2A and 2B). Larvae weaned on the 12th day (A12D), with a total length of ~10mm and 10mg wet weight, were efficiently able to metabolize the dry food; the onset of free choice of dry food (A+D) also occurred from the 12th day, but in this case, even being able to use the nutrients from that food source, the animals continued to

select preferably the live food for at least another 12 days, and then started to ingest the dry food substantially, as evidenced by more expressive changes in the isotopic signatures.

Late weaning (at 12 days) did not affect the animals' growth as occurred in early weaning, at three and six days of feeding. The highest survival rates (>70%, $P<0.05$), were obtained in treatments A; A+D; and A12D. In treatments D, A3D, and A6D these rates were about 12, 34, and 37%, respectively.

Conclusions

The results indicate that, in the first week of exogenous feeding, pacu larvae consume preferentially *Artemia* nauplii, even when dry food is available; however, in the absence or restriction of live food, the animals ingest and assimilate nutrients from the dry food. Voluntary selection began between the 12^{th} and 18^{th} day, but a significant intake of dry food occurred much later, when the animals were at the juvenile stage, at about 30 days.

The stable isotopes technique was very useful, revealing intrinsic and consistent responses, which allowed the detection of the individual participation of *Artemia* nauplii and dry diet as food sources, by means of the assimilation and retention of carbon and nitrogen atoms from the corresponding foods in the larval tissues.

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BIOCONTROL OF PATHOGENS IN SHRIMP HATCHERIES

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Microflora associated with larval stages of shrimp could affect the health of the larvae. In spite of extensive water treatment in hatchery systems, bacterial flora build up in larval rearing tanks. As the larval age increases, generally the bacterial counts also increase. *Vibrio* spp. constitute an important component of the bacterial flora in hatchery systems. Under certain situations, the luminous *V. harveyi* dominate and cause mass mortalities. Our earlier studies have shown that *V. harveyi* can persist in hatchery environments by forming biofilms on various surfaces and biofilm bacteria are highly resistant to sanitizer and antibiotic treatments.

Bacteria with anti-vibrio activity could be potential candidates for biocontrol of luminous *Vibrio* spp. in hatcheries. Some of the bacteria that have been found to produce anti-vibrio compounds are *Bacillus* spp. and marine *Pseudomonas* strains. In experimental systems, these bacteria showing anti-vibrio activity were not harmful to shrimp larvae at levels up to $10^4 \cdot ml^{-1}$ in water and they prevented proliferation of luminous *V. harveyi*.

To develop alternatives to antibiotics in aquaculture, we have been isolating bacteriophages lytic against pathogenic *Vibrio* spp. We have isolated over 8 phages against *V. harveyi*. Most of the phages have a broad host range being lytic for over 70% of 150 *V. harveyi* isolates from different parts of India. All the phages were double-stranded DNA phages. The phages were tested for their ability to induce virulence by inoculating the phage with host to *Penaeus monodon* postlarvae. The percentage mortality was reduced in phage-treated tanks.

In the laboratory microcosm, the ability of bacteriophages to reduce populations of luminous *V. harveyi* was studied. At 200ppm treatment with a phage preparation, the luminous bacterial counts were reduced from $10^5 \cdot ml^{-1}$ to undetectable levels by 72h. Trials were conducted in hatchery systems where natural outbreaks of luminous bacterial disease was observed. Bacteriophage-treated tanks showed over 80% survival while untreated tanks showed 100% mortality. This

observation suggests that bacteriophage treatment would be an effective alternative to antibiotic treatment in hatchery system to control luminous bacteria.

All the bacteriophages isolated were tested for the presence of a putative virulence gene that was reported from another bacteriophage earlier. All the bacteriophages isolated in this study were negative for this gene.

Biofilm formation is one of the strategies for survival of luminous *V. harveyi* in hatchery systems. Bacteriophage treatments of biofilm formed on concrete and polyvinylchloride (PVC) surfaces were found to reduce biofilm cells by over 80% in 72h. This suggests that bacteriophages could be potential biocontrol agents against biofilm forming *V. harveyi*.

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PHENOTYPIC PLASTICITY OF THE REARED BLACK SEA TURBOT: EFFECT OF DIET CAROTENOIDS?

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AN INTENSIVE HIGH-DENSITY LARVAL REARING SYSTEM FOR THE LARGE-SCALE SIMULTANEOUS PRODUCTION OF FAMILIES OF THE PACIFIC OYSTER *CRASSOSTREA GIGAS*

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Introduction

One of the most significant limitations to the power of a shellfish selective breeding programme is the difficulty of producing large numbers of families using conventional static larval rearing systems. A greater number of families allows increased selection intensity and reduced build up of relatedness, resulting in a faster rate of genetic gain. A system is described here for the reliable production of cohorts of 64 families or more simultaneously.

Materials and methods

The system comprises a modular bank of 64 moulded plastic tanks operating on a continuous flow-through basis. Each individual tank has a volume of 2.5 litres and typically holds one family. Nylon mesh filters are used to retain the developing larvae within each tank. Larvae are normally screened and tanks cleaned every second day and individual tank filters are washed daily.

A header tank ensures a constant supply of 1-µm-filtered seawater into which microalgae are added via a peristaltic pump. Typical flow rate is 100ml per 2.5-litre tank per minute. Up to 2 500 000 D-larvae are introduced to each tank at the start of larval rearing, with a final yield of up to 500 000 eyed larvae per tank.

Results and discussion

Two family cohorts have been reared using the system. Of the 64 families in each cohort loaded into the system as D-larvae, 60 and 62 families survived (94 and 97% respectively) to produce at least 5000 spat per family following settlement; sufficient numbers to be used in Cawthron's selective breeding programme (King, 2004). The ability to produce large cohorts of families enables a greater selection intensity to be applied for a given level of relatedness in the offspring. Where complex mating designs are used, the reliability of the system

means that the mating design is more likely to remain intact through larval rearing, rather than becoming unbalanced through the loss of families.

The small size of the system reduces the hatchery resources required and minimises the effort required for larval rearing. The total work area required for the tank system is approximately 30m^2 (excluding seawater pre-treatment, etc). Total labour requirement for tank cleaning, larval screening, and servicing the system is four person-hours per day on average. The reduction in effort required, combined with the reliability of the system, provides a significant saving in the cost per family over conventional shellfish family rearing systems.

Conclusions

The use of intensive continuous flow-through systems for larval rearing can improve both the cost efficiency and genetic gain achievable in shellfish selective breeding programmes.

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INTEGRATED REARING SYSTEMS FOR FINFISH LARVAE

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Introduction

Larvae culture in general, and specifically larvae nutrition, are considered to be the 'bottle-necks' for marine finfish culture. Fish larval rearing experiments are carried out in various systems, from small beakers to very large commercial tanks, making it difficult to compare data across systems. A need for a standardized, easily replicable system is evident.

A continuous supply of live or dry feeds and a controlled environment – i.e., temperature, filtration, photoperiod, oxygen, and pH – are essential for any experimental or commercial system. These environmental factors are best controlled automatically in order to minimize variation between tanks and between experiments. However, only a few automatic systems have been developed for marine finfish hatcheries.

During the last three years, the authors have developed an integrated rearing system for marine fish larvae. It includes all the necessary components for larval culture – i.e., larvae tanks, live food delivery, microdiet (MD) feeders, and an adjunct live food enrichment system. All systems are automatic or semi-automatic and have the flexibility of multi-rearing protocols – i.e., individual feeding regimes for groups of replicated tanks.

Larvae rearing system

The system includes twenty-four 270-l conical tanks with up-welling or bottom-draining flow-through water dynamics (Kolkovski et al., 2004a). The inlet water passes through a gas exchange column that saturates the water with dissolved oxygen (DO) and stabilizes the pH. The gas exchange column is extremely efficient in saturating the inlet water with DO (from 43±2% to 103±2% at 20°C). It also removes other gases such as carbon dioxide, which increases the water pH (pH 7.6-7.95) and nitrogen, to prevent supersaturation and gas bubble disease

prior to the water reaching the tank. This eliminates the need for vigorous aeration inside the tank, which may stress fragile fish larvae.

The system was originally designed for nutritional experiments using formulated feeds and the use of an up-welling water inlet method extends the suspension time of inert particles in the water column. The system enables the operator to change the water flow direction from up-welling to bottom-draining as the larvae grow and metamorphose into juveniles. A unique outlet filter was developed that eases the daily routine of replacing screens when enriched live food is used (Kolkovski et al., 2004a). The filter is made from PVC foam and is square shaped. The sides of the box are open in order to allow the screens to slide into place using grooves cut into the inside of the box. There are several mesh size screens ranging from 49μm to 500μm for 'day' and 'night' use as well as for different larval stages. At the bottom of each side of the filter box there is a porous tube, which provides an 'air curtain', in order to prevent the screens from becoming blocked. This filtration design not only acts as a filter, preventing larvae and live and/or dry food to be flushed from the tank, it also acts as a surface skimmer, preventing an oil film on the water surface. A clean water surface facilitates swim bladder inflation, promotes better growth and survival, and reduces deformities.

The system is fully controlled by a single programmable logic controller (PLC). Light intensity, photoperiod, dimming time, live food, and algae pumping intervals are all automated, substantially reducing labour requirements.

Artemia hatching and enrichment system

Live food such as *Artemia* is considered to be an essential part of any marine finfish hatchery. Standard methods have been developed for hatching cysts and enhancing the nutritional value of the *Artemia* nauplii by using different enrichment products. Although there are a variety of commercially available products in the market, further research on more specific enrichments to meet specific nutritional requirements in finfish larvae is still required. A simple, compact experimental system was developed in order to provide a reliable platform for nutritional experiments for variety of live food organisms (Kolkovski et al., 2004b). The system was built as a compact, all-in-one unit consisting of eight 50-l conical tanks situated within a temperature controlled water bath. This arrangement reduces variation between the replicates (tanks) resulting from individual heaters and aeration. It reduces the manpower needs via simple procedures for harvesting, washing, and refilling all of the tanks synchronously and allows automated addition of enrichments.

It also includes switchable bottom (submerged in the water bath) or upper lighting to help concentrate the *Artemia* nauplii during harvest. The system has been

used for a variety of experiments, comparing commercial and experimental enrichments, bacterial monitoring, and evaluation of different *Artemia* procedures.

Automatic microdiet feeding system

Currently there are very few, if any, commercially available feeders that are able to continuously dispense small amounts of MD. The ones that are available are designed mainly for the ornamental market rather commercial fish hatcheries, or they are too large and suitable only for relatively large amounts of large diet particles. The automatic MD dispenser (AMD™) is designed to periodically administer a small amount of MD (20-80mg) to larval rearing tanks, in order to spread the allocation of the required daily amount of feed evenly across the whole day. This prevents the need to manually feed the larvae, and provides a more constant availability of feed across the whole photoperiod, and outside of working hours. This should also reduce opportunities for bacterial proliferation on unconsumed feed particles. The feeder can cope with a diet particle size range between 70μm and up to 1.5mm. Each feeding event, the same amount of MD (±1%) is released without the need to weigh the diet each time. The AMD's are PLC controlled and can be programmed to any feeding interval schedule needed. The AMD mechanism is based on two stainless steel slotted plates. One is static and the other slides above it via solenoid control. In a normal position the slots in both plates are covered preventing the MD from spilling into the tank. When the solenoid is activated, the top plate slides and aligns the slots as it passes. At that time the MD particles drop into the tank. A manifold jets constant airflow beneath the plates in order to scatter the MD particles across the water surface. The AMD system is suitable for research and/or a commercial hatchery, giving flexibility for different feeds and feeding protocols.

Conclusion

The experimental tank system is simple and easy to operate. It provides a powerful tool to execute and reproduce most larval experimental designs, while minimising unintended variation within the system. It allows operation with minimal manpower due to the automation of the feeding system and the ease of the daily routine – i.e., filter exchange. Several fish species ranging from temperate to tropical (pink snapper *Pagrus auratus*, yellowtail kingfish *Seriola lalandi*, and barramundi *Lates calcarifer* (Curnow et al., 2004; Kolkovski et al., 2004c)) were reared successfully in the system. A variety of factors including different live food enrichments, MD, and environmental settings were assessed.

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USE OF 'TAILOR-MADE' LIVE FEED ENRICHMENTS AND ADDITIVES IN FISH LARVAE NUTRITIONAL RESEARCH

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Introduction

A major problem in intensive culture of marine fish, especially larvae, is inadequate food quality leading to reduce growth, deformities, and mortality. During the last decade, strong attention has been paid to the roles of specific nutritional components such as essential fatty acids (EFA), especially highly unsaturated fatty acid (HUFA), phosphoglycerides, vitamins C and E, carotenoids, immunostimulants, and some others components in larval nutrition. The majority of these studies have focused on the role of EFA n-3 HUFA and especially eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3), and arachidonic acid (AA, 20:4n-6).

These essential nutritional components are usually incorporated into 'bioencapsulation' or enrichment products served to the live food (rotifers and *Artemia*). The live food is given to the fish larvae after the enrichment period, thereby transferring the essential ingredients to the larvae. Many oil-based emulsions or dry powders are commercially available.

In many cases, determination of nutritional requirements of fatty acids and other nutritional components is done by comparing commercially available enrichments differing in their EFA or other nutrients. The disadvantage of this approach is that these commercial products usually differ from each other in more than one component. Some research institutes have their own 'in-house' enrichments to be used in fish larval nutrition studies. However, these enrichments are not available commercially.

Live food enrichment

Today, there is large variety of commercially available enrichments with different levels and ratios of EFA, anti-bacterial agents, vitamins, etc. However, in

some cases, especially with new species or susceptible larvae, different levels and/or other ingredients are needed in the enrichments. For example, immune-stimulants and 'mega' doses of vitamins E and C are used to increase stress resistance and reduce deformities. In public aquaria and ornamental fish hatcheries, stress resistance and coloration enhancement is needed. These can be achieved with high levels of carotenoids.

Currently, there are no commercially available 'tailor-made' enrichments aimed at research institutes as well as commercial hatcheries and public aquaria. A new service is now available aimed at optimising live food enrichments to meet the nutritional requirements of marine fish species. The 'tailor-made' enrichment can be designed to suit any nutritional and/or experimental requirements. For example, different levels and ratios of EFA, vitamins, therapeutics immune-stimulants, carotenoids, and pro-biotics can be included. Specific nutrients and/or ingredients are incorporated into the oil/water matrix of the globules (liposome), so they are available to the live food and not dissolved in the water. Three studies which used this service are highlighted below.

Dietary requirement for 22:*6*n-3 of striped trumpeter larvae

A study to determine the dietary requirement for 22:6n-3 of striped trumpeter *(Latris lineata*) larvae during early development was conducted by the Marine Research Laboratories, Tasmanian Aquaculture and Fisheries Institute (Bransden et al., 2004; 2005). A novel dose-response design seldom used in larval marine fish studies was employed. Seven experimental emulsions were formulated with increasing concentrations of 20:5n-3 and 22:6n-3 and used to enrich rotifers. Enriched rotifer 22:6n-3 concentrations ranged from approximately 2-16 mg/g dry matter (DM). Enriched rotifers were fed to striped trumpeter larvae from 5-18 days post hatch (dph). Significant positive regressions were observed between most dietary and larval fatty acids at 10, 14, and 18 dph. Conversely, an inverse relationship was found between dietary and larval 22:5n-3. Larval 22:5n-3 is an intermediate product in the conversion of 20:5n-3 to 22:6n-3, and its presence in high amounts when dietary 22:6n-3 was low, indicated elongation of 20:5n-3 in an unsuccessful attempt to produce sufficient 22:6n-3 in order to meet physiological requirements. A dose-response trend between dietary 22:6n-3 and larval 22:5n-3 has not been so clearly demonstrated in other studies on marine fish. This relationship enabled calculation of the dietary requirement of 22:6n-3 of 12.7mg.g^{-1} DM for striped trumpeter larvae during the rotifer feeding period.

Effect of PUFA-enriched *Artemia* on western rock lobster

Western rock lobster, *Panulirus cygnus*, phyllosoma were grown from hatch to stage IV (Liddy et al., 2005). Larvae were fed with *Artemia* enriched with 'tailor-made' enrichments as follow: (1) Base enrichment (Base) containing 52%

squid oil, (2) Base enrichment supplemented with docosahexaenoic acid (DHA) rich oil, (3) Base enrichment supplemented with arachidonic acid (AA) rich oil, or (4) Base enrichment supplemented with DHA and AA (D+A) rich oils. Total survival of phyllosoma to stage IV was high, with no significant difference between treatments. The major lipid class (LC) in all phyllosoma (stages II and III) was polar lipid (PL) (88.9–92.4%), followed by sterol (ST) (6.2–9.7%). Triacylglycerol (TAG), free fatty acid (FFA) and hydrocarbon/wax ester (HC/WE) were minor components (≤1%). In contrast, the major LC in all enrichments and enriched *Artemia* was TAG (76.3–85.1% and 53.4–60.2%, respectively), followed by PL (11.4–14.8% and 30.6–38.1%, respectively). The main fatty acids (FA) in phyllosoma were 16:0, 18:1n-9c, 18:1n-7c, 18:0, AA, EPA, and DHA. Addition of AA, and to a lesser extent DHA, to enrichments resulted in increased levels of those FA in *Artemia* and phyllosoma compared to the Base enrichment. This was particularly evident for stage III larvae. Comparatively, elevated growth and survival rates for phyllosoma to stage IV were achieved with DHA and AA enriched diets.

Effect of 'mega' doses of vitamins C and E on yellowtail kingfish larvae

Kingfish (*Seriola lalandi*) are identified by industry in Australia and New Zealand as a promising aquaculture candidate. However, early indications of jaw deformity and low levels of swim bladder inflation are currently limiting commercial production. The incidence of cranial malformations and jaw malformations in larvae of other species has been related to broodstock and larvae nutrition, including insufficient dietary phospholipids, HUFA's and vitamins C and E as well as vitamin A metabolites. Previous work suggests that high doses of vitamins C and E may increase stress resistance in larvae and reduce deformities. An experiment was designed to test the effect of 'mega' doses of vitamins C and E supplementation in rotifers and/or *Artemia* enrichments (Kolkovski et al., 2004). Two 'tailor-made' enrichments were manufactured; base enrichments with standard levels of vitamins C (ascorbic acid) and E (α tocopherol) and 'mega' dose enrichment (3% each, on DM basis). Four feeding treatments were conducted. (1). Base enriched rotifers followed by Base enriched *Artemia*, (2). Base enriched rotifers followed by 'mega' dose enriched *Artemia*, (3). 'mega' dose enriched rotifers followed by Base enriched *Artemia* and (4). 'mega' dose enriched rotifers followed by 'mega' dose enriched *Artemia*. Early results revealed that 'mega' doses of vitamins C and E supplementation in the *Artemia* enrichments significantly reduced the susceptibility of larvae to stress (dip net and freshwater) and the incidence of specific deformities.

'Tailor-made' enrichments as well as broodstock additives are currently available free-of-charge to cooperating research groups (supplied by Nutra-Kol, Western Australia). The service has been funded by the Fisheries Research and

Development Corporation (FRDC) and the Department of Fisheries, Western Australia.

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STORAGE OF UNFERTILIZED RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) EGGS IN POLYETHYLENE BAGS

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Introduction

For conducting breeding programs in fish, storage and transportation of gametes is advantageous. For the storage of rainbow trout eggs, a maximum height of 4 layers and an atmosphere of O_2 have been shown to be critical (Stoss et al., 1980; Jensen and Alderdice, 1984; Komrakova and Holtz, 2004a). Salmonid eggs are commonly shipped in polyethylene (PE) bags inflated with air or O_2. In previous studies, Komrakova and Holtz (2004b) found that, when storing unfertilized rainbow trout eggs in vials, a covering of polyethylene foil (freezer bag foil, 30µm thick, Toppits, Melitta, Germany) permitted sufficient gas exchange to warrant survival. This raises the question whether it might be possible to package unfertilized rainbow trout eggs in polyethylene bags. This question is addressed in the present investigation.

Materials and methods

Eggs and sperm were obtained by stripping 3 to 4-year-old breeders from our own breeding unit at the University Experimental Farm Relliehausen at the height of spawning season. Pooled good quality semen from 5-6 males at a time was cryopreserved in pelleted form (Holtz, 1993) to be used during the fertilization trials. Unfertilized eggs from several females were pooled and supplemented with antibiotics at concentrations of 125IU penicillin and 125µg streptomycin per g of eggs and coelomic fluid. Eggs were sealed in PE bags of size 7×3cm or 7×8cm. When filled, the bags were 1.4cm thick. The bigger bags contained about 850 eggs, the smaller bags about 120 eggs. One big or 7 small bags were placed into a large (3 l) PE bag filled with a moisture-saturated O_2 or N_2 gas. The O_2 atmosphere was renewed either at 5 or 10d intervals or not at all. Storage temperature was +2°C. After 5, 10, 15, and 20 days of storage, respectively, eggs were fertilized with frozen-thawed semen at 8×106 spermatozoa per egg (Stoss and Holtz, 1981). The experiment was conducted with 5 replications. Five control groups (100 eggs each) were fertilized on day 0 of storage and

served as controls. The fertilization rate of stored eggs was expressed as percent of the proportion of eyed eggs obtained in controls. Statistical analyses were performed using the program "SAS version 8.1". Differences among treatments were tested by two-way analysis of variance with size of bag and gas regime as main effects. Each time period was analyzed independently. Significance of various effects was assessed by applying F-test ($P<0.05$), differences between individual means by Student's t-Test ($P<0.05$).

Results and discussion

The proportion of eyed eggs in the control group (day 0) averaged 81% (SEM 6). As shown in Table I, in all treatment groups, fertilization capacity decreased with increasing storage time. In eggs stored under O_2, repeated gas exchange exerted a slightly deleterious effect, in particular with small sized bags. When no exchange of O_2 occurred, differences in bag size did not matter. Differences were not very conspicuous and no statistical significances were observed.

With eggs stored under an atmosphere of N_2, those contained in big PE bags had deteriorated significantly within the first 5 days ($P<0.05$). The further decline was no more rapid than in the other groups. On the contrary, eggs in small PE bags stored under N_2 retained their fertility better than those in any of the other treatment groups, although differences were not statistically significant.

Oxygen consumption was found to be essential for viability of rainbow trout eggs (Czihak et al., 1979). Furthermore, previous work has indicated that storage of eggs in the absence of O_2 or O_2 deficiency significantly reduces fertility of stored eggs (Stoss et al., 1980). Since, in the present investigation, the eggs had no direct contact with the respective gas atmosphere, the PE foil appears to permit gas exchange. This confirms observations by Komrakova and Holtz (2004b), indicating that the fertility of eggs stored under O_2 in vials covered with PE foil did not decline more than in uncovered vials. The eggs enveloped in PE bags and maintained under an atmosphere of N_2, appeared to have a sufficient O_2 supply from atmospheric O_2 contained in a dissolved state in their own cytoplasm and the coelomic fluid they are immersed in. This interpretation is at variance with reports by Withler and Humphreays (1967) who found that sockeye or pink salmon eggs stored in closed plastic vessels lost their fertility rapidly.

With the thickness of the bags used in the present study, the maximum of 4 layers of eggs recommended by Stoss et al. (1980) and Komrakova and Holtz (2004a) was not exceeded. Hence our data support the conclusion that the number of layers might be one of the major factors prolonging egg viability while the volume of stored eggs was observed to be of less importance.

In conclusion, chilled storage of unfertilized rainbow trout eggs in sealed polyethylene bags was successful in preserving their viability for up to 20 days. This could provide a practical method of increasing the storage time of unfertilized eggs and maintaining the viability during storage and transportation.

Table I. Percent of eyed eggs stored in PE bags of 2 sizes (7×3×1.4cm or 7×8×1.4cm) at 2°C under moisture-saturated atmosphere of O_2 or N_2 renewed either at 5 or 10d intervals or not at all expressed in relation to freshly collected eggs (81% fertilized). (5 replications/treatment). Different superscripts are significantly different ($P<0.05$, Student's *t*-Test). (–) = No gas exchange

Gas atmosphere	Interval of gas exchange (d)	Days of storage	Big bags		Small bags	
			Mean	SEM	Mean	SEM
O_2	–	5	92^b	2	92^b	3
		10	78	7	81	7
		15	66	9	64	9
		20	52	10	48	12
O_2	10	5	88^b	3	87^b	3
		10	75	6	77	7
		15	62	8	57	10
		20	48	10	39	10
O_2	5	5	90^b	2	91^b	2
		10	79	6	79	6
		15	64	11	55	10
		20	44	10	35	10
N_2	–	5	67^a	6	87^b	1
		10	56	5	79	5
		15	50	8	68	9
		20	37	10	57	10

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RECENT PROGRESS IN LARVAL NUTRITION OF MARINE FISH AND CRUSTACEANS IN JAPAN

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In recent years the studies on larval nutrition on marine fish and crustaceans in Japan have been progressed on determining nutrient requirement and developing efficient microdiets for larval stages. This paper reviews the recent works that were conducted in Japanese research institutions. The following areas are included in the review: energy budget of kuruma shrimp, red sea bream, and Japanese flounder at *Artemia* feeding stages, Vitamin C requirement of larval and postlarval kuruma shrimp, development of peptide and fatty acid/calcium-based microdiet for red sea bream and Japanese flounder, pattern of marine fish larval feed intake, stocking density, and protein supplement for larval mortality.

Artificial microdiets have been developed for determining the nutrient requirements of larval aquatic animals. More practically, these are used for replacing live feeds such as rotifers and brine shrimp in seed production operation. The determination of the optimum nutrient levels in microdiets is not successful without the data of feed intake. Previously reported methods for estimating the feed intake and nutritive values of microdiets for fish and crustacean larvae demonstrated that one of the convenient methods for measuring microdiet intake is to use cholestane and dotriacontane as inert markers.

The microdiet, which was made based on a combination of a peptide from milk casein and fatty acid-calcium containing a large amount of docosahexaenoic acid obtained from fish oil, has been developed, and tested for larval red sea breams and Japanese flounders. These studies suggest the potential of casein hydrolysates as a partial replacement for live foods from the early developmental stages in larval red sea bream and Japanese flounders. Another study using soybean protein isolate (SPI), soybean peptides, stearoyl calcium lactate (SCL), and FOCS indicated that soybean peptides with molecular weights of 1000-3000 and FOCS are superior to SPI and SCL as microdiet nitrogen and lipid sources for red sea bream and Japanese flounder larvae and early juveniles.

There have been several studies on the role of vitamin C (ascorbic acid) associated with the promotion of growth, survival, feed efficiency, molting, stress re-

sistance, and immune response in juvenile penaeid shrimp. On the other hand, very few studies are available on the effect of ascorbic acid on larval performances of penaeid shrimp. Accordingly, Moe et al. (2004) conducted the study to evaluate and compare the efficacy of L-ascorbyl-2-monophosphate-Mg (AMP-Mg) and L-ascorbyl-2-monophosphate-Na/Ca (AMP-Na/Ca) on larval development and stress tolerance of kuruma shrimp, and to determine the dietary optimum levels of ascorbic acid in microdiet for the fastest development and growth, and highest stress tolerance of kuruma shrimp larvae. This study demonstrated that AMP-Na/Ca seems to be more effective than AMP-Mg on body weight, whole body ascorbic acid concentration in shrimp, and stress tolerance. The optimum dietary ascorbic acid level was found to range from 43-71mg.kg^{-1} diet.

Highly unsaturated fatty acid (HUFA) enrichment is common practice in seed production of marine cultured species due to the lack of HUFA in rotifers and *Artemia* nauplii. On the other hand, determining the energy partitioning can clarify the efficiency of energy utilization in relation to larval performance, and such information will aid in better understanding the physiology of larval aquatic animals. Although positive effects of enrichment of *Artemia* on larval growth are well documented, comparisons of an energetic analysis between larvae fed HUFA enriched and non-enriched *Artemia* have not been made. There are two studies on the energy budget of Japanese flounder larvae and kuruma shrimp post-larvae when fed *Artemia* nauplii. These studies demonstrated that HUFA enrichment of *Artemia* allocated more energy into growth, with a lowering of feces energy loss in both species. Furthermore, animals fed EA exhibited higher energy allocation for metabolism than those fed NEA, leading to higher growth rates through greater physiological and biochemical processes in EA fed groups.

The effects of larval density, rearing media, and protein additions on Japanese flounder larval survival were investigated by using small containers. They proposed a convenient method for assessing mortality in early stages of marine teleost larvae. Application of the methodology demonstrated that the addition of protein effectively reduces mortality due to handling, and that the mortality at the time of first feeding is not due to starvation in the Japanese flounder larvae.

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GROWTH KINETICS IN THREE STRAINS OF THE *BRACHIONUS PLICATILIS* SPECIES COMPLEX

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Introduction

In the present study, numerical response experiments were performed using three strains representing different taxa of the *Brachionus plicatilis* species complex that are widely used in aquaculture. The aim was to assess the competitive ability of the strains and predict the outcome of competition under different conditions in nature and mass cultures.

Materials and methods

Three strains of the *B. plicatilis* species complex were used: a SINTEF strain ('SIN22') belonging to *B."*Nevada", a strain coming from 'Maricoltura di Rosignano Solvay' hatchery ('MRS10') classified as *B."*Cayman", and a *B. plicatilis s.s.* strain ('10') obtained from the University of Valencia, Spain. The cultures were kept in darkness in a temperature-controlled chamber. The temperature and salinity were 25^{o}C and 25ppt, respectively.

Each strain was cultured separately. Standard food concentrations were 0, 0.0025, 0.005, 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.3, 2.6, 5, 10, and 20×10^{6} cells.ml^{-1} of *Nannochloropsis oculata*. Fifty amictic female rotifers, chosen randomly from stock cultures at exponential growth phase, were put into stoppered tissue culture flasks with 50ml of food suspension on a shaking table at 100rpm. At 24-h intervals, the food suspension was completely renewed and females and eggs were counted. Fifty animals of every experimental flask (or all those alive when the number was <50) were randomly chosen and pipetted back into the new medium. The experiment was terminated when the intrinsic daily growth rate (r) stabilized for at least 3d (coefficient of variance ≤0.1).

The intrinsic daily growth rate (r) for daily intervals was calculated from the exponential model. The egg female ratio (EF) was estimated as the number of eggs divided by the number of females. Monod curves, with a threshold for zero-growth, were fitted by iterative nonlinear regression (Systat v.10) to describe the relationship between food concentration and growth rate (as well as EF):

$$r = r_{\max} \frac{F - F_o}{F - F_o + K}$$

where r is the population daily growth rate, r_{max} is the maximal population growth rate, F the food concentration, F_o the threshold food concentration for zero population growth, and K the Monod constant (Rothhaupt, 1993).

Statistical differences were tested using ANOVA (Statgraphics Plus v.5).

Results and discussion

In the three strains tested, the modified Monod equation successfully described the relationship between the growth rate and food concentration (Fig. 1 and Table I). The same has been shown in previous studies (Rothhaupt, 1988; 1990; Ciros-Perez et al., 2001). The curves were characterized by a curvilinear increase with food concentration asymptotically approaching a maximal growth rate. The strain with the highest growth rate was MRS10 (significantly higher r over the range of 2.6-20×10^6 *N. oculata* cells.ml^{-1}, $P<0.05$). Below those food levels, strain 10 showed a marginally less negative growth rate (significantly higher r over the range of 0-0.02×10^6 cells.ml^{-1}, $P<0.05$). However, a population can persist only for a relatively short period of time at such low food levels. The maximum growth rate (r_{max}) derived from the regression analysis was above 1 in all cases and significantly higher in MRS10 compared to the other two strains ($P<0.05$; Table I). The threshold food concentration (F_o) was different from zero in all cases, and lower in MRS10 and 10, but no significant differences were found. According to the results, the best competitor appears to be *Brachionus* "Cayman" (MRS10), both at high (r_{max}) and low food (F_o) concentrations. *B.* "Nevada" (SIN22) was the worst competitor of the three over the whole range of food concentrations tested. However, the most striking is the similarities in performance of the three strains at moderate growth rates.

The modified Monod equation also successfully described the relationship between the egg female ratio (EF) and food concentration (Fig. 2 and data not shown). In the plateau region of the curve, EF was above 0.8 for SIN22 and MRS10, and close to 1 for strain 10 (significantly higher EF over the range of 2.6-20×10^6 cells.ml^{-1}, $P<0.05$). The inconsistency in maximal values of growth

rate and EF among the strains suggests that there must be other differences – possibly in life history traits – that could account for the observed results.

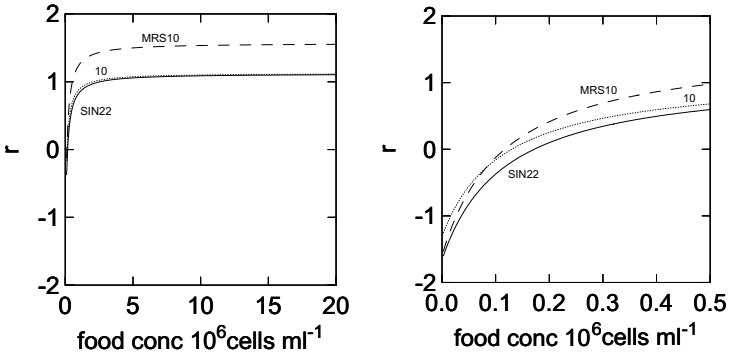

Fig. 1. Relationship between *Nannochloropsis oculata* concentration and growth rate (r, d^{-1}) for the three strains of the *Brachionus plicatilis* species complex. The curves were fitted by iterative nonlinear regression calculated from a modified Monod equation. The graph on the right shows detail for low food concentration.

Table I. Estimates ± ASE (and confidence intervals) for the parameters of the modified Monod equation describing the relationship between population growth and food concentration for three strains of *Brachionus plicatilis* species complex. Monod curves were fitted by iterative nonlinear regression ($P < 0.05$). Asterisk indicates significant difference ($P<0.05$). F_o, K, and K_c in 10^6 cells.ml^{-1}, r in d^{-1}. $K_c = F_o + K$.

	SIN22	**MRS10**	**10**
r_{max}	1.12 ± 0.08	1.57 ± 0.07*	1.13 ± 0.04
	(0.94 – 1.29)	(1.41 – 1.72)	(1.04 – 1.21)
F_o	0.17 ± 0.02	0.12 ± 0.01	0.13 ± 0.01
	(0.12 – 0.22)	(0.09 – 0.14)	(0.11 – 0.15)
K	0.29 ± 0.04	0.23 ±0.03	0.24 ± 0.02
	(0.19 – 0.38)	(0.17 – 0.29)	(0.20 – 0.29)
K_c	0.46 ± 0.06	0.35 ± 0.04	0.37 ± 0.03
	(0.32 – 0.60)	(0.26 – 0.43)	(0.31 – 0.44)
R^2	0.98	0.99	0.99

Conclusions

According to our results, competition would favour *B.* "Cayman" out of the three strains tested. The outcome of competition would, of course, be influenced by abiotic factors (T, S), environmental fluctuation, and mixis patterns. The differences in competitive ability will increase with increasing population growth rates. In the case of mass cultures in hatcheries, the abiotic factors are controlled to a greater extent compared to nature and the outcome of competition could be largely based on food availability. Since in such cultures food concentrations are

considerably higher than those encountered in nature, it is predicted that *B.* "Cayman" would be the most successful competitor out of the three. Oscillations in food availability due to non-continuous or constant supply of food may modify our conclusions.

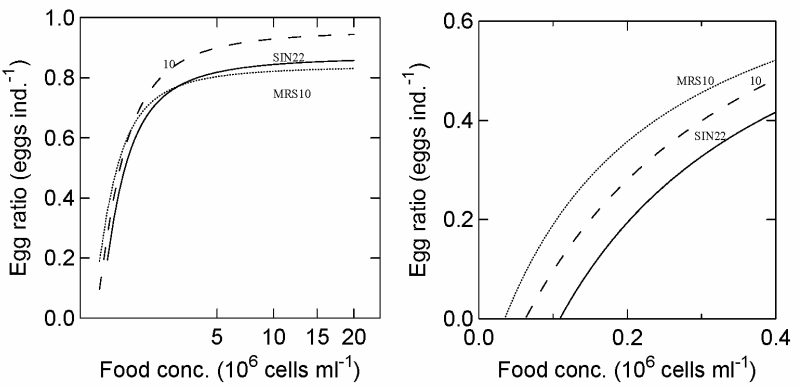

Fig. 2. Relationship between *Nannochloropsis oculata* concentration and egg:female ratio (EF) for the three strains of the *Brachionus plicatilis* species complex. The curves were fitted by iterative nonlinear regression calculated from a modified Monod equation. The x axis of the graph on the left has been square-root transformed for ease of presentation. The graph on the right shows detail for low food concentration.

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CULTIVATION AND APPLICATION OF COPEPODS AND CLADOCERANS FOR LARVAL REARING IN JAPAN: PAST, PRESENT AND FUTURE

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Zooplankton as live food for marine finfish larviculture is still important in aquaculture. Although *Artemia* nauplii have been introduced as live food since the 1940s, it was already known that their nutrition, especially its DHA-EPA contents, was poor for larval fishes to culture. And *Artemia* nauplii were too large (400 μm -) to apply to larval fishes of some species just after hatch. In order to search the smaller size and more abundant nutrition of live food, various species of zooplankton were tested in marine larval rearing worldwide, also in Japan. Many species of zooplankton were collected from natural seawaters, and then fed to larval fishes directly in hatcheries. Although their nutritional value for larval fishes was excellent, it was difficult to culture most of them. The harvested quantities from natural seawaters fluctuated and the supplies for larvae, therefore, were unstable. It was possible to culture some species of copepods and cladocerans in open ponds. Those cultures were also unstable, and they needed large areas, such as open ponds, and a great deal of labor to maintain them.
Euryhaline rotifer *Brachionus* was introduced as first live food for larval fishes in the 1960s in Japan. Rotifer culture was easier and more stable and many hatcheries began to use it as live food. In the 1990s, most hatcheries of the world were using rotifer and *Artemia* as live food for larval fishes, and copepods and cladocerans seldom got used. Moreover, micro artificial diet for larval fishes were developed and the feeding system mainly performed in present, rotifer-*Artemia*-micro artificial diet, was established. In Japan, many studies of feeding or culturing of copepods and cladocerans used to be performed but not so many in present. Little hatcheries in Japan are feeding copepods collected from wild to larval fishes, but many avoid it to prevent fishes from the risk, such as disease including VNN. Therefore most hatcheries are according to the feeding system, rotifer-*Artemia*-artificial diet.

Recently, *Artemia* eggs have got difficult to be supplied stably and they have got more expensive. Copepods and cladocerans are expected as the replacement

live feed of *Artemia*. Frozen copepods gets distributed commercially and used as the supplemental feed during *Artemia* feeding. Although the use of frozen copepod is getting to spread, it is still expensive and its quality is variable. Moreover, some problems, e.g. skeletal deformity and malpigmentation, have been still insoluble with the feeding system, rotifer- *Artemia*- artificial diet, or the improvement of the nutrition contents of the present feeding system. Some studies got rid of the malpigmentation by feeding on copepod, and some reported the success of the cultivation of small mouth larval fish such as grouper larvae by feeding on the nauplii of copepods. In each study, copepods were collected from open seawater, or cultured in open ponds. In order to supply copepods and cladocerans stably with good quality to larval fishes, it is necessary to develop in-door intensive culture technology.

The following items are important to develop intensive culture of copepods and cladocerans and to supply them stably: 1) screening the species of micro algae and concentration of the culture, 2) how to maintain the culture water quality and 3) mass production of their resting eggs for storage.

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THE IMPACT OF DIET ON THE EFFECTIVITY OF REARING PIKE-PERCH, *SANDER LUCIOPERCA* (L.) LARVAE OBTAINED FROM OUT-OF-SEASON SPAWNING

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Introduction

Appropriate larval nutrition ensures proper individual growth and development in a given species. High mortality, low growth rates, and body deformations are all seen during the pike-perch larval rearing. In an attempt to improve larval rearing of pike-perch (*Sander lucioperca* (L.)), the impacts of various commercial starter diets used to rear marine and freshwater fish were tested and compared.

Materials and methods

The experimental material was comprised of pike-perch larvae five days post hatch (5DPH) that were obtained from out-of-season spawning (Zakęś and Szczepkowski, 2004). The hatch, with an initial mean body weight of 0.7mg and an initial mean total body length TL of 5.1mm, were reared in 0.2-m^3 rotation tanks in a recirculating system. The rearing parameters were optimal for this species (Craig, 2000; Szkudlarek, 2004). Water temperature was 20°C. Mixed feeding was applied during rearing, i.e., *Artemia* sp. + artificial feed: two marine fish starters (groups SF1 and SF2); one freshwater fish starter (group FF). The duration of the experiment was four weeks, during which samples were taken at weekly intervals to determine growth.
The growth rate, survival, share of larvae with inflated swim bladder, and larval body deformities of the pikeperch larvae from out-of-season spawning were determined. The results were analyzed with one-way analysis of variance (ANOVA) and Tukey's test ($P<0.05$).

Results and discussion

The results obtained indicated there were significant differences between the analyzed rearing parameters among the three diet regimes (Table I). The final

mean body weight ranged from 0.17g (in group FF) to 0.35g (in group SF1). These differences were determined to be statistically significant (Fig. 1).

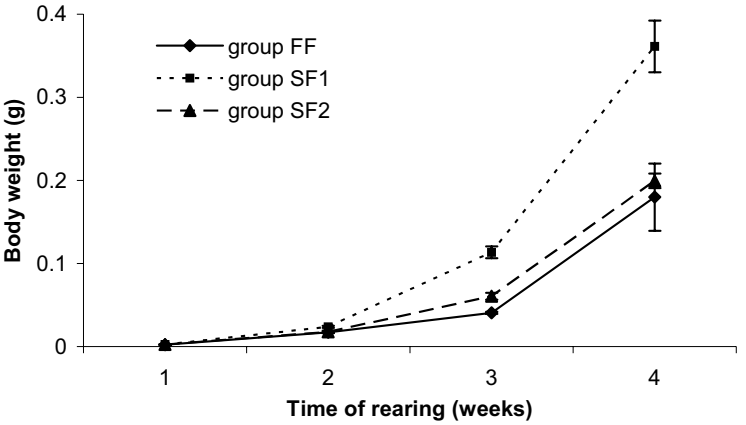

Fig. 1. Body weight gain of larval pikeperch during four weeks of rearing among three dietary regimes (mean ±SD).

In the second week of rearing, differences were noted in the specific growth rate (SGR). The index for group SF1 was 25.3%.d^{-1} and was significantly higher than in group FF, which was 22.9%.d^{-1}. The trend was maintained for the duration of the experiment. The final coefficient of body weight variation (CV_{BW}) within the three dietary regime groups was similar. Larval survival following four weeks of rearing was significantly the highest in groups SF1 and SF2 at 16% (Table I.). The percentage share of fish with inflated swim bladders (13dph, i.e., during the period when the pneumatic duct closed and the bladder inflated) was significantly highest in group SF1 at 70%. This figure for the other two groups was 42% (group FF) and 40% (group SF2) (Table I).

Body deformations were noted in all the fish groups and were observed in both fish with inflated and uninflated swim bladders (this phenomenon was especially evident in group SF1; inter-group differences were statistically significant; $P<0.05$; Table I).

The nutrition studies of the application of *Artemia* and artificial feed during the early stages of rearing pike-perch larvae argue for the application of mixed feeding (Hilge and Steffens, 1996). Using commercial feed developed for marine species had a beneficial impact on pikeperch larval survival and growth rate.
However, numerous body deformities, especially in group SF2, indicate that the feed does not fully meet the nutritional requirements of pikeperch larvae. The spinal deformities (scoliosis, lordosis) observed may have resulted from a deficit of nutritional components such as vitamin C and polyunsaturated fatty acids.

Fortifying *Artemia* with these elements might prevent skeletal deformities and have a beneficial impact on the larval growth of this species (Xu et al., 2003).

Table I. Growth, survival, percentage of swim bladder inflation, and body deformities in pike-perch larvae fed three types of feed: FF, SF1, SF2 (mean±SD, each performed in duplicate); data in the same row with the same superscript do not differ significantly statistically (P>0.05).

Specification	Feed		
	FF	SF1	SF2
Final body weight (g)	0.17^{a} (±0.04)	0.35^{b} (±0.03)	0.19^{a} (±0.00)
Specific growth rate SGR (%.d^{-1})[1]	19.8^{a} (±0.80)	22.3^{b} (±0.30)	20.2^{b}(±0.15)
Final coefficient of body weight variation CV_{BW} (%)[2]	35.0 (±1.14)	30.9 (±5.22)	33.7 (±14.16)
Survival (%)	9.3^{a} (±2.02)	16.1^{b} (±2.77)	15.9^{b} (±0.56)
Share of larvae with inflated swim bladder (13dph, %)	42.5^{a} (±3.53)	70.0^{b} (±21.21)	40.0^{a} (±21.21)
Share of larvae with body deformity among fish with uninflated swim bladder (%)	45.8^{a} (±5.89)	93.9^{b} (±5.02)	66.9^{a} (±6.30)
Share of larvae with body deformity among fish with inflated swim bladder (%)	3.6^{a} (±0.18)	15.0^{b} (±21.21)	1.7^{a} (±2.35)

[1]SGR=(ln final body weight (g)-ln initial body weight (g)×time of rearing^{-1}(days))×100
[2]CV_{BW}=(mean body weight×SD^{-1})×100

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DIGESTIVE ENZYME STUDY OF HYBRID OF SILVER CARP (*HYPOPHTHALMICHTHYS MOLITRIX*) × BIGHEAD (*ARISTICHTHYS NOBILIS*) DURING ONTOGENY

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Introduction

Cross of silver carp (*Hypophthalmichthys molitrix*) and bighead (*Aristichthys nobilis*) is widely accepted in aquaculture due to having the positive heterosis for growth rate (Bartley et al., 2001). It is well documented that at the early developmental stage, fishes experience a crucial metabolic phase during the shifting of feeding. The present study was focused on the fate of major functional digestive enzymes found in the gut of the hybrid fish during early ontogeny. Efforts have been made to characterize the digestive proteases.

Materials and methods

Larvae in three replicates were stocked (45 000 fish.m^{-3}) in 450-l tanks in recirculating system. Larvae were fed with live food (*Brachionus* spp., *Ceriodaphnia* spp., *Mesocyclops* spp.) ad libitum up to 14 days post-hatching (dph). From d14 onward, mixed feeding with artificial diet (40% protein) was started at the rate of 4% of body weight. The whole digestive tract of individual fish was used for enzyme assay. Amylase activity was measured by Bernfeld (1955) method. Total protease activity was assayed using 1% azocasein as substrate (Garcia-Carreno, 1992). Protease class was evaluated by treating the enzymes with different specific inhibitors (Garcia-Carreno and Haard, 1993). Trypsin and chymotrypsin activities was evaluated according to Erlanger et al. (1961) using N-α-benzoyl-D_L-arginine-*p*-nitroanalidine (BAPNA) and Suc-Ala-Ala-Pro-Phe-p-nitroanalidine (SAPNA) as substrates, respectively. Lipase activity was measured following the method of Winkler and Stuckman (1979). SDS-PAGE was performed according to Laemmli (1970). The protease composition and their classes were evaluated by substrate SDS-PAGE as described by Garcia-Carreno et al. (1993). For protease class evaluation, enzyme extracts having 5mU activity were incubated with different inhibitors like SBTI (250µM), PMSF (100mM), TLCK (10mM), and EDTA (20mM) prior to loading on to the wells.

Results and discussion

On d4, the specific amylase activity was 0.078±0.01mg maltose per mg protein.h^{-1}. The activity showed a 74% increase in 8th day larva than 4-day-old one. The enzyme activity in 34dph larva was 0.109±0.12mg maltose per mg protein.h^{-1}. Total protease activity in 4dph larva was 14.37±2.21mU per mg protein.min^{-1} Total protease activity increased with the age of fish. Trypsin activity was 11.38±1.6mU per mg protein.min^{-1} in 4-dph fish. Chymotrypsin activity was low (2.8±0.5mU per mg protein.min^{-1}) at the beginning of the study. Trypsin and chymotrypsin activities were maximum in 32-dph larvae. In 4-dph fish, the lipase activity was 2.3±0.1mU and the maximum activity was recorded in 32-dph (7.4±0.8mU) larva.

Table I. Percentage of inhibition of protease activity by different inhibitors in silver carp and bighead carp hybrid during ontogenesis.

Age of Fish (dph)	SBTI	PMSF	TLCK	TPCK	EDTA
28	92	81	54.2	47	7.1
30	92.4	77.4	46.1	48.4	10.3
32	84	71.7	52.3	49.7	11.5
34	82.8	80.7	55.7	39	6

Inhibition of protease activity by various inhibitors showed the presence of serine proteases (Table I). The molecular weight of protein bands found in the digestive extracts ranged from 20.9-123.4kDa. In 4-dph larva two activity bands of proteases (73.7 and 43.6kDa) were recorded. Total 9 activity bands ranging from 19.1-119.6kDa were observed. The intensity and number of bands increased with the age of fish. Treatment of enzyme samples with various inhibitors reduced the number of bands. SBTI and PMSF inhibited 2 and 5 activity bands, respectively. TLCK inhibited two trypsin-like enzyme activity bands whereas, TPCK treated samples showed inhibition of four chymotrypsin-like enzyme. In the present experiment, the decrease in amylase activity from high value after first week was possibly due to developmental changes in the gut morphology and increased protein in the tissue. Cahu et al. (2004) suggested that regulation of amylase is post-transcriptional in early larval stages of sea bass *Dicentrarchus labrax* (to 25dph) and become transcriptional towards the end of larval period. The increase in protease activity from 18 days after initial low activity suggested that the modulation for adaptation to digest greater protein content in the food. Chymotrypsin is more pronounced than trypsin during ontogenesis, which may be associated with the regulation of trypsin and chymotrypsin in the gut of the fishes. The increased lipase activity at the fourth week in this study may be attributed to the adaptation of the larvae to better digestion and utilization of lipids. The results obtained from the SDS-PAGE and substrate SDS-PAGE suggested the appearance of high molecular weight proteins at the beginning of the

development followed by lower molecular weight one. Inhibition of activity bands by SBTI and PMSF suggested the presence of serine proteases. Inhibition of activity bands by TLCK and TPCK suggested the presence of trypsin and chymotrypsin-like proteases.

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EFFECTS OF PROTEIN HYDROLYSATE IN FEED FOR ATLANTIC COD (*GADUS MORHUA*) AND ATLANTIC HALIBUT (*HIPPOGLOSSUS HIPPOGLOSSUS*) AT WEANING

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Introduction

Hydrolysed protein in weaning diets is found to enhance the performance of marine fish larvae (Cahu et al., 1999). Sea bass larvae improved both survival and growth when 25% of the dietary protein was hydrolysed compared to lower and higher substitution levels. In addition, an optimal level of hydrolysed protein seems to induce a more adult like digestion, indicated by increased activity of the brush border enzymes alkaline phosphatase (AP) and leucine aminopeptidase N (Lap) compared to the activity of the cytosolic enzyme leucine-alanine peptidase (Leu-ala; Cahu et al., 1999). Also carp larvae performed better when fed 25% hydrolysed protein (Carvalho et al., 2004).

Materials and methods

Cod (*Gadus morhua*; initial weight 10.7±2.1mg; 32 dpff) and halibut (*Hippoglossus hippoglossus*; initial weight 221.5±69.0mg; 63 dpff) larvae were reared at the Institute of Marine Research, Storebø, Norway. At start of the experiments (d0), the larvae were transferred to 50-l triplicate tanks and weaned on the experimental diets. Cod and halibut were fed for 41 and 56 days, respectively, and cod were co-fed *Artemia* during the first five days of the experiment.

The experimental feeds were heat-coagulated microbound particles based on fish mince and squid belly (Hamre et al., 2001). The diets were dried and crushed into appropriate particle sizes; <0.3mm and 0.3-0.6 mm for cod and 0.3-0.6mm and 0.6-1.0mm for halibut. The dietary protein was substituted with varying levels of pepsin hydrolysed fish mince as described by Kvåle et al. (2002). The percentages of dietary fish mince that were hydrolysed with pepsin were 0, 10, 20, 30, and 40 in the cod experiment and 0, 15, 30, and 45 in the halibut experiment.

The diets and hydrolysates were based on saithe and cod for cod and halibut, respectively.

Wet weights were registered at the start, intermediate sampling (only halibut), and end of the experiments. At the intermediate sampling and end, activity of the enzymes Leu-ala, AP, and Lap were analysed in accordance with Cahu et al. (1999). AP and Lap are markers of enterocyte development and thus indicate ability of a more adult-like digestion. Leu-ala is thought to have higher activity at larval stages (Zambonino Infante and Cahu, 2001). One replicate was missing in the H10 and H20 group in the cod experiment, and one group in the H30 group in the halibut experiment. The data were statistically analysed by one-way ANOVA followed by Tukey HSD.

Results and discussion

The dietary level of hydrolysed protein affected the performances of cod and halibut larvae, but in different ways. Cod offered the diet with the highest level of protein hydrolysate, H40, achieved 18.4% survival, while the larvae offered 10% or no hydrolysate achieved less than 9% survival ($P<0.04$; Tab I). The ratio of Lap vs. Leu-ala was higher in the H30 group compared to the H0 group ($P<0.04$). A high incident of mortality took place after the co-feeding period with *Artemial* (d5), while the enzyme activities were measured at the end of the experiment (d41). It might be that the optimal level of hydrolysed protein was reduced during the experiment. Carvalho et al. (2004) found that carp larvae improved growth and survival when weaned on a diet were part of the protein was soluble, but after two weeks of feeding the diets with and without soluble protein seem equally utilized.

Table I. Survival rates and ratio of activity of leucine aminopeptidase N (Lap; mU.larvae^{-1}) and alkaline phosphatase (AP; mU.larvae^{-1}) vs. activity of leucine-alanine peptidase (Leu-ala; μU.larvae^{-1}) in the intestines of Atlantic cod larvae fed different level of protein hydrolysate.

	H0	**H10**	**H20**	**H30**	**H40**
Survival	7.1±1.3^{b}	8.1±4.0^{b}	10.7±6.7^{ab}	11.8±0.7^{ab}	18.4±1.6^{a}
Lap/Leu-ala	169±89^{b}	338±7^{ab}	370±15^{ab}	380±70^{a}	285±85^{ab}
AP/Leu-ala	785±47	910±286	736±165	865±72	921±87

In the experiment with halibut, the survival rate of larvae offered the non-hydrolysed diet was 56.5%, while in those offered 15% hydrolysate or more, less than 35% survived ($P<0.04$; Tab II). The activity of the brush border enzymes AP and Lap confirm the result, as the H0 group had higher activity than the H30 (only AP; $P<0.02$) and H45 group ($P<0.03$).

The differences between cod and halibut might be due to different feeding habits in the two species. Cod larvae are regarded as much faster in catching feed particles, while halibut larvae are rather slow. Thus, the protein level might be suboptimal for halibut larvae as hydrolysed protein will leak at higher rates than intact protein (unpublished data). The results may also have been influenced by different ages of the cod and halibut larvae at weaning. Cod was weaned early in this experiment, while halibut was lately weaned. This suggestion agrees with Carvalho et al. (2004) which found that soluble protein improved the performance of carp larvae only during the first two weeks of feeding and thereafter there was no effect of protein solubility. However, in a previous study where halibut was weaned at 40dpff, the optimal level of protein hydrolysate was found to be 10% or lower (Kvåle et al., 2002), and this support the result that cod and halibut have different optimal level of hydrolysed protein at time of weaning.

Table II. Survival rates and specific activity(U.(mg protein)$^{-1}$) of brush border leucine aminopeptidase N (Lap) and alkaline phosphatase (AP) in Atlantic halibut larvae fed different level of protein hydrolysate.

	H0	**H15**	**H30**	**H45**
Survival	56.6±8.6^{a}	35.0±5.9^{b}	28.6±9.7^{b}	22.4±6.5^{b}
Lap	1.34±0.28^{a}	1.20±0.20^{ab}	0.61±0.31^{ab}	0.65±0.09^{b}
AP	6.27±1.27^{a}	4.36±1.52^{ab}	1.26±0.46^{b}	1.57±0.33^{b}

In this study growth was not affected by the diets. Cod had a final weight of 311.2±61.0mg after 41 days of feeding, while halibut had achieved a final weight of 969.3±558.3mg after 56 days of feeding.

Conclusion

In this study, hydrolysed protein in weaning diets for Atlantic cod and Atlantic halibut had different effects. A high level of protein hydrolysate (40%) promoted survival in cod larvae, as opposed to a non-hydrolysed diet in halibut. These results were confirmed by analyses of activity of intestinal enzymes. There was no effect of protein hydrolysate on growth in neither species.

Acknowledgements

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DO COPEPODS MEET THE AA REQUIREMENTS OF THE WHITE GROUPER LARVAE BETTER THAN ROTIFERS AND *ARTEMIA*?

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Introduction

The white grouper (*Epinephelus aeneus*) is considered a potential new species for aquaculture. Rearing protocols to achieve good growth during on-growing under intensive conditions have been reported as well as significant strides forward in brood stock management (Hassin, et al., 1997). The main bottleneck for the farming of this species remains larval rearing.

Two critical phases are usually identified during this period, one occurring at first feeding and the second one during metamorphosis climax. First feeding remains problematic due to the small size of larval mouth gape and the point of no return is thought to be extremely short (Ben-Atia et al., 2003). Metamorphosis occurring at latter stages is characterized by heterogeneous growth, poor pigmentation and cannibalism; it is known to be controlled via thyroid hormones (TH). It has been hypothesised that live feed provided at these stages are deficient in some amino acid (AA) precursors of this hormones such as phenylalanine and tyrosine. Fish larvae are known to display changes in their AA content along their ontogeny, and an imbalanced diet may result in lower growth and developmental abnormalities.

A possible alternative to the rotifers and *Artemia* commonly used in aquaculture is copepods which comprise most of the larva's natural prey in the sea. Numerous species present nauplii stages sufficiently small to be ingested by fish larvae and their and which contain a biochemical composition that is believed to be ideal. Moreover, copepods are usually rich in nutritional-precursors of TH. *Acartia tonsa* is a calanoid species successfully cultivated at CCMAR and could be a possible candidate as live preys for *E. aneus* larval rearing.

The aim of this work was to determine indispensable amino acid (IAA) profiles of *E. aeneus* larvae during ontogeny and to compare them with IAA content of various live preys (*B rotundiformis*, *Artemia* spp., and *Acartia tonsa*), in order to evaluate this copepod species, in terms of satisfying the AA requirement of the larvae of white grouper.

Materials and methods

All cultivations and enrichment of live preys took place using standard conditions described by the manufacturer. INVE strain BE 480 (INVE Aquaculture, Belgium) was used to obtain newly hatched *Artemia* nauplii. Rotifers *Brachionus rotundiformis* were enriched for 24h with DHA Protein Selco and metanauplii *Artemia* (RH Artemia cysts) were enriched for 24h with Super Selco

Acartia tonsa was cultivated semi-intensively in outside 9-m^3 tanks located at the experimental station of Ramalhete (Faro, Portugal) and fed using induced blooms of *Isochrysis galbana* and/or *Chlorella minutissima*. Nauplii (stages N1-N3) and copepodits (stages C4-C6) were sampled using the 55-180µm and 180-500µm fraction respectively.

White grouper (*Epinephelus aeneus*) were cultured at the National Centre for Mariculture (Eilat, Israel) according to standard procedures. Two-day-old yolk sac larvae were stocked in 1500-l conical V-tanks with a 100% daily water exchange rate of filtered (10µm) seawater (25%) at 27±1°C. All samples were collected in duplicate, quickly rinsed, frozen in liquid nitrogen and stored at -80°c until analysis. They were then freeze dried and dry weight determined. 1mg DW sample was processed for protein extraction using trichloro-acetic (TCA 6%, 4°C, 24h). Acid hydrolysis was performed using HCl (HCl 6M + 0.5% phenol, 108°C, 24h). Hydrolysed samples were then passed through a 0.45µm Acrodisk syringe filter before injection in a reversed-phase Waters HPLC (Breeze system) connected to a Waters UV/Visible detector. Norleucine was used as an internal standard.

All AA data are expressed as weight percentage of the proteinic AA pool. A/E ratio was calculated. A/E ratios of *E. aeneus* were plotted against those of food for detection of AA imbalances (Conceição et al., 2003).

Results and discussion

Changes in AA profile are already apparent in early development of *E. aeneus* larvae. Regarding indispensable amino acids (IAA), significant changes were noticeable for lysine, leucine, valine, and methionine; changes in dispensable amino acids (DAA) were significant for glycine, proline, isoleucine, and serine.

The lysine content decreased significantly at 22 and 39dah when compared to 2, 3, and 11dah. Leucine and valine appear to follow same pattern. They both have a significantly higher contribution at 2dah compared to 39dah. Methionine content appears to be low during the first stages (2, 3, and 11dah) and then increases, especially during the latest stages (22 and 39dah).

Glycine and proline content significantly increased during ontogeny, being significantly higher at 39dah when compared to other stages.

Acartia tonsa N1-N3 and C4-C6 stages IAA content as well as the one of *Brachionus plicatilis* and *Artemia* spp. nauplii and metanauplii stages was determined and the related A/E ratio calculated. Data for amino acid content of the two last live preys were taken from Aragão et al. (2004). Comparison of A/E ratios was performed at various points of the ontogeny and for the different live preys analyzed (Figs. 1 and 2). In these figures, AA located above the bisecting line are in deficiency in the live prey for white grouper larvae of the given age.

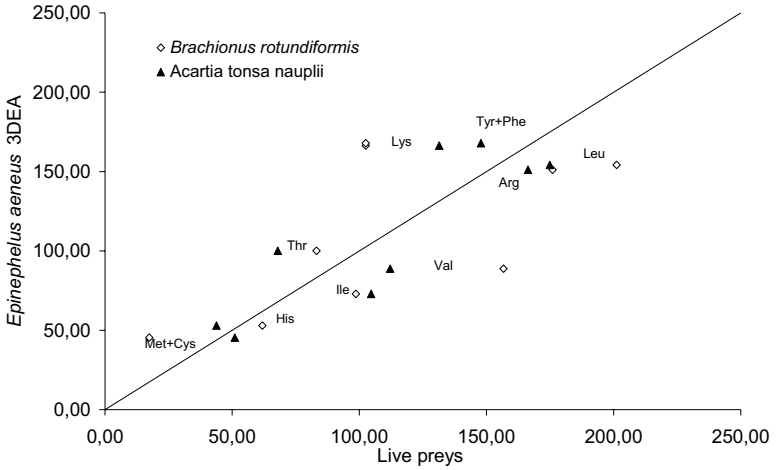

Fig. 1. A/E ratios of 3-dah *E. aeneus* and live prey.

Acartia tonsa nauplii seems to be better balanced in terms of AA compared to *B. rotundiformis* for first feeding larvae (3dah) *E. aeneus* (Fig. 1). This is especially true regarding lysine. However, *A. tonsa* nauplii appear to be deficient in methionine and cystein and may not fulfill larval sulphur AA requirement, what seems to contradict data on other copepod species (Helland et al., 2003.).

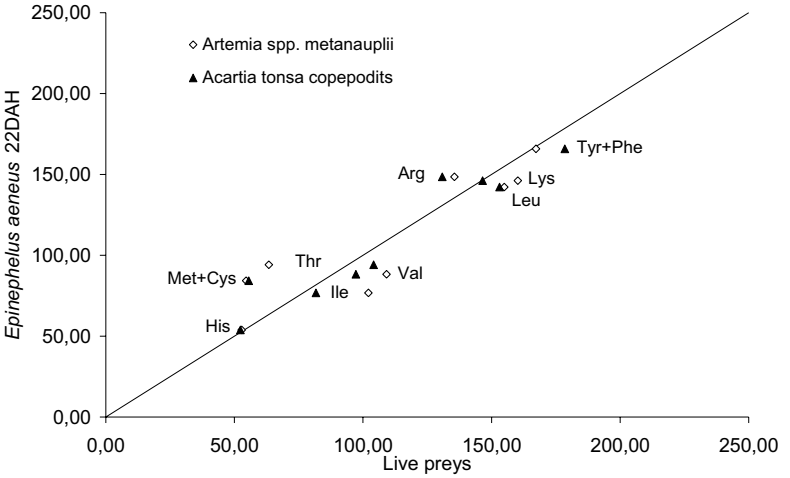

Fig. 2. A/E ratios of 22-dah *E. aeneus* and live prey.

Artemia spp. metanauplii, but not *A. tonsa* copepodites (Fig. 2), seem deficient in threonine in 22dah *E. aeneus* larvae. Both live preys seem again unbalanced in terms of sulphur AA, as well as arginine.

The results from the present study suggest that *A. tonsa* nauplii and copepodites may be a more suitable diet in terms of AA requirements compared to commonly used live prey types.

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COMPLEX MICROPARTICLES FOR DELIVERY OF LOW-MOLECULAR-WEIGHT, WATER-SOLUBLE NUTRIENTS TO MARINE FISH LARVAE

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Larvae of many marine fish species are entirely dependent on live feeds such as rotifers and *Artemia* for successful development to metamorphosis. Early stages of altricial marine fish larvae typically show low proteolytic digestive capacities and may require dietary supplies of low molecular weight peptides and amino acids. Dependence on live feeds may be because artificial diets do not effectively deliver such low-molecular-weight, water-soluble (LMWS) nutrients to larvae due to rapid leakage losses.

A complex microparticle composed of lipid spray-beads, containing LMWS nutrients, bound within a zein matrix was developed to deliver LMWS materials to fish larvae. Lipid spray beads were prepared by spraying a mixture of ground particles or a solution of LMWS nutrients suspended in molten menhaden stearine (MS) into a chamber chilled with vapors from liquid nitrogen. The molten lipid hardened to form beads containing the LMWS nutrients. To form complex particles, lipid beads were mixed with other nutrients – such as proteins, starch, and lipid – in a 6.7% w/v solution of zein dissolved in 90% v/v aqueous ethanol. The zein mixture containing the lipid beads was then atomized into a chamber at room temperature to evaporate the alcohol, resulting in the formation of complex microparticles containing lipid-spray beads.

We found that retention of the highly soluble amino acid glycine ($250 g \cdot l^{-1}$ at 25°C) by lipid beads prepared with 75/25% w/w MS/sorbitan monopalmitate depended on the physical form of the incorporated glycine. Glycine incorporated when dissolved in aqueous solution was retained by beads suspended in water at a significantly higher efficiency (time for 50% loss of encapsulated core [T_{50}]=90 min) compared with glycine incorporated in particulate form (T_{50}=4min). In contrast, the less soluble amino acid tyrosine ($0.45 g \cdot l^{-1}$ at 25°C), when incorporated in particulate form, was retained at a high efficiency

(T_{50}=2280min) by beads suspended in water. The advantage of incorporating LMWS materials in particulate form rather than in aqueous solution is that it is more likely that high incorporation efficiencies can be obtained; e.g., an incorporation efficiency of 23%w/w was obtained using particulate tyrosine.

Amino acid retention was significantly greater using complex particles compared with zein-microbound particles, prepared by simply adding amino acids to zein without first incorporating them into lipid beads. This result indicated that lipid beads were effective in reducing leakage of amino acids from complex particles. Retention of amino acids by complex particles was directly dependent on the aqueous solubility of the amino acid, with less soluble amino acids being better retained (e.g., tyrosine T_{50}=37min) than more soluble amino acids (e.g., glycine, alanine, and serine T_{50}=2min).

The digestibility of zein-bound complex particles containing lipid beads prepared with 100% MS was qualitatively determined by feeding particles to 3-day-old clown fish larvae and microscopically observing release of a colored dye (Poly-Red 478) from ingested particles into the gut. Ingested particles were observed in the foregut and particle breakdown and release of Poly-Red 478 was observed in the hindgut.

In summary, complex particles, consisting of zein-bound lipid spray beads containing LMWS nutrients, provide a more effective means of delivery of this type of material to marine fish larvae and other suspension-feeders than using simple microbound zein particles. However, additional experiments are required to further improve encapsulation and retention efficiencies of complex particles for highly water-soluble nutrients, such as glycine.

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CHARACTERIZING THE DIGESTIVE PHYSIOLOGY OF CALIFORNIA HALIBUT (*PARALICHTHYS CALIFORNICUS*) LARVAE: TOWARDS DEVELOPING SUITABLE MICRODIETS AND WEANING PROTOCOLS

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Introduction

The California halibut (*Paralichthys californicus*), distributed from Washington to Southern Baja California, presently supports commercial and sport fisheries in the United States and Mexico. As for many marine fish stocks, annual landings have decreased over time which has stimulated research in aquaculture and stock enhancement of this species on both sides of the border. One of the principal research areas in the field of marine larviculture is to replace or reduce the use of live prey, which is labour-intensive and expensive, with microdiets tailored to the nutritional requirements and digestive capacity of the larvae of the species of interest. The development of adequate microdiets to replace live foods requires a thorough understanding of the digestion processes occurring during ontogeny. Thus the objectives of this study were to utilize a multidisciplinary approach to asses the proteolytic capacity of larvae using biochemical and histological analysis and to evaluate the efficiency of various microdiets and weaning protocols to support adequate growth and survival of California halibut larvae.

Materials and methods

Eggs were collected from natural spawns of California halibut broodstock held in the California Halibut Hatchery, Redondo Beach, CA, USA. Newly hatched larvae were reared either at the Fish Nutrition Laboratory of the Department of Aquaculture, C.I.C.E.S.E., Ensenada, México, or at the Center for Aquatic Biology and Aquaculture (CABA), University of California.

Characterizing protease activity
Newly hatched larvae were reared in a semi-closed recirculating system and fed HUFA-enriched rotifers (5.ml^{-1}) from hatching until nineteen days post-hatch (dph) and HUFA-enriched *Artemia* nauplii (5-7.ml^{-1}) thereafter. Total and specific activity of trypsin and leucine-aminopeptidase and acid and alkaline proteases activities were assessed throughout development (until 44dph) using spectrophotometry (Erlanger et al., 1961; Appel, 1974; Sarath et al., 1989).

Experimental microdiets
This trial was performed to evaluate three microdiets produced by different manufacturing techniques. Briefly, following hatching, 2000 larvae were stocked in a 150-l round fibreglass tank at a density of 13 larvae.l^{-1}. Larvae were fed HUFA-enriched live prey from first-feeding (3dph) to 16dph and *Artemia* thereafter. The microdiets were fed in combination with *Artemia* from 20dph and larvae were completely weaned by 26dph. Two types of experimental microdiets were prepared: a microbound diet (MCB) using *Artemia* cysts as the protein source (Garcia-Ortega et al., 2003) and a spray-dried microbound diet (MED) using fresh fisheries products (Lazo et al., 2004). A commercial diet (ALH) was used as a reference treatment and a live-prey-only treatment as a control (CTR). Three replicate tanks per diet were used. Growth, mortality, and pigmentation of the larvae were used as response variables to evaluate the microdiets

Weaning performance
The last feeding trial was designed to evaluate the ability of California halibut larvae to respond to weaning performed at various stages of development. Weaning onto a commercially available microdiet was performed at 16, 26, 36, and 46dph. Larvae were stocked into replicate tanks where *Artemia* was replaced by the microdiet over a 3-d period. For each weaning period, growth, survival, and enzyme activity of alkaline and acid proteases and leucine-aminopeptidase were measured several days after weaning and compared to a control *Artemia*-fed group.

Results and Discussion

Characterizing protease activity
Trypsin-like activity, leucine-aminopeptidase, and alkaline protease activities were detected shortly after hatching and before the opening of the mouth. Acid protease activity was not detected until 36-40dph, concomitant with the development of the gastric glands. The specific activity of trypsin-like enzyme and leucine-aminopeptidase showed two distinct peaks at 8 and 20dph. The second peak coincided with the shift from rotifers to *Artemia*. Hence, newly hatched California halibut larvae possess alkaline proteolytic activity before first-feeding.

Experimental microdiets

California halibut larvae accepted the experimental diets after several days of co-feeding (observed under a microscope) and were completely weaned by 26dph. Growth in terms of standard length, wet weight, and dry weight was significantly ($P<0.05$) lower for the microdiets compared to the CTR treatment, but no significant differences among them were found. However, significantly higher mortality was found for treatments ALH (n=482) and MCB (n=683) compared to MED (n=282) and CTR (n=23) treatments. Based on visual observations, larvae fed the microdiets had lower ingestion rates compared to the live-prey-fed larvae, which would explain the lower growth and survival observed for these treatments. Interestingly, the CTR treatment produced a significantly higher percentage of albinism (68%) compared to the microdiets (27%)

Weaning performance

Survival was significantly ($P<0.05$) lower in all the microdiet-fed treatments. Growth was significantly reduced in all weaning treatments compared to the control except for the 46dph group. The better performance at the later date has been reported for other flatfish (Bengston et al., 1999). No differences in alkaline proteases and aminopeptidase specific activity were detected between microdiet and *Artemia*-fed larvae at 16 and 26dph, but the specific activities were higher for the microdiet-fed larvae at 36 and 46dph. No acid proteases activity was detected for the 16 and 26dph groups. No differences in acid proteases specific activity was recorded between the larval groups fed microdiet or *Artemia* at 36 and 46dph. Additionally, low ingestion rates of the microdiet were observed when the larvae were weaned at 16 and 26dph.

Conclusions

Based on the digestive capacity evaluated in this study, the timing of the development of the functional stomach and the weaning performance trial, we propose that California halibut can be adequately weaned to formulated diets before completion of the metamorphosis around 36dph. The lack of weaning success at an early date cannot be entirely due to the absence of a functional stomach or the lack of proteolytic digestive enzymes but could be related to low ingestion rates. More research is needed to increase ingestion rates of the microdiets in other to provide sufficient nutrients for the fast growing larvae. We suggest the use of protein hydrolysates of marine organisms, such as krill hydrolysates, to increase ingestion rates (Kolvkoski et al., 1997). In addition, a longer co-feeding period is suggested to increase acceptance and digestion of the microdiets.

Acknowledgements

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ENZYME ACTIVITY AND mRNA LEVELS DURING EMBRYONIC DEVELOPMENT AS MARKERS OF EGG QUALITY IN SPOTTED WOLFFISH

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Introduction

In aquaculture, good-quality eggs are defined as those exhibiting low mortalities at fertilization, eyeing, hatching, and first-feeding. In new emerging aquaculture species, egg quality variability is frequently encountered. It has been demonstrated that larval performances are dependent on egg quality (Kjorsvik et al. 2003). In addition to inheritance of desirable reproductive traits in individual broodfish, egg quality is affected by environmental and rearing conditions. We propose to further investigate physiological and biochemical changes during embryonic development and first-feeding to better define those parameters that correlate with egg viability. On practical grounds, the use of early indicators of egg quality could allow early detection of "faulty" egg batches.

Yolk composition and selected enzymes have been correlated with egg viability in a wide range of species (Lahnsteiner et al., 1999; 2001; 2003; Carnevali et al., 2001). Taking into consideration that 1) oocyte developmental competence is the ability to be fertilized and complete normal embryonic development; 2) the early stages of development in fish are characterized by high growth rates; and 3) that this capacity relies on well-tuned enzymatic systems involved in digestion and protein synthesis (Lemieux et al., 2003), we propose to 1) measure the level of activity of selected digestive and metabolic enzymes (EA) and 2) quantify selected messenger RNA during the embryonic development of spotted wolffish using egg batches of different quality. Spotted wolffish is an aquaculture species of interest in Québec, Canada (Le François et al., 2002). Large egg size (8mm) compared to other marine fish, enables us to measure several parameters on a single individual egg.

In an earlier experiment conducted by Tveiten et al. (in progress) using the same experimental fish, a significant positive relationship between egg survival until

hatch and start-feeding success was found. Survival until hatch explained as much as 55% of the variation in start-feeding survival. Our experiment investigated the potential of improving this estimate based on EA and the quantifying functional and structural proteins expression in fertilized spotted wolffish eggs.

Material and methods

Spotted wolffish eggs were obtained from a captive broodstock reared at the Troms Steinbit AS fish farm (Senja, Norway). Fertilized eggs were obtained from 12 families and incubated. During development, egg samples from each spawn were taken (n=5) at 110d post-fertilization (~730dd°) (hatching=1000dd°) and kept at -80°C until analysed. Survival was monitored as a measure of egg quality. A range of qualities was induced by injection of 17,20 ß-P during ovarian maturation to a group of spawning females and compared to a control group sham-injected.

Enzyme activities were measured on individual eggs. All assays were performed in duplicate and activity results were expressed in U.g egg^{-1} and in U.g $protein^{-1}$, where one U (units) represents one µmol substrate transformed per minute (µmol.min^{-1}). The enzymes measured are the following: PK, LDH, AAT, TRY, and HOAD. A portion of the homogenate was refrozen at -80°C for protein determination and RNA/DNA and mRNA quantification. Nucleic acid content and relative quantification of mRNA myosin and trypsin using RT-PCR techniques are in progress.

Results and discussion

A positive relation exists between survival at eyed egg stage, hatching and first-feeding (Figure 1a,b). Survival rate is markedly different between families and ranged from 9.4 to 50.7% (figure 2). Descriptive statistics of EA are presented in Table 1 (expressed in U mg $prot^{-1}$). Statistical analysis indicates no significant relation between individual EA and survival at any period (survival to eyed-egg, at hatch or at first-feeding). However, when more enzymes are used, a significant regression is found (survival at hatch = 31.5 – 4.16 LDH – 4.25 PK + 37.6 HOAD). The weakness of the relation ($R^2 = 0.238$) suggests that the sole utilization of EA during embryogenesis to predict survival at hatching is unlikely to be sensitive and precise. mRNA quantification and expressions of EA in U.mg DNA^{-1} or RNA^{-1} might be more reliable egg quality indicators.

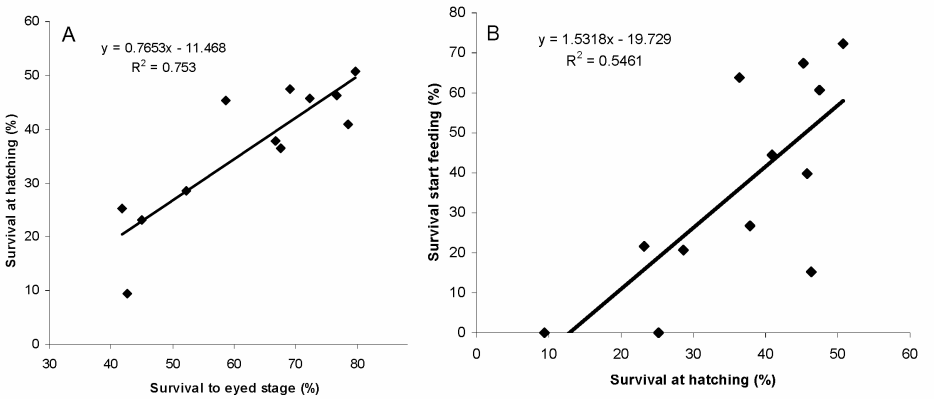

Fig. 1. A) Survival at hatching (%) in relation to survival to eyed egg stage; B) survival at first feeding in relation to hatching rate (%) in 12 families of spotted wolffish of variable quality.

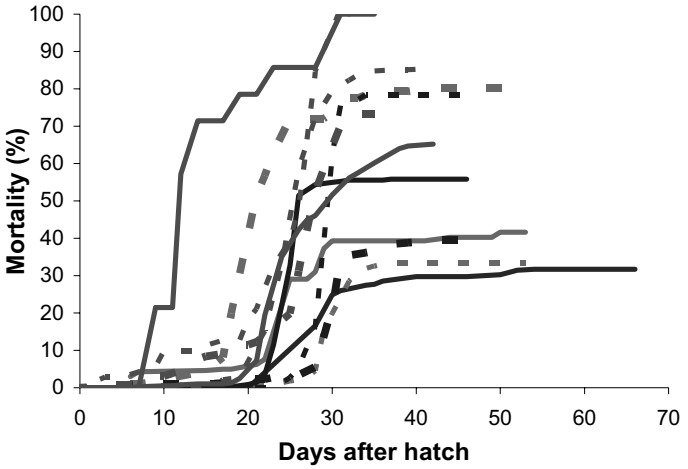

Fig. 2. Post-hatched cumulative mortality (%) of 12 families of spotted wolffish of variable quality.

Table I. Descriptive statistics of enzymatic activities and survival rate at hatching (SHACH).

	TRYP	PK	HOAD	AAT	LDH	SHACH
N of cases	60	60	60	60	60	60
Minimum	0.21	3.53	1.23	3.98	7.00	9.40
Maximum	0.44	12.17	2.28	7.76	13.91	50.75
Mean	0.34	5.88	1.91	6.06	10.09	36.43
Standard Dev	0.06	1.61	0.26	0.91	1.51	12.02
Coefficient of variation	0.16	0.27	0.14	0.15	0.15	0.33

A parallel must be made with work on *Anarhichas lupus*, a closely related species, that associated higher levels of metabolic or digestive EA in juvenile fish on hatching day to superior survival and growth performance during first-feeding (Lamarre et al., 2004). A cumulative post-hatch mortality of 0.5% corresponded to 31, 16, 40, 38% higher EA for TRY, AAT, LDH, and PK, respectively, measured on hatching day compared to low quality eggs (34% mortality). Carnevali et al. (1999) reported that mRNA of cathepsin D and activity were highest in non-viable eggs in *Sparus aurata*. We suggest that the use of EA as a predictive method for quality and performance assessment might be restricted to post-hatch and first-feeding stages.

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QUALITY OF HATCHERY-REARED JUVENILES FOR MARINE FISHERIES STOCK ENHANCEMENT

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The potential for stock enhancement by release of hatchery-reared juveniles continues to be a topic of interest to researchers and fisheries managers. While in many studies the focus has tended to be on the technology for production of juveniles, the need for a more multidisciplinary approach is now becoming accepted. Ideally, this includes studies of population dynamics and recruitment-limitation of wild stocks, environment-stock interactions, habitat availability, genetic studies of wild and released stocks, and integration with appropriate fisheries management.

While it may be relatively straightforward to culture large numbers of seed animals, the quality of hatchery-reared juveniles may limit the effectiveness of any release programme. The quality of juveniles may be defined either by their ability to attain the age and size to recruit to a commercial fishery or their fitness to survive to contribute to the spawning stock. Many factors will inevitably influence batch-batch variability in the viability of hatchery-reared juveniles and their ability to compete in the wild. Selection of broodstock can represent a bottleneck and such issues as variance in parental and family contributions and impacts on effective population size must be given careful consideration, using sufficient numbers of animals that reflect the genetic identity of the stocks to be enhanced. In addition, environmental conditions and husbandry practices within the hatchery as well as broodstock and larval nutrition can all fundamentally influence the quality of offspring. Further conditioning and/or selection during nursery culture may also be critical in maximising the physiological and behavioural fitness of wild juveniles post-release.

Although evaluation of long-term performance of individual batches of juveniles requires considerable effort or may be impossible in some cases, this type of quantification is likely to be an important component in the determination of the

effectiveness of release programmes. This paper reviews effects of hatchery and nursery practice on larval and juvenile fitness for stock enhancement and presents examples of comparisons of quality of wild and hatchery-reared juveniles and effect of pre-release conditioning on subsequent survival and growth.

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FOOD VALUE OF CYCLOPOID COPEPOD, *PARACYCLOPINA NANA* FOR FLOUNDER *PARALICHTHYS OLIVACEUS* LARVAE

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Introduction

In marine finfish aquaculture, the use of copepods as live food for larvae not only increases growth and survival of the fish larvae (Doi et al., 1997; Payne et al., 2001) but also reduces malpigmentation (McEvoy et al., 1998), compared to larvae reared with rotifers and *Artemia*. The objective of this study was to evaluate growth, survival, and malpigmentation rate of flounder (*Paralichthys olivaceus*) larvae reared with the following diet combinations: 1) *Paracyclopina nana* nauplii, 2) rotifers, 3) *Artemia* nauplii, 4) *P. nana* nauplii and rotifers (1:1), and 5) *P. nana* adults and *Artemia* nauplii (1:1).

Materials and methods

The study focused at two different feeding stages; 1-12dah (rotifer feeding stage) and 14-30dah (*Artemia* feeding stage). Either *P. nana* nauplii, *P. nana* adults, rotifers, *Artemia* nauplii, or a combination of two of these prey (see below) were used as live feed. *P. nana* was cultured with *Tetraselmis sueci,* and rotifers and *Artemia* nauplii were enriched with Super Selco.

For the rotifer-feeding stage, the day before hatching, 1200 eggs were transferred to a 50-l rear tank (working volume 40 l) and kept at 18±0.5°C. Larvae were fed with either *P. nana* nauplii (C), 50% copepod nauplii and 50% rotifer (CR), or rotifer (R) in triplicate tanks. Every three days starting from hatching, 30 larvae were sampled for the measurement of the standard length and dry weight (DW). Larval survival was determined at 13dah.

For the *Artemia*-feeding stage, 14-day-old larvae were transferred to 25-l experimental tanks (working volume 10 l) and kept at 18±0.5°C. Larvae were fed with either copepods (C4 stage adults) (C), 50% copepod adults and 50% *Artemia* (CA), or *Artemia* (A) in triplicate tanks. Every two days, five larvae were

sampled for standard length and dry weight measurements. Metamorphosis and malpigmentation rates were measured on 30dah.

Fatty acid profiles of live food organisms fed to *P. olivaceus* larvae were also analyzed. Growth, survival, pigmentation characteristics and fatty acid profiles were analyzed using one-way ANOVA. Differences between means were compared using Tukey's test.

Results and discussion

Most marine fish species require the essential fatty acids such as EPA and DHA for their growth and survival and it is important to supply these fatty acids to fish larvae for successful production (Sargent et al., 1999; Payne and Rippingale, 2000). In this study, during the rotifer-feeding stage, survival and growth of the flounder larvae fed with *P. nana* nauplii were significantly higher than larvae fed with rotifers (Table I).

Table I. Survival and growth of flounder *P. olivaceus* fed different diets from 1-12dah.

Diets	Survival rate (%)	Total length (mm)	Width (mm)	Dry weight (mg)
R	20.0 ± 2.23^{a}	5.5 ± 0.06^{a}	1.5 ± 0.03^{a}	0.12 ± 0.001^{a}
CR	28.5 ± 2.41^{ab}	5.9 ± 0.09^{b}	1.7 ± 0.06^{b}	0.13 ± 0.002^{a}
C	33.2 ± 1.74^{b}	6.3 ± 0.07^{c}	1.9 ± 0.05^{b}	0.17 ± 0.007^{b}

In *Artemia* feeding stage, flounder larvae fed with *P. nana* adult and *P. nana* adults + *Artemia* nauplii had significantly better growth, pigmentation rate, and metamorphosis rate than larvae fed with *Artemia* alone on 30dah. However, no significant difference between three treatments was found in survival of flounder larvae on 30dah (Table II).

Table II. Growth, survival, malpigmentation rate, and metamorphosis rate of flounder *P. olivaceus* fed the different diets from 14-30dah.

Diets	Total length (mm)	Width (mm)	Dry weight (mg)	Survival rate (%)	Malpigmentation rate (%)	Metamorphosis rate (%)
C	16.7 ± 0.15^{b}	9.0 ± 0.12^{b}	6.6 ± 0.20^{b}	58.0 ± 3.59^{a}	3.2 ± 1.99^{a}	86.7 ± 1.94^{b}
CA	16.4 ± 0.12^{b}	8.6 ± 0.12^{b}	6.6 ± 0.10^{b}	62.3 ± 3.80^{a}	8.9 ± 1.03^{a}	81.4 ± 4.43^{ab}
A	15.1 ± 0.18^{a}	7.7 ± 0.13^{a}	5.0 ± 0.23^{a}	55.1 ± 1.03^{a}	18.2 ± 2.00^{b}	65.2 ± 3.50^{a}

n-3 HUFA content of *P. nana* nauplii and adult was sufficient for normal growth of marine fish larvae with 3.5% and 4.8%, respectively (Table III).

Table III. n-3 HUFA contents (% dry weight) of organisms fed to *P. olivaceus* larvae

Food organisms	EPA	DHA
Rotifers	0.9±0.11[a]	0.9±0.03[b]
Artemia	1.4±0.02[b]	0.3±0.06[a]
P. nana (nauplii)	0.6±0.13[a]	3.5±0.11[c]
P. nana (C4~adult)	0.5±0.02[a]	4.8±0.07[d]

McEvoy et al. (1998) reported that pigmentation of marine fish larvae is affected by the essential fatty acid content. Results from our study showed that the HUFA content of *P. nana* would be sufficient for successful pigmentation and metamorphosis of flounder larvae. Our results indicate that the brackishwater cyclopoid copepod, *P. nana* is an effective live food organism for larval production of flounder *P. olivaceus*.

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A COMPARISON OF THE EFFICACY OF DISINFECTANTS FOR CULTURED YELLOWTAIL KINGFISH (*SERIOLA LALANDI*) EGGS

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Introduction

Production of farmed *Seriola* spp. (amberjacks and kingfish) is a developing industry worldwide, valued in excess of USD$3.8M p.a. (FAO, 2002). In New Zealand, production of the yellowtail kingfish, *S. lalandi* is in its infancy, but is recognised as having substantial growth potential. The availability of high quality eggs obtained from captive broodstock is considered a potential bottleneck to New Zealand's kingfish production, and research is therefore being directed towards addressing this.

Disinfection of eggs has a role in the maintenance of egg quality in two areas: the prevention of vertical transmission of pathogens (Hirazawa et al., 1999); and the treatment of opportunistic pathogens during incubation (Tendencia, 2003). At the NIWA Bream Bay Aquaculture Park, kingfish eggs are routinely disinfected by a brief immersion treatment in chloramine T (Sigma) prior to their transfer to hatchery facilities. The present study was undertaken to assess the effectiveness of this protocol and to compare the relative efficacy of some other commercially available products for egg disinfection.

Materials and methods

Eggs were collected using a skimmer and upweller following non-induced, tank-based spawnings. Spawning occurred approximately 18h prior to collection and embryonic development was at the gastrula stage. Following collection, eggs were rinsed in 1-µm filtered, UV-sterilised seawater for several minutes. Aliquots of 45 000 eggs were then placed in 2.5 l filtered seawater in 50-l upwelling plastic tanks, and disinfectant treatments conducted as detailed below.

Treatments used were: formalin (100ppm formaldehyde), an iodophor, povodine-iodine (Betadine® 100ppm iodide), hydrogen peroxide (100ppm), chloramine T (50ppm) and Virkon® (Antec Ltd., Suffolk, UK; 0.05% w/v). Con-

trol tanks received no disinfectant treatment. After treatment for 15 minutes, water flow to the tanks was commenced at 5 $l.min^{-1}$ for 10min, and then reduced to 1 $l.min^{-1}$ for the duration of the experiment. Tanks were lightly aerated, and water temperature maintained at 23±1°C using 300W immersion heaters.

Samples for bacterial enumeration were first collected after the completion of treatment, immediately the tanks reached 50 l volume (0h), and then at 6, 12, 24, and 43h. Triplicate 100-µl samples were collected from each tank, briefly vortexed and serially diluted, and plated on marine agar and TCBS medium, for counts of total bacteria and presumptive *Vibrio* spp., respectively. Plates were incubated at room temperature for 48h prior to counting. Hatch rate was determined at 43h post-treatment, and the developmental status of non-viable eggs was also assessed at this time. The effects of disinfectant treatment on hatch rate and bacterial numbers were analysed by one-way ANOVA following arcsin and log transformation respectively.

Results and discussion

Hatching rate varied considerably between both disinfectant treatments and duplicate tanks (Table I). However, in the present study, none of the disinfectants were effective, at the doses tested, in increasing hatch rates of kingfish. Other studies of egg disinfection have demonstrated some increased survival to hatch, following immersion treatment of grouper, *Epinephelus coioides,* eggs with iodophor (Tendencia, 2003). In contrast, studies of spotted halibut, *Verasper variegatus* and red sea bream, *Pagrus major* using iodophor, hypochlorite and hydrogen peroxide produced more equivocal results (Hirazawa et al., 1999).

Table I. Hatching rate of duplicate tanks of yellowtail kingfish eggs following a 15-min disinfectant treatment.

disinfectant	**control**	**chloramine T**	**formalin**	**hydrogen peroxide**	**iodophor**	**Virkon**
dose	-	50ppm	100ppm	100ppm	100ppm	0.05%w/v
hatching rate	20.2	53.7	18.7	21.7	0.0	0.0
(%)	24.0	23.8	89.0	92.2	0.0	0.0

Elevated mortality of developing embryos, such as occurred following treatment with iodophor and Virkon in the present study, has been reported in similar studies of a variety of finfish species (Hirazawa et al., 1999; Tendencia, 2003; Peck et al., 2004). Similarly, elevated rates of developmental abnormalities have been recorded following disinfected treatment in spotted halibut (Hirazawa et al., 1999). Both the timing of treatment and dose rate have been shown to be determining factors in the production of normal larvae (Hirazawa et al., 1999; Tendencia, 2003; Peck et al., 2004). Although the disinfectant dose rates used in the present study had been previously demonstrated not to cause immediate mor-

tality (P. Lee and S. Pether, unpublished data); operational constraints at Bream Bay Aquaculture Park limited the time at which initial treatments could be applied. Timing may be a factor in the observed results with iodophor and Virkon treatment, as the majority of eggs developed to an advanced embryo stage, before failing to hatch. The effects of the timing of disinfectant treatment on hatch rate and on the incidence of developmental abnormalities in kingfish are yet to be investigated.

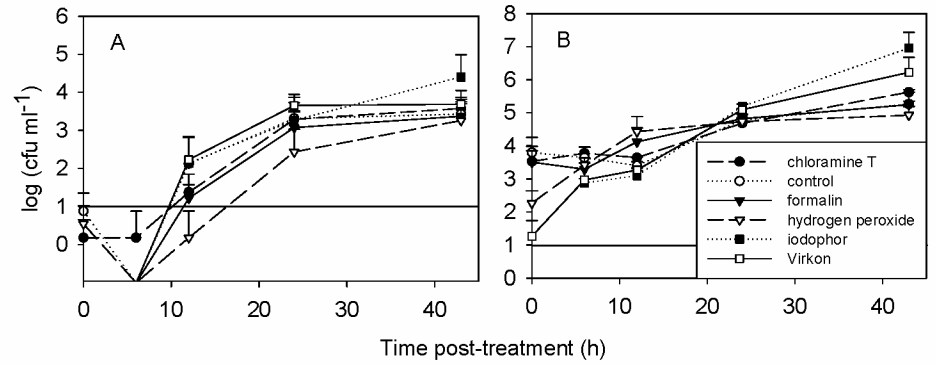

Fig. 1. Bacterial counts (cfu.ml^{-1}) of A) *Vibrio* spp. and B) total, from 0-43h post–treatment with disinfectants as shown. Data are mean + range for duplicate tanks. Horizontal lines indicate detection threshold.

Counts of presumptive *Vibrio* increased substantially in the period 12-43h after treatment, while total bacterial numbers had increased 100-fold by 12h (Fig. 1) and there were no effects of the disinfectant treatments on either total or presumptive *Vibrio* counts at any of the sampling periods. This contrasts with other studies (Hirazawa et al., 1999; Tendencia, 2003) where bacterial loads were significantly reduced at some point after disinfection. These differences may be due, in part to the effects of handling and rinsing eggs after transfer (Hirazawa et al., 1999; Tendencia, 2003).

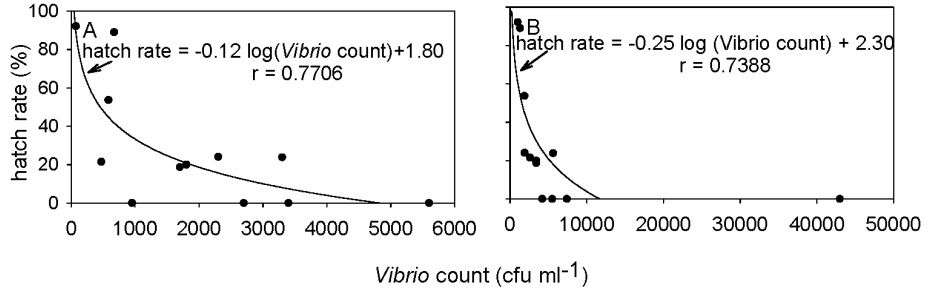

Fig. 2. Relationship between kingfish hatch rate and presumptive *Vibrio* count at A) 24h and B) 43h post-treatment with disinfectants.

There were significant relationships between hatch rate and presumptive *Vibrio* counts at 24 ($r=0.77$, $p<0.05$) and 43h ($r=0.74$, $p<0.05$) after treatment (Fig. 2) for data pooled across all treatments. This relationship suggests that a further reduction in bacterial loads, by repeated treatments, may enhance hatching success and further studies are planned to investigate this.

Conclusions

This study has demonstrated that the current practice of routine disinfection offers few benefits in terms of hatching success. However, the practice does not have a detrimental effect on egg survival and does maintain a level of biosecurity against vertically transmitted pathogens or the incidental transfer of pathogens from broodstock to hatchery areas and, for these reasons, may be regarded as a sound commercial practice.

Acknowledgments

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EFFECTS OF DIFFERENT WATER TEMPERATURES DURING EARLY STAGES ON THE DEVELOPMENT OF DEFORMITIES IN ATLANTIC COD (*GADUS MORHUA* L.) AND ATLANTIC HALIBUT (*HIPPOGLOSSUS HIPPOGLOSSUS* L.)

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Research on causes of deformities in farmed fish has been a priority for AKVAFORSK for many years with emphasis on Atlantic salmon, rainbow trout, and Atlantic halibut. Different deformities still cause problems in the farming of many fish species. Increased mortality, decreased growth rates, and downgrading at slaughter are often experienced in deformed fish, causing major economic losses. In addition, high frequencies of deformities are an important ethical issue for the industry which will probably become even more important in the future.

Most of the knowledge about environmental effects on the development of deformities in coldwater species has so far been gained from Atlantic salmon and Atlantic halibut. Considering the very different environments of these two species, there are still many similarities between environmental effects in salmon and halibut. Factors like water temperature, salinity, water current, etc. are likely to affect the development of important organs in several species. Temperature has proven to be a highly potent causative factor for induction of deformities both in salmon and halibut. In halibut, the frequency of deformities is highly correlated with temperature during the yolk sac stage. In an experiment where the larvae were incubated at 4, 6, 8, 10, or 12°C, the frequency of normal larvae was significantly higher at the 4°C compared to all other temperatures. Survival during the yolk sac stage was not affected by temperature when kept below 12°C. In another experiment the temperature was raised from 4°C to 8 or 10°C at different ages after hatching. It was demonstrated that the halibut larvae were most susceptible to develop deformities when the temperature was raised within the first three weeks post hatching. Thereafter there was no negative effect of increasing the temperature to either 8 or 10°C. About three weeks after hatching most major structure are developed in halibut. Gaping jaws and yolk sac oedema were the most common deformities observed in these experiments.

In a preliminary experiment on cod, one group of eggs from each of Arctic and Atlantic cod were incubated at three different temperatures (4, 8, and 12°C) until first feeding and thereafter first-fed at 8°C. Another group of Atlantic cod eggs from the same batch as the first experiment were incubated at 4°C until first feeding and thereafter first-fed at three different temperature regimes (8°C, 12°C, and a gradual increase from 6-12°C, 4 replicates per treatment). There was no significant effect of incubation temperature on survival during egg incubation or frequencies of deformed larvae at hatching and 415 day-degrees post first-feeding. When larvae were first fed at different temperature regimes, the highest frequencies of normal larvae was obtained when the temperature was gradually increased from 6 to 12°C compared to 8 or 12°C during the entire first-feeding period. Larvae first-fed at 12°C showed significantly higher frequencies of skeletal deformities than larvae fed at the two other temperature regimes. Oedema was most frequently observed at 8°C and lowest at 12°C. This was the most frequent deformity observed in this experiment. There was no significant difference in growth between the three temperature regimes up to 415 day-degrees post first-feeding. This experiment was terminated at 415 day-degrees post first-feeding due to problems with the water quality.

Result from an ongoing experiment with cod where effects of three different incubation temperatures (4, 6, and 8°C) and three different temperature regimes during first-feeding (8°C, 12°C, and a gradual increase from 6-12°C) are investigated and will be presented. Results from staining of bone structures in halibut juveniles raised at different temperatures (7, 12, and 13°C) will also be presented.

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BACKYARD HATCHERIES FOR FISH AND SHELLFISH – THE ASIAN PHENOMENON

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With an average fish consumption per capita of over 20 $kg.yr^{-1}$, fishery products are a major and valuable part of the diet for people in many parts of subtropical and tropical Asia. Aquaculture now represents a large and rapidly growing proportion of total fishery production and is characterized by its extreme biodiversity in terms of food production – literally hundreds of species are being reared in freshwater, brackish water, and marine environments. Nevertheless a core group of freshwater species including common carp, Chinese and Indian carps, various tilapias species and their hybrids, snakehead, *Pangasius* and *Clarias* catfish, and *Macrobrachium* prawn make up most production. Saltwater production is dominated by penaeid shrimp, milkfish, sea bass, groupers, and mollusks (mussels, clams, cockles, abalones, oysters). Consistent seed supply for these species has supported the development and growth of their culture and has evolved from a reliance on wild catch to domesticated hatchery production. Presently, with few exceptions of marine species such as eels, tuna, crabs, and lobsters, virtually all others can be reproduced in hatcheries. In general, hatchery development requires suitable environmental conditions, appropriate production technologies, skilful human resources, investment capital, and market demand. The scale of hatchery operations ranges from small backyard producers to large corporate enterprise, but the former appear to dominate the sector. Backyard hatcheries are characterized by their low-cost, low-tech, and often family-based operations. In order to share resources, knowledge, and markets, backyard hatcheries are often geographically clustered

The rapid development of inland aquaculture in South and Southeast Asia over the last three decades reflects past investment in developing the capacity of the private sector to produce fish seed. Fish seed production and marketing systems for backyard hatcheries are diverse typically linked to food fish farmers through complex networks of intermediaries. Both production and demand for seed is highly seasonal dictated by climatic conditions with grow-out cycles. Several types of consolidation and division of labour appear to be underway as the sector matures. Large-scale hatcheries are emerging for species such as marine shrimp,

groupers and abalones that require costly maintenance of broodstock and sophisticated spawning/hatchery techniques, or for species such as tilapia that requires large pond facilities and for which economies of scale are important. Meanwhile, backyard operations assume downstream operations including egg hatching and larval rearing, early or advanced nursing. This staged seed production system complements financial and expertise requirements among producers. Concentration of enterprises, particularly those nursing seed, continue to dominate production in many areas as physical proximity appears to favour information flow between producers and potential customers, but improved and affordable infrastructures (roads, transportation, telecommunications) open opportunities for seed production local to new centers of aquaculture. A vibrant private sector is increasingly unaffected and independent from the public sector, however, despite clear potential benefits of closer association and information exchange. These issues are assessed and discussed in the light of likely future trajectories for inland aquaculture.

In Asia, backyard hatcheries still play a predominant role in seed production for many species, and their value-added, knowledge-driven activities also contribute significantly to employment opportunities and livelihoods in rural areas. However, such household-level operations may lack capacity for controlling and improving seed quality and backyard operators may also be less aware of issues related to emerging bio-security and environmental concerns. This raises opportunities for strategic business alliances between larger and smaller enterprises in the future.

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AN ORAL NNV VACCINE AND ITS EFFICACY TEST IN THE LARVAE OF MALABAR GROUPER (*EPINEPHELUS MALABARICUS*)

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Nervous necrosis virus (NNV) is a serious pathogen that infects grouper and other fish in the larval stage. This infection often causes mortality rates higher than 90% and total loss to the hatchery, hence, it is important to develop a vaccine for its prevention. However, with the early onset of this disease at the larvae stage, small size of fish larvae and sensitivity to handling make the vaccination by injection or immersion impossible. Oral vaccination is generally believed to be the most desirable method because it is non-stressful for fish, user-friendly for farmers, and permits mass vaccination. Among several oral vaccination methods that have been developed, bioencapsulation of killed pathogenic bacteria in live *Artemia* appears as one of the most desirable options, where the antigen is encapsulated in the natural starting feed (*Artemia*) of the larvae to facilitate antigen uptake, and since the antigen is encapsulated it will not contaminate the delicate larval-rearing environment. Unfortunately, since *Artemia* only eat bacteria, this method can only be used to develop bacterial vaccines, but not NNV vaccines. In addition, protective immunity of this oral vaccine has not been demonstrated.

In this report, we described an oral vaccine method in which the viral antigen gene was cloned and expressed in the bacteria, which were then fed to the *Artemia*. To test the efficacy of this oral vaccine, grouper larvae were vaccinated using *Artemia* encapsulated with *E. coli* comprising recombinant NNV VP protein. We found by immunohistochemical observation that antigen delivered with this method could avoid digestion and subsequently be absorbed at the hindgut of grouper. The anti-NNV VP specific antibody in the vaccinated larvae increased 7 days after vaccination as assayed by ELISA; fourteen days after vaccination, the vaccinated larvae were challenged with NNV at a dosage of 10^7 per fish (LD_{50}), and the survival rates of control and vaccinated groups were 45% and 80%, respectively, giving a RPS of 63.6%. These results demonstrate this NNV oral vaccine is an effective way to immunize grouper larvae, and it could be expanded for the development of other oral vaccine.

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CRYOPRESERVATION OF FISH OOCYTES: ACHIEVEMENTS AND PROSPECTS

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The growth of intensive aquaculture requires efficient and effective methods to preserve gametes for higher flexibility in broodstock management, genetic improvement programs, and preservation of genetic diversity. While methods for cryopreservation of fish spermatozoa are well-known, preserving important maternally inherited genetic factors has yet to be achieved. Methods for cryopreservation of yolk-laden fish embryos remain elusive so far, mainly due to their relative large size, the presence of egg shell envelope structure, and their relatively high water content. Therefore, our main objectives were to develop technologies for cryopreservation of fish oocytes while ensuring their viability after cryogenic storage and thawing. Studies were performed on the zebrafish (*Danio rerio*; ZF) as a model for freshwater fish and the gilthead seabream (*Sparus aurata*; GSB) as a model for marine fish producing pelagic floating eggs.

Multidisciplinary studies included: (1) Investigations on the formation and structure of the egg shell envelopes as they may form a barrier for permeation of cryoprotective agents (CPAs) and exit of water during cryopreservation procedures. Four ZPs proteins were identified in the GSB; ZPBa, ZPBb, ZPC, and ZPX. The novel ZP isoform ZPX was highly expressed in the ovary while the other isotypes were highly expressed in the liver. The composition of the eggshell was analyzed and the antibodies generated against the specific isoforms will be used to determine if the eggshell is morphologically compromised by cryopreservation. (2) Studies into the process of oocyte hydration of pelagic

eggs, as it is envisaged as a major obstacle during oocyte cryopreservation. Investigations led to the discovery of a novel subfamily of water channels (aquaporins, AQPs), named AQP1o in marine teleosts. In GSB, the AQP1o is predominantly expressed in the ovary and appears to be involved in water uptake into the oocyte during meiosis resumption. These results have uncovered one of the key molecular events during oocyte hydration in marine fish. (3) Identification of specific biological markers to monitor oocyte viability after manipulation and/or cryopreservation, including; (a) Development of genomic markers through identification of ZF stripped oocyte transcriptome, using the Serial Analysis of Gene Expression (SAGE) technology. Oocyte integrity after cryopreservation was determined by using cellular repartition of selected segregated maternal mRNAs inside the oocytes. (b) Proteomic profiling of oocytes developmental stages generated protein profile catalogues for ZF and GSB oocytes, revealing changes taking place during oocytes maturation. A stage dependent shift in the pattern of cleavages and processing of vitellogenin (VTG) was found in oocytes and this pattern was unique to each of the studied species. (c) Biochemical markers included monitoring the expression and activity of cathepsins that are involved in VTG and yolk processing in ZF and GSB oocytes, during maturation from early to late vitellogenic stages. Stage dependent changes in the expression and activity of cathepsins B, D, and L were revealed for ZF and GSB oocytes. (4) Development of in vitro incubation procedures to promote oocyte maturation and ovulation after thawing of cryopreserved oocytes. The production of biological active GSB-rLH was initiated and results indicated that rLH significantly stimulated the secretion of E_2 in sliced GSB ovaries, the frequency of GSB oocytes undergoing GVBD (Germinal Vesicle Breakdown) in a dose dependent manner and significantly attenuated the occurrence of atresia among cultured oocytes. (5) Development of cryopreservation procedures for oocytes. The effect of permeating and non-permeating CPAs on viability of oocytes was assessed by vital stains [trypan blue (TB) and thiazolyl blue tetrazolium bromide (MTT)] and by the occurrence of GVBD in mature oocytes. Permeability of oocyte envelopes to CPAs was determined by uptake of ^{14}C-methanol and estimation of membrane permeability coefficients. Slow and fast cooling procedures revealed 4-17% survival in stage III ZF oocytes, after thawing from LN2, by TB staining. Survival after thawing from LN2 depended on oocytes size in the GSB, with ~80% survival of early vitellogenic oocytes by MTT staining, 2h after thawing. Only 5-8% of the mature oocytes survived briefly after thawing from LN2 and none survived after 2h of incubation. Genomic, proteomic and biochemical tools were used to determine the damage incurred by CPAs and cooling procedure.

These results signify the prospects and future directions that will be required for achieving viable oocytes after cryogenic storage. This study was EC project CRYOCYTE #Q5RS 2002-00784.

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EFFECT OF FEEDING ON STRESS RESISTANCE IN THREE SPECIES OF NEOTROPICAL FISH LARVAE

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Introduction

Success in aquaculture may be directly related to management and the type of food used, and more resistant or susceptible animals can be produced. Among the methods used to evaluate larval quality, the air exposure test has been utilized with animals reared under different management of food (Kraul et al., 1993; Kanazawa, 1997). This work had the objective of evaluating the effect of feeding on stress resistance (air exposure test) in larvae of three species of neotropical fish: oscar (*Astronotus ocellatus*), pacu (*Piaractus mesopotamicus*), and spotted surubim (*Pseudoplatystoma coruscans*).

Materials and methods

Larvae of different species were reared in tanks containing 100 l water, with continuous water flow and a temperature of 28.9±0.8°C.

Preliminary tests were conducted to determine the best air exposure times for each species. In those tests, the larvae were fed *Artemia* nauplii and were submitted at different ages to the following treatments: Test 1 – E_0 (without air exposure); $E_{0.5}$ - 30sec; E_1 - 1min; E_3 - 3min; E_5 - 5min; and E_7 - 7min air exposure on drying paper; and Test 2 – R_0 (without air exposure); $R_{0.5}$ – 30seg; R_1 – 1min; R_3 – 3min; R_5 – 5min; R_7 – 7min; R_{10} – 10min; R_{12} – 12min; R_{15} – 15min; R_{17} – 17min; and R_{20} – 20min air exposure on a sieve. Based on these results, the experiments were performed by testing different diets and feeding schemes.

Oscar larvae, 6dah, were submitted to three feeding treatments: *Artemia* nauplii, artificial microdiet (MD, Fry Feed Kyowa), or maintained on fasting. After feeding for the first five days (11 days of life), the larvae were submitted to test E_5.

At 5dah, the pacu larvae were submitted to the following feeding treatments: *Artemia* nauplii at increasing amounts, *Artemia* nauplii at increasing amounts on the first two days followed by mixed feeding (*Artemia* nauplii at decreasing

amounts plus MD), MD only, and larvae maintained on fasting. After treatment for three, six, and 10 days, the larvae were submitted to tests E_1 and R_{20}.

Spotted surubim larvae beginning exogenous feeding (3dah) received *Artemia* nauplii for three days. After this period, the animals started to receive different feeding treatments: *Artemia* nauplii, forage fish larvae (10 *Colossoma macropomum* larvae per surubim larvae), or MD. After treatment for three days, the larvae were submitted to tests E_5 and R_{20}.

In the stress resistance tests, the animals were separated in 1-litre beakers (between 10 and 15 larvae per beaker), which were maintained in a thermostatic bath system under constant aeration. The larvae were submitted to a fasting period of six to eight hours under these conditions. After this period, the animals were carefully placed onto drying paper (Test 1), or maintained on the sieve (Test 2). Each treatment consisted of three to four replicates.

After the corresponding air exposure periods, the animals were returned to the same beakers with aeration and their survival rate was recorded after 24 hours; this value was considered the Stress Resistance Rate (Re).

The experiments involving oscar and spotted surubim were conducted in a completely randomized design, and the results were analyzed by ANOVA. For pacu larvae, analysis in a subdivision scheme was used, where the main treatments were the different food items, and the secondary treatments were the larval ages at the time when the tests were conducted. Whenever significant differences between treatments were found ($P<0.05$), the means were compared by Tukey test at the 5% probability level. The Re percentage values were transformed to arcsine in the analyses.

Results and Discussion

The use of *Artemia* nauplii and MD for oscar larvae during the first five days of feeding provided greater Re values ($P<0.05$) when compared with fasting animals (Table I).

The Re results for pacu larvae after feeding for three days, at an exposure time of one minute on drying paper did not show differences ($P>0.05$) between treatments (Table II). However, after treatment for six or 10 days, larvae given MD or maintained fasting showed smaller Re values ($P<0.05$) than larvae that received *Artemia* or a mixed feeding. With regard to the age of larvae when the tests were applied, no Re differences were observed as the animals developed. In test R_{20}, the use of *Artemia* nauplii and a mixed feeding produced better results than other treatments. The use of MD and fasting showed a decrease in Re with time, demonstrating that the condition of the larvae deteriorated.

Table I. Mean Stress Resistance Rate (Re) values (±SD) for oscar *A. ocellatus* larvae submitted to the air exposure test applied for five minutes on drying paper (E_5). Different letters indicate significant differences (P<0.05) by Tukey test.

Treatments	Re (%)
Artemia	62.5 ± 10.4^{A}
Artificial microdiet	45.3 ± 12.5^{A}
Fasting	15.7 ± 7.8^{B}

Table II. Mean Stress Resistance Rate (Re) values (±SD) for pacu *P. mesopotamicus* larvae submitted to air exposure tests for one minute on drying paper (E_1) or for 20 minutes on a sieve (R_{20}). Different letters (capital on the vertical and small on the horizontal) indicate significant differences (P<0.05) by Tukey test between different food treatments, in different tests.

Treatments	E_1 – air exposure for 1min on drying paper		
	3rd day	6th day	10th day
Artemia	60.0 ± 36.0^{Aa}	96.6 ± 5.7^{Aa}	100.0^{Aa}
Mixed feeding	40.0 ± 43.5^{Aa}	76.6 ± 11.5^{Aa}	76.6 ± 23.0^{Aa}
Artificial microdiet	6.6 ± 11.5^{Aa}	10.0 ± 10.0^{Ba}	8.3 ± 7.6^{Ba}
Fasting	10.0 ± 10.0^{Aa}	6.6 ± 5.7^{Ba}	0.0^{Ba}
	R_{20} - air exposure for 20min on a sieve		
Artemia	93.3 ± 11.5^{ABa}	96.6 ± 5.7^{Aa}	96.6 ± 5.7^{Aa}
Mixed feeding	100.0^{Aa}	83.3 ± 15.2^{Aa}	80.0 ± 10.0^{Aa}
Artificial microdiet	63.3 ± 28.8^{Ba}	0.0^{Bb}	0.0^{Bb}
Fasting	38.7 ± 36.4^{Ba}	3.3 ± 5.7^{Bab}	0.0^{Bb}

Both tests on spotted surubim larvae demonstrated that forage fish larvae provided the best Re rates just three days after the food was changed (Table III).

Table III. Mean Stress Resistance Rate (Re) values (±SD) for spotted surubim *P. coruscans* larvae submitted to air exposure tests for five minutes on drying paper (E_5) or for 20 minutes on a sieve (R_{20}). Different letters indicate significant differences (P<0.05) by Tukey test, between different tests.

Treatments	E_5 – air exposure for 5min on drying paper
Artemia	60.0 ± 10.0^{B}
Forage fish larvae	93.3 ± 5.7^{A}
Artificial microdiet	3.3 ± 5.7^{C}
	R_{20} - air exposure for 20min on a sieve
Artemia	63.3 ± 5.7^{AB}
Forage fish larvae	76.6 ± 15.2^{A}
Artificial microdiet	46.6 ± 5.7^{B}

The air exposure test has been used by several authors to evaluate the quality of food on the physiological response of larvae to stress. Air exposure times of 30-120s on a sieve have been used for different fish species, and it has been observed that the quality of food during the larviculture affects larval resistance to

air exposure (Kraul et al., 1993; Kanazawa, 1997), in agreement with observations in this work for three neotropical species. However, the species studied in this work proved rugged and resistant, and thus require a much longer air exposure time to manifest the stressing effect. Luz (2004) exposed *Hoplias lacerdae* larvae on drying paper for 10 min; this time is much higher than the 1- and 5-min times that were applied to pacu and spotted surubim, and oscar larvae, respectively. The results highlight the importance of ideal exposure time determination for each species.

The tests employed in this work were effective to demonstrate the effect and importance of feeding on Stress Resistance in oscar, pacu, and spotted surubim larvae. Although lengthy, the test on a sieve for 20 minutes seemed less drastic than exposure on drying paper (1-5min), where the animals loss of protection mucus due to the contact with the drying paper.

Conclusions

The effect of previous feeding on larval resistance was observed for all species. Oscar larvae fed *Artemia* nauplii or MD showed similar Re. The same was verified for pacu larvae, which showed similar stress resistance values when fed *Artemia* or under mixed feeding (*Artemia* + MD) than others that received MD. For spotted surubim the use of forage fish larvae provided more resistant individuals than those treated with *Artemia* or MD.

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BENEFICIAL EFFECTS OF NUCLEOTIDES ON CRUSTACEANS: AXENIC *ARTEMIA* CULTURE AS A TEST CASE

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Introduction

Nucleotides are low molecular weight biological compounds with major roles in most biological processes. The significance of exogenous nucleotides in improving growth and health status has been demonstrated in salmonids (Burrells 2001a; Burrells 2001b). The present study used axenic *Artemia* cultures to 1) evaluate effects of nucleotides on growth and disease resistance in crustaceans, as well as 2) to value the use of axenic conditions in crustacean larviculture research.

Materials and methods

Axenic culture of *Artemia* was achieved by a modification of a technique described by Sorgeloos et al. (1986). 10g of cysts (EG grade, INVE Aquaculture NV, Baasrode, Belgium) were hydrated for 1h in 89-ml well-aerated tap water, followed by addition of 3.3ml sodium hydroxide (32%) to keep pH above 10. Then 50ml of sodium hypochlorite was added to initiate decapsulation which was monitored under a stereomicroscope until 80% complete and then stopped by adding 50 ml of sterile sodium thiosulphate. All reagents were cooled overnight to avoid damage to the embryos and hence poor hatching as the decapsulation process is exothermic. Decapsulated cysts were rinsed with autoclaved seawater (Instant Ocean, Aquarium Systems, Sarrebourg, France), put into sterile 50-ml falcon tubes (Becton Dickinson Labware, Lincoln Park, New Jersey, USA) containing 30ml of sterile seawater and then placed on a rotor with sufficient light and incubated for 24h. Hatched sterile *Artemia* nauplii were placed in sterile falcon tubes at a density of 1.5 nauplii.ml^{-1} and kept on a rotor at 25°C. All procedures were conducted in a laminar flow to ensure sterility. Xenic culture was achieved following a technique described by Sorgeloos et al. (1986).

Sterilised diet (Prolon®, Inve Technologies NV, Baasrode, Belgium) was supplemented with nucleotides (Ascogen®, Chemoforma, Augst, Switzerland) at 0%, 0.002%, and 0.005%. To determine effect on growth, a 6-day feeding trial was conducted after which individual body length was measured. Culture media and feed were plated every two days to check for contamination. Effect on disease resistance was determined by challenging four day old axenically cultured *Artemia* with 10^3 cells.ml^{-1} of *Vibrio proteolyticus* and survival measured after 24 and 48h with uninfected treatments serving as control. Rationale for axenic conditions was determined by comparing growth under both axenic and xenic conditions, included a treatment in which *Artemia* were starved. All treatments were carried out in triplicates with one-way ANOVA used to determine differences between treatments ($p<0.05$) and in case of significant differences Duncan's multiple range test was used for post-hoc analysis.

Results and discussion

Generally higher inclusion levels of dietary nucleotides resulted in improved growth and disease resistance. Ascogen® at 0.005% resulted in significantly higher growth, almost 18% better than the control (Fig. 1). Ascogen® provided fast multiplying cells with sufficient supply of nucleotides highly in demand hence sparing energy which would have been used for their synthesis (Quan, 1992; Borda et al., 2003). Also exogenous nucleotides might have improved feed uptake, absorption and utilisation due to enhanced intestinal morphology (Burrels, 2001a).

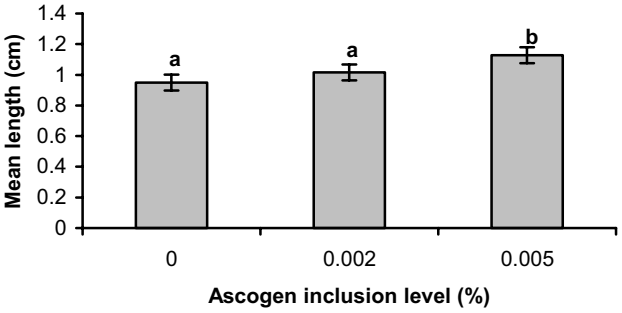

Fig. 1. Mean body length of axenically cultured *Artemia* after 6 days of culture. Error bars indicate standard error. Different letters above each bar denotes significant differences in length ($p<0.05$).

After 24h of challenge test, *Artemia* in all infected treatments showed signs of swimming difficulties but without any significant difference in survival (Fig. 2). After 48h, survival in the 0.05% Ascogen® treatment was significantly higher compared to the control. Survival in all uninfected treatments was higher than

80%. It was suggested that exogenous nucleotides are key nutrients for the immune system in shrimp (Devresse, 1999). They influence the activity of natural killer cells and macrophages (phagocytosis) which play a key role in the non–specific immune system. They also enhance gut function through rapid repair and an improved microbial flora (Burrels, 2001b).

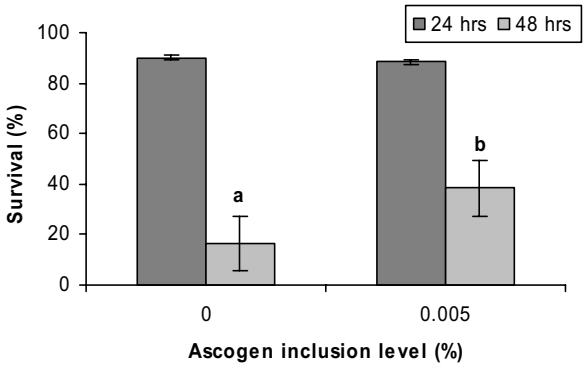

Fig. 2. Survival of axenically cultured *Artemia* after 24h and 48h challenge with *Vibrio proteolyticus*. Error bars indicate standard error. Different letters above each bar denotes significant differences in length ($p<0.05$).

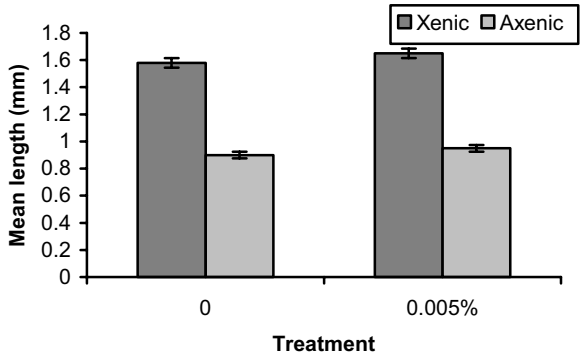

Fig. 3. Mean body length of *Artemia* cultured under xenic and axenic conditions. Error bars indicate standard error.

Growth performance under xenic conditions was more than 50% higher compared to axenic conditions (Fig. 3). *Artemia* starved under xenic conditions also survived for six days while those under axenic survived for three days only. Xenic conditions harbour microbial populations which increase nutritional value of feed (Verschuere et al., 1999). They synthesise nutrients from dissolved organic matter which are either released into the media or incorporated into microbial cells available for consumption by *Artemia*. This could explain the longer survival of starved *Artemia* in xenic culture. Microbial populations may also

prevent proliferation of opportunistic pathogens through release of chemical substances with bactericidal or bacteriostatic effects (Verschuere et al., 2000). These findings underscore the importance of eliminating microbial populations in evaluation of feed additives using an *Artemia* assay.

Conclusion

This study has shown that exogenous nucleotides can significantly improve growth and disease resistance in *Artemia*. These findings may have important application in larviculture of crustaceans particularly penaeid shrimps. However further studies should be done to understand the exact mode of action, dose – response relationship and duration of effects after intake. Axenic *Artemia* culture proved to be a valuable tool for testing immunostimulatory compounds.

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INFLUENCE OF DIETARY INULIN AND OLIGOFRUCTOSE AS PREBIOTICS FOR YOUNG TURBOT, *PSETTA MAXIMA* (LINNAEUS, C. 1758)

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Introduction

The main challenge in European turbot production is to improve feed formulation, in order to optimise growth and fish resistance through the development of health promoting diets. The introduction of probiotics in aquaculture to modulate the intestinal microflora has known encouraging results (Gatesoupe, 1999). However, the probiotic strains remain dominant in the gastrointestinal tract only during the dietary treatment. It is unlikely that exogenous administration of a single probiotic strain will result in long-term colonisation of the gut. Therefore, it is necessary to develop innovative strategies in order to establish a stable and healthy community of gastrointestinal microbiota in fish. The aim of this study is to examine the effects of dietary inulin (Raftiline ST) and oligofructose (Raftilose P95) on the growth of young turbot and on the microbial ecology of the digestive tract both at the early larval stage and from weaning stage onward.

Materials and methods

The larval rearing was conducted at IFREMER, Brest, France, according to the method described by Gatesoupe (1990).

The first experiment investigated inulin and oligofructose as prebiotics for turbot larvae (7 days post-hatch: dph). Larvae were fed for 14 days (three replicates per treatment) with *Artemia*, treated with 6 substrates including inulin at concentrations of 10 and 60mg.l^{-1} (diets S1 and S6, respectively) and oligofructose at concentrations of 10, 30, and 60mg.l^{-1} (diets P1, P3, P6, respectively).

In the second experiment, inulin and oligofructose were tested during 26 days as dietary prebiotics for turbot from weaning onward (29-55dph). Turbot larvae

received three experimental diets (three replicates per diet). Test diets contain 2% inulin (diet S) or 2% oligofructose (diet P). In the control diet (C), 2% cellulose is included.

In both experiments, mortality and growth were monitored. Bacterial flora was analysed by standard procedures and selected isolates were analysed for genotypic comparison by amplified ribosomal DNA-restriction analysis (ARDRA) as described by Gatesoupe (2002).

Results and discussion

In the first experiment, none of the treatments improved larval growth significantly. The survival rates (data not shown) recorded were high in all groups (including the control). Both inulin and oligofructose did however significantly increase ($P<0.01$) the bacterial load of the larvae (Table I). There was an overall dominance of *Vibrio alginolyticus* at the end of the experimental period.

Table I. Effect of prebiotics on microbiota in turbot larvae during the feeding on *Artemia* (bacterial counts: means±SE, with different superscripts for significantly different means, Kruskall-Wallis ANOVA on ranks, followed by Dunn's test; $P<0.01$). nd: not detected; dph: days post-hatch..

Group	11dph	25dph					
	Initial	C	S1	S6	P1	P3	P6
Petrifilm counts (log cfu.larva^{-1})	5.4±0.1^{ab}	4.7±0.3^{b}	5.3±0.2^{ab}	5.3±0.3^{ab}	3.6±1.8^{ab}	5.8±0.1^{a}	5.9±0.1^{a}
TCBS counts (log cfu.larva^{-1})	5.3±0.1	4.9±0.4	5.5±0.1	5.7±0.3	3.9±2.0	5.8±0.2	5.8±0.2
Vibrio sp. S12411[§] (%)	28	nd	33	nd	nd	nd	nd
V. alginolyticus P61224[£] (%)	16	94	67	100	100	100	100
Other *Vibrio* spp. (%)	42	nd	nd	nd	nd	nd	nd
Other bacteria (%)	14	6	nd	nd	nd	nd	nd

The identification was based on alignment with nucleotide sequences currently available in NCBI database; [§] accession numbers of a homologous fragment: AF293974, position of the first and the last nucleotides corresponding to the fragment in the referenced sequence: 43-605; [£] Accession number: AJ704375, sequence deposited for this study.

In the second trial, the growth rate (Table II) of larvae fed P diet was significantly higher than for the other groups ($P<0.01$). The bacterial load (Table III) was highly variable and no significant differences were observed between the groups. Before weaning (29dph) bacterial flora was dominated by *Vibrio* spp. which represented 96% of the isolates. At the end of the experiment (55dph), a dominance (>60%) of *Vibrio* spp. was still observed in the larvae fed the inulin (S) and control (C) diets. Although in the oligofructose group (P) still 41% *Vibrio* spp. are present, 14% of the isolates were identified as *Bacillus* sp.

L171P95. This species is closely related to *B. subtillis* KL-077 and KL-073 (Venkateswaran et al., 2003). Further in vitro experiments showed that this strain grew faster using oligofructose as single source of carbon, instead of glucose. This isolates inhibited in vitro growth of the pathogenic *V. anguillarium* strains isolated from turbot.

Table II. Effect of prebiotic administration on turbot at the weaning stage.

Treatment	Final mean weight (g)	SGR[1]	Survival rate (%)
C	0.41 ± 0.02^{a}	8.4 ± 0.4^{a}	88.6 ± 1.7
S	0.41 ± 0.02^{a}	8.4 ± 0.2^{a}	82.1 ± 1.8
P	0.50 ± 0.02^{b}	9.2 ± 0.2^{b}	87.4 ± 1.3

Values are means±SE; Mean weight, n=90; Specific growth rate and survival rate, n=3; different superscripts indicate significantly different means (Tukey's test; $P<0.01$)

[1]Specific Growth Rate = [(LnFW – LnIW)/t] × 100; FW: final mean weight for each replicate; IW: initial mean weight (46 ± 2mg); t = time (26 days).

Table III. Effect of prebiotics on intestinal microbiota in weaning turbot (bacterial counts: means±SE, Kruskall-Wallis ANOVA on ranks not significant, n=6). nd: not detected.

Group	29dph	55dph		
	Initial	C	S	P
Petrifilm counts (log CFU g^{-1})	3.2 ± 1.4	3.6 ± 0.3	2.8 ± 0.3	3.0 ± 0.4
TCBS counts (log CFU g^{-1})	3.1 ± 1.4	2.7 ± 0.9	3.8 ± 0.9	1.8 ± 1.2
Vibrio sp. S12411[§] (%)	nd	28	22	16
Vibrio sp. L2C55[£] (%)	nd	36	44	25
Other *Vibrio* spp.(%)	96	nd	nd	3
Bacillus sp. L171P95[#]	nd	nd	nd	14
Other bacteria (%)	4	36	34	42

The identification was based on alignment with nucleotide sequences currently available in NCBI database; [§] accession numbers of a homologous fragment: AF293974, position of the first and the last nucleotides corresponding to the fragment in the referenced sequence: 43-605; [£] Accession number: AJ634477, sequence deposited for this study; [#] accession numbers of a homologous fragment: AY030331, position of the first and the last nucleotides corresponding to the fragments in the referenced sequence: 53-785.

From the second experimental set up it is clear that oligofructose exerts a pronounced positive effect on growth rates of weaned turbots. In addition oligofructose stimulated the presence of *Bacillus* sp. up to 14% of the isolates. This may play a role in the beneficial effect that oligofructose exerts on turbot growth, since *Bacillus* spp. have been documented as probiotics in fish (Irianto and Austin, 2002) and shrimp (Vaseeharan and Ramasamy, 2003).

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INFLUENCE OF TEMPERATURE ON BODY SIZE AND YOLK-SAC VOLUMES OF THREE LARVAL RHEOPHYLIC CYPRINIDS (GENUS *LEUCISCUS*)

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Introduction

The determination of the thermal conditions of incubation and rearing of fish larvae is a current practice to evaluate its requirements under intensive rearing conditions, especially when related to new species in larviculture. Previously, progress in propagation of riveline fish from the genus *Leuciscus* (Kujawa et al. 1998, Kucharczyk et al. 2000, Kujawa et al. 2000, Harzevili et. al. 2003) focused the interest of aquaculturists and conservationists on this group of cyprinid fishes.

The present work intends to determine the effects of egg incubation in similar temperature conditions on body size parameters of three rheophylic cyprinid *Leuciscus* spp., important for the European conservational aquaculture.

Materials and methods

Dace (*Leuciscus leuciscus*), ide (*L. idus*), and chub (*L. cephalus*) were artificially reproduced with hormonal stimulation (Ovopel) to obtain viable embryos. Fertilised eggs were incubated in 40-dm^3 aquaria in different stable temperatures (7.5, 9.5, 12.3, 15.7, 19.0, 23.0, 25.0, and 27.5°C). The hatched larvae were reared in the same temperatures to resorption of yolk-sac and fed ad libitum with live *Artemia* nauplii. Samples (n=30) were taken to measure total length and yolk-sac volume at hatch, the beginning of external feeding, and resorption of yolk-sac. Pictures of live fish were taken by digital camera, and body parameters were measured to the nearest 0.01mm with using DP-Soft analytical program. The yolk-sac volume was calculated according to Blaxter and Hempel (1963).

The experiments were carried out in two replicates, and significant differences were determined by a Duncan's multiple comparison test.

Results and discussion

The influence of water temperature on dace or ide eggs incubation has been studied, among others, by Kennedy (1969), Florez (1972), Mills (1980), and Rechulicz et al. (2002). Data on chub are presented by Penaz (1968), Penaz and Sterba (1969), Economou et al. (1991), and Calta (2000). No comparative studies on this problem in similar temperature conditions are available. Our results show that the body size and yolk-sac volumes of three cyprinids from the genus *Leuciscus*, incubated in the same temperature regimes, were differentiated at particular stages of early development (Figs. 1 and 2).

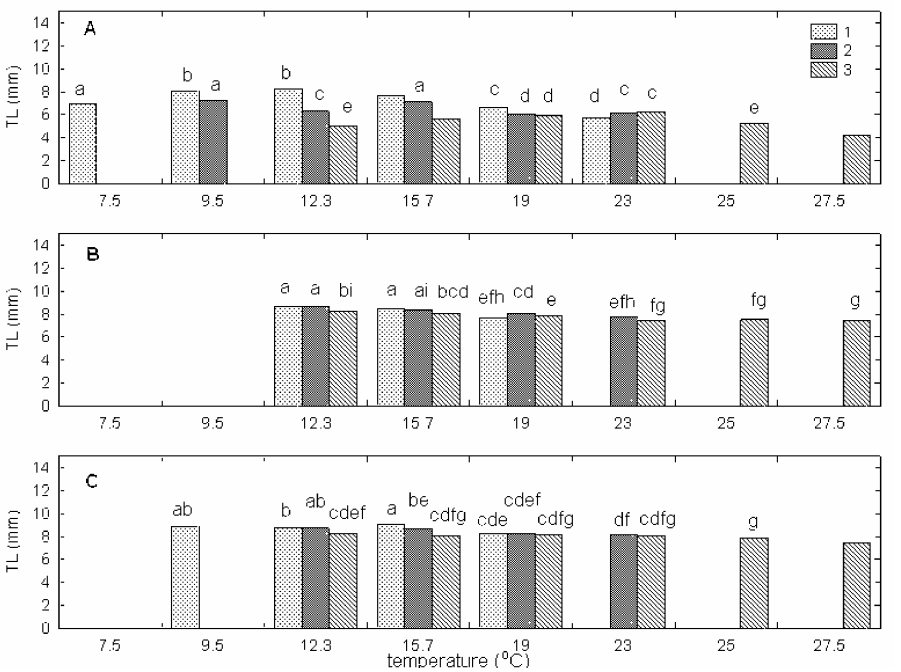

Fig. 1. The effect of incubation temperatures on body length of three cyprinids (1 – *Leuciscus leuciscus*, 2 – *L. idus*, 3 – *L. cephalus*) at hatch (A), the beginning of external feeding (B), and yolk-sac resorption (C). All values are the average of two replicates. SD varied from 0.06 to 0.49. Means having the same letter(s) are not significantly different (P<0.05).

Total body length of dace at hatch ranged in different temperatures from 5.78-8.30mm. Their yolk-sac volumes ranged from 0.33-0.62mm^3. The length of ide hatchlings ranged from 6.18-7.23mm, and their yolk-sac volumes varied from 0.66-1.13mm^3. Similar size of embryos was noted by Florez (1972) after egg incubation at 22.0°C. The body size of chub in these conditions ranged from 4.20-6.28 mm, with yolk-sac volumes varying from 0.38-0.80mm^3. Hatched dace embryos had the largest body size, whereas chub were the smallest. How-

ever, both species had the smallest yolk-sacs, not significantly different. Dace embryos started external feeding after 10-22d (depending on temperature), and after 12-23d utilised their yolk-sacs. Ide started feeding after 6-21d, whereas their yolk-sac was utilised after 7-23d. In chub embryos respective values for feeding time and resorption of yolk-sac were 5-34d and 5-35d.

In the beginning of external feeding the body length of embryos was more consistent (Fig. 1). However, particular species had significantly different yolk-sac resources in dependence to temperature conditions (Fig. 2), but with no such large differences as at hatch.

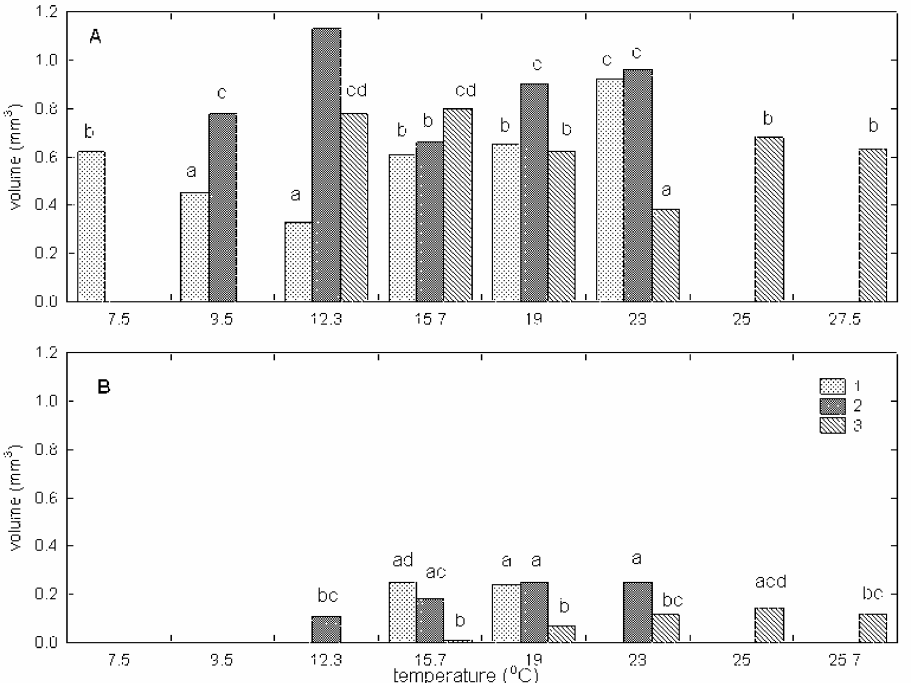

Fig. 2. The effect of incubation temperatures on yolk-sac volume of three cyprinids (1 – *Leuciscus leuciscus*, 2 – *L. idus*, 3 – *L. cephalus*) at hatching time (A) and the beginning of external feeding (B). All values are the average of two replicates. SD varied from 0.00-0.22. Means having the same letter(s) are not significantly different (P<0.05).

Conclusions

The ide tolerate temperatures from 9.5-23.0°C for egg incubation, whereas dace and chub range between 7.5-23.0°C and 10.0-27.5°C, respectively. Larvae hatched in these conditions were the best developed and had the largest body size with the smallest yolk-sac volumes.

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HOW INITIAL SIZE DIFFERENCES AFFECT CANNIBALISM AND GROWTH IN EURASIAN PERCH *PERCA FLUVIATILIS* LARVAE

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In some fish species, intra-cohort cannibalism is an important issue that can induce significant losses of initial stock during the larval stage. Despite numerous studies on quantitative characteristics of larval stocks, the determinism for emergence of cannibalism is still unclear. There are consistent reports that initial stocking density is an important imposed factor affecting emergence and intensity of cannibalism and that high density inhibits cannibalism rates in Eurasian perch larvae, whereas in post-larval stage, cannibalism rate is higher at elevated densities. Other factors have received little attention or are reported inconsistently depending on fish species. While some studies demonstrated that initial size heterogeneity is a key factor to cannibalism and growth dynamics of larval stocks in some fish species, results from a recent experiment indicate that such factor has no obvious impact on further cannibalism and growth of young juveniles of Eurasian perch and European seabass, *Dicentrarchus labrax*.

Therefore, three experiments were conducted to study the effects of early history on further quantitative characteristics of young juvenile Eurasian perch after mixing different progenies and changing stocking densities, and to investigate the role of some potent factors (ratio of potential cannibals and high initial size heterogeneity) in inducing cannibalism. Eggs from two or three females were fertilized with sperm from a single male to produce half-sibling groups. Then settings compared different size classes of post-larvae (smallest, medium, and largest [potential cannibals]) from either pure progenies or mixed ones during one or two months.

The results demonstrate that initial weight (W_0) and its variation (CV_0) at restocking affect further stock quantitative characteristics in direct or indirect pathways. W_0 negatively affects growth rate just after restocking, afterwards this relationship interferes with other abiotic factor, such as the dynamics in stocking densities since average weight was positively affected by W_0 after two months in

the present study. Despite considerable differences at the onset of each experiment (CV: 35-220%), initial CV_0 had no impact on further heterogeneity. However, CV_0 positively affected type II cannibalism, which caused higher mortalities than type I cannibalism in all trials in agreement with previous studies. Therefore, CV_0 has an indirect effect on survival and biomass through type II cannibalism, especially when restocking is processed 2 or 3 weeks after hatching. Indeed, the overall cannibalism was low compared to previous studies and cannibalism was not the most important factor affecting survival and growth characteristics, when young juveniles of 30-day-old were used. In this case, other abiotic factors may interact on the correlation between intra-cohort cannibalism and population dynamics.

Mixing different progenies at restocking is an important issue for rearing perch post-larvae but the results of the present study show that such practice has no significant effects on final quantitative characteristics including cannibalism rate, whatever the developmental stage of larvae. Nevertheless, this may be related to a low genetic variability of half siblings used in this study although it has been show that maternal gene pool has major impact on growth characteristics in some fish species.

Concerning the relationships between cannibals and preys, cannibalism rate and size heterogeneity did not differ when only medium size post-larvae were restocked with increasing number of cannibals, or even if the smallest fish were mixed with 8-15 times larger ones, and whatever the earlier history of cannibals. Nevertheless, a slight decrease in cannibalism rate was observed at initial lower proportion of cannibals. These results may indicate that, out of the dominance by largest larvae in the population, some larvae can exhibit cannibalism behaviour independently of their size classes. Moreover, they confirm previous studies that the ratio between prey larvae and predators is one of the major factors regulating the emergence of cannibalism.

The results indicate that initial weight affects significantly growth and survival rates during the first stage after restocking but this impact is progressively overlapped by the effect of overcrowding. Initial heterogeneity has no significant impact on further intra-cohort heterogeneity but affects significantly the emergence of cannibalism. The study also suggests that the initial proportion of cannibals does not affect further cannibalism whatever the presence or absence of smallest larvae in the initial stock or the earlier live history of cannibals. Moreover, it also seems that cannibalism emerges not only because of the opportunity coming from the size differences, but also due to other factors, such as an innate cannibalistic behavior. So, an investigation of parental effects including neomales is ongoing and comparisons will be made between normal and monosex progenies.

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ENHANCEMENT OF ANDAMAN SEA BLACK TIGER SHRIMP STOCK FOR SUSTAINABLE PRODUCTION OF QUALITY BROOD-STOCK: A CONCEPT

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Introduction

Due to the decline of Andaman Sea black tiger shrimp (*Penaeus monodon*) wild broodstock, Thai government approved a large amount of fund for a domestication and selective breeding program for this species. By now more than half a decade has been spent, but still there is no positive impact on black tiger shrimp seed production. Our research group believes that there should be an alternative and faster way to ensure quality broodstock. We recommend a stock enhancement program in the Andaman Sea to ensure long lasting supply of quality broodstock for this industry. Andaman Sea broodstock used for shrimp farming in Thailand resulted in the earning of more than USD$20 billion during the past two decades.

Rationale and methodology

Research in the past has shown that it is possible to control breeding of *P. monodon* in captivity; i.e., by pond rearing. However, wild broodstock always produced significantly better fecundity and hatchability than pond-reared broodstock (Menasveta et al., 1993).

Molecular genetic studies (Klinbunga et al., 1999) have revealed that there were two distinct black tiger shrimp (*P. monodon*) populations in Thai waters, one in the Andaman Sea and another in the Gulf of Thailand (Fig. 1); the former supporting the shrimp farming industry (more than 90%) during the past two decades. Shortage of broodstock was a bottleneck up to three years ago, and the price of gravid females went up to more than USD$250 per individual. However, with the introduction of *P. vannamei*, demand for *P. monodon* broodstock declined, though the price is still at USD$50-75 per female.

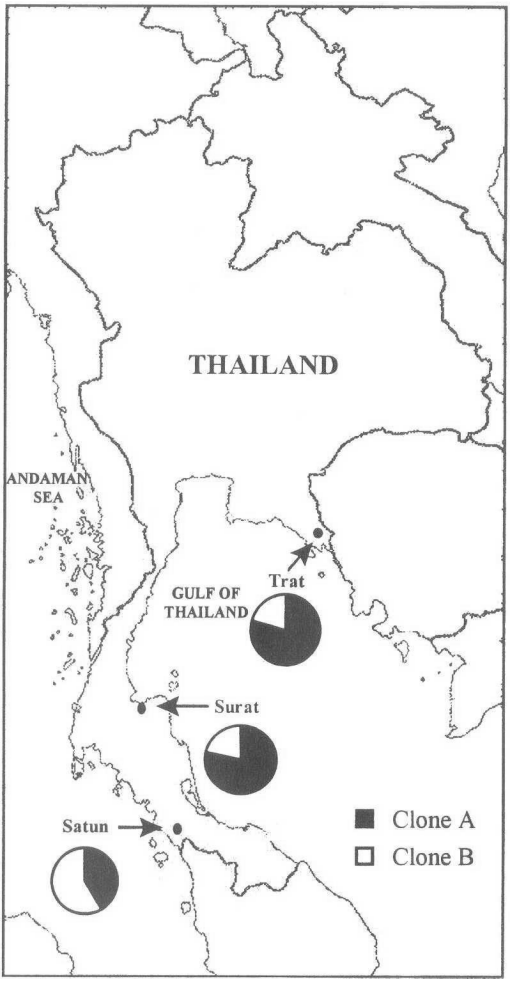

Fig. 1. Geographic distribution of major mtDNA phylogenetic clone A and B among the populations of *P. monodon.* Geographical heterogeneity analysis indicated population differentiation between Andaman Sea stock and the Gulf of Thailand stock, $p<0.0001$. (Klinbunga et al., 1999).

Several shrimp stock enhancement programs in Asian waters resulted in the recapture of adult shrimps at the rate ranged from 2-15% (Devenport et al., 1999; Su amd Liao, 2001; Qing-Yin et al., 2004). The meta analysis also revealed that shrimp recruitment related directly with the quantity of spawners (Ye, 2000). According to Thailand fishery statistics, the catch of Andaman Sea black tiger shrimp during 1988-1995 averaged 172 tons.yr^{-1}. Assuming the catch was 50% of the total stock and the average weight of shrimps was 100g, the total stock would be 344 tons, with a population of 3 400 000 shrimps in Andaman Sea. In 2000, 250 000 broodstock were captured from the sea, accounting for 7.2% of the total stock. Catching the broodstock at this rate would make the stock vulnerable for collapse.

Enhancing the small stock should result in a fast increase of the population. At present, we have all basic knowledge of the population characteristics such as DNA markers of this stock (Klinbunga et al., 1999; Tassanakajon et al., 1998; Supungul et al., 2000).

A stock enhancement program should be carefully carried out; for instance, parent stocks should be indigenous to the area and not carry pathogenic virus. There should be the development of larviculture and nursery techniques to make sure we can produce healthy fingerlings of 1.5-month-old before release. Assuming 2% recapture, the release of 10 million fingerlings would result in 200 000

broodstock. A re-stocking program every year for 2-3 years would eventually increase the proportion of disease-free broodstock in the population. If it works with black tiger shrimps, it can also be applied to other species. With this proposed methodology the total cost to produce one broodstock would be approximately USD$1.20.

Discussion and conclusion

Experience from Australia suggests that post-release survival depends on the release habitat, release time, and size at release (Loneragan et al., 2004). Larger size at release was seemed to increase post-release survival (Qing-Yin et al., 2004). In this regard, we propose to release the shrimps at 1.5 months old, or 1-2g. Shrimps at this size can withstand nature better than the post-larval size, seen in previous releases at the post-larval size.

This conceptual project has several beneficial aspects: 1) it will be an alternative way to increase shrimp broodstock; 2) it provides an increase of recruitment for capture fisheries; 3) it is a clean and cheap technology complying with the principle of species diversity conservation; and 4) it applies application of molecular genetic techniques into fisheries and aquaculture management.

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DETERMINATION OF PROSTAGLANDIN E_2 (PGE_2) IN POLYCHAETES (*PERINEREIS* SP.) AND ITS EFFECT ON *PENAEUS MONODON* OOCYTE DEVELOPMENT IN VITRO

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Introduction

Polychaetes are the best maturation feed for prawn broodstock due to their high nutritional value and some other unknown factors, especially hormone-like substances. Which polychaete hormones that influence development of the reproductive system of prawn broodstock have never been characterised to date. Vitellogenesis of prawn is governed by numerous hormones, for example, eyestalk neuropeptides, biogenic amines, ecdysteroids, and a juvenile hormone-like compound, methyl farnesoate. This experiment aimed to verify the existence of prostaglandin E_2 (PGE_2), the spawning hormone of prawn, in polychaetes and to assay the PGE_2 extracted from polychaetes on prawn oocyte development.

Materials and methods

PGE_2 was extracted from sand polychaetes (*Perienereis* sp.) and blood polychaetes (*Marphysa* sp.) collected from beaches in the eastern part of Thailand. Other sources of live feed for prawn broodstock – i.e., squid, mussels, and clams – were also tested for PGE_2 determination. Physiological level of PGE_2 in prawn broodstock was identified from reproducing *Penaeus monodon* females at different stages of ovarian development.

The samples were homogenised in 0.4% NaCl and extracted with ethanol and acetic acid (Amersham, 2000). The mixture was centrifuged at 2500rpm for 5min and the supernatant was eluted with hexane and ethyl acetate onto Sep Pak (C_{18}) column. The eluant was collected and dried under nitrogen gas and stored at -85°C for further analysis by either enzyme immunoassay or HPLC.

For measurement of low concentration PGE_2, the test kit for PGE_2 by Amersham (RPN 222) was used. The dried samples was resuspended in methanol and pipetted into 96 pre-treated wells. The colour developed as a result of binding reaction was monitored by spectrophotometer (microplate reader) at the wavelength of 450nm.

For measurement of high concentration PGE2, the sample was analyzed by Reversed-Phase High Performance Liquid Chromatography (RHPLC) (Tahara and Yano, 2003). The dried sample was re-dissolved in ethanol and injected onto the Prevail C18 column (0.46×15cm, Alltech). An isocratic solvent containing 17mM phosphoric acid/acetonitrile (7:3, v/v) was used at a flow rate of $1.5ml.min^{-1}$. The effluent was monitored at 195nm to determine the elution of PGE_2 (Tahara and Yano, 2003).

Ovary at stage I of prawn broodstock was dissected and washed in PBS and then incubated in M199 (plus salt) with or without PGE_2 extracted from polychaetes or synthetic PGE_2. After 24 hours, the experiment was terminated and the ovary was preserved in Davidson's fixative for histological examination (Bell and Lightner, 1988). The diameter and percentage of oocyte stages in hormonal treated ovary was evaluated.

Results

The results showed that polychaetes contained PGE_2 at levels higher than any other live feeds. Concentration of PGE_2 varied according to sex, age, and sources. Eight-month-old polychaetes contained higher PGE_2 (18.16±5.82µg $PGE_2.mg^{-1}$ protein) than 2-, 4-, or 6-month-old ones (100.71±10.38; 114±1058; 160.8±37.09µg $PGE_2.mg^{-1}$ protein, respectively) (Fig. 1).

Ovarian extracts, muscle and haemolymph of female shrimp also possessed significant amounts of PGE_2 with varying level related to the degree of maturation. Ovary at the cortical rod stage displayed highest amount of PGE_2 (30.30±4.05µg $PGE_2.mg^{-1}$ protein) which rapidly declined after spawning (Fig. 2).

Prawn ovary in vitro incubation with various treatment of PGE_2 showed that PGE_2 stimulated uptake of vitellogenin into oocytes. After 24h incubation with this hormone, immature oocytes developed into matured and ready to spawn oocytes (Fig. 3). PGE_2 extracted from polychaetes at $5ng.ml^{-1}$ had a more prominent effect on oocyte development over synthetic PGE_2 at the same concentration.

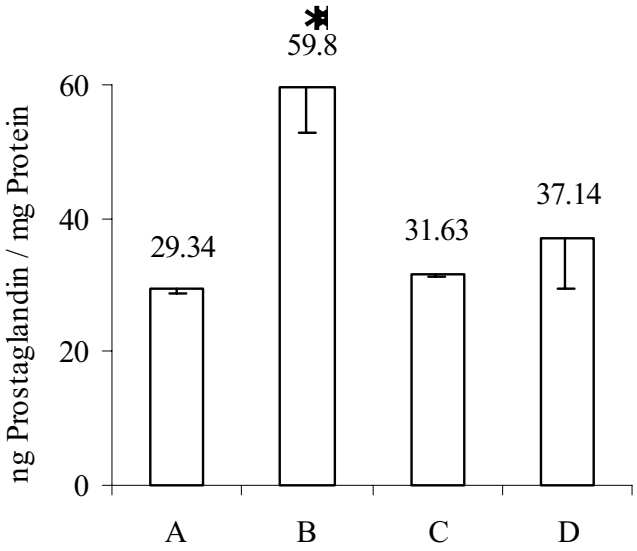

Fig. 1. Levels of PGE_2 in sand worms (*Perinereis* sp.) compared to blood polychaetes (*Marphysa* sp.) at age difference. Treatments having the asterisks are highly significant different ($P<0.05$). A = 4-month-old *Perinereis* sp.; B = 8-month-old *Perinereis* sp.; C = 6-month-old *Marphysa* sp.; D = 8-month-old *Marphysa* sp.

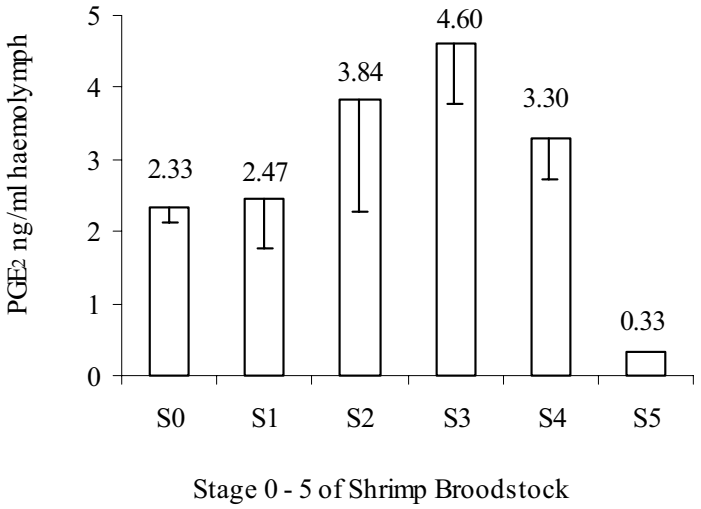

Fig. 2. Levels of PGE_2 in haemolyph of female *Penaeus monodon* broodstock at different ovarian maturation stage.

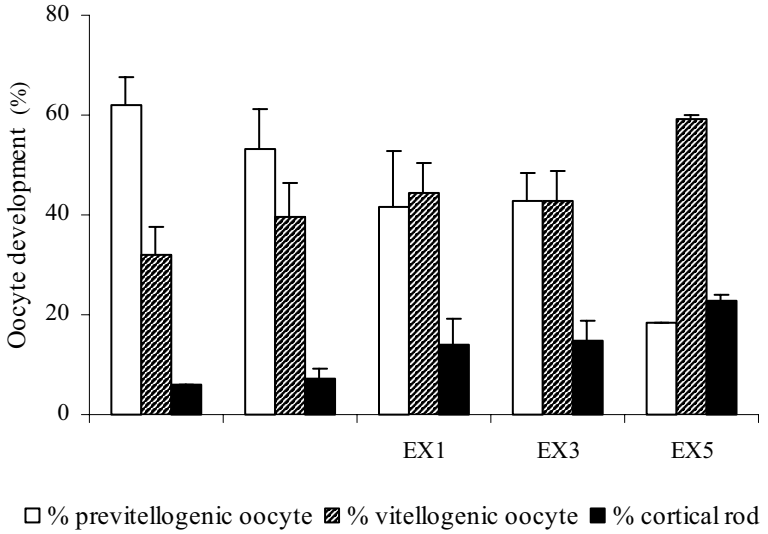

Fig. 3. Percent of oocytes at different stages after incubates with various concentration of PGE_2 extracted from polychaetes for 24h. EX1 = PGE_2 at 1ng.ml^{-1}, EX3 = PGE_2 at 3ng.ml^{-1}, and EX5 = PGE_2 at 5ng.ml^{-1}.

Conclusions

Polychaetes displayed the highest PGE_2 concentration than any other live feed for prawn broodstock. The level of polychaete PGE_2 varied according to age, feed intake, and sources. Polychaete PGE_2 could enhance maturation process of prawn oocytes. The effective concentration of PGE_2 from polychaetes was at the same level with PGE_2 level in haemolymph of prawn with vitellogenic oocytes.

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EFFECTS OF CHOLINE SOURCES ON POSTLARVAL KURUMA SHRIMP, *MARSUPENAEUS JAPONICUS*, IN THE PRESENCE OF DIETARY PHOSPHATIDYLCHOLINE

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Introduction

Choline is essential for the structure and function of all cells in terrestrial and aquatic animals (Canty et al., 1996), especially crustaceans (Camara et al., 1997) to sustain the good growth and other biological functions. Still the interactive relations between choline and other nutrients or even between different choline sources need to be investigated as many contradictions were found among the findings of the previous studies (Kanazawa et al. 1976; Deshimaru and Kuroki, 1979; He, 1993; Gong et al., 2003). The present study was conducted to evaluate the effects and interaction of two choline sources (choline chloride [CC] and soybean phosphatidylcholine [SPC]) on the growth and the biological features of postlarval kuruma shrimp, *Marsupenaeus japonicus*.

Materials and methods

A 30-day feeding experiment was conducted as a 2×2 factorial design. The postlarval shrimp (PL-15; 25-days after hatching) with mean initial body weight of 0.004 ± 0.0002g were randomly allocated to 15 30-l tanks, as triplicates per each dietary treatment. The shrimps were fed three times per day with four κ-carrageenan micro-bound diets with casein (vitamin-free) as the main protein source, contained two levels of CC (0.06 and 0.12%) and two levels of SPC (2 and 4%), in addition to the control (basal) diet without supplemental choline sources as shown in Table I.

Results of growth, feed efficiency and biochemical composition were tested by super ANOVA (1.11 statistical package for Mac). One and two-way ANOVA were applied sequentially for the above mentioned parameters to check the pure and the interactive effect of the choline sources on these parameters. Survival data were arc-sin transformed before the statistical evaluation.

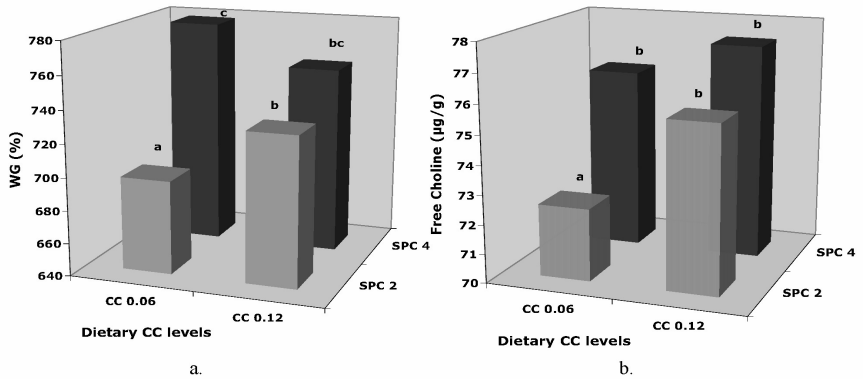
Table I. Formulations, proximate, and SPC composition of the experimental diets.

Ingredients	Experimental diets (g.100g^{-1})				
	1	2	3	4	5
Casein	50.0	50.0	50.0	50.0	50.0
k-Carrageenan	5.0	5.0	5.0	5.0	5.0
L-Tryptophan	1.4	1.4	1.4	1.4	1.4
L-Arginine	3.9	3.9	3.9	3.9	3.9
L-Lysine	1.0	1.0	1.0	1.0	1.0
Squid Liver oil	4.6	4.6	4.6	4.6	4.6
Vitamin mix	0.8	0.8	0.8	0.8	0.8
Mineral mix	8.6	8.6	8.6	8.6	8.6
α-Starch	10.0	10.0	10.0	10.0	10.0
α-Cellulose	4.8	2.7	2.6	0.7	0.6
CMC	2.0	2.0	2.0	2.0	2.0
Sucrose	5.0	5.0	5.0	5.0	5.0
Cholesterol	0.5	0.5	0.5	0.5	0.5
Attractants[a]	2.4	2.4	2.4	2.4	2.4
CC[b]	0.0	0.06	0.12	0.06	0.12
SPC	0.0	2.0	2.0	4.0	4.0

[a]Sodium citrate (3); sodium succinate (3); glucosamine HCl (8); Betain (3); Taurine (3); IMP (1); Glutathione (1); Glycine. [b]Nacalai Tesque Inc. Kyoto, Japan.

Results and discussion

The addition of CC and/or SPC significantly ($P < 0.01$) improved the parameters such as WG (Fig. 1a), SGR, AFER, APER, and SR, (Table II) and increased the whole body content of crude protein, free choline (Fig. 1b) and PC and decreased the body lipid content as shown in Table II. Furthermore, a significant ($P < 0.05$) interaction was detected between both choline sources in terms of WG, SGR, AFER, APER, in addition to the whole body contents of lipid, free choline and PC. No significant ($P > 0.05$) interaction between CC and SPC in terms of SR and carcass content of moisture and protein could be detected.



a. b.

Fig. 1. Effects of different levels (%) of CC and SPC on: a. Weight gain (WG%); b. Free choline content of the whole body of post-larval kuruma shrimp.

In the shrimp groups received the lower level of SPC (2%), the increment of CC level from 0.06 to 0.12% significantly ($P < 0.01$) increased the WG, SGR, AFER, APER, and the whole body content of free choline and PC, in the same time, lowered the whole body lipid content. However, the increment of CC level at the same low SPC level (2%) did not affect the SR or the carcass content of both moisture and protein.

Table II. Effect of different % levels of CC and SPC on various performances of post-larval *M. japonicus*.

Variables[a]	Control (0,0)	SPC (2) CC (0.06)	SPC (2) CC (0.12)	SPC (4) CC (0.06)	SPC (4) CC (0.12)
SR (%)	57[a]	81[b]	80[b]	80[b]	84[b]
WG (%)	440 ± 12.0[a]	697 ± 17.3[b]	731 ± 20.2[c]	778 ± 11.3[d]	754 ± 18.9[cd]
WG (g)	0.90 ± 0.01[a]	2.03 ± 0.02[b]	2.10 ± 0.01[c]	2.27 ± 0.02[d]	2.23 ± 0.12[cd]
SGR (%.day^{-1})	4.70 ± 0.05[a]	6.80 ± 0.05[b]	6.90 ± 0.04[c]	7.10 ± 0.10[d]	6.90 ± 0.11[cd]
AFI (g)	1.50 ± 0.01[a]	2.39 ± 0.01[b]	2.39 ± 0.01[b]	2.55 ± 0.01[d]	2.42 ± 0.02[c]
AFER	0.60 ± 0.01[a]	0.85 ± 0.01[b]	0.88 ± 0.01[c]	0.92 ± 0.01[d]	0.89 ± 0.02[d]
APER	1.40 ± 0.10[a]	2.20 ± 0.02[b]	2.30 ± 0.01[c]	2.43 ± 0.01[d]	2.40 ± 0.02[d]
Carcass analysis					
Moisture (%)	80.19 ± 0.5[a]	79.39 ± 0.9[a]	78.27 ± 0.7[a]	78.42 ± 0.7[a]	79.18 ± 0.2[a]
Protein[b] (%)	52.45 ± 3.1[a]	64.19 ± 4.4[b]	60.09 ± 4.1[b]	60.77 ± 3.7[b]	64.28 ± 4.5[b]
Lipid[b] (%)	16.00 ± 0.5[d]	11.90 ± 0.2[c]	10.45 ± 0.1[b]	7.73 ± 0.3[a]	8.09 ± 0.1[a]
Free choline	41.11 ± 0.2[a]	72.48 ± 0.1[b]	75.70 ± 1.5[c]	76.21 ± 1.5[c]	77.38 ± 0.9[c]
PC	35.65 ± 0.1[a]	43.22 ± 0.6[b]	47.95 ± 1.0[c]	49.15 ± 0.9[c]	49.28 ± 0.7[c]

[a] Same letters within each row are not significantly different ($P>0.05$) and different letters means significant difference.

[b] Protein and lipid contents (%) were given in dry weight basis. Free choline and PC were given in μg.g^{-1}.

When shrimps received the higher level of SPC (4 %), the increment of CC level from 0.06-0.12% did not result in any significant increase/decrease of the above-mentioned parameters. Thus, the increment of CC level in diets showed a significant effect at the lower SPC level (2%) but not at the higher level (4%). Moreover, the increment of SPC levels from 2-4% significantly ($P < 0.01$) increased/decreased the above same parameters as shown in Table II and Fig. 1 a&b. As for the shrimp, information available on the necessity of choline is fragmentary and conflicting even in the same species; for example, Kanazawa et al. (1976) have shown that *M. japonicus* juveniles required 0.06% CC for normal growth, whereas, Deshimaru and Kuroki (1979) did not find any beneficial effect of CC-inclusion on the same species at the juvenile stage. Camara et al. (1997) reported that the supplementation of 1.5% and 3.0% SPC with 95% purity (containing 0.2-0.4% choline group) were enough to maintain optimum growth of larval and post-larval *M. japonicus* when no other choline source was added to the diet. As for *L. vananamei*, He (1993) has found that 0.09% CC should be supplemented to the diet to sustain growth and survival. Gong et al. (2003) have revealed that the white shrimp *L. vannamei* required choline when

commercial SBL were not included in the diet, but did not when adequate levels of the SBL were supplied to the diet. This result apparently differs from the earlier finding of supplemental effect of CC on the same shrimp species (He 1993), as also observed in kuruma shrimp; *M. japonicus* (Kanazawa et al. 1976; Deshimaru and Kuroki 1979). We assumed that these contradictory results in earlier studies might be partly due to the interaction between CC and PC and/or among the nutrients like methionine and betaine acting as methyl group donor.

Conclusions

In conclusion, the present study showed that CC and SPC were needed for sustaining good growth and survival of post larval shrimp (PL-15 to PL-45). Moreover, the effects of supplemental CC and SPC were dependent on the dietary level of each other's.

Acknowledgements

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CHANGES IN EGG LIPID AND FATTY ACID PROFILE DURING EMBRYONIC DEVELOPMENT OF *LABEO ROHITA* (HAM.)

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Introduction

Indian freshwater aquaculture is comprised mainly of finfish, particularly carp, and there is enormous demand for healthy fry for stocking. Timely availability of quality larvae continues to be a serious impediment. Larval production largely depends on egg hatchability, egg quality, and larval survival, but variable reproductive performance and unpredictable reproductive failures of broodstock have proven to be major limiting factors for mass larval production. Various reproductive physiological aspects are intricately linked to gross nutrient availability and the regulating role of nutrition on reproduction has profound effects on fecundity, egg size, egg chemical composition, hatchability, and viability of larvae. In all oviparous fishes, the developing embryo is totally dependent on nutrients, especially lipids and fatty acids (FA) stored in egg yolk, for successful development, egg viability, and survival (Izquierdo et al., 2001). In this context, an attempt has been made to study the changes in egg lipids and fatty acid compositions during embryonic development of Indian major carp *Labeo rohita* (Ham.).

Material and methods

Eggs were stripped from individual pond-reared females (weighing 2.0-2.2kg) and fertilized with the milt from two to three males. Fertilized eggs from each female were kept separate in individual Petri dishes and transferred to incubators (28-29°C). Groups of 20-30 egg samples were collected nine times at different stages of embryonic development comprising blastodisc, cleavage, morula, yolk invasion, yolk plug, embryo elongation, head differentiation, and twitching (0, 14.25, 21.370, 42.75, 114, 142.5, 171, 199.5, and 513 degree days, respectively) between the time of the eggs stripped and hatching for analysis of chemical composition. Dry matter (DM) content was determined after oven-drying at 60°C to constant weight. Lipid extraction was carried out following Bligh and Dyer (1959). Fatty acid methyl ester (FAME) derivative was prepared by adding 20% boronitrifluoride (BF_3) in methanol to lipid and heating for a half an hour at

100°C. After cooling, water (equivalent volume as BF_3) and hexane (½ volume of BF_3) were added, centrifuged at 3000rpm for 5min, and allowed to separate for 5-10min in a separating funnel. The upper phase was recovered and FAME in hexane was dried with sodium sulfate, which was then removed by filtering through a Pasteur pipette containing glass wool. The operating conditions of the gas chromatograph (PYE UNICAM, GC 104) consisted of a Flame ionization detector (FID), stainless steel column packed with 10% Diethylene glycol succinate polyester (DEGS), column temperature 195°C (maintained for 10min isothermal), injection port temperature 210°C, detector temperature 210°C, N_2 carrier gas at 35-40ml.min^{-1} flow rate, recorder chart speed of 640mm.h^{-1}. The methyl ester peaks were identified by comparative chromatography with external standard FAME (Sigma Chemical Co, USA) mixtures by comparing their retention time and quantified by a Spectraphysic SP 4270 integrator.

Results

Both the amount and relative proportions of lipids declined during incubation (Fig. 1) and the content ranged from 18.21-28.17% of DM. There was a sharp decline in the DM proportion after incubation whereas lipid-free DM remained the same (Fig. 2) with some decrease in the amount after incubation. Changes in FA composition are presented in Table I. The saturated fatty acids (SAFAs) shared about 18.50±12.97% of the total FAs in which 14:0 and 18:0 were more abundant than 16:0 in freshly stripped eggs. Among the monounsaturated fatty acids (MUFAs), 18:1n-9 was dominant (6.34%) and the PUFAs arachidonic acid (ArA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3), and docosahexachoic acid (DHA, 22:6n-3) made up 0.96, 1.84, and 2.55%, respectively, of total FAs in freshly stripped eggs. During embryonic development, relative proportions of 16:0, ArA, and DHA increased whereas SAFA 14:0, 18:0, and MUFA 18:1n-9 decreased. There was little change in the relative percentage of EPA during the course of incubation. Egg survival to hatching was positively correlated with ArA:EPA and DHA:EPA ratio in freshly stripped eggs (Fig. 3).

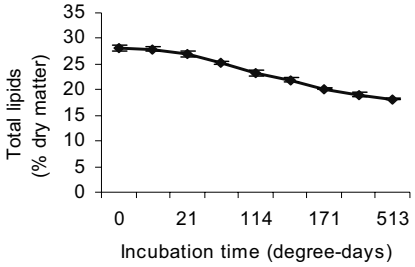

Fig. 1. Changes in total lipid (% dry matter) content in *L. rohita* eggs during embryonic development (mean ± SD).

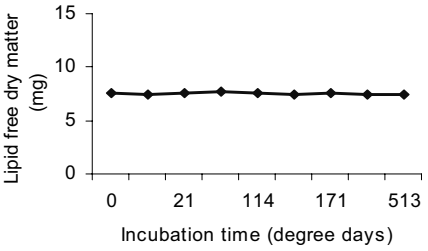

Fig. 2. Changes in lipid-free dry matter content in *L. rohita* eggs during embryonic development

Table I. Fatty acid composition (% of total fatty acids) in egg lipid of *L. rohita* during embryonic development (mean ± SD).

Fatty acid	Incubation period (degree-days)								
	0	**14.5**	**21.37**	**42.75**	**114**	**142.5**	**171**	**199.5**	**513**
14:0	24.24 ±0.40	23.27 ±0.54	20.59 ±0.41	20.25 ±0.52	18.30 ±0.24	18.09 ±0.07	17.88 ±0.30	17.06 ±0.06	16.11 ±0.01
16:0	1.51 ±0.03	1.80 ±0.01	1.85 ±0.02	1.92 ±0.02	2.79 ±0.13	7.03 ±0.65	10.94 ±0.97	12.12 ±0.41	15.6 ±0.20
18:0	29.76 ±1.08	28.47 ±0.40	27.13 ±0.02	26.92 ±0.35	26.04 ±0.14	25.3 ±0.29	24.17 ±0.05	23.15 ±0.13	22.09 ±0.07
16:1 n-7	0.65 ±0.09	0.75 ±0.06	0.63 ±0.05	0.67 ±0.07	0.66 ±0.08	0.68 ±0.13	0.70 ±0.04	0.67 ±0.11	0.63 ±0.05
18:1 n-9	6.34 ±0.15	6.29 ±0.18	6.07 ±0.14	5.65 ±0.13	4.96 ±0.06	4.75 ±0.09	3.68 ±1.20	3.74 ±0.37	5.33 ±2.21
18:2 n-6	2.84 ±0.03	2.85 ±0.05	2.8 ±0.04	2.81 ±0.03	2.77 ±0.04	2.77 ±0.10	2.84 ±0.03	2.85 ±0.04	2.80 ±0.09
18:3 n-6	0.18 ±0.02	0.13 ±0.02	0.14 ±0.02	0.14 ±0.01	0.13 ±0.03	0.09 ±0.03	0.13 ±0.05	0.13 ±0.02	0.12 ±0.03
20:4 n-6	0.96 ±0.02	0.96 ±0.03	1.03 ±0.10	1.07 ±0.10	1.07 ±0.12	1.19 ±0.05	1.24 ±0.14	1.25 ±0.25	1.63 ±0.45
18:3 n-3	5.88 ±0.07	5.79 ±0.33	5.86 ±0.07	4.17 ±2.93	5.88 ±0.05	5.86 ±0.06	5.9 ±0.02	5.88 ±0.10	5.87 ±0.04
20:3 n-3	3.91 ±0.1	3.73 ±0.14	3.75 ±0.17	3.71 ±0.17	3.73 ±0.14	3.71 ±0.14	3.62 ±0.15	3.66 ±0.19	3.73 ±0.27
20:5 n-3	1.84 ±0.05	1.84 ±0.05	1.83 ±0.05	1.84 ±0.05	1.84 ±0.04	1.86 ±0.04	1.85 ±0.02	1.84 ±0.06	1.79 ±0.07
22:5 n-3	8.43 ±0.25	8.27 ±0.35	8.51 ±0.07	8.53 ±0.24	8.63 ±0.10	8.39 ±0.28	8.47 ±0.36	8.45 ±0.32	8.42 ±0.33
22:6 n-3	2.55 ±0.03	2.69 ±0.05	2.99 ±0.18	3.43 ±0.48	3.85 ±0.03	3.95 ±0.04	4.54 ±0.46	4.85 ±0.05	4.96 ±0.06
Σ SAFA	18.50 ±12.97	17.85 ±12.24	16.52 ±11.36	16.36 ±11.21	15.71 ±10.25	16.80 ±7.97	17.66 ±5.75	17.44 ±4.79	17.95 ±3.11
Σ MUFA	3.49 ±3.11	3.52 ±3.03	3.35 ±2.98	3.16 ±2.73	2.81 ±2.35	2.71 ±2.23	2.19 ±1.79	2.20 ±1.69	2.98 ±2.93
Σ n-6	1.33 ±1.18	1.31 ±1.20	1.32 ±1.17	1.34 ±1.17	1.32 ±1.15	1.35 ±1.16	1.40 ±1.18	1.41 ±1.19	1.51 ±1.18
Σ n-3	4.52 ±2.47	4.46 ±2.40	4.59 ±2.44	4.33 ±2.57	4.78 ±2.39	4.75 ±2.29	4.87 ±2.31	4.93 ±2.29	4.95 ±2.29

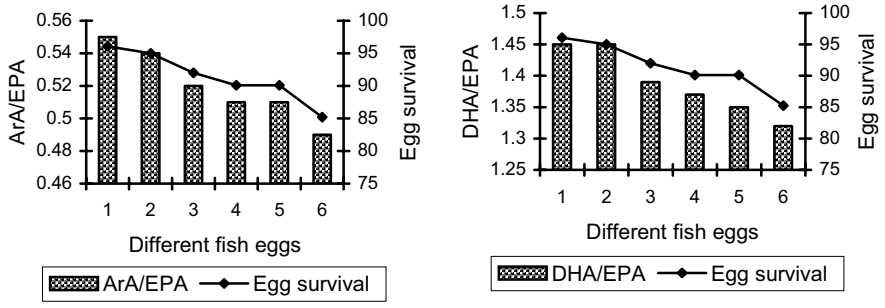

Fig. 3. Relationship between egg survival and ArA/EPA (left) and DHA/EPA (right) ratio in freshly stripped eggs of *Labeo rohita*.

Discussion

Lipid proportion declined during rohu egg development, indicating that egg lipids were preferentially mobilized and catabolised to provide energy for the developing embryo. The high amount of SAFAs of freshly stripped eggs was mainly 14:0 and 18:0, contrary to most marine fish species with up to 30% mainly as 16:0 (Tveiten et al., 2004). The percentage of ArA in the lipids of the freshly stripped eggs was low but increased during embryonic development, similar to other fish species (Tveiten et al., 2004). It might have contributed to the very complex regulation of cell multiplication during embryonic development by acting as a mediator on protein kinases and the two enzymes regulating the synthesis and degradation of cAMP. Fish eggs usually contain substantial amounts of n-3 PUFAs, which are the essential constituents of the cell membrane phospholipids (PL), particularly phosphatidylcholine during embryogenesis and larval development, through cell differentiation and proliferation. Among the n-3 PUFAs, there was little change in the relative percentage of EPA during incubation over time in the present study. EPA has well established roles in the structure and function of membrane PL, and modulation in formation of eicosanoids from ArA by competing with the enzyme system; it might have served as an energy source during rohu embryonic development without being selectively catabolized. This is also similar with the findings of Tveiten et al. (2004). Increase in the relative proportions of DHA (22:6n-3) during embryonic development provided evidence of selective DHA retention in the structural PL and the developing rohu larva might have incorporated it in neural cell membranes of the eye and brain tissues for its normal development (Sargent et al., 2002). Since seed production performance of fish largely depends upon egg hatchability, quality, larval survival, and vigor, this may have great relevance for female broodstock nutrition in the context of variable reproductive performance and unpredictable reproductive failures during mass seed production, the main goal of hatcheries.

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THE USE OF LIPOSOMES AS NUTRIENT DELIVERY SYSTEM TO MARINE FISH LARVAE

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Introduction

Nutritional deficiencies of live prey used for exogenous first-feeding in marine larval fish rearing necessitate their supplementation with certain nutrients in order to fulfill the requirements of the fish. In most cases, larval nutrition studies have been focused on the essential fatty acid (EFA) demands, especially eicosapentaenoic (EPA) and docosahexaenoic acids (DHA), but other compounds as phospholipids, vitamins, and free amino acids have been proposed as crucial elements in the early development stages of several marine fish species. As such, this paper reports the studies conducted at the IATS on the use of liposomes – phospholipid vesicles enclosing an internal aqueous space – as both hydrophilic and lipophilic supplements to emulsions in live prey enrichments, but also as a system to deliver both nutrient types to the larvae by means of liposome baths.

Liposomes as live prey enrichment supplements: bioencapsulation

Bioencapsulation consists of the delivery of substances to larvae through their incorporation inside live prey used for exogenous first-feeding. The process makes use of the non-selective feeding behavior of rotifers and *Artemia* to enrich their body composition in therapeutic or nutritional substances. Emulsions, the enrichment product mostly employed in marine larviculture, display the ability of providing only lipophilic substances, among which EFA, vitamins, and carotenes are the most important. Hontoria et al. (1994) proposed liposomes as a novel delivery system to provide both lipophilic and hydrophilic molecules encapsulated in *Artemia* nauplii, demonstrating their ability to incorporate a hydrosoluble marker enclosed inside a phospholipid bilayer. These findings were confirmed when Ozkizilcik and Chu (1994) studied liposome ability to enrich *Artemia* nauplii in phospholipid and free glycine. Special attention must be made to phospholipids, since they are considered to be a suitable way for providing EFA

to the larvae because these molecules facilitate the absorption of lipids in the larval gut, promote growth, and reduce the potential entry of lipid peroxides through the nauplii as a consequence of their antioxidant properties. Despite the fact that the enhancement of polar lipid fraction has been considered not to be possible (Rainuzzo et al., 1994), McEvoy et al. (1996) and Monroig et al. (2003) obtained higher polar lipid contents in nauplii enriched with liposomes compared to nauplii treated with emulsions rich in neutral lipids. Among hydrosoluble nutrients, amino acids represent the major substrates of aerobic metabolism during the development of embryo and yolk-sac larvae of many marine fish species. Given that few amino acid commercial boosters exist, liposomes become a unique way to improve the amino acid content of live preys. Thus, Tonheim et al. (2000) obtained free methionine contents 60× higher in nauplii enriched with liposomated methionine than in unenriched ones. Extensive literature has demonstrated the benefits of different vitamins to the correct development of fish larvae.

Experiments conducted in our lab feeding liposome-treated *Artemia* nauplii to sea bream larvae tested the supplementation of nauplii with polyunsaturated fatty acids (PUFA) and polar lipids by means of liposomes prepared with krill phospholipid. Other experiments were carried out bioencapsulating liposomated vitamins and amino acids in *Artemia* nauplii that were used as food for sea bass larvae. In this last case the nutrients assayed to enhance nauplii nutritional value were retinyl palmitate, sodium ascorbate, and free methionine. In general, the content of the different nutrients used on the enriched nauplii was increased to a certain extent, proving that the liposomes were able to modify the live prey nutritional content. However, the fish larvae fed the treated nauplii do not reflect the above mentioned differences with emulsion controls. It is worthy to mention that in all experiments, fish larvae showed similar survival and growth to that of the positive controls. The absence of apparent physiological benefits when liposome-treated nauplii are offered to fish larvae can be attributed to several causes. Perhaps a longer follow-up after the treatment period is needed to evidence some effect on the physiological state of the larvae. It is also necessary to pay attention to more specific variables related to each nutrient in order to find physiological changes derived from the nutritional boosting. It is also possible that the high growth rate makes the need for nutrients so demanding that any additional amount of them is rapidly depleted and hence undetectable.

In addition, preliminary studies have elucidated that bioencapsulation procedures using liposomes can vary from those of self-emulsifying products. Incubation time, naupliar density, aeration, lipid concentration, and dosage are variables that have been studied in order to optimize the enrichment with liposomes. In this sense, experimental results indicate a different behavior of different types of liposomes (multilamellar, extrusion unilamellar, and detergent solubilization unilamellar vesicles) when their ability to enrich *Artemia* nauplii in EFA and

phospholipids is evaluated. On the other hand, naupliar density levels seem not to differ from those employed for normal emulsion enrichments. Special attention must be paid to aeration, since the study of the effect of different air flows and air diffusion systems has revealed important implications in the final enrichment efficiency, presumably related with the tensioactivity of liposomes. Other experiments in progress, which are focused on the setting of optimal liposome concentration and its dosage during the incubation period, will be helpful in the optimization of liposome enrichment procedures.

Liposomes as direct nutrient delivery system to fish larvae: liposome baths

A novel technique to transfer nutrients to fish larvae consists of the immersion of larvae in liposomated substances, obviating their bioencapsulation in the live preys and avoiding their consequent metabolic activity over the administered substances. This strategy was firstly followed by Fox et al. (1990) in order to develop a radiotracer method for studying PUFA requirements in marine fish larvae. Koven et al. (1999) studied the efficacy of liposomes as a nutrient supplement in first-feeding of two species of marine fish larvae, concluding that the consumption of liposomes in both species was measurable. More recently, several studies have been carried out in our laboratory with the purpose of detecting the incorporation of lipophilic (EFA) and hydrophilic (ascorbate and methionine) nutrients into marine larvae by means of liposome baths. In the case of hydro-soluble substances, results have been compared to the incorporation achieved exposing the animals to equivalent concentrations through aqueous solutions. At the moment, results would indicate a poor entry of liposomated nutrients into the larvae, although analyses seem to reflect slightly higher average contents of EFA and ascorbate nutrient in larvae treated with liposomes compared to control non-treated larvae. These results could be confirmed optimizing the experimental designs.

Conclusions

The use of liposomes as a tool for the delivery of different nutrients to fish larvae is possible and allows growing experimental larval cultures without differences compared to emulsion controls. Liposomes are able to change the content of *Artemia* nauplii in different nutrients that are delivered through them. The improvements in delivering hydrophilic nutrients encapsulated in lipidic vesicles remain a promising possibility of the use of the liposomes. Although they are incorporated in the nauplii enriched with them, the effects detected in the larvae are not conclusive. Direct liposome baths show evidence pointing to the incorporation of some of the nutrients without live prey mediation.

Acknowledgements

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DIETARY NEUTRAL LIPID LEVEL AND SOURCE AFFECT FOOD INTAKE, NUTRIENT ABSORPTION, GUT STRUCTURE, ENZYMATIC ACTIVITY AND GROWTH IN MARINE FISH LARVAE

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The requirements for essential fatty acids (FA) have been extensively studied in marine fish larvae but few studies have examined the effect of total lipid levels in larval diets. Attempts to meet larval requirements using poor sources of essential FA (predominantly neutral lipids) may result in excessive lipid content and a number of authors have reported poor larval growth associated with high dietary lipid content. Several effects of high dietary neutral lipid levels which may have a potential negative influence on larval growth can hypothesized: at the digestion and absorption level (assuming that a high dietary lipid content may eventually result in a lower efficiency or decreased activity of digestive enzymes and reduced absorption efficiency) and at the ingestion level (if a lipid-rich diet results in a lower food and thus protein intake, i.e., if a mechanism of regulation of food intake according to dietary energy level exists in larvae, as has been shown in juvenile and adult fish). The present work reviews studies carried out on commercially valuable farmed species that investigated the effects of neutral lipid level and lipid source on some of these key factors influencing larval growth.

Growth, gut histology, and nutrient absorption were studied in Senegalese sole (*Solea senegalensis*) larvae fed either non-enriched *Artemia* (NEA) or *Artemia* enriched on a soybean oil emulsion (EA). Feeding the higher neutral lipid diet (EA) resulted in a lower growth and an increased accumulation of lipid droplets within the gut epithelium, which might be hypothesised to physically affect lipid absorption. In fact, by tube-feeding a series of radiolabelled lipids and FA, it was demonstrated that the higher enterocyte lipid accumulation was associated with a lower FA absorption efficiency, which might explain the lower growth of larvae fed EA. However, when FA differing on chain length and degree of saturation

were tube-fed, they were not equally affected and particularly DHA absorption did not appear to have been influenced by enterocyte lipid droplet accumulation. In addition, larvae were fed ^{14}C-*Artemia* in which the radiolabel was mostly incorporated in the protein fraction. These trials showed that although the lipid accumulation observed within the enterocytes does not appear to affect total amino acid (AA) absorption efficiency, the absorption rate appears to be slower which, in continuously feeding larvae, may result in a slower clearance of the gut lumen with potential effects in the reduction of food intake.

The effect of neutral lipid level (7.5 or 15% of oil in microdiet) and source (fish oil, triolein, and coconut oil) on growth and lipase activity was examined in seabass (*Dicentrarchus labrax*). Growth was affected by dietary lipid source being, in this case, slightly increased at the higher lipid level (except for the coconut oil diet in which a high supply of medium-chain triacylglycerols may have deleterious effects). These results may be explained by a higher energy intake, if feeding is not regulated by total lipid level. As for the lipase activity, it was significantly affected by the source of dietary lipid but not by its quantity. Differences in the FA composition of the diet, related to the specificity of lipase towards FA differing in chain length and degree of saturation, may explain these results. However, growth was not related to lipase specific activity, suggesting that lipase synthesis is not a limiting factor for growth.

Food intake and nutrient absorption of ^{14}C-labelled diets were investigated in seabream (*Sparus aurata*) larvae. In one experiment testing *Artemia* enriched on a fish oil emulsion at higher and lower doses (HF and LF), no significant differences were found in larval growth. However, the larvae fed the high fish oil (HF) diet showed a significantly higher food intake and a significantly lower nutrient absorption, supporting the existing idea of an inverse relationship between food intake and nutrient absorption efficiency. In another experiment where larvae were co-fed a microdiet and *Artemia* enriched on two levels of soybean oil emulsion (HS and LS), a significantly higher dry weight was achieved by larvae fed on the low soybean oil (LS) diet, which was also significantly more ingested and absorbed. These experiments showed that dietary lipid level significantly affects larval food intake and absorption efficiency but the effect was dependent on lipid source, suggesting that dietary FA composition might be a more determinant factor than total lipid level in the regulation of food intake, through pre- or post-absorptive mechanisms, such as through effects on palatability, digestibility, and stimulation of neuroendocrine pathways.

In conclusion, it is clear that lipid level in diets for marine fish larvae may have an important role in influencing larval growth and development. However, dietary lipid supply cannot be dissociated of its FA composition, which appears to play a central and determinant role on the nutritional and physiological impacts of dietary lipid inclusion, at the ingestion, digestion and absorption levels.

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ONTOGENETIC DEVELOPMENT OF METABOLISM IN LARVAL YELLOWTAIL (*SERIOLA LALANDI*)

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Introduction

The early development of yellowtail is poorly understood due to difficulties in finding eggs and larvae in the wild. Yellowtail culture in the southern hemisphere is solely reliant on hatchery-reared juveniles and offers an opportunity to study the early developmental biology of this species. Unlike many northern-hemisphere-cultured fish species, there is little information concerning the deployment of metabolites used to fuel development prior to first-feeding in yellowtail. We have investigated a number of aspects of yellowtail egg and larval developmental biology including the effects of temperature on developmental rate, respiration, morphometrics, and the use of metabolic fuels.

Methods

A series of experiments were carried out to assess the effect of temperature on 1) developmental rate, mortality, and respiration; 2) egg and larval morphometrics and, 3) the change in egg and larval metabolites such as glycogen, protein, and free amino acids. Experiments were carried out at temperatures between 17 and 24°C.

Results

Water temperatures above 17°C are required to trigger spawning in the spring months. A single spawning event (1-2 females) produces 50 000-500 000 eggs. Yellowtail produced relatively small eggs, 1.4mm in diameter, which hatched after around 105h at 17°C and had a survival at hatch of 40-60%. At 23°C, the time to hatch decreases to around 50h and survival was between 10-30%. The

oxygen consumption rate of eggs and larvae generally increased in a linear fashion with development. Temperature had no effect on egg volume during development, but did have an effect on larval length at hatch, which was around 5mm at 18°C. Eggs incubated at 18°C produced larvae that were up to 20% longer than at 24°C. Larvae tended to hatch smaller at warmer temperatures (22 and 24°C) and continued to grow until first-feeding, whereas at lower temperatures (18 and 20°C) larvae did not increase in size after hatch. Oil droplet volume decreased considerably during embryo and larval development for all temperatures, but tended to plateau after hatch at cooler temperatures. There was a negative correlation between temperature and oil droplet volume at hatch. Ontogeny and temperature did not influence egg or larval protein, glucose or glycogen content to any great extent. Free amino acid content decreased markedly during ontogeny, from an initial concentration around 180nmol.individual^{-1}.

Discussion

Yellowtail eggs and larvae appeared to develop more slowly compared to other species with similarly sized eggs at equivalent temperatures. As with many other pelagic fish studied to date, free amino acids appeared to be an important metabolic fuel during embryogenesis. The depletion of the oil globule throughout development indicated that this was a continual source of energy which appears to be partially conserved at cooler temperatures during the first feeding window. These data indicate that the best practice larviculture method would include incubating eggs and larvae at cooler temperatures (17-18°C) as this should result in higher survival, larger larvae at hatch, and better conservation of yolk constituents through first feeding.

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IS SEAWATER A RELIABLE SOURCE OF IODINE FOR ATLANTIC HALIBUT (*HIPPOGLOSSUS HIPPOGLOSSUS* L.)?

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Introduction

Abnormal development is almost absent in halibut larvae when they are fed copepods, their natural prey organisms (Shields et al., 1999). Several nutritional differences are found between *Artemia* and copepods (Bolker and Hill, 2000, Hamre et al., 2002). Halibut larvae fed commercially enriched *Artemia* contain lower levels of iodine and thyroid hormones and they have less-developed pituitary and thyroid glands (Solbakken et al., 2002; Pittman et al., unpublished data). This may be a reflection of the low iodine content in *Artemia* compared to copepods, which may contain up to 700× higher level of iodine (Solbakken et al., 2002). Iodine is essential in all vertebrates for the production of the thyroid hormones thyoxine (T_4) and triioodothyronine (T_3), containing 4 and 3 iodine molecules, respectively. The iodine requirement has not been investigated in marine fish larvae, but a possible deficiency may lead to the malformations observed during early life stages of many marine species.

Fish in general may satisfy their iodine requirement through seawater and diet. Both iodate (IO_3^-) and iodide (I^-) are present in seawater and the total amount of iodine (I) shows little variation (ca. 0.5µM). However, the relationship between iodate and iodide varies with depth and geographical location. Iodide, the form that is bioavailable, dominates the euphotic zone, while iodate dominates beneath (Wong, 1991). Pumping water up from beneath the euphotic zone may lead to too-low levels of accessible iodide. Furthermore, early marine larvae do not have fully developed gills, the organ where absorption of iodide is shown to occur in fish (Hunn and Fromm, 1966). Whether the larvae are able to absorb iodide from the seawater through organs such as skin or intestine is unknown. If seawater iodide is sufficient under natural circumstances, the level necessary to satisfy the iodine requirement must be documented. In this project we want to quantify the possible uptake of iodide from seawater in Atlantic halibut larvae.

Materials and methods

Atlantic halibut larvae were reared at the commercial hatchery Norsk Kveite AS, Fromeide, N-5314 Fromgarden, Norway. The larvae were fed enriched *Artemia*. 24 larvae from 6, 23, and 45 days post-first-feeding were individually incubated in 12ml of water with increasing levels of iodide (15, 60, 120, 300, 600, and 1000nM) with a constant level of $^{125}I^-$ (13 333Bq.ml^{-1}) for three hours. The larvae were then thoroughly rinsed in distilled water and killed with an overdose of MS-222. The intestine was dissected and carcass and intestinal tube was counted separately using a Liquid Scintillation Counter (Tri-Carb, Packard Instrument Company) with 4ml of Solvable and 10ml of Ultima Gold XR (Packard Instrument Company).

The levels of $^{127}I^-$ in the water are to be determined by HPLC coupled ICP-MS. Influx (J_{in}) of iodide is calculated using the following formula:

$$J_{in} = \frac{Q_{larva}}{X_{out} \times t}$$

where Q_{larva} is the radio activity per larva, X_{out} is the specific activity of the incubation water (cpm.nmol^{-1} I^-), and T is the incubation time (Chang et al., 1997)

Results and discussion

Preliminary results show an increasing influx of iodide in larvae with increasing concentration of I^- in the incubation water.

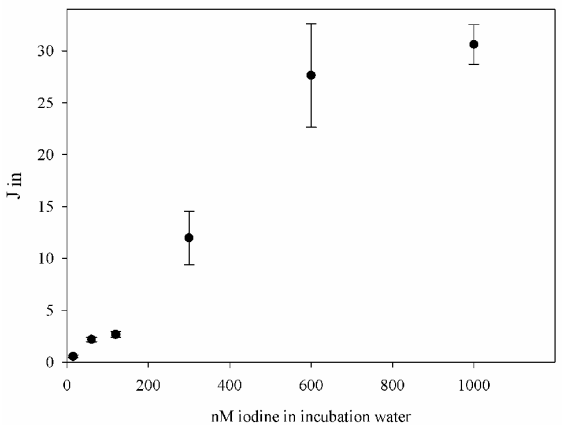

Fig. 1. Preliminary curve for average uptake of iodide in halibut larvae (6 days post-first-feeding) at 6 different iodide concentrations. Intestines were dissected out prior to counting. X-axis shows theoretical levels of I^- (nM) in the incubation water (the actual level is at the time of writing not yet analysed). Y-axis shows the influx (J_{in}) calculations using theoretically levels of $^{127}I^-$.

The data (Fig. 1) do not fit either the Michaelis Menten plot or the linear plot, expected in typical active or passive uptake, respectively, but may possibly be fitted to a sigmoidal curve, indicating an allosteric system with positive correlation. Results from uptake trials at day 23 and 45 with an increased number of iodide concentrations may reveal the shape of the actual curve. These results will be presented and discussed in relation to whether seawater can be considered an important source of iodine.

Acknowledgements

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IMMUNITY OF FISH LARVAE

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To rigorously revise and compare the scarce but heterogeneous – and sometimes apparently contradictory – information available on the development of the fish immune system and immunity, one must bear in mind the interspecific variations in the fish ontogenesis process. We will roughly assume the sequence embryo-larval-juvenile, characterised, respectively, by endogenous feeding (yolk)-transition (yolk plus exogenous feeding)-post transition (exogenous feeding). A preliminary consideration should be about the convenience of concentrating future research on fish species of scientific or commercial interest. The use of the powerful zebrafish model together with new research technologies, particularly genetic and chemical manipulation, is providing definitive new data for better understanding the ontogeny of fish immunity.

The embryo immunity seems to consist of maternally transferred specific and non-specific immune factors (antiprotease, lisozyme, C_3, IgM), which remain in the yolk sac. Namely, antibody raised in the maternal circulation is incorporated into oocytes in the ovary and transferred from the yolk sac into larval circulation, where it is gradually metabolised by larvae. This seems to indicate that immunisation of broodstock before spawning may serve to reduce the early mortality in newly hatched larvae.

From the larval to adult period, two immunity stages should be established. Firstly, a stage of non-specific immunity, roughly similar to that of the invertebrates, followed by a second stage, which starts when the immune system tissues become morphologically and functionally useful, after which the larva show progressively more elaborate non-specific and specific immunity, finishing with the immune features of the adult fish.

The short first stage of non-specific immunity is characterised by two haemopoietic domains specified by *draculyn* gene expression: the well-known caudal embryonic blood island giving rise to erythroblasts and endothelial cells, and the ventrolateral mesoderm giving rise to macrophage-like mononuclear phagocytes. Immediately, derived anteriolateral mesoderm *flk*-expressing cells converge as

two thin bands to delineate the primordia of the head vessel, whereas macrophage progenitors (*dra*-expressing cells, which appear at least as early as erythroid cells) converge, stop expressing *draculin*, migrate to the yolk sac and differentiate, and invade the mesenchyma of the head, some joining the blood circulation. It has been suggested that these early cells arise through a non-classical, rapid differentiation pathway, which bypasses the monocytic series, and occurs before any other type of leukocyte appears in the embryo. The "primitive", "pre-macrophages", or "young macrophages" display fast motility and interact with proerythroblasts, but still have no phagocytic experience. They differentiate into "early macrophages", which phagocytose apoptotic bodies and large amounts of bacteria. They also secrete an impressive array of growth factors, cytokines, and proteins for remodelling the extracellular matrix, regulating aspects of organogenesis and promoting angiogenesis. The question arises whether they are replaced later by "classical" macrophages or whether they are long-living residents in adult tissues.

Regarding the second stage of immunity (from larva to adult), head-kidney, spleen, and thymus, which develop in this order in marine fish, seem to be the main tissues involved. Avoiding interspecific variation, they show mixed "red" and "white" cell populations. Leukocytes are the predominant cell type in head-kidney and red cells in the spleen. There is no information on ontogenesis of the myeloid series. Cellular defence mechanism seems to develop earlier than the corresponding humoral one. The thymus appears to be the primary organ for T-lymphocytes and the head-kidney the primary organ for B-lymphocytes. It is thought that stem cells in thymus come from head-kidney and that mature T-lymphocytes from thymus seed the spleen. "Cell bridges" between the kidney and thymus have been observed. In cyclostomes, evidence exists for thymus-dependent immune responses, although the thymus is absent. Chondrichthyans are the earliest evidencing thymic tissue. In teleosts, thymic anlage appears about two days post fertilization. Microscopically two layers of epithelial cells appear and, immediately, some of the other thymic cell types. Studies using monoclonal antibodies, flow cytometry, and immunocytochemistry indicate that early (pre-) T-cells have their origin in a different compartment and subsequently mature in the thymus, expressing a four-chained TCR, homologous to those of higher vertebrates. Recombination activating genes (*Rag*) are also present in the early thymus indicative of the earliest T-cells.

The development of B-cells is influenced by weight, age, and season. Mature B-cells and IgM neo-synthesis are detected in two-day-old larvae. Although a few B-cells are found at two days, only from one month onward are (sIg+) plasma cells detected in kidney but not (or occasionally) in spleen. Mature B-cells, plasma cells, and strong IgM synthesis are present when lymphomyeloid organs are fully developed (50-day-old sea bass larvae).

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EARLY WEANING TRIALS EXAMINING SURVIVAL, GROWTH, AND GUT HISTOLOGY OF ATLANTIC COD LARVAE

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Introduction

The development of an artificial diet to control larval nutrition is necessary for expansion of marine aquaculture production. This study examines the survival and growth of larval Atlantic cod, *Gadus morhua*, fed our zein-microbound experimental diet. Two studies were conducted to determine if early weaning with our experimental diet was possible, or if supplementation with low levels of *Artemia* sp. would better increase growth and survival.

Materials and methods

Atlantic cod larvae were reared in 22-l tanks connected to a 1500-gallon recirculating system containing artificial seawater (33ppt). Eggs were stocked into rearing tanks immediately prior to hatch at a density of 75 larvae.l^{-1} and maintained at 10°C for the duration of the experiments. *Brachionus plicatilis* were enriched with Algamac and fed to cod larvae from hatch to 21 days post-hatch (dph). The experimental diet was fed every hour from 8dph to the termination of the experiment at 36dph. The experimental diet was compared to a commercial diet (Biokyowa 250A) and a live food treatment (fed rotifers to 21dph and *Artemia* sp. until the termination of the experiment on 36dph). On 36dph, larvae were collected and preserved for histological analysis.

In the second experiment, larvae were reared in the same recirculating system to 50dph. The first experiment was repeated on another hatched group of Atlantic cod larvae with an additional treatment added. A fourth group of larvae was fed rotifers and diet until 21dph and then, rather then being fed solely the diet, the larvae were given a 35% reduction of *Artemia* sp. as compared to the live food treatment.

Standard lengths and dry weights were measured at hatch, 8, 22, 36, and 50dph. Survival was calculated by counting all remaining fish at the conclusion of the

experiment. The experiments were carried out in triplicate and results were analyzed by one-way ANOVA. Percent data were transformed (arcsine) before conducting analysis of variance. Tukey's HSD test was used to determine significance differences among the means ($p<0.05$) with SYSTAT^{TM} software (Snedecor and Cochran, 1993).

Results and discussion

Cod larvae fed the experimental diet (ZMPD) did not differ significantly in standard length from those receiving Biokyowa 250A or live food at 36dph ($p<0.05$), but were not as heavy as the Biokyowa 250A group or the live food group at the termination of the experiment (Table I). Survival was 34%, 24%, and 34% for the ZMPD experimental diet, Biokyowa 250A, and live food group, respectively (Table I).

Table I. Survival, standard length, and dry weight of cod larvae reared on an experimental diet (ZMPD), Biokyowa 250A, and live food to 36dph. Values represent the mean ± SD of three replicates.

Diet	ZMPD	Biokyowa	Live Food
Survival (%)	34.46 ± 11.54^{a}	24.88 ± 13.09^{a}	34.18 ± 5.81^{a}
Standard Length (mm)	10.94 ± 1.11^{b}	11.37 ± 1.22^{b}	12.60 ± 1.53^{a}
Dry Weight (mg)	1.42 ± 0.34^{c}	1.90 ± 0.27^{b}	2.81 ± 0.52^{a}

In the second study carried out to 50dph, the larvae fed the experimental diet with 35% of the *Artemia* fed to the live food control did not differ significantly from the live food control relative to standard length, dry weight, or survival ($p<0.05$; Table II). Early weaning to rotifers without any *Artemia* use yielded lower growth and survival ($p<0.05$).

Table II. Survival, standard length, and dry weight of cod larvae reared on an experimental diet (ZMPD), experimental diet with reduced *Artemia* (35% of live food group), and live food only to 50dph. Values represent the mean ± SD of three replicates.

Diet	ZMPD	ZMPD + 35%A	Live Food
Survival (%)	12.32 ± 1.21^{b}	22.71 ± 3.53^{a}	17.80 ± 8.70^{ab}
Standard Length (mm)	11.49 ± 1.35^{b}	13.28 ± 2.17^{a}	13.20 ± 2.74^{a}
Dry Weight (mg)	2.21 ± 2.13^{b}	3.51 ± 1.23^{ab}	4.44 ± 1.99^{a}

The addition of salmon roe to the experimental diet as a phospholipid source increased the performance of the diet in comparison to earlier work in the lab (Baskerville-Bridges and Kling, 2000). Considerable research shows that preformed phospholipids are needed by developing larval marine fish for development and growth (Kanazawa, 1985). The gut histology, when compared to ear-

lier research in our lab, shows the disappearance of "lipid vacuoles" with the addition of dietary phospholipid to the diet (refer to figure on poster).

Conclusions

Survival and growth in the first experiment to 36dph looked promising, and the second study was needed to see if our zein-microbound experimental diet could support larval cod through the many stages of larval development. The results of the second experiment show that more research is needed to understand the changes in nutritional requirements and digestive capacity through the entire larval period. At this time, our diet will not completely replace the requirement for live food in an aquaculture setting due to the need for cost-effective rearing (higher survival). These studies do support, on the basis of growth, survival, and gut histology, that early weaning of larval Atlantic cod to a microparticulate diet is possible with significantly less live food. Our more recent studies in the apparent digestibility of our experimental diets will provide a novel technique to evaluate microdiets through the entire larval stage and accelerate our progress.

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SKELETAL DEFORMITIES IN THE EARLY STAGES OF SEVEN-BAND GROUPER *EPINEPHELUS SEPTEMFASCIATUS*

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Introduction

Skeletal deformities in reared fish are a major concern in aquaculture. The deformities in the juvenile stage have an important role in commercial aquaculture, as they reduce the market value of fish product and require manual sorting. Many studies on skeletal deformities in jaws and vertebrae have been reported for larvae and juvenile reared under intensive aquaculture.

The seven-band grouper, *Epinephelus septemfasciatus* (Thumberg), is a commercially important species, occurring in shallow waters from Japan, Korea, and China (Heemstra and Randall, 1993). This species is recognized as a potential new species for aquaculture in Japan. The recent improvement in rearing techniques for this species has led to increase in larval survival. However, a high proportion of cultured juveniles exhibit jaw and vertebral malformations. The aim of this study is to describe the ontogeny of different types of skeletal deformities in intensive cultured seven-band grouper.

Materials and methods

Artificially fertilized grouper eggs were obtained following Shein et al. (2004). The eggs were then distributed into two circular 100-kl tanks (Tank A and B) before hatching with initial stocking density of ca. 10 eggs.l^{-1}. They were supplied with running UV-treated, sand-filtered seawater. Throughout the experiment, the eggs and larvae were reared at water temperature of 19.9-26.5°C for

Tank A and 20.1-26.9°C for Tank B. From 2 days after hatching (dah), larvae were reared under green-water conditions. Larvae were fed with SS-type rotifer at a density of 10 ind.ml^{-1} from 3-9dah and L-type rotifer at a density of 5 ind.ml^{-1} from 10-45dah. From 24-55dah, *Artemia* were given at a density of 1 ind.ml^{-1}. A formulated artificial feed was supplied from 40dah.

To monitor larval morphology, ca. 100 larvae per tank were taken every 2 or 3 days from each tank and fixed with 10% seawater-buffered formalin. Samples were then transferred to 70% ethanol for subsequent observation. Standard length (notochord length for preflexion and flexion larvae), and upper jaw length was measured under a binocular microscope. Osteological observations were done using clearing and staining method for larvae and soft X-ray photographs for juveniles.

Results and discussion

The high incidence of skeletal deformities was observed in jaw and vertebral skeletons of the cultured seven-band grouper. Jaw deformities were seen after flexion larval stage (20dah), representing 20-98% of the fish sampled for Tank A and 0-20% for Tank B during 20-82dah (Fig. 1). Most of the jaw deformities were explained by abnormal maxilla and premaxilla curvature. The clearing and staining method showed these deformed elements (except for supramaxilla) had ossified prior to 14dah, at which jaw skeletal deformities were observed.

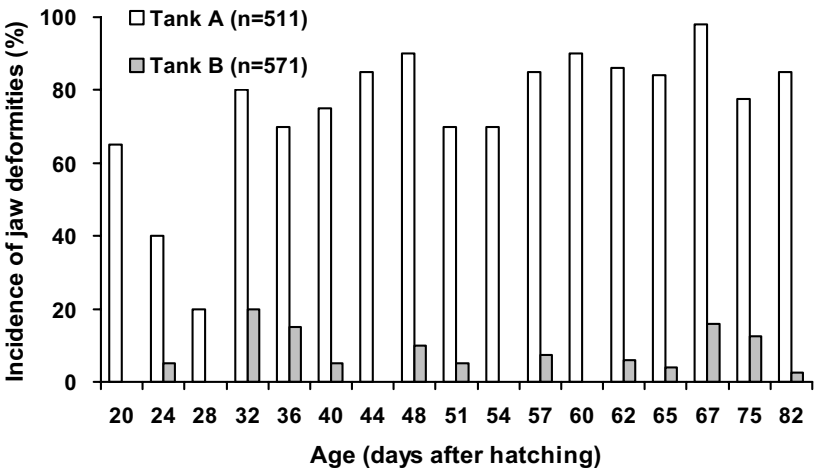

Fig. 1. Incidence (%) of jaw deformities observed in Tank A and B during 20-82 days after hatching.

Deformities in the vertebrae were observed from the transformation stage and they became more frequent in the juvenile stage. The incidence of the deformi-

ties ranged from 2.0-70.2% in Tank A and 0-61.9% in Tank B during 57-100dah (Fig. 2). The most common deformity observed was lordosis, being characterized by distortions of vertebral centrum, neural spine and haemal spine. The lordosis generally occurred on the positions between precaudal and caudal vertebra (the 8-11th vertebra).

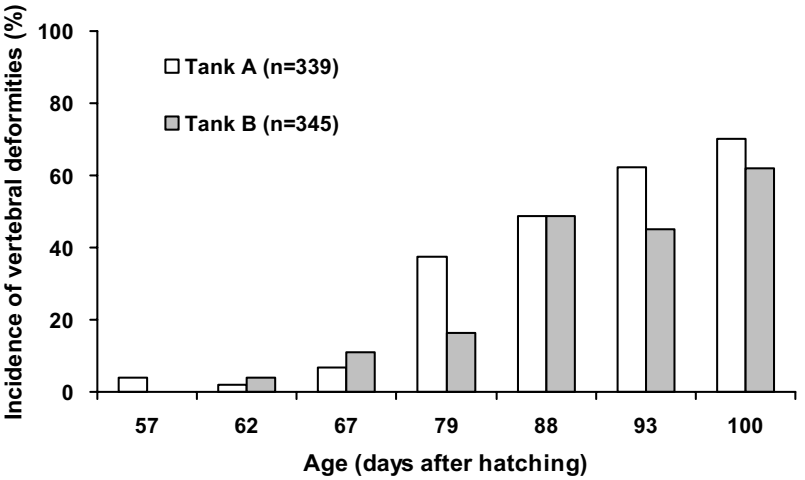

Fig. 2. Incidence (%) of vertebral deformities observed in Tank A and B during 57-100 days after hatching.

Other deformities included lack of inter-nostril epidermis and skull skin, being observed from the postflexion stage. The former showed a frequency of 100% in Tank A and 46.0-100% in Tank B during 44-72dah. The latter was observed only in Tank A, exhibiting 10.0-60.0% of the examined fish during 51-82dah.

This study is the first description on normal and abnormal skeletal development of early life stages of reared seven-band grouper. The deformed skeletal elements, and the ontogenetic stage where deformities develop were identified. There are several possible explanations causing skeletal deformities in intensive aquaculture (e.g., Chatain, 1994; Divanach et al., 1997; Cahu et al., 2003). However, causative factors of deformities could not be identified in this study due to various external parameters in large scale rearing tank. Further studies are required to determine the parameters (nutritional and physical environmental conditions) affecting skeletal development.

Acknowledgements

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APPLICATION OF EUROPEAN REARING TECHNIQUES IN FRY PRODUCTION OF LARGE YELLOW CROAKER *PSEUDOSCIAENA CROCEA* (RICHARDSON) IN THE PEOPLE'S REPUBLIC OF CHINA

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Introduction

Artificial breeding of large yellow croaker *Pseudosciaena crocea* (Richardson) was first accomplished in 1987 (Liu, 1999). After this success, the number of artificially produced fry increased exponentially and culminated in the period of 1995-2000, with a maximum of 1.3 billion fry produced in 2000 (Hong and Zhang, 2003). The traditional Chinese breeding technique has been mainly copied from the shrimp hatchery practices and has been reviewed by several researchers (Su et al, 1997; Liu, 1999; Chen, 2001). In the present study the traditional Chinese rearing technique is compared with a more intensive culture strategy inspired on European concepts as live food enrichment and weaning on artificial diets.

Materials and methods

The main differences between the European rearing techniques (ET) and the Chinese rearing techniques (ET) are given in Table I.

In the broodstock trial, large yellow croaker spawners (64) consisting of 19 males and 45 females were reared in a 50-m^3 tank as described by Chen (2001). After 3 days of acclimation, the fish were divided by a net, half of the population receiving the control diet consisting of fresh oyster meat (CT broodstock), the other half (ET broodstock) receiving the commercial broodstock diet Breed-M (INVE, Belgium). The fishes were hormonally induced with LRH-A3 on day 35 and eggs were collected on day 37 as described by Chen (2001). The survival and quality of larvae from both egg batches were evaluated after a standard lar-

val rearing on the same diet of rotifers (Culture Selco as food and DHA Protein Selco as enrichment).

Table I. Description of the European and Chinese rearing techniques used in the large yellow croaker experiment

Rearing stage (duration)	Characteristics		European rearing techniques	Chinese rearing techniques
Broodstock (37 days)	Diet		Breed-M	Fresh oyster meat
	Daily feeding (% of body weight)		1%	3%
Hatchery (35 days)	Rotifer	Tank volume	2000 l	50 000 l
		Culture method	Batch (4 days per run)	Continuous (10-15 days per run)
		Density (rotifers.ml^{-1})	170 to 500	10 to 200
		Food	Artificial diet	Bakers' yeast
		Enrichment	During culture	*Chlorella* for 6-11h
		Contamination	Free swimming ciliates	Free swimming ciliates, *Vorticella,* bacterial red spots on tank walls
	Artemia nauplii		Enriched with DC DHA Selco (25 h)	Newly hatched
	Water exchange (% per day)		Flow-through (20-200%)	Stagnant (10-100%)
	Larval feeding regime		Rotifers (d3-15) Artemia (d10-13) Copepods (d12-20) Weaning diet (d20-35)	Rotifers (d3-15) Artemia (d10-13) Copepods (d12-35) No weaning
Nursery (60 days)	Source of larvae		ET tanks	CT tanks
	Feed		Diet NRD3-NRD6	Eel feed, fish paste

For the hatchery phase, the floating eggs obtained from each broodstock were incubated for hatching in 2 tanks of 50m^3 and further reared until day 35 following Chen (2001). The rotifers fed to ET tanks were cultured in 2-m^3 tanks with an experimental combined rotifer culture and enrichment diet (INVE, Belgium); the rotifers fed to CT tanks were cultured in 50-m^3 tanks with baker's yeast and enriched with *Chlorella* for 6-11h prior to their transfer to the fish tanks. Newly-hatched *Artemia* nauplii (Great Salt Lake, Utah, USA) were fed to the CT tanks. The ET tanks were fed with enriched *Artemia* (DC-DHA Selco, INVE, Belgium). Copepods (collected from the wild) were fed to the larvae of both treatments from day 12 onwards. Larvae of the ET treatment were weaned on the artificial diet (NRD, INVE, Belgium) starting from day 20. Fish of the CT treatment were not weaned and were further fed with copepods.

In the nursery trial, fish from the CT treatment were stocked in 2 cages (3×3×3m) at 30 000 larvae per cage and fed on a local eel feed for the first 7 days followed by trash fish paste. The larvae originating from the ET treatment

were also stocked at the same density in similar cages and received a nursery diet (NRD, INVE, Belgium). The growth, survival and stress condition of the fish were evaluated after 60 days.

The following parameters were evaluated at the end of the broodstock, hatchery, and nursery phases: number of eggs, egg diameter, number of oil globules per egg; percent egg hatching, larval/fry survival, total length, and weight of larvae and fry. All data were analyzed by a one-way ANOVA and significant differences determined by a Tukey multiple comparison test. The stress test was performed in 50-g.l^{-1} seawater as described by Dhert et al. (1992).

Results and discussion

The broodstock fed on oysters (CT) and on the artificial diet (ET) matured synchronously and produced fertilized eggs with similar output and egg quality characteristics. No differences were noticed in egg diameter or oil globule characteristics among the treatments. The quality of the larvae originating from both treatments was evaluated at the end of the rotifer feeding stage. Survival of the larvae fed on Breed-M (ET) had a higher survival (84%) than the larvae originating from the oyster-fed broodstock (61%). The stress resistance was not significantly different among the treatments. These results show that yellow croaker broodstock can perfectly be adapted to artificial diets and produce high quality offspring. The off-the-shelf availability and standard quality of Breed-M could be applied to reduce labour cost in food preparation and prevent possible disease transfer via natural feeds.

Chinese and European rearing and enrichment techniques for rotifers differ considerably. In Europe, higher rotifer concentrations (max. 500.ml^{-1}) can be produced in smaller tanks; furthermore, the application of combined culture and enrichment techniques yield cleaner and healthier rotifers (i.e., less free swimming ciliates, less *Vorticella,* no bacterial red spots on tank walls).

The analysis of highly unsaturated fatty acids (HUFA) performed on all live feeds showed significant differences in their FAME patterns. Rotifers enriched following the ET treatment were richer in n-3 HUFA and showed a higher DHA/EPA ratio. ET-*Artemia* (enriched under local Chinese conditions, suboptimal conditions as compared to applications in Europe) showed a lower n-3 HUFA profile than copepods. The latter had a lower DHA/EPA ratio but contained more short chain FA than *Artemia*.

HUFA analyses of the larvae revealed that the fatty acid profile of the live prey clearly affected the composition of the fry; i.e., ET larvae richer in HUFA during the rotifer stage, but the difference eventually gets smaller when larvae received copepods in the CT treatment.

The concept of weaning of the fish fry is not applied in the traditional yellow croaker nurseries where fish are constantly kept on trash fish. The differences among both treatments were spectacular as ET fish were growing significantly faster and their survival was higher. Furthermore significant differences were observed in the quality of the fish during the nursery and ongrowing stages, i.e. mortality due to stress was considerably lower, coloration of the fish was natural (yellow) and the swimming behaviour of the fish was different with ET fish swimming at the bottom of the cage, while CT fish were close to the surface.

Food conversion ratios (FCR) calculated at the end of the experiment were 3 times lower in the ET treatment as compared to the CT treatment.

In conclusion it can be stated that the Chinese technique is relying exclusively on live preys and trash fish and is more exposed to disease vectors. The European technique offers more guarantees in the quality of the end product and is less polluting but is relying on a more intensive approach with a higher demand especially in the aeration supply of live food which is not always compatible with the infrastructure of local Chinese hatcheries.

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CHANGES IN PLANKTONIC STRUCTURE IN REARING WATER DURING THE INTENSIVE LARVICULTURE OF *PAGRUS MAJOR*

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Introduction

There are prey-predator interactions between many kinds of organisms in a rearing tank of fish larvae. Thus, the rearing tank of fish larvae can be regarded as a kind of artificial ecosystem with larvae at the top. In the present study, we characterized quantitatively the entire planktonic structures in rearing water during intensive larval production of the red sea bream *Pagrus major*, and discussed relationships between changing planktonic structures and survival of larvae.

Materials and methods

Larval cultivation of *P. major* was conducted from 22 May to 27 June 2004 in two different-volumed square concrete tanks: Tank 1 is 30kl and 1m deep and Tank 2 is 50kl and 1.6m deep. The density of eggs introduced into each tank was 15 eggs.l^{-1}. Larvae were fed rotifers *Brachionus rotundiformis* from day 3 after hatching. After day 21 in Tank 1 and day 24 in Tank 2, *Artemia* nauplii were offered to larvae, and *Nannochloropsis* sp. was supplied throughout.

Plankton structure was analysed in the rearing water with a modified method described by Tanaka and Rassoulzadgan (2002). The plankton in the rearing water was microscopically classified into prokaryotes (bacteria and cyanobacteria) and eukaryotes. The eukaryotes were further classified into autotrophic and heterotrophic groups and the plankton in each group was classified by size: pico-sized (<2μm), nano-sized (2-20μm), and micro-sized (20-200μm). Rotifers and *Artemia* nauplii were quantified as carbon weight.

Results and discussion

Among the environmental factors, lighting was different between Tanks 1 and 2. Mean light intensity in Tank 2 during the rearing period was 62% of that in Tank

1. It was reported that the larvae reared in higher light intensities had higher prey capture than larvae reared in lower light intensities (Puvanendran and Brown, 2002). This result implied that light condition influenced feeding on prey and subsequent survival rate of larvae. Therefore, survival rate in Tank 2 might be lower than that of Tank 1 under low light condition.

Survival rates of *P. major* at the final day of rearing were 41.3% in Tank 1 and 12.5% in Tank 2. There were significant differences in the length of *P. major* between both tanks after day 21.

In Tank 1, autotrophic nano-plankton (ANP), mostly *Nannochloropsis*, was highest at day 3 (>72%) and decreased toward the final day of rearing (0.4%). Contrarily, rotifers increased from 20% at day 3 to >80% at day 24. In Tank 2, ANP was less than 72% until day 7. On the other hand, rotifers in the rearing water dominated after day 10, and were more than 56% of total plankton biomass, except for at Stn A at day 3 (61%). It has been suggested that *Nannochloropsis* may act as a supplementary feed for the remaining rotifers in the larval rearing tank (Lubzens et al., 1995; Yoshimatsu et al., 1995). Moreover, *Nannochloropsis* added into rearing tanks has been considered to control water quality (e.g., Yoshimatsu et al., 1995). ANP dominated in the early days in Tank 1 but not in Tank 2. Light condition of Tank 2 for *Nannochloropsis* might also be worse than that of Tank 1. Therefore, *Nannochloropsis* activity and its controlling effect of water quality may have contributed to the tank differences.

Nutritional values, especially omega-3 highly unsaturated fatty acid (ω3-HUFA), of rotifers added as live prey influence growth and survival rates of larvae (Watanabe et al. 1989; Takeuchi et al. 1990; Watanabe 1993). When *Nannochloropsis* was introduced to a rearing tank at a concentration of $2.5\text{-}5.0\times10^5$ cells.ml^{-1}, the rotifer ω3-HUFA content remained constant for 3 hours, but decreased when *Nannochloropsis* was less than 2.5×10^5 cells.ml^{-1} (Yoshimatsu et al. 1995). In the present study, abundance of ANP, mainly *Nannochloropsis*, in the rearing water at early stages of rearing of larvae was $2.2\text{-}5.1\times10^5$ cells.ml^{-1} in Tank 1 and $0.5\text{-}2.7\times10^5$ cells.ml^{-1} in Tank 2. These results suggest that nutritional values of rotifers in Tank 2 decreased linearly after being transferred into rearing tank. Thus, differences in the nutritional value of rotifers in the rearing water might have influenced survival rates of *P. major* larvae.

In Tank 1, the biomass ratio of autotrophs:heterotrophs in the rearing water was highest at day 3, and then decreased exponentially toward the final day of rearing (Fig. 1). In Tank 2, the ratio was highest at day 7, and a small peak was found on day 28. This ratio in Tank 1 at day 3 was much higher than that of Tank 2.

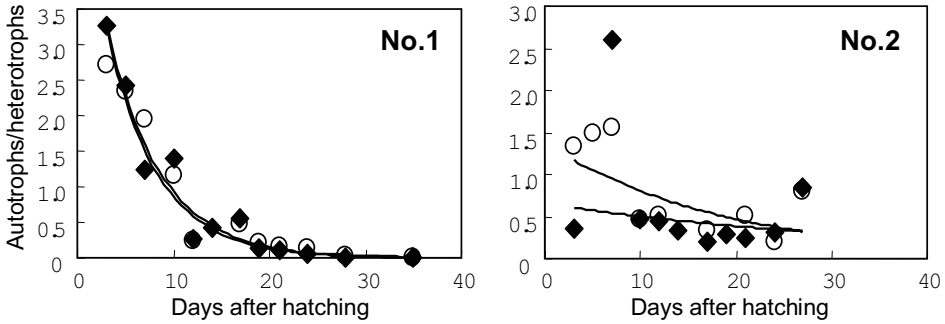

Fig. 1. Changes in biomass ratio of autotrophs:heterotrophs in the rearing water at Stns. A (circles) and B (diamonds) in Tank 1 (left) and Tank 2 (right) during the rearing periods.

In the present study, autotrophic components, especially ANP, dominated and might be active in the rearing tank in early stage, and the planktonic structure shifted exponentially from autotrophic to heterotrophic, mainly rotifers, in Tank 1. In Tank 2, however, autotrophs did not dominate in the rearing water at day 3 when rotifers and *Nannochloropsis* were introduced for the first time, and the pattern of change from ANP to rotifers was different than in Tank 1. The survival rate in Tank 1 was much higher than in Tank 2. The present study suggests that larvae can survive and grow at a higher rate because of the controlling effect of *Nannochloropsis* on water quality, the nutritional value of rotifers in rearing tank when autotrophic components – especially ANP – dominate and are active in the rearing tank in early stage, and the exponential shift in planktonic structure from autotrophic components to heterotrophic components – mainly rotifers – as larvae grow.

Conclusions

The present study focused on environmental condition and plankton ecology in the early stages of larval rearing, as the adaptability to environmental factors of larvae just after hatching is weaker than older ones. The planktonic structure, especially the biomass ratio of autotrophs:heterotorophs, may be a suitable indicator of the biological environment quality in the rearing water.

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DOES BURROWING AFFECT THE PERFORMANCE OF *FARFANTEPENAEUS PAULENSIS* BROODSTOCK?

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Introduction

Like most species of penaeid shrimps, *Farfantepenaeus paulensis* burrows in the substratum during the day and emerges at night (Iwai, 1978). One of the advantages of burrowing is a reduction in energy requirements due to a decrease in activity (Dall et al., 1990). However, when maintained in captivity for reproductive purposes, broodstock animals are stocked in hard-bottomed tanks where they are unable to burrow. A decline in production over time, usually termed "reproductive exhaustion", is routinely observed in the captive reproduction of penaeids (Palacios et al., 1999). Furthermore, the incapacity to burrow may also affect their mating rate and increase the occurrence of external damage to appendages and exoskeleton, as has already been demonstrated for *Penaeus esculentus* juveniles (Sellars et al., 2004). This study examined the physical condition and the reproductive performance of wild-caught *F. paulensis* broodstock kept in hard-bottomed tanks or in tanks provided with sand substrate.

Materials and methods

Mature *F. paulensis* were captured by otter trawl off southern Brazil (27°30'S). After one week in the laboratory, groups of 30 males and 40 females in the intermoult period were randomly selected and stocked in 10-m^3 tanks. Upon stocking, females were unilaterally eyestalk ablated. Two experimental tanks filled with 5000 l of seawater were provided with or without a sand substrate. In the tank provided with substrate, a 10-cm sand layer was placed over a plastic mesh over a drain board. The net mesh prevented sand particles from falling down through the drain board and clogging the system. Fourteen 50-cm-long air lifts allowed water to recirculate through the sand layer. Water was exchanged at 50%.day^{-1}. Ammonia, pH, temperature, and salinity levels were monitored daily and remained within acceptable levels (temperature 26°C, salinity 31-32ppt). Shrimp were fed to satiation 4× daily with fresh/frozen mussel (*Perna perna*), fish (*Macrodon ancylodon*), squid (*Illex argentinus*), and a commercial matura-

tion diet (Breed S, INVE). Uneaten feed, moulted exoskeletons, and dead shrimp were removed every morning. Photoperiod was set at 15h of light per day.

Females with mature ovaries were sourced out daily and transferred to separate 120-l spawning tanks and returned to their respective maturation tanks the following morning. The number of eggs was estimated from three 100-ml replicated samples of the spawning tank water, collected after homogenization. Fertilization rates were determined microscopically. Additionally, the interval between eyestalk ablation and first spawn (latency period), intervals between successive spawns, and spawning frequency were recorded.

The experimental period lasted 50d. On day 1 and 50, physical condition of males and females from both treatments were estimated according to a methodology adapted from Sellars et al (2004). The level of external damage to appendages and exoskeleton (lesions, necrosis in the uropods, carapace, and abdomen, as well as missing or broken thoracic or abdominal appendices, antennae or antennules) formed an index of physical condition, which was expressed by the physical condition score (PCS). PCS was calculated as the sum of cumulative damages observed in each animal and thus higher occurrence of physical damages results in higher PCS values.

Reproductive performance data were statistically assessed using Student's t-test to identify significant differences ($p<0.05$) between treatments. Data on fertilization rates were arcsine transformed before analysis, but untransformed values are presented here. Three-way analysis of variance followed by a Tukey test were used to evaluate significant differences in PCS, using sex (male and female), period of time (initial and final), and tank bottom type (hard-bottom or sand substrate) as independent variables.

Results and Discussion

At the end of the experimental period, survival rates of females and males from the tanks with and without substrate were 75.0 and 47.5%, and 83.2 and 100%, respectively. Multi-factorial analysis detected significant differences in the physical condition score (Table I).

Regardless of tank bottom type, physical condition of males improved from the beginning to the end of the experiment. Conversely, physical condition of females from the tank with sand substrate remained stable throughout the experiment, whereas PCS of females in the hard-bottomed tank declined from the beginning to the end of the experimental period. Although several practices routinely employed in the maturation of penaeids (such as sourcing/transference and maintenance of ready-to-spawn females in comparatively smaller spawning tanks), and spawning itself, may have a significant impact in the deterioration of

female's physical condition, the use of a sand substrate resulted in a significant reduction in the damage sustained by shrimp females. On the other hand, sourcing of mature females in the tanks with sand substrate was more time-consuming as many females were still buried in the substrate after lights were turned off. This may also help to explain the apparently lower number of spawns among the group of females in the tank with sand substrate (Table II).

Table I. Initial and final physical condition score (PCS) of *F. paulensis* males and females kept in tanks with sand substrate or hard-bottom for 50 days. Superscript letters indicate significant differences ($p<0.05$).

	Sex	Time	PCS
Sand substrate	Male	Initial	14.5^{b}
		Final	12.2^{a}
	Female	Initial	15.0^{b}
		Final	14.6^{b}
Hard-bottom	Male	Initial	14.8^{b}
		Final	12.8^{a}
	Female	Initial	15.2^{b}
		Final	17.6^{c}

Table II. Mean (± SD) reproductive performance parameters of *F. paulensis* maintained in tanks provided with sand substrate or hard-bottom for 50 days. Superscript letters indicate significant differences ($p<0.05$).

	Sand substrate	Hard bottom
Number of spawns	91	111
Latency period (ablation-1st spawn; days)	12.9 ± 11.3 b	5.3 ± 3.3 a
Period 1st spawn - 2nd spawn (days)	13.7 ± 8.1 b	7.9 ± 6.1 a
Period 2nd spawn - 3rd spawn (days)	9.1 ± 8.2	9.6 ± 8.9
Eggs/spawn (103)	139 ± 67	162 ± 55
Fertilization rate 1st spawn	87.4 ± 9.6	85.4 ± 15.1
Fertilization rate 2nd spawn	61.5 ± 22.9	65.2 ± 36.6
Fertilization rate 3rd spawn	45.6 ± 17.2	77.7 ± 23.4
Spawns per female	2.7 ± 1.6	3.1 ± 1.5

In their study with *P. esculentus* juveniles, Sellars et al. (2004) found that the use of substrates reduced significantly the occurrence of body damages. Similarly, Arnold et al. (2005) report that the use of substrates improved the survival rate of penaeid juveniles reared at high densities. The present results therefore agree with these studies as the use of sand substrate in the maturation tanks reduced physical damages of *F. paulensis* females. Although the experimental design does not allow any conclusion regarding the effect of tank bottom on survival rates, the present results indicate the possibility that mortality of females may be reduced in tanks provided with sand substrate.

Overall, no major differences were observed on the reproductive performance of *F. paulensis* females from the different treatments (Table II). Number of eggs

per spawning event and spawning frequency (spawns per female) were not significantly different between treatments. However, females in the tank with sand substrate had a significantly longer latency period between ablation and first spawn, and a similar longer period of time between first and second spawns. These differences are probably related to an earlier peak of moulting that was observed for this group of females one week after eyestalk ablation. Although this finding suggests the possible existence of a relationship between burrowing and moulting, the exact reasons for this are unknown. Regardless the experimental treatment, a decrease in the fertilization rate was observed after the first moulting period probably because of lower mating success. Nevertheless, females kept in the tank with the sand substrate had a few fertilized spawns after the first moulting period compared to none from those in hard-bottomed tanks.
In conclusion, results from the present study indicate that the effects of burrowing are rather related to the maintenance of female physical condition than to improvements in their reproductive performance. Although a slight change in female moulting period and improvements in survival of females and mating success may be related to the presence of sand substrate, further studies are necessary to elucidate these observations.

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HATCHERY PRODUCTION OF BABYLON SNAILS (*BABYLONIA AREOLATA*) IN VIETNAM

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MARINE MOLLUSC HATCHERY IN THAILAND: COMMERCIAL SEED PRODUCTION DEVELOPMENT

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Introduction

Research and development on marine mollusc hatchery seed production in Thailand was initiated in 1984. The country's first marine mollusc hatchery was established in Prachuap Khiri Khan by the governmental Department of Fisheries and has been operating since 1986. Successful breeding, larval rearing, and nursery techniques for mass seed production have been achieved for several species of bivalves and gastropods. Hatchery and culture development were limited within governmental research and academic institutes with little attention from private sectors, due to a rather long culture period and unattractive market value of molluscs compared to shrimps. Hatchery-produced seeds were mostly released for natural stock enhancement while only small portion was used in culture research experiment. Hatchery seed production was carried out by private farms for the pearl oyster industry only. One pearl farm run by Thai people developed its hatchery with particular success on *Pinctada maxima* seed production since the 1990s, and still continues operation. Another big joint venture company was set up by foreign businessmen for *P. maxima* seed production, but unable to make a good profit, it was closed after few years' operation.

In the past 6-7 years, the abalone *Haliotis asinina* and Babylon snail *Babylonia areolata* turned out to be very popular as new potential species for commercial culture. A number of private hatcheries have been established, mostly involved with grow-out. Hatchery techniques have been simplified to suit commercial-scale operation. Recirculation is one of the required practices to overcome the problem of insufficient good seawater in some areas.

Abalone seed production

There are increasing numbers of private hatcheries set up both in the Gulf of Thailand and the Andaman Sea. Two species, the native donkey's ear abalone *H. asinina* and the exotic *H. diversicolor* imported from Taiwan, are presently pro-

duced. Most hatcheries for *H. asinina* are operated to serve their own grow-out facilities while seed productions of *H. diversicolor* are mainly for export to Taiwan at the size of 1cm. Many private hatcheries have been established, but some had failed and consequently closed. At present, only a few hatcheries are known to be in successful operation with profitable seed production.

Broodstock of *H. asinina* are either collected from nature or selected from cultured ones. Abalones are fully mature and can be used for breeding after 1.5-2 years of culture. Natural broodstock are mostly superior to the cultured ones in egg quality and number. Although artificial feed has been successfully developed to enhance gonad maturation (Boonyaratpalin et al., 2003), the result has not yet been utilized commercially. Breeding is successfully controlled by a12L:12D photoperiod. Rearing, settlement, and nursery techniques for mass seed production are modified and simplified by using large concrete tanks with easy-made plastic collectors. A semi-closed recirculating water system has been developed and applied in a government hatchery, while most private hatcheries having good water supply basically rely on flow-through systems.

Babylon snail seed production

There are 2 species of Babylon snails, *Babylonia areolata* and *B. spirata*, in Thai waters. The first attains higher demand with higher price in seafood markets while the latter is for local consumption. Only the spotted Babylon *B. areolata* is subjected to commercial hatchery seed production. Commercial culture of this species has been rapidly developed after successful intensive research and training by governmental institutes. Many simple private hatcheries have been established, but only 1 very successful big farm in the eastern Gulf of Thailand and a few other small to middle size hatcheries are successfully operating to date.

Babylon snail seed production relies mainly on wild broodstock. Although cultivated adults can also be used for breeding, they are inferior to the natural mature ones in terms of egg production, as in the abalones. Broodstock held for a long time in the hatchery usually decrease their reproductive efficiency, so newly collected adults are occasionally needed to replace the old ones. Mating and spawning is easily induced corresponding with water change. In most Babylon snail hatcheries, 20-30% daily change of water is applied for veliger larval rearing throughout the 2-3-week rearing period. The method is easily applied but sometimes leads into deterioration of condition and contamination, while 100% water change every other day applied in the government hatchery results in better sanitation and mostly higher yield of seed production. As in abalones, a semi-closed recirculating system has been developed and applied for post-settlement in the government hatchery with promising success, but most private hatcheries still rely on flow-through system.

Bivalves hatchery seed production

Only for the pearl oyster *P. maxima* has hatchery seed production been considered and started by private companies. Pearl oyster is of very high value so seed production is much needed for the pearl industry. But only one farm has operated with some success and still continues its hatchery operation today. Broodstock are maintained to maturation in the sea. Ripe adults are stimulated to spawn by aerial exposure combining with the seawater manipulation method. Larval rearing is modified from conventional bivalve larval rearing techniques. Post-settlement spat are maintained in culture tanks until about 0.5cm size, and then transferred to nursery in the sea. Seed producing farms mainly supply themselves in the pearl culture industry; however, some have been supplied to other farm for culture trials occasionally. Since bivalve hatcheries require more complicated techniques than gastropods, there is no other private hatchery developed for bivalves besides this single one for pearl oysters.

Government action in mollusc hatchery seed production

There is increasing demand for abalone and Babylon snail seeds to supply newly operated and small culture farms. While private hatcheries' production does not yet meet the demand, government hatcheries still play an important role in seed production to promote commercial mollusc culture. In another practice to reduce the pressure of overharvesting the wild stock, or to manage stock selection, the government hatchery supports maintaining good quality broodstock to produce healthy veligers for farmers who can further accommodate remote settling. Hence private farms do not have to deal with broodstock management but still have an access to competent veligers for settlement. The hatchery techniques for bivalves, though having been developed and applied successfully for many species in Thailand over the past 20 years, have yet to be well implemented by the private sector. There is increasing interest in potential species of bivalves, e.g., scallop (*Chlamys senatoria*), oriental hard clam (*Meretrix meretrix*), etc. Thus, a government hatchery is still necessary for bivalve hatchery seed production.

Acknowledgements

Cooperations of several private mollusc hatcheries in Thailand and their wills to share their experiences are truly appreciated for making this paper possible.

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EFFECT OF POLYAMINES ON AMYLASE AND IMMUNOGLOBULIN M GENE EXPRESSION IN SPOTTED SAND BASS LARVAE (*PARALABRAX MACULATOFASCIATUS*)

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Introduction

Large-scale egg production of marine fish has been a bottleneck in aquaculture systems. Low survival rates in rearing systems are associated with the incapacity of the larvae to adapt to the feeding sequence during the first weeks of culture (Kolkovsky et al., 1997). The change from endogenous to exogenous digestion is concomitant with the morphological and functional transformations of the digestive tract in larvae. Previous reports indicate that some components of the artificial diets, as well as live prey, regulate some digestive enzyme genes. Low levels of some cytosolic enzymes, associated with an increase in enzymes of the brush border membranes, dictates enterocyte maturation in mammals and fish. Contrary to the increasing information on digestive enzymes as maturation digestive indicators, this is the first report which considers the immunoglobulin M as a maturation parameter in marine fish larvae. Also, we consider expression of certain genes could be used as maturation markers of the digestive tract (Tovar-Ramirez et al., 2002).

Materials and methods

Eggs were obtained from natural spawns of spotted sand bass broodstock maintained under controlled conditions in the Laboratory of Experimental Biology at CICIMAR-IPN (Rosales-Velázquez, 1997). The larvae were reared in a closed recirculating system. Microalgae *Nannochloropsis oculata* (300 000 cells.ml^{-1}) was added until 12dah. Larvae were fed rotifers *Brachionus plicatillis* (1-10 rotifers.ml^{-1}) from 2-15dah, with *Artemia sp.* nauplii after 15dah, and juveniles (2-6 nauplii ml^{-1}) as a control treatment. Three types of microcapsules were supplied as experimental treatments on 15dah, with 0%, 0.1%, and 0.3% espermine. Larvae were fed live prey and microcapsules for 5 days. We obtained relative quan-

tification of amylase and immunoglobulin M gene expression in larvae of spotted sand bass by RNA extraction and quantitative polymerase chain reaction (Q-PCR). TRIzol® reagent (Gibco BRL) was used for total RNA extraction from larvae samples. Then, 5µg total RNA was reverse-transcribed to cDNA with the Ready-To-Go T-Primed First-Strand Kit (Pharmacia Biotech). The expression of amylase and immunoglubulin M from larvae was studied by real-time PCR, using Gene Expression assays from the Assays-by-Design SM (Applied Biosystems), consisting of a mix of unlabeled PCR primers and TaqMan® MGB probes (FAM™ dye-labeled). The TaqMan® probes were designed based on the amylase and immunoglobulin M partial sequences of *P. maculatofasciatus*. The eukaryotic 18S rRNA (Applied Biosystems) was used as the endogenous control for normalizing mRNA levels of the target gene. For the enzyme amylase, the forward primer was 5′GTCTGGTCGGTCTGTTGGA-3′ and the reverse primer was 5′CTTGTTCATGAAGTCAGCAACCTT-3′. For immunoglobulin M, the forward primer was 5′TTCAAAACTGCAGACTGGAACAGT-3′and the reverse primer was 5′CACAGTTCCTTGATGGACTCATGAT-3′. Thermal cycling and fluorescence detection were conducted with the 7000 Sequence Detection Systems (Applied Biosystems). Thermal cycler conditions were as follows: Initial setup 2min at 50°C followed by 10min at 95°C for one cycle; denature for 15s at 95°C, followed by 1min at 60°C for annealing/extending for 40 cycles. Analysis was made by the ABI Prism 7000 SDS software.

Results and discussion

Amylase and IgM expression levels of spotted sand bass larvae fed with live prey show a decrease from 5-10dah (Fig. 1), followed by a significant increment, probably a consequence of digestive tract differentiation. Alvarez-González et al. (2001) reported amylase activity in the spotted sand bass larvae at 1dah, reaching highest activity at mouth opening. Similar amylase activity has been reported in *Dicentrarchus labrax, Coregonus lavaretus*, and *Plecoglossus altivelis altivelis* (Tanaka et al., 1972; Rösch and Segner, 1990; Breuil et al., 1997). From 10-15dah, the amylase and IgM expression in larvae fed live prey increased, and the same pattern was observed in larvae fed microcapsulated diet, but only from 15-25dah. (Figs. 1 and 2). Growth (data not shown) correlates with levels of IgM and amylase expression observed until 25dah in microcapsulated feed provided to larvae. While previous reports indicate that amylase and IgM abundance depends on complete yolk absorption, those reports did not show amylase and IgM falling after 5dah, as we observed.

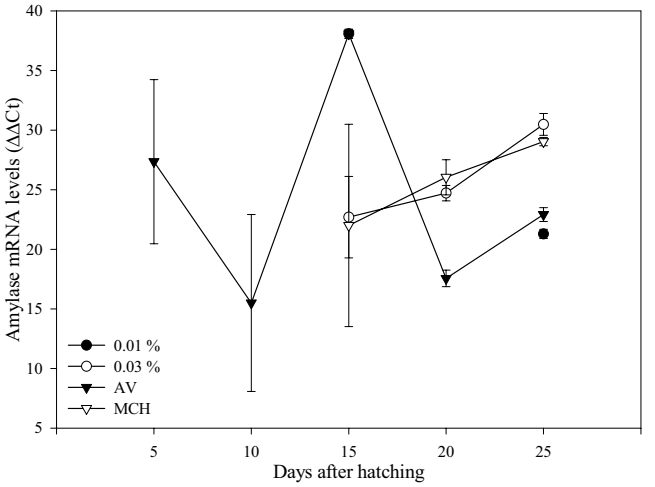

Fig. 1. Levels of amylase expression related to eukaryotic 18s rRNA of spotted sand bass larvae fed with two different concentrations of spermine. Means ± SD (n = 3).

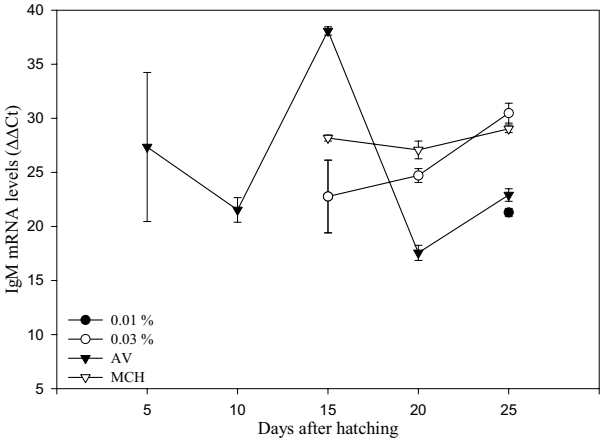

Fig. 2. Expression of IgM levels related to eukaryotic 18s rRNA of spotted sand bass larvae fed two different concentration of spermine. Means ± SD (n=3).

Conclusions

We believe that the levels of expression of the selected genes coincides with the formation of the pancreas (data not shown), about 15dah and the fact what the gastric glands and the first pyloric caeca were observed after 16dah (Peña et al., 2003) and thymus and head kidney development (Breuil et al., 1997). Finally, we suppose that the IgM pattern correlates with growth and development of some digestive organs, as was recently observed, and this contributes to the determination of the maturation status of fish larvae.

Acknowledgements

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EFFECT OF DIFFERENT PHOSPHOLIPID SOURCES AND LEVELS IN FORMULATED DIETS ON LARVAL DEVELOPMENT OF ATLANTIC COD (*GADUS MORHUA)*

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Introduction

Skeletal deformities are frequent in cultured fish and represent a significant economic problem. In hatchery-reared cod in Norway, high levels of spinal malformations have been reported with occurrences up to 60%. Environmental and genetic factors as well as pathology and nutrition are attributed in the literature to skeletal deformities in fish larvae reared in hatcheries. Anomalies have been associated with deficiencies in lipids, especially the highly unsaturated fatty acids eicosapentaenoic acid, docosahexaenoic acid, and arachidonic acid. Furthermore, whether these fatty acids occur in the phospholipid or neutral lipid fraction seem to have some effect on the larval efficiency to utilize the HUFA (Cahu et al., 2003). This experiment aimed at investigating the larval quality of Atlantic cod regarding skeletal development with emphasis on ossification of the spinal column and the incidences of malformations, after feeding with experimental compound diets with differences in quality and levels of phospholipids.

Materials and methods

The experiment was conducted in black coned 150-l rearing tanks and lasted 45 days post-hatching (dph). Eggs were disinfected with glutaraldehyde two days prior to hatching, and were incubated in the experimental tanks at 8°C at a density of 150 eggs.l^{-1}. The temperature was raised to 12°C at the start of feeding. The experiment was carried out with three tanks per treatment. Larvae were fed yeast-Marol E enriched rotifers from 3-16dph followed by a co-feeding period with rotifers and experimental diets from 17-24dph. From 25dph, larvae received only the respective microdiets until 45dph. Algal paste (*Nannochloropsis* sp.) was added to the tanks from 1-22dph.

Three experimental diets (from IFREMER, France) in which the phospholipid sources were either marine or vegetable (soybean lecithin) were used (Cahu et al., 2003). In two diets, similar dietary HUFA levels were either in the marine phospholipids (PM7) fraction or in vegetal neutral lipids (TG7). In the third diet, a higher HUFA level from marine phospholipids (PM14) was tested. Samples of 10-15 larvae for each analysis (dry weight (DW), standard length (SL), ossification of the spine, and deformities) were taken from each tank at each sampling time throughout the experimental period. Larvae were preserved in 5% formalin until later staining with the Alcian Blue - Alizarin Red Staining Technique for bone and cartilage (Balon, 1985). Mean dry weight, standard length, ossification, and malformation values were compared by a one-way ANOVA (significance level at $P<0.05$) followed by Tukey's pairwise comparisons. Dry weight and standard length values were log10 transformed while percentages of ossification and malformation were arcsine ($x^{1/2}$) transformed before the ANOVA analysis.

Results and discussion

Ossification of the 1st pair of neural arch was observed on 21dph with statistical significant differences between the groups (Fig. 1). Highest percentage of larvae starting to ossify belonged to the group fed diet TG7 (43%), followed by larvae fed on PM7 (17%) while none of the larvae fed on diet PM14 had begun to ossify. On 31dph, ossification of the vertebral segment bodies was observed in larvae from all treatments with significant highest ossification rate of the spine reached by larvae fed on marine phospholipid source diets, PM14 (16%) and PM7 (10%), while the group fed on diet TG7 had only ossified 3% of the spine. At the end of the experiment, between 77-80% of the spine was ossified with no significant differences between the groups.

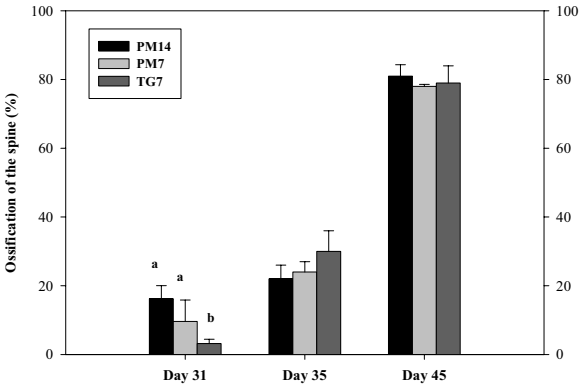

Fig. 1. Ossification rate of the vertebral segment bodies of the spine on 31, 35, and 45 dph in larvae fed on three different micro diets (Means and standard errors, n=12 per mean). Different letters indicate significant differences in ossification rate between treatments ($p<0.05$).

Spinal malformations (lordosis, scoliosis, fused notochord segments, jaw, and bent-neck) were observed in low numbers at early stages (Fig. 2). The incidences of these deformities increased from 35dph, especially bent-neck and fused vertebral segment bodies (star watcher). These were the two main deformity types observed in larvae from all treatments with no statistical significant differences regarding occurrence between them. A significant correlation between the presence of spinal malformations and standard length and myotome height was found. Malformed larvae were smaller and had shorter myotome height compared to normal larvae.

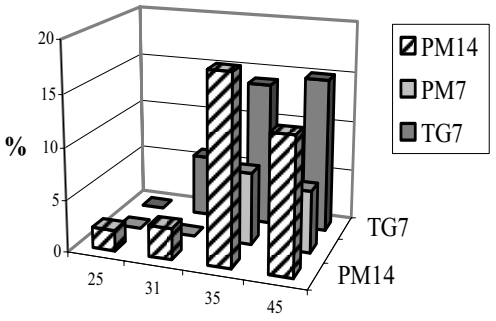

Fig. 2. Percentage of larvae with deformities in Atlantic cod on 25, 31, 35, and 45dph (n=15×3 for each column).

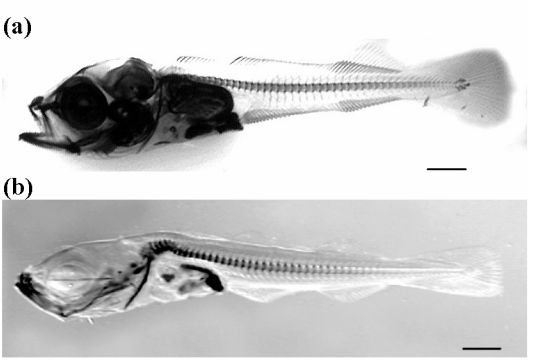

Fig. 3. (a) Normal larva (45dph). SL = 12.13mm MH = 2.0mm Bar = 1mm. (b) Larva with bent-neck (45dph). SL = 12.13mm MH = 1.52mm

Significant differences in SL were only found on 25dph, a day after the co-feeding period was ended. Larvae from both treatments receiving marine phospholipids PM14 (8.05±0.21) and PM7 (7.87±0.22) were bigger than larvae receiving vegetal phospholipids (7.60±0.14). On 45dph the larvae in the TG7-group had a lower dry weight than in the PM7 and PM14 groups. However, dif-

ferences in dry weight between treatments were not significant. The survival of the cod larvae was not significant different at the end of the experiment. The flexion stage was observed on 45dph, with highest values in larvae fed on TG7, followed by PM14 and PM7 with 29, 23, and 19% respectively (not statistically significant).

Differences in growth and anomalies, especially bent-neck and fused vertebral segment bodies, were first apparent from 35dph. The results indicate that addition of vegetal phospholipids in the diets did not affect malformation rate, although growth was decreased. However, 45 days may be too short a period to find notable differences among the treatments.

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WEANING OF ATLANTIC COD (*GADUS MORHUA*) WITH MICROPARTICULATE DIETS AT DIFFERENT TEMPERATURE AND WITH DIFFERENT AMOUNT OF MARINE PHOSPHOLIPIDS

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Introduction

To formulate a diet for marine fish larvae, lipids are one of the ingredients needed. Sargent (1999) recommends 10% marine phospolipds in the diets and Cahu et al. (2003) recommend 12% soya phospholipids to sea bass. The requirements seem to vary depending on species (Geurden et al., 1998a,b,c). Cod is a coldwater species and might have a different requirement from warmwater species.

Materials and methods

The effects of different amount of marine phosholipids and different temperature on growth, survival, and liver index of *Gadus morhua* were studied in two experiments. Cod larvae were start-fed on rotifers/*Artemia* and weaned to formulate feed. In the first experiment the start mean wet weight was 26mg and 50-l tanks with 100 fish in each tank were used. The amount of marine phospholipids was 7.2; 5.2; 3.3, and 2.1%. Control diet was a commercial weaning diet. Four different temperatures were tested on the different diets: 6, 8, 10, and 12 °C. The different groups were fed until 252 day degrees. This experiment was conducted without replicates. To confirm the results the experiment was repeated with three diets (7.2, 5.2, and 2.1% phospholipids) and two temperatures (8 and 12°C), and with three replicates in each group.

Results and conclusions

In general, the results from the first experiment showed lower mortality and higher growth with higher marine phospholipid content. The results of the second experiment confirmed the result from the first experiment. At the end of the

experiment at 12ºC, the highest (457mg) mean weight was found in the 7.2% diet group compared to (401mg) in the 2.1% diet group and survival were 76% and 69%, respectively. At 8ºC, final mean weight was 438mg in the 7.2% diet group compared to 344mg in the 2.1% group and survival was 78% and 60%, respectively. There were small differences in liver index in the different groups and it varied between 4.3 and 5.0%.

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THE EFFECTS OF FORMULATED DIETS ON DEVELOPMENT AND SURVIVAL OF PIKE-PERCH LARVAE

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Introduction

A number of studies were carried out to find a satisfactory, formulated diet that would substitute for natural food (rotifers, *Artemia* sp.) in larval rearing of various fish species (Dabrowski et al., 2003; Takeuchi et al., 2003). Feeds used as first food during fish larval development must be fine-grained, acceptable, digestible, and utilized for body protein/lipid synthesis by the larvae. They should also show an optimum composition of nutrients to achieve high survival and growth rate, and correct development (metamorphosis) of fish.

Material and methods

Larvae from Fish Farm Kołoniec were brought to the laboratory, and randomly divided into groups, 200 individuals per tank (5 treatments × 5 replicates). The fish were fed from the 5 day post-hatch (day 0 of the experiment) with: *Artemia* nauplii, two commercial diets: Aglo Norse (AN) (Larvae Feed Ewos – Bergen, Norway), BioKyowa (BK) (Kyowa Hakko Koygo, Tokyo, Japan), and two experimental, formulated diets "C" (non-hydrolyzed casein) and "CH" (25% of protein as casein hydrolysate) (Carvalho et. al., 2004). Five larvae from each aquarium were sampled on the days: 0, 7, 14, 21, 28, and 35 of the experiment for wet weight measurements, and for histological analyses. Histological procedures and morphometric analysis were done according to Ostaszewska et al. (2005). Survival was calculated as average for 5 aquaria (mean±SD, n=5). Statistical analysis was done using SPSS 12.

Results and discussion

The highest survival (58.4±8.29%) after 35 days of the experiment was observed in group fed *Artemia* nauplii, while the lowest (21.6±3.58%), in the group fed CH. Highly significant differences occurred between the CH and C groups and

other feeding groups ($p<0.05$). After 5 weeks of rearing, the average body mass of fish were 212± 32mg, 207±39mg, 194±46mg, 53.8±6.8mg and 56,4±4.1mg for *Artemia*, AN, BK, C, and CH diet group respectively (mean ± SD, n=10).

Histological observations show that in the enterocytes of the posterior intestine section, the supranuclear areas showed absorptive vacuoles with PAS-positive inclusions (Fig. 1a-c). In the BK- and AN-fed fish, the absorptive vacuoles were considerably larger (Fig. 1b) comparing to the other groups. The enterocytes absorptive vacuoles of fish fed AN and BK diets considerably decreased after the 28th day of rearing. The absence of absorptive vacuoles in the posterior intestine indicates starvation (Crespo et al., 2001). However, excessive vacuolization indicates inhibition of intracellular digestion in the enterocytes (Fig. 1b), and inhibited transport of digestion products into circulation (Ostaszewska et al., 2005). Reduction of number and size of vacuoles in the supranuclear area of enterocytes in fish fed BK and AN diets probably resulted from the onset of HCl and pepsinogen secretion by the gastric glands (Ash, 1985).

Livers of fish fed *Artemia* nauplii, AN and BK diets had a considerably larger hepatocytes (area) comparing to the fish fed C and CH diets. The differences were statistically significant ($p<0.05$). In the hepatocytes cytoplasm of fish fed *Artemia* nauplii, and AN diet, light lipid vacuoles and glycogen storage areas (PAS positive) were similar (Fig. 1d). In hepatocytes of fish fed BK diet, glycogen storage areas were larger than lipid vacuoles (Fig. 1e). Moreover, the livers of fish fed CH showed dispersed necrotic areas consisting of 3-5 hepatocytes. Nucleus fragmentation and destruction (caryorhexis) was also observed. Hepatic tissue of fish fed CH diet showed dilated blood vessels, blood flow congestion, and areas of fatty degeneration of hepatocytes (Fig. 1f). The level of secretion of proenzyme granules in pancreas was similar in all experimental groups (Fig. 1g). Histological analysis revealed that in pike-perch fed AN and BK diets (Fig. 1h), adipose cell aggregations were present among the acinar cells. The changes observed in pancreas were probably caused by the excessive lipid content in the diet (Assimacopoulos-Jeannet, 2004). The use of hydrolyzed casein as main protein source for larvae freshwater fish (Carvalho et al., 2004) enhanced development and growth rate, and increased survival. In the present study, the casein-based diet (C) and the one containing casein hydrolysate (CH) did not bring about as satisfactory results as commercial feeds. The pike-perch larvae fed these diets showed significantly lower survival as well as growth and development rate, comparing to the other groups. The areas of liver fatty degeneration, poorly developed intestinal folds (Fig. 1c), smaller hepatocytes (Fig. 1f) in these fish indicate their poor nutritional condition.

The results of the present study suggest that pike-perch larvae may be successfully fed commercial diets from the very moment of mouth opening, until the juvenile stage. The histological observations showed that larval development of

fish fed these diets, in spite of same changes in digestive tract, was very similar to the development of fish fed live *Artemia* nauplii.

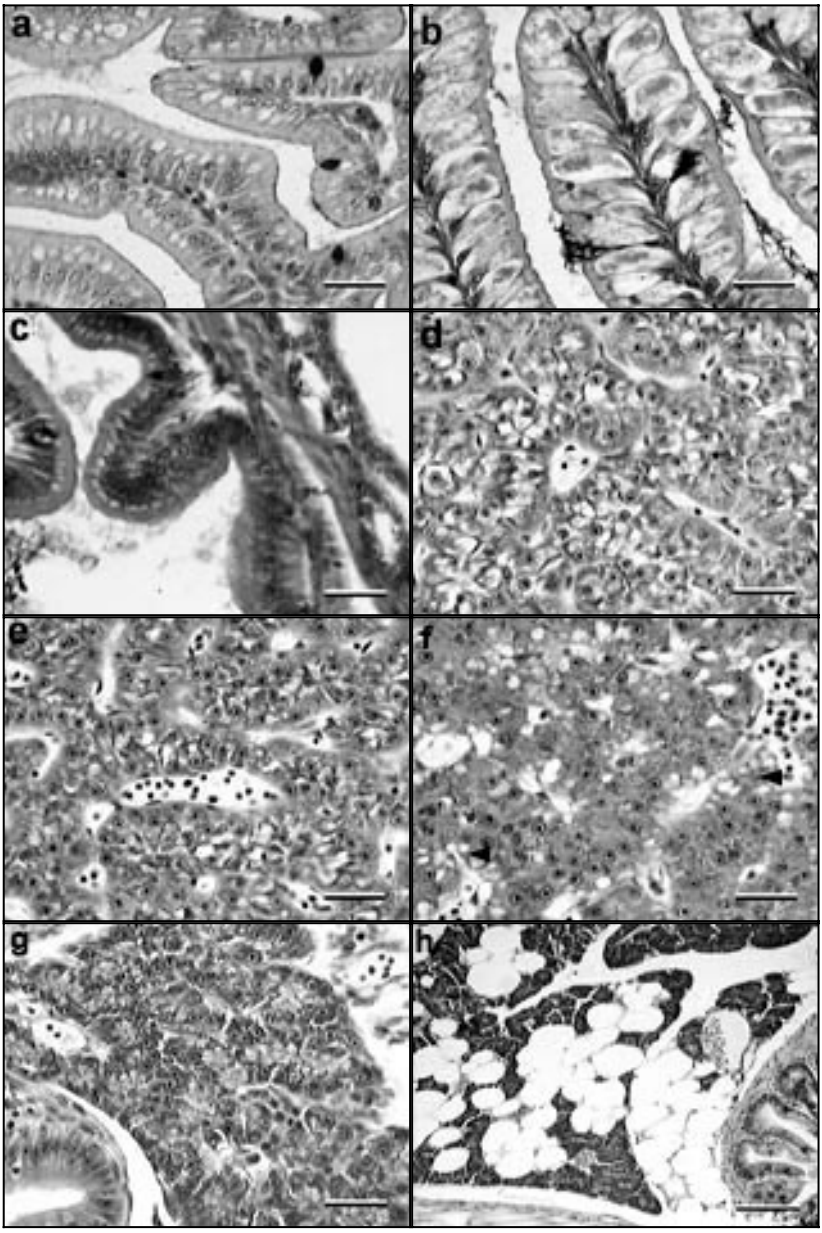

Fig. 1. Posterior intestine epithelium of fish fed: (a) *Artemia* nauplii, (b) AN diet, (c) C diet. Liver of fish fed: (d) *Artemia* nauplii, (e) BK diet, (f) CH diet (areas of fatty degeneration of hepatocytes = arrow head), Pancreas of fish fed (g) *Artemia* nauplii, (h) AN diet. (AB/PAS staining). Scale bars=25µm.

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FAMILY AND STRAIN VARIATION IN GROWTH AND SURVIVAL OF COMMUNAL REARED LARVAL AND JUVENILE COD

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Introduction

The introduction of a new species into aquaculture requires background information about genetic structure of the wild stock. This is to reduce potential environmental effects from escapees, but also to select the broodstock that is best suited for aquaculture in the region. During this experiment we compare performance of cod larvae and juveniles from different families/strains that were reared in an identical environment since hatch.

Materials and methods

Broodstock of coastal Atlantic cod (*Gadus morhua* L.) were collected from four regions along the Norwegian coast during spring 2002 (Otterå et al., in press). During spawning experiments the following two years, family groups of cod eggs were produced and hatched (op. cit.). Only larvae that hatched within a limited time frame in all families were used. These were reared during their larval and juvenile stages according to intensive rearing protocols established for cod. Two rearing temperatures, 6 and 12°C, were compared in triplicate tanks to mimic the natural variation in temperature the larvae would have experienced in their regions of origin. The larvae and juveniles were sampled during the rearing experiment, and their family origin identified by DNA fingerprinting. Four of the ten microsatellites used to genotype the broodstock (Dahle et al., in press) – Gmo34, Gmo35, Gmo132, and Tch11 – were used for family identification of each larva.

Results and discussion

The genotyping of the material is still ongoing, and larval mortality and growth according to family and strain will be presented.

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SALINITY STRESS RESPONSE IN SHRIMP POSTLARVAE: RELATION TO FURTHER PERFORMANCE AND PHYSIOLOGICAL BASIS

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The salinity stress test (SST) is traditionally applied to shrimp postlarvae (PL) to determine their readiness for transfer to growout ponds: PL with higher survival are considered to be of better quality. A higher survival to SST is supposed to be associated to higher survival and growth (performance) during growout, but this has not been tested. This is surprising, given the wide use of the SST in hatcheries and the general belief that it is predictive of performance. Moreover, the underlying physiological bases of PL tolerance to salinity exposure are poorly understood.

To test if a higher survival to SST is associated to better performance during growout, we used 40 *Litopenaeus vannamei* individual spawns (batches) of the same age that were cultured simultaneously under the same conditions. The activity of Na^+/K^+-ATPase was higher in 15 day old PL subjected to lower salinities, but no relation was found with the SST. Survival to a SST was positively correlated to PL length. The batches that had higher survival to the SST also had higher survival during stocking. However, it was not correlated to survival during growout or to size of juveniles during growout, and thus it was not reliable as predictive indicator of performance during growout.

The survival of PL to the SST increases with age, because osmoregulatory capacities increase. Survival of PL can also be modified by nutrition and culture conditions. To establish the effect of energy reserves on survival rate of PL of the same age submitted to a SST, we compared fed and starved 20-day-old PL. The activity of Na^+/K^+-ATPase increased 63% in both anterior and posterior gills in shrimp subjected to a salinity challenge, and was higher in posterior gills. Starvation did not affect these osmoregulatory responses, although starved PL had a significantly lower survival rate when exposed to a SST. These results indicate that the lower survival rate from a salinity stress test on starved PL cannot be fully explained by impairment of the osmoregulatory mechanisms. Levels of energy reserves, mainly lipids, in several tissues suggest a mobilization of lipids to satisfy the energy demands of the osmoregulatory response or an increase in metabolism as part of a general stress response.

Several studies report a higher survival to a SST in PL offered a diet containing high levels of highly unsaturated fatty acids (HUFA). However, it is not clear if this increase is a result of a better physiological condition as a result of enhanced nutritional status, to a specific effect of the HUFA on osmoregulatory mechanisms, or both. Variations in the proportion of fatty acids of cellular membranes can affect the Na^+/K^+-ATPase activity in rats and some crustaceans. We analyzed if HUFA-rich diets could modify the fatty acid composition of membranes in gills, and if this change in composition could affect the activity of the Na^+/K^+-ATPase pump and carbonic anhydrase in relation to a SST. PL1 were fed *Artemia* sp. nauplii enriched with HUFA and after 20 days, no significant differences were observed in survival rates during culture, but survival to a SST was higher and gill area was larger in PL20 fed high HUFA, which was also reflected in fatty acids of tissues. Na^+/K^+-ATPase activity was higher in PL submitted to a SST, especially when fed high HUFA. The beneficial effect of HUFA supplementation in the diet on survival to SST is partially related to modification of fatty acid composition of gills, a larger gill area, and higher activation of Na^+/K^+-ATPase, effects which in turn enhances total osmoregulatory capacity.

HUFA are accumulated in a higher proportion in marine compared to freshwater organisms. Feeding HUFA to PL might be more effective to increase their tolerance to high salinities, compared to low salinities. We fed PL high and low levels of HUFA during 3 weeks and found that PL fed the high HUFA diet had bigger gill area and a higher survival to a high and low SST, but there was no effect on performance during culture. However, PL fed the low HUFA diet had a better osmoregulatory capacity measured in whole organisms at low salinities.

To analyze hemolymph osmolarity studies using juveniles were necessary. Juveniles can be exposed to very low salinities during inland farming or to high salinities in regions where temperatures are high and water exchange low. Juveniles were fed high and low HUFA-enriched diets and submitted to a chronic (3 weeks) or acute (hours) salinity challenge. Juveniles grown at 5psu during 3 weeks had lower survival than shrimp grown at 30 and 50psu, and no significant effect of HUFA was found. In contrast, growth was significantly lower for shrimp grown at 50psu, but this effect was compensated by the HUFA enriched diet. HUFA supplementation in the diet did not affect osmotic pressure or Na^+-K^+-ATPase activity, although gill membrane fatty acid composition was strongly influenced. It is suggested that changes in permeability due to higher HUFA content in membrane phospholipids reduce the energy cost of osmoregulation and in turn allows higher energy allocation for growth.

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DEVELOPMENT AND DISTRIBUTION OF THE INTESTINAL ENZYMATIC ACTIVITY IN *PARALABRAX MACULATOFASCIATUS* LARVAE FED LIVE PREY

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FLUORESCENT MARKERS TO MEASURE INGESTION IN FISH AND SHELLFISH LARVAE

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Introduction

The small size of marine fish larvae and the small quantity of food ingested during the first weeks after the commencement of exogenous feeding posed great difficulties for the accurate measurement of food intake and nutrients digestibility. Fluorescent markers are cheap and safe alternatives to radiotracers that could be applied to assess ingestion rates of small aquatic organism. The aim of the present study was two-fold: (1) to quantify ingestion for compound and live diets with precision using fluorescent markers, and (2) to trace the fate of fluorescent-tagged proteins after ingestion.

Materials and methods

Fluorescein isothiocyanate (FITC)-labeled proteins and chlorophyll-*a* (chl a) were used as fluorescent tracers for inert and live food, respectively. FITC was cross-linked to dietary protein and encapsulated into alginate-chitosan particles. For the encapsulation, 1.6% (w/v) alginate was dissolved in water and mixed with FITC-labelled protein. The mixture was loaded in a syringe fitted with a 21-ga needle enclosed in an air nozzle. The alginate solution was dropped directly into a chitosan/$CaCl_2$ solution which produces a coacervated capsule with a membrane of alginate-chitosan and a gelatinized core of alginate. The capsules were thoroughly washed in distilled water before use. A range of capsule sizes were obtained by controlling the extrusion rate of the alginate solution and the air flow through the nozzle.

Unfed cultures of *Artemia* ($100.ml^{-1}$) were incubated with live cultures of *Tetraselmis* sp. Five hundred individuals were sampled at regular intervals and rinsed with filtered seawater. The chl a within the guts was then extracted overnight with 90% acetone at 4°C in the dark. Total pigment content per nauplii was

then used to determine the number of algae cells present within the gut by factoring in the amount of pigment per algae cell. Maximum feeding rate was computed for the interval required to reach half the maximum gut fullness. Results were analyzed using nonlinear regression in Systat V.10.

In a second set of experiments, groups of 9 and 21-d-old plaice (*Pleuronectes platessa*) larvae were randomly transferred to 1-l glass beakers and deprived of food for 3-6h period. Microcapsules of appropriate size were offered at a concentration of 5-8 capsules.ml^{-1}. Twenty larvae were collected at 1 and 2h after adding the capsules. Feeding was scored under epifluorescent illumination. When present, larvae with positive fluorescence were mounted in glass slides and optical sections of the gut area collected in a Zeiss LSM 510 laser scanning confocal microscope (LSCM).

Results and discussion

Alginate-chitosan capsules demonstrated good water stability and could be manufactured at sizes ranging from 30-750µm in diameter. The alginate core could be easily solubilized and chitosan membrane split open after capsules swell in citrate buffer. Property that allows for easy fluorescence quantification of encapsulated tracers. Different proteins were effectively marked with FITC. The fluorescent tag enabled the detection of approximately 10-µg capsules.

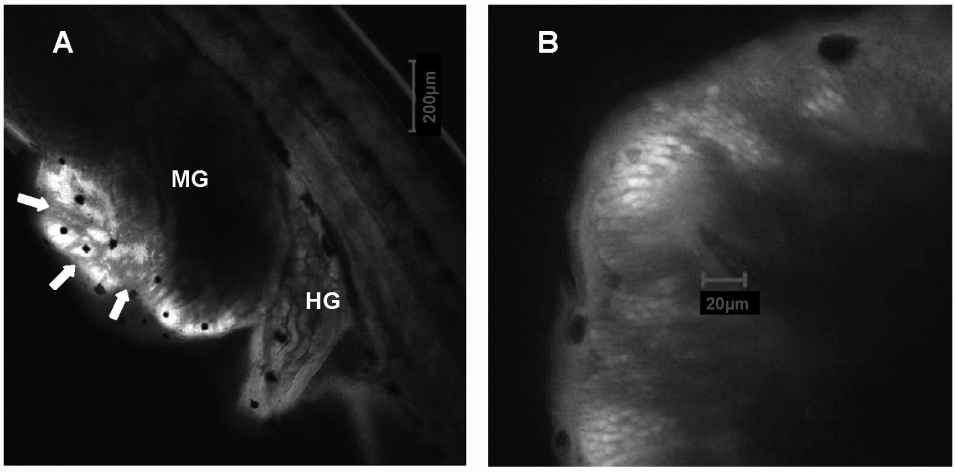

Fig. 1. (A) Confocal image showing the mid gut area of a 9-d old plaice larva after 2h of ingesting microcapsules loaded with FITC-casein. Mid gut (MG) and hind gut (HG). The arrows indicate intestinal folds delimiting areas of strong fluorescence. (B) Detail of the epithelium of the posterior mid gut area showing strong fluorescence within presumptive absorptive cells.

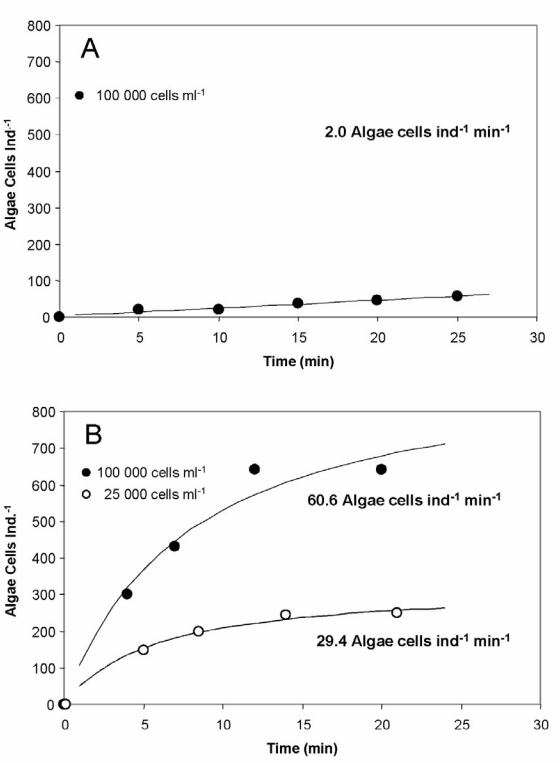

Fig. 2. Ingestion rates of *Artemia* sp. at 20°C. (A) *Artemia* nauplii (3h post hatch) and (B) *Artemia* metanauplii (16h post hatch). Food concentration and ingestion rates are given. Regression lines were generated with a linear (A) and a rectangular hyperbolic model (B), respectively.

No differences in feeding incidence on microcapsules were detected between 9- and 21-d-old plaice larvae during the first hour after the capsules were offered and remained around 10-15% to the end of the trials. Although small compared to 100% feeding incidence on *Artemia*, it allowed us to explore the effectiveness of the formulated microcapsules as a delivery vehicle to explore the digestion of dietary ingredients. Intact capsules were detected in the midgut and foregut. After 2h the entire gut was filled by a fluorescent solution and no distinct capsules were discernible. Observations of the capsule behaviour in the incubation beaker confirmed that after 1h most capsules settled at the bottom and hence were not accessible to the planktonic larvae. It is probable that any fluorescent material found in the larvae after 2h had been ingested within the first hour of the experiment. Since the stability of the capsules in seawater was determined to be greater than 12h and leaching accounted only for about 4-5% during the first 6h, the presence of fluorescence within the gut confirmed capsules breakdown. A closer LSCM examination of the gut revealed the presence of fluorescence inside presumptive absorptive cells suggesting digestion and assimilation of the tracer protein included in the alginate-chitosan microcapsule. FITC react mainly with lysine and terminal amino groups of proteins. Extracellular digestion of tagged proteins would generate a majority of small peptides and free amino acids with lost fluorescence and hence undetectable. Labelled compounds would mainly remain in the gut lumen since the presence of the fluorescent tag can hinder their recognition by membrane transport proteins. The fluorescence observed inside cells could be alternatively explained as a result of pinocytosis of protein-derived material from gut lumen characteristic of the larval stages of marine fishes (Govoni et al., 1986; Tytler et al., 1997).

Newly hatched *Artemia* (3h post-hatch) showed a much lower feeding incidence than *Artemia* metanaupii (16h post-hatch). *Artemia* metanauplii responded with only two-fold increase in response to the four-fold increase in food density. This is a direct and quick assessment based in actual ingested prey by filter feeders feeding on an autotrophic organism, that could be apply to different prey-predator combination including fish larvae (Tytler et al., 1997). Ingestion of DAPI-labelled heterotrophic protists have also been measured and used to follow its ingestion by pollock larvae (*Theragra chalcogramma*; Lessard et al., 1996). Minute amounts of fluorescent-labelled food can be detected with precision in short, easy-to-conduct assays (Kelly et al., 2000). We are currently optimizing the tagging-encapsulation process to measure ingestion in single larvae.

Conclusions

The suitability of a particular feedstuff has to be judged based on the larval ability to digest and assimilate it. LSCM allows a direct qualitative assessment for determining the digestibility of feed ingredients. Moreover a range of fluorescent tags are available allowing for differential labelling of multiple dietary compounds that could be used to simultaneously assess different nutrients or diets. This information is fundamental to development and demonstration of new technologies for the commercial farming of marine species.

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CAPTIVE BROODSTOCK OF *LITOPENAEUS STYLIROSTRIS* IN NEW-CALEDONIA: AN EXAMPLE OF FUNDAMENTAL RESEARCH APPLICATION IN PRODUCTION

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Introduction

In New Caledonia, the aquaculture industry produced 2200 tons of top quality shrimp in 2004, with significant prospect for industry growth. All the production is based on a single species (*Litopenaeus stylirostris*) introduced in the early 80s and reproduced in captivity for 28 generations. Twenty thousand broodstock (male and female) reared with specific conditions determined as "broodstock conditions" are necessary to insure the supply of the 200 million post-larvae for grow-out ponds. Due to the climate seasonality (tropical-subtemperate), the transfer of broodstock from earthen outdoor ponds to indoor maturation tanks is often associated with significant mortalities, especially during the cold season.

Materials and methods

Shrimp broodstock of 50-60g used in these experiments were reared in earthen ponds. Broodstock culture was carried out in two steps: (1) from post-larvae to 20g, shrimp were reared at 2 animals.m^{-2} for 4 months and (2) they were transferred in another pond at a lower density (0.5 animals.m^{-2}) and grown to 50-60g for an additional 4 months. When the shrimps were ready to breed, they were transferred into the maturation tanks in hatchery. The effects of different transfer conditions on survival and reproductive performances of the broodstock had been tested four times.

For this study, the broodstock were divided in two batches for each trial: (1) control animals transferred in standard conditions where they were fed immediately following the transfer at ambient temperature (22°C in winter) and at seawater salinity (35ppt) and (2) treated shrimps transferred in experimental conditions

where they were unfed for 2 days and water was heated (26°C), with lower salinity (26ppt). These conditions were maintained for one week in order to study the effects of transfer conditions on the female survival rate following the eyestalk ablation. The reproductive performances of the different batches were studied when standard conditions were restored in all the batches.

Aside from the experimental approach, data were collected from a commercial hatchery where the experimental protocol has been applied since the end of 2003.

The survival results were analysed by a one-way ANOVA. Data were normalized by an arcsin transformation and significant differences determined by a 5% PLSD Fisher using Statview software.

Results

Three days after stocking in maturation tanks, the average survival rates were 77.6 % and 88.4% for the control animals and the treated ones, respectively. The broodstock survival was significantly improved in the experimental treatment (Table I, Figs. 1a, 1b).

Table I. Broodstock survival rate three days after the transfer in the standard and experimental batches.

	1st trial	2nd trial	3rd trial	4th trial
Standard transfer	62.5%	80.6±7.5%	91.6±4.3%	75.6±1.8%
Experimental transfer	78.6%	92.5±1.9%	93.7±3.2%	89.0±4.1%

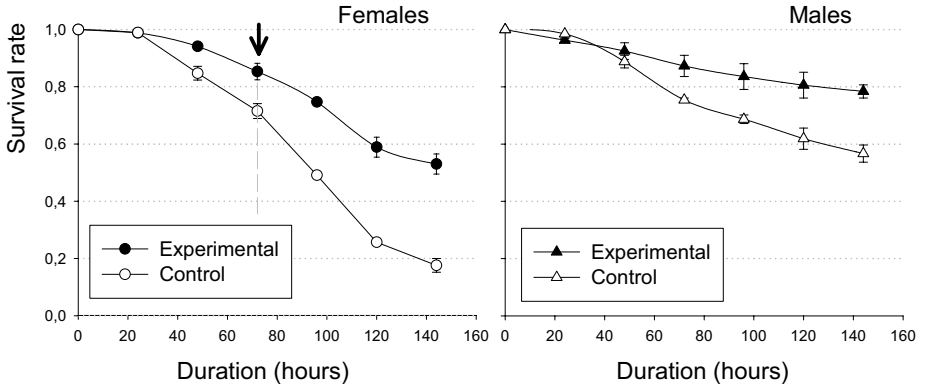

Fig. 1. Survival rate of the broodstock in the hatchery for females (a) and males (b). Arrow signifies eyestalk ablation of the females.

Following the eyestalk ablation, females showed another mortality peak the third day which was significantly lower for shrimps transferred in experimental conditions (Fig. 1a). Regarding to the reproductive performance of females, the experimental group displayed 40% more spawns per female compared with the control group. Whenever, the fecundation rate and hatching rate were in the same range for the two groups. In summary, the treatment improved the spawning rate without affecting the eggs quality.

Fig. 2 shows the broodstock survival following their transfer into the hatchery before 2003 and after the application of the new protocol. Survival rate was improved by 22% and 29% for the males and the females, respectively.

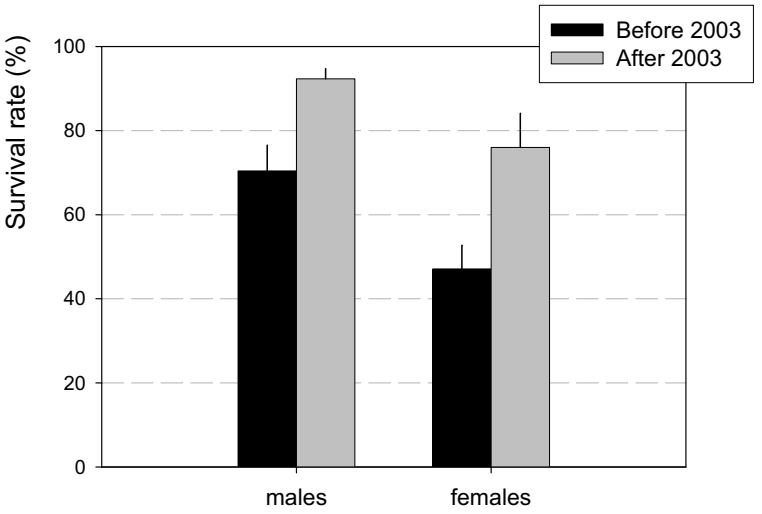

Fig. 2. Broodstock survival rate in commercial hatchery in standard conditions (before 2003) and according the new protocol (after 2003).

Discussion

In New Caledonia, broodstock mortality in winter requires three conditions: presence of *Vibrio peneacida*, low temperature (below 22°C), and physiological disturbance. By removing one of these factors, it is possible to overcome this mortality (DAC, unpublished data). We carried out the experiments above according our observations on physiological disturbances of shrimp *Litopenaeus stylirostris* due to handling stress during winter (Wabete et al., 2003).

Those disturbances are characterized by a drop of the shrimp osmoregularity capacity (medium osmotic pressure minor shrimp blood osmotic pressure) leading to a loss of the blood affinity for oxygen (Wabete et al., unpublished). This affinity loss is compensated for the first 24 hours by a hyperventilation which in-

creases the partial oxygen pressure in the hemolymph (Wabete et al., 2004); after this period, the heart would take over by increasing its flow for several days. Shrimp are probably weakened by this last phenomenon, which could explain high mortality after their transfer in standard conditions in winter. We designed our protocol in order to reduce the handling stress of broodstock in winter by transferring them in iso-osmotic (26ppt) heated water (26°C) allowing them to recover their ionic homeostasis faster and given no feed in the first days.

Conclusions

Fundamental research on physiological disturbances of shrimp during their transfer allows design of an applied protocol which dramatically improves the survival rate of the broodstock in the hatchery. This new procedure, based on a better control of salinity and temperature conditions, has been transferred successfully to private hatcheries and may contribute to increase profitability of Caledonian shrimp industry.

Acknowledgements

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ARCTIC CHAR *(SALVELINUS ALPINUS)* EGG QUALITY IN RELATION TO FATTY ACID COMPOSITION

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Introduction

Interest in Arctic char in the aquaculture industry is increasing, especially in Northern Europe, but an increase in the Central European market is expected. The advantage of the species is its preferences for cold, clear water where an increased nutritional load is expected to be of minor importance for water quality (a governmental investigation: Swedish aquaculture- a future industry, Department of Agriculture, Ds 2000:42).

A selection program on Arctic char started 1984 in Sweden and is an example of a successful selection program leading to enhanced growth and postponed sexual maturation (Nilsson, 1992). A drawback in the demand of eggs for fish farmers is a high and unpredicted variation in hatching success of the eggs. The selection program as such cannot be the cause as a high variation in egg survival also occurs in other unselected stocks where the parent fish have been raised in farms and fed commercial salmonid feed. An increased mortality can to some extent be caused by increasing water temperatures in summer and early autumn but also farms that are supplied with groundwater never exceeding 8ºC have a high variation in mortality.

The n-3:n-6 polyunsaturated fatty acid ratio in the natural diet of all limnic organisms differs between freshwater and marine environments, being much lower in freshwater compared to seawater (Arts, 2001; Henderson and Tocher, 1987; Kaitaranta and Linko, 1984; Pickova et al., 1997). This has been mirrored in the composition of fatty acids in lipids of fish eggs. The lipid-rich char eggs contain a high proportion of storage lipids, which is a requirement in eggs with long incubation times (Wiegand, 1996). Landlocked Atlantic salmon (*Salmo salar*) eggs have been investigated by Pickova et al. (1999) with regard to lipid composition in eggs of farmed and wild females. A significant difference was found

between the fatty acid composition and hatching rate between the egg production from females of different origin. The egg lipids from farmed female salmon were richer in n-3 fatty acids, which was not in line with the findings from the lipid composition of free-living females in the Lake Vänern. The hatching success was much greater in the wild offspring (approx. 90%). The results from that study indicate that fatty acid composition of the broodstock diet has to be optimized. In the present study, Arctic char (*Salvelinus alpinus*) have been investigated. Char have been reared over the past few decades, allowing a comparison between the eggs from both wild females feeding on a diet based on lipids of limnic (natural food chain) origin and eggs from females fed an artificial diet (in farms) of marine origin. In a review by Jobling et al. (1998), variability in egg quality was mentioned as one of the main problems that have arisen. This problem has yet to be solved. Jobling et al. (1995) investigated the importance of temperature on gonad development and the fatty acid composition. Unfortunately that study did not compare fish feeding on different diets and the main fatty acid of interest, arachidonic acid, was not identified. The aim of the present study is to explore if a change in dietary fatty acid composition will influence the hatching percentage and survival at early life stages of Arctic char.

Materials and methods

Wild fish were caught in one freshwater lake and farmed broodstock fish from two farms were sampled and all were stripped, the gametes were collected, and eggs were fertilized. The eggs from individual females were incubated separately in incubation boxes in flow-through systems. The eggs were hatched and the hatching rates were recorded. Eggs from all females of different origin were sampled (1-2g per female), lipids were extracted, and fatty acids of phospholipid and triacylglycerol fractions were analysed (Pickova et al., 1997). Feed used in the farmed fish was analysed for fatty acid composition of total lipids.

Results

Hatching rates varied greatly between the wild- and farmed-origin eggs. Lipid analyses showed significant difference in composition of fatty acids. The content of 20:5n-3 in phospholipid fraction was higher in eggs from reared females compared with eggs from natural population. Further, in PL fraction, 20:4n-6 levels in these eggs were significantly lower (1.5% versus 8.0%). Also, the content in the triacyl-glycerol fraction differed greatly between wild and eggs from reared females, whereas this fraction showed almost no corresponding difference in EPA content. In addition, the level of 22:6n-3 differed greatly between the two groups: 31% in the farmed fish eggs vs. 19.1% in the wild (Table I). The fatty acid composition of feed based on marine lipids had a ratio between arachidonic and eicosapentaenoic acids, totally in disagreement with the natural conditions if compared with published freshwater data (Arts 2001, Kaitaranta

and Linko, 1987; Pickova et al., 1999; see Table I). The hatching rates of farmed char were significantly lower, with a large variation, when compared to the stripped wild collected char eggs.

Table I. Fatty acid composition in % of total identified fatty acids in phospholipid fraction of wild and farmed broodstock char and total lipid of feeds. AA: arachidonic acid, EPA: eicosapentaenoic acid, PUFA: polyunsaturated fatty acids, LCPUFA: long chain polyunsaturated fatty acids.

	Wild	**Farmed 1**	**Farmed 2**	**Feed**
14:0	2.6	1.2	1.4	7.5
16:0	20.8	18.0	17.2	17.8
18:0	8.1	6.4	3.4	3.1
18:1 n-9	9.9	11.3	9.2	9.9
18:1 n-7	7.4	4.6	3.8	2.6
18:2 n-6	1.6	1.4	2.1	4.5
18:3 n-3	0.9	0.3	0.3	1.2
20:1	2.0	6.6	6.6	5.6
20:4 n-6	8.0	1.3	1.7	0.5
20:5 n-3	9.6	7.2	8.1	10.4
22:5 n-3	3.5	2.1	1.8	1.1
22:6 n-3	19.1	31.4	35.2	11.3
AA/EPA	0.83	0.18	0.21	0.05
PUFA n-3	33.1	41.0	45.4	24.0
PUFA n-6	9.6	2.7	3.8	5.0
PUFAn-3/PUFAn-6	3.45	15.2	11.9	4.8
LCPUFA n-3	32.2	15.2	11.9	22.8
LCPUFA n-6	8.0	1.3	1.7	0.5
LCPUFA n-3/ LCPUFA n-6	4.0	31.3	26.5	45.6

Conclusions

Arachidonic acid is more than 15 times higher in the naturally produced eggs of Arctic char compared to the fed broodstock char. In addition, the ratio between long chain polyunsaturated fatty acids of n-3 and n-6 groups is significantly more altered that the ratio of n-3 and n-6 polyunsaturates, which gives an indication that the elongation and desaturation ability in this salmonid species is very limited. These results indicate that the natural balance of essential fatty acids in terms of eicosanoid precursors and thereby membrane properties are greatly altered, plausibly being the one cause of the low hatching success in the farmed eggs. The changed balance of essential fatty acid is probably not the only cause behind high mortality of eggs from farmed females but may lower the resistance to environmental constrains such as high water temperature, considering the bioactivity of fatty acid derived eicosanoids in the immunity, inflammation, reproduction, and other mechanisms (Calder, 2001). The effect of global warming is expected to change the geographical limit of Arctic char both as wild population but in particular limiting the suitable location of fish farms specialized in Arctic

char. We conclude that an economically feasible farming protocol requires an improved diet with higher content of n-6 fatty acids, especially arachidonic acid, to achieve a better egg quality in char.

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ISOLATION AND CHARACTERISATION OF BACTERIA FROM THE GUT OF COD LARVAE FOR CONSIDERATION OF PROBIOTIC EFFECTS

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Introduction

The aquaculture industry is facing problems concerning growth, survival, and reproducibility during first-feeding of fish larvae, with microbial aspects in the rearing tanks as one big bottleneck. A possible solution to this problem is the use of probiotics, which are micro-organisms with a health benefit to the host (Gatesoupe, 1999). Probiotics may enhance growth and survival in fish larvae by production of substances inhibiting detrimental bacteria (Austin et al., 1995), by adhering to intestinal mucus and wall surfaces (Westerdahl et al., 1991), by competing for chemicals and nutrients (Ringø and Gatesoupe, 1998), or by enhancing the immune system of the larva (Vadstein, 1997). A common selection criterion for putative probiotic strains has been the in vitro production of inhibitory compounds towards known pathogens for the considered fish species.

Materials and methods

Cod larvae (*Gadus morhua* L.) used for the study were sampled from Brattøra Research Center (NTNU) in Trondheim and Fosen Aquasenter at Fosen, Central Norway. The larvae from Brattøra were all sampled at 16 days after hatching (dah), while the larvae from Fosen were sampled on 10 and 18dah. Ten of the larvae originated from intensive flow-through aquaculture systems (FTAS) while the 2 others came from a recirculation aquaculture system (RAS).

The larvae were homogenised and plated on marine agar and incubated at 15ºC in darkness. Colony forming units (cfu) were counted after visible colonies were formed and 120 dominant isolates were selected by random for further study. All isolates on the plates were tested for antagonistic activity against *Vibrio anguillarum* (Hjelm et al., 2003). In total 31 isolates with antagonistic effect were de-

tected. All 151 isolates were grown in Marine Broth containing glycerol and stored at -80ºC until further use.

All isolates were characterised with respect to colony morphology (colour and shape), Gram-reaction, oxidase reaction, shape, motility, 0/129 sensitivity, Ox-Ferm test, and growth in 6% NaCl. Based on these tests the isolates were roughly classified to genus or group using a standard identification scheme (Bagge-Ravn et al., 2003). In addition, cluster analysis was done to group the isolates on the basis of all tests. Nine dominant and 10 antagonistic isolates were selected for further analysis and were tested for properties such as maximum growth rate, tolerance for bile (Nikoskelainen, 2001), production of enzymes (API ZYM), hemolytic properties, antagonism against 3 dominant isolates and 4 pathogenic bacteria (Hjelm et al., 2003), adhesion to mucus (Nikoskelainen, 2001), and competition in mucus.

Results and discussion

The antagonism test yielded 3-6 antagonistic isolates per larva. Results show that the cfu.larva^{-1} varied considerably between larvae from the same processing plant (Fig. 1). This was also true for larvae from different processing plants. The CFU levels were generally higher in larvae from FTAS than in larvae from RAS.

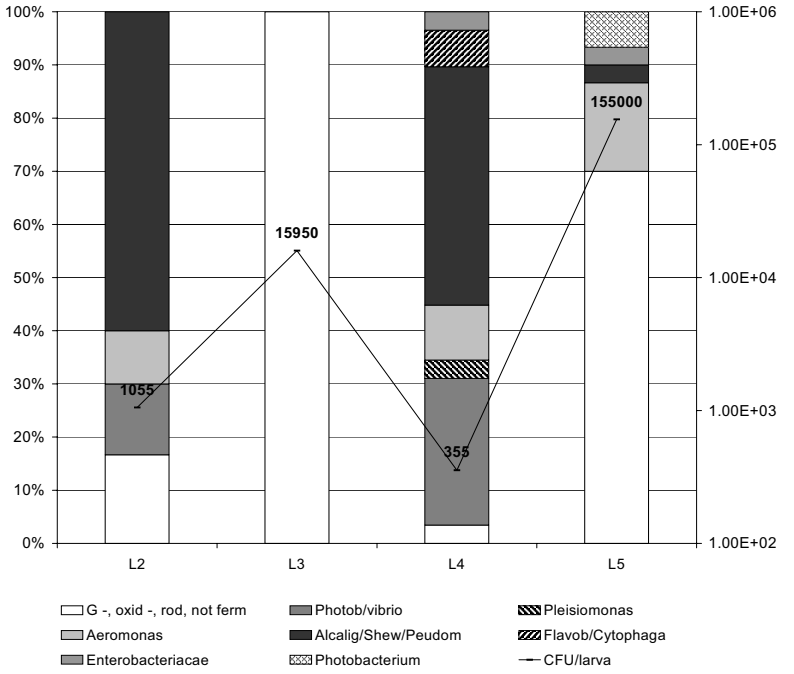

Fig. 1. Total cfu.larva^{-1} and percentage of various taxonomic groups of the dominant bacterialflora in larvae from Brattøra (30 bacteria isolated from each larva).

Figs. 1 and 2 reveal that there were obvious differences in the bacterial composition between individual larvae. These individual differences were apparent within the same tank as well as between the two different systems. The cfu levels reflect that there was no correlation between the original bacterial number in the cod larvae and the number of bacterial bio-types present in the gut (Fig. 1). It is possible that not all bacteria/bacterial-types grow under the experimental conditions given. Furthermore, the scheme used in the classification did not include all the tests executed, and thus led to an underestimation of the diversity.

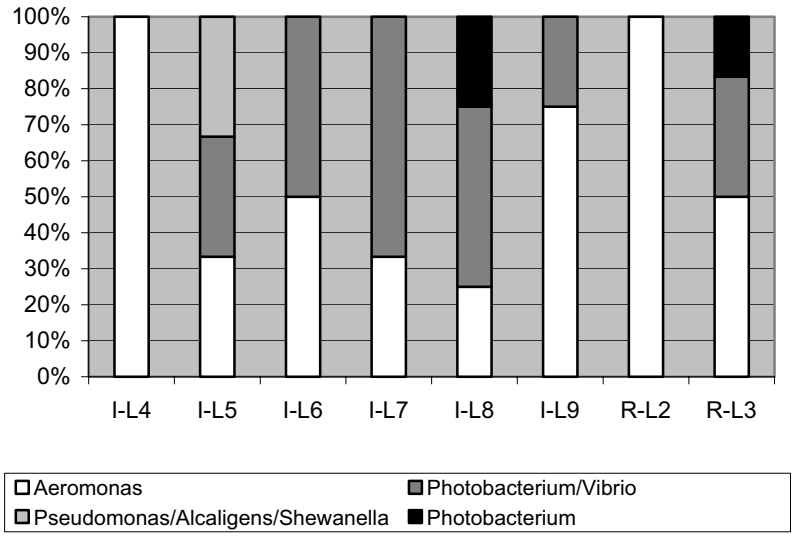

Fig. 2. Percentage dominance of various taxonomic groups of bacteria of the isolates with antagonistic effect. The bacteria were isolated from 8 different larvae. I-L4 and I-L5 originates from Brattøra FTAS, I-L6 – I-L9 originates from Fosen FTAS, and R-L2 – R-L3 are from Fosen RAS.

The tests carried out on the 19 selected isolates revealed that all isolates survived the exposure to bile and about half of the isolates were haemolytic. Most isolates with antagonism towards *V. anguillarum* showed antagonism against the selected dominant isolates and the other pathogenic bacteria. The further characterization of these isolates is still in progress.

Conclusions

The cfu.larva^{-1} differed considerably between individuals, and generally, bacterial levels were higher in larvae from flow-through compared to recirculation systems.

The results from further experiments are still in progress. A couple of isolates with the desired properties will be selected as possible probiotic candidates.

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NEW MICROALGAE FOR MOLLUSC HATCHERIES

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Introduction

The number of microalgal strains, and particularly strains of flagellate species, currently used as food for larvae in mollusc hatcheries is low. This fact negatively affects shellfish production and, accordingly, new species are sought. In this study, four microalgal species (the prymnesiophytes *Imantonia rotunda*, *Emiliania huxleyi*, *Pseudoisochrysis paradoxa*, and the pavlovophyte *Diacronema vlkianum*) were tested as food for *Crassostrea gigas* larvae. These species were selected from fifteen different species belonging to different algal classes (Prymnesiophyceae, Pavlovophyceae, Prasinophyceae, Dinophyceae, Dictyocophyceae, Cryptophyceae, Bangiophyceae) screened according to their productivity and cytomorphological characteristics. *E. huxleyi* and *I. rotunda* led to poor growth while *P. paradoxa* and *D. vlkianum* supported good larval performances. The latter two microalgae were then characterised (dry weight, ash, gross composition, fatty acids) at different stages of growth and their productivity in standard hatchery conditions (300-l cylinders) was assessed in order to verify their potential for use in commercial hatcheries.

Materials and methods

Microalgae, all belonging to the ALGOBANK microalgal collection of the University of Caen, were initially cultured in 250-ml glass flasks and then in 2-l glass carboys and 300-l Perspex cylinders. Seawater (salinity: 33-34‰) was 1µm-filtered, enriched with sterilized Conway medium, and either autoclaved (≤2 l) or UV-treated (300 l). Temperature and continuous illumination ranged between 19-20°C and 35-50µmol photons.m^{-2}.s (flasks) to 22-23°C and 180-220µmol photons.m^{-2}.s (carboys and cylinders). Carboys and cylinders were aerated with a 3% CO_2:air mixture.

During two feeding trials, larvae of *C. gigas* were fed with bi-specific diets composed of one of the new species associated with the diatom *Chaetoceros calcitrans* forma *pumilum* (feeding ratio: 80% flagellate and 20% diatom), according to the methods detailed by Ponis et al. (2003). Moreover, positive (*C. calcitrans* f. *pumilum* + *Isochrysis aff. galbana* clone T-Iso) and negative (unfed larvae) controls were added. Larvae were sampled at the beginning (day 2 after fertilization) and end (day 16) of the trial and assessed for mortality and growth by means of image processing.

Phytoplankton growth was estimated daily using a Malassez haemocytometer, while cellular size, expressed in diameter equivalent, was determined with a Coulter Counter. Growth rate was calculated as $\mu = [(\ln N_1 - \ln N_0)/(t_1 - t_0)]/\ln 2$, where N_1 = measurement at time 1 (t_1), and N_0 = measurement at time 0 (t_0).

For dry weight, 50ml of biomass were centrifuged and rinsed with 20ml of 0.5M ammonium formate. Pellets were then harvested and weighed.

For biochemical analysis, *D. vlkianum* and *P. paradoxa* cultures were sampled during both exponential and stationary phases. Proteins, lipids and carbohydrates were measured according to Lowry et al. (1951), Bligh and Dyer (1959), and Dubois et al. (1956), respectively. Fatty acid analysis was performed according to Marty et al. (1992), using a gas-chromatograph (Hewlett-Packard, HP6890) equipped with a J&W 65 DB Wax column (30m × 0.25mm, 0.25-µm film thickness) and using C23 as the internal standard.

Data were analyzed by ANOVA and significant differences were detected by Scheffe's tests ($P<0.05$); data expressed in percentages were transformed (arcsin square root $x_i.100^{-1}$) before statistical analysis.

Results and discussion

Larvae fed with *D. vlkianum* or *P. paradoxa* clearly exhibited high survival (>84%) while growth did not greatly differ from positive control Cp+Ti (Fig. 1).

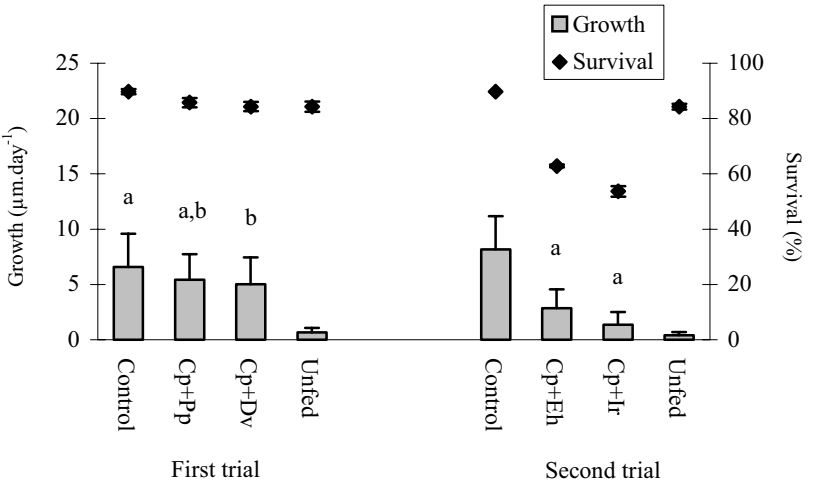

Fig. 1. Growth (average ± SD, $n \geq 200$) and survival (average ± SD, $n=3$) of *C. gigas* larvae fed with different microalgal regimes. Control = *C. calcitrans* f. *pumilum* + T-Iso*;* Cp+Pp = *C. calcitrans* f. *pumilum* + *P pinguis*; Cp+Eh = *C. calcitrans* f. *pumilum* + *E. huxleyi*; Cp+Ir = *C. calcitrans* f. *pumilum* + *I. rotunda*. Columns sharing a common superscript letter are not significantly different (P <0.05).

In contrast, larvae fed with *E. huxleyi* or *P. paradoxa* showed poor development with low survival (<63%). *D. vlkianum* and *P. paradoxa* exhibited a well balanced biochemical profile, particularly in PUFA content, *D. vlkianum* being rich in EPA (20:5n-3) and DHA (22:6n-3) and *P. paradoxa* in DHA (Table I).

Table I. Main characteristics of *D. vlkianum* and *P. paradoxa* cultures at different stages of growth ($n \geq 2$; Exp.= exponential phase; St.= stationary phase; * $P<0.05$).

	D. vlkianum		***P. paradoxa***	
	Exp.	**St.**	**Exp.**	**St.**
Size (µm)	4.37 ± 0.42	4.57 ± 0.58	3.98 ± 0.34	4.64 ± 1.23
Dry weight (pg.cell^{-1})	14.53 ± 0.75	19.27 ± 0.39*	10.32 ± 1.09	12.68 ± 0.11*
Ash (pg.cell^{-1})	0.72 ± 0.32	0.45 ± 0.17	1.21 ± 0.08	1.15 ± 0.13
Proteins (% organic matter)	24.65 ± 0.06	29.83 ± 1.50*	48.75 ± 1.40	30.67 ± 0.55*
Carbohydrates (% o.m.)	31.49 ± 0.97	23.40 ± 6.64	11.41 ± 0.52	29.16 ± 0.88*
Lipids (% o.m.)	33.58 ± 3.43	39.16 ± 3.69	29.97 ± 0.36	35.50 ± 0.03*
Total fatty acids (% o.m.)	18.12 ± 1.98	24.89 ± 0.41	18.02 ± 0.63	20.03 ± 0.37*
Total SAT (% fatty acids)	28.32 ± 5.18	30.53 ± 1.86	32.17 ± 1.88	36.31 ± 0.54
Total MONO (% f.a.)	23.06 ± 2.43	28.71 ± 1.20	15.69 ± 0.34	26.18 ± 0.61*
Total POLY (% f.a.)	48.32 ± 7.93	40.51 ± 0.32*	51.79 ± 1.50	37.26 ± 0.12*
EPA (% f.a.)	21.84 ± 3.68	16.80 ± 0.63*	1.41 ± 0.05	0.75 ± 0.17*
DHA (% f.a.)	7.24 ± 1.37	3.95 ± 0.01*	39.71 ± 0.34	25.78 ± 0.30

The total polyunsaturated fatty acids and particularly EPA and DHA contents were higher in *D. vlkianum* during the exponential phase, while dry weight and

proteins were higher during the stationary phase. *P. paradoxa* was richer in proteins, total polyunsaturated fatty acids, and EPA during the exponential phase of growth; in contrast, dry weight, as well as carbohydrates, lipids, total fatty acids, and monounsaturated fatty acids were higher in *P. paradoxa* harvested in stationary phase. In order to verify the productivity of these species, batch cultures were carried out in 300-l Perspex cylinders. Both species reached high concentrations ($>10 \times 10^6$ cell.ml^{-1}) with good daily productivities (0.8 and 0.5 divisions.day^{-1}, for *D. vlkianum* and *P. paradoxa* respectively). *P. paradoxa* is a microalga which has been previously cited for its potential use in aquaculture (Chu and Dupuy, 1980) but to our knowledge remains unused, while *D. vlkianum* was unknown as feed for molluscs. This study emphasizes the interest of both species for nutrition of oyster larvae and illustrates the feasibility of their culture in commercial mollusc hatcheries.

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LARVAL REARING OF FOUR *SPARIDAE* SPECIES

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Introduction

Species diversification is presently a concern amongst aquaculture researchers and producers trying to find new profitable species for commercial production. Some sparid species which are common along the Iberian Peninsula coast fall within the most appreciated and highly market-valued species in Portugal. For this reason, the larval rearing of four sparid species of commercial interest (*Diplodus sargus, D. cervinus, D. vulgaris*, and *D. puntazzo*) has been conducted in order to develop efficient production techniques and feeding strategies, mainly during the larval stage. Furthermore, good encouraging production results obtained for *D. sargus* (Koumoundouros et al., 2001; Pousão-Ferreira et al., 2001) supported the interest to evaluate the potential of other valued *Diplodus* species. In this study, the growth and survival rate, feeding regimes and schedules, and fatty acid profile for each one of the *Diplodus* species are presented. Results are subsequently compared with reference results of *Sparus aurata* rearing techniques, growth, and survival performances, with the aim to evaluate production efficiency for each species.

Materials and methods

Larval rearing experiments were carried out over 30 days using eggs obtained by natural spawning and local photoperiod and temperature conditions from captive broodstock of *D. sargus, D. cervinus*, and *D. vulgaris* and by hormonal induced spawning (LHRHa, 15µg.kg^{-1}♀) of *D. puntazzo* maintained at the IPIMAR-CRIPSul (Portugal) hatchery. Broodstock diet was composed of fresh food (squid and sardine) supplemented with commercial pellets (Sorgal S.A. Portugal) on a daily basis. Stocking densities were 2.8kg.m^{-3} (811±307g.fish^{-1}, n=31), 0.6kg.m^{-3} (1004.7±768g.fish^{-1}, n=6), 1.1kg/m^{3} (466±93g.fish^{-1}, n=25), and 5.6kg.m^{-3} (2135±539g.fish^{-1}, n=28) for *D. sargus, D. cervinus, D. vulgaris*, and *D. puntazzo*, respectively. The spawning season occurred in winter/spring for *D. sargus* and *D. vulgaris*, spring for *D. cervinus*, and was induced in *D. puntazzo* in the winter (Fig. 1). Eggs were placed in 200-l incubation tanks, at a density of

1-2g.l^{-1} and temperature similar to the broodstock tank of 18±1ºC, until hatching (±36h). The larvae were reared in 1500-l blue cylindrical fibreglass tanks, in an open-water circulation system, with mechanical, biological, and UV filtration. The tanks were gently aerated from the bottom and the "green-water" method was used (1.5-3×10^5 cells.ml^{-1}). Salinity and temperature were measured daily and maintained at 38±1ºC and 19±1ºC, respectively. Dissolved oxygen was kept close to the saturation level. The photoperiod was 14L:10D, and larval density ranged between 25-75 larvae.l^{-1}.

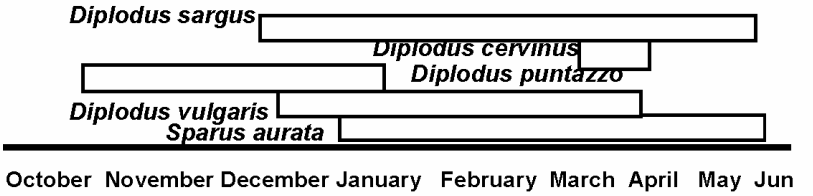

Fig. 1. Spawning season for the four *Diplodus* species and *Sparus auratus*.

Larval feeding schedules started with live food followed by gradual weaning to inert food. Rotifers (*Brachionus plicatilis*) were cultured on baker's yeast and enriched with Protein Selco® (INVE S.A. Gent, Belgium) before being fed to the larvae three times a day at a density of 5-10 prey.ml^{-1}. *Artemia* (A.F. and E.G., INVE) nauplii and enriched metanauplii (DHA Super Selco®, INVE) were kept at a density of approximately 0.1-1 prey.ml^{-1} in the larval tanks. Twenty individuals from each tank were sampled at hatching (0 days after hatching, dah), mouth-opening (2-3dah), and 10, 20, and 30dah for determination of total length. Determination of food ingestion was based on prey observation in the larvae digestive system under a stereomicroscope and the feeding schedule was then established. Swimbladder inflation was observed using a stereomicroscope on a daily basis during that period. The experimental feeding regimes for the four species are presented in Fig. 2, while Fig. 3 provides growth results. The fatty acid composition was determined in eggs and compared with available data for *S. aurata (*Fig. 4).

Results and discussion

The results demonstrate that the existing rearing techniques used for the most common species produced in aquaculture (*S. aurata, Dicentrarchus labrax*) can be easily adapted to the *Diplodus* species. *D. puntazzo* was the only species in which hormonally induced spawning was required. Eggs diameter averaged 0.9±0.01mm for *D. sargus* and *D. puntazzo* and 1.07±0.02mm for *D.vulgaris* and *D. cervinus.* Egg viability was lowest (30%) for *D. puntazzo,* intermediate (40-50%) for *D. sargus* and *D. vulgaris*, and highest (>75%) for *D. cervinus*.

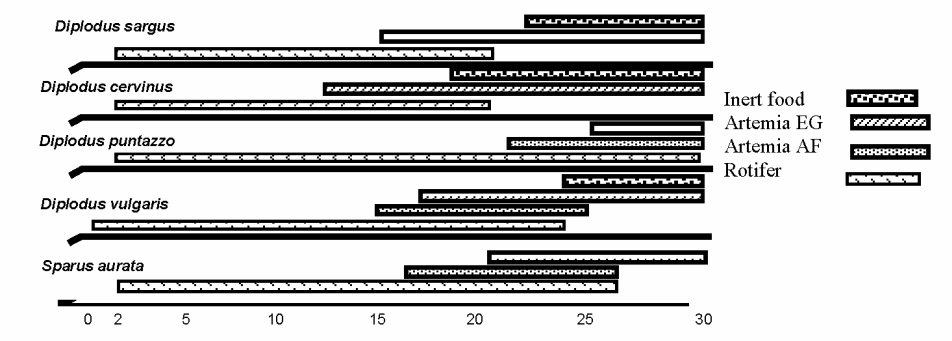

Fig. 2. Feeding regimes for the four *Diplodus* species and *Sparus auratus*

In Fig. 2 the feeding schedule for the four species reared is presented. Results for *D. sargus* and *D. cervinus* indicate an earlier ingestion of larger preys, intermediate for *D. vulgaris*, while *D. puntazzo* shows a delayed pattern. Based on Fig. 3, *D. cervinus* had the best growth performance while *D. puntazzo* had the lowest. Statistical analysis revealed that growths were significantly different for each species of *Diplodus* ($p<0.0001$). *D. sargus*, *D. cervinus*, and *D. vulgaris* larval survival was in the expected range for the sparidae species (5-20 % from hatching to 30dah), but *D. puntazzo* showed a lower survival during the same period. This worse result could be related to the artificial induction of spawning in this species. Also, difficulty to separate the oily film in the larval tanks resulted in a decrease of swimbladder inflation for all species.

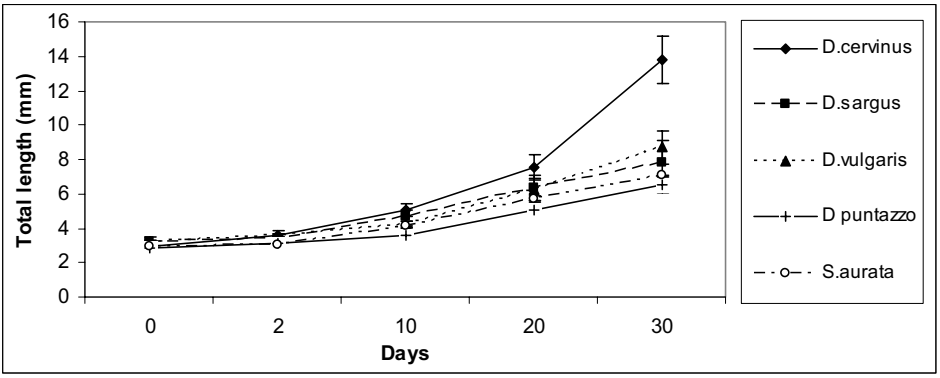

Fig. 3. Linear growth of *Diplodus sargus*, *D. cervinus*, *D. vulgaris*, *D. puntazzo*, and *Sparus aurata* larvae.

Fig. 4 compares the fatty acid profile of three *Diplodus* species eggs with *S. aurata*. The fatty acid profiles of *D. sargus, D. cervinus*, and *D. vulgaris* eggs show high absolute amounts of DHA and EPA, slightly superior when compared with *S. aurata* eggs. The DHA/EPA ratio is similar for all species.

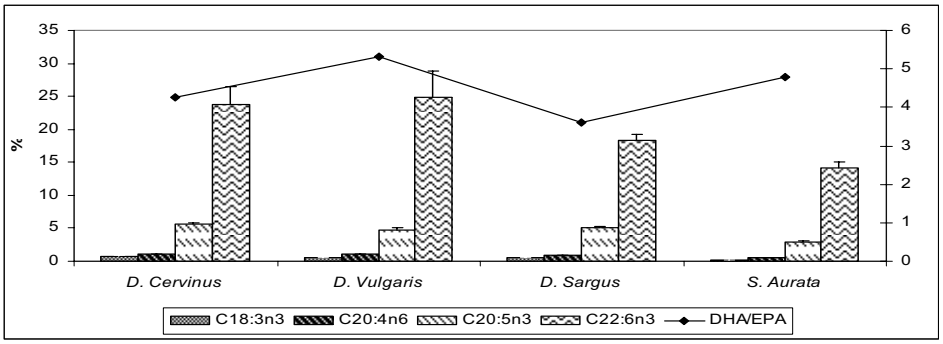

Fig. 4. Fatty acid composition (μg.mg^{-1} of dry weight) of three *Diplodus* spp. eggs and *Sparus aurata*.

Conclusions

The methodology and facilities used for the *Diplodus* species studied was similar to the ones used in *S. aurata*. Based on each particular growth rate, a specific feeding schedule was established for each species. Not matching spawning seasons favours the diversity of cultures, which contributes to higher profits in facility production.

Higher fatty acid profile in eggs and faster growth rate for *D. sargus, D. cervinus*, and *D. vulgaris* leads to special care on live prey enrichment and formulation of inert diets. Further, special focus should be given to the levels of essential FAs included in the diet, particularly for DHA and DHA/EPA ratio.

The present study shows that these species are potential candidates for aquaculture diversification or stock enhancement.

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UNDERSTANDING AND REMOVING THE BARRIERS TO DOMESTICATING THE BLACK TIGER PRAWN *PENAEUS MONODON*

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The black tiger prawn *Penaeus monodon* is the dominant prawn species farmed in Australia comprising 65% of the total annual farm production value of $55 M. The industry is currently totally dependent on wild broodstock. The supply of wild broodstock is erratic both seasonally and annually and can lead to severe shortages in broodstock and postlarvae. Reliance on wild *P. monodon* broodstock is a high-risk practice and it precludes the opportunity to enhance production through selective breeding and controlling the spread of disease. Experience with other species, particularly *Litopenaeus vannamei*, has clearly demonstrated the benefits of progressing from reliance on wild broodstock to the use of domesticated, high-health, selectively bred broodstock.

In 2002 an Australian research consortium, led by the Australian Prawn Farmers Association (APFA), was formed to develop the knowledge and technology to enable commercial-scale domestication of *P. monodon*. The research has focused on comparing and improving broodstock rearing environments and methods, optimizing reproductive output, establishing effective viral screening tests, and examining genetic predisposition to domestication.

The broodstock rearing environments tested were tanks, raceways, and ponds. The results demonstrated progressive improvements in the growth and survival in successive generations of domesticated *P. monodon* in each of the rearing environments. The temperature-controlled environments of tanks and raceways permitted male and female *P. monodon* to reach reproductive maturity by 11 months, in synchrony with the annual cycle of farm production in Australia. The comparatively lower water temperature in the broodstock ponds during the winter months resulted in slower growth rates and an increase in the time taken to reach maturity. The number of eggs spawned by the best performing females (upper 30%) of tank-, raceway-, and pond-reared broodstock was above a target goal set by the APFA (based on performance of wild broodstock of a similar size). However, the mean hatch rates (number of viable nauplii produced) of the best-performing domesticated females (upper 30%) were lower than the target

goal. Overall the reproductive performance (eggs spawned and hatch rates) demonstrated the need to significantly improve hatch rates in the domesticated stocks. The results of reciprocal matings between wild-caught and domesticated, tank-reared *P. monodon* males and females demonstrated that variation in reproductive performance was more dependent on the source of females than the source of males. These results indicate significant potential to improve the reproductive output of tank-reared *P. monodon* through improvements in the quality of the female broodstock. An analysis of the results of *P. monodon* domestication over three generations prior to the commencement of the project demonstrated that there is a genetic component to suitability for reproduction in captivity. However, the estimation of heritabilities showed relatively low values for percentage hatch rates (0.08+0.12) and nauplii number (0.07+0.13).

Screening wild founder broodstock at the start of the project confirmed that gill-associated virus (GAV) and Mourilyan virus (MoV) were endemic in wild *P. monodon* broodstock from the east coast (EC) of Queensland. Broodstock from this region are the dominant source of postlarvae and farm stocks are universally infected with GAV. Mourilyan virus was also detected in farm stocks at high prevalence, but the source(s) of infections are yet to be determined. The results demonstrated that infection of domesticated broodstock with GAV and MoV did not prevent successful growth, survival, and reproduction. However, the elimination of these viruses from the domesticated stocks remains a high priority.

The implications of these results for the prospects of progressing beyond domestication to the use of high-health, selectively bred *P. monodon* for the Australian industry will be discussed.

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EVALUATING THE EFFECT OF POTENTIAL SYNBIOTICS ON IN VITRO ANTAGONISM AGAINST A PATHOGEN USING DGGE

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Introduction

One of the most important modes of action of probiotic organisms is to limit the growth of pathogenic organisms. Many probiotic selection studies have focused on in vitro antagonism tests, and most were limited to single-strain agar-plates-based methods such as cross-streak and double-layer methods. We used DGGE, a DNA-based community fingerprinting technique, to evaluate the performance of probiotic strains in mixed cultures with a *Vibrio* strain when offered different well-characterised (dead yeast cell wall mutants) putative prebiotic substrates.

Materials and methods

Three probiotics (GR8, forming yellow stained colonies in marine agar; GR10, a spontaneous rifampicin resistant mutant; and LVS2) were chosen based on their previously demonstrated positive effect in *Brachionus* culture, as well as one pathogen *V. harveyi* 002. They all have distinct 16SrDNA DGGE migration patterns and they were cultured separately in marine broth 2216E. We harvested cells at exponential phases and mixed with GR8, GR10, LVS2, and *V harveyi* 002 at 1:1:1:0.01 ratios. Three different null mutants of yeast mnn9, fks1, chs3 (isogenic deletion strains derived from BY4741 – resulting either in reduced mannose, glucan, or chitin content, respectively, of the yeast cell wall) – and the wild type strain (WT; genotype described in Marques et al., 2004) were grown in YNB (2% glucose, 0.67% Yeast Nitrogen Base) supplemented with necessary amino acid and uracil until stationary phase. Yeast cells were harvested, autoclaved, and subsequently used as substrate in the bacteria culture.

Bacterial mixtures were inoculated at 10^5cfu.ml^{-1} at day 0. The experiments were performed in triplicate in closed sterile Falcon tubes (TRPR, γ-irradiated) with 20ml of filtered (0.22µm) and autoclaved seawater (FASW) on a shaker (25ºC, 150rpm). The initial amount of WT yeast added to the tubes was 10^5 cells.ml^{-1}; for other yeast mutants, adjusted amounts were added on the basis of ash-free dry weight of each yeast mutants (Marques et al., 2004). Samples were collected on day 3 and day 6 for marine agar plating, TCBS agar plating and DNA extraction. On day 3, 100µl of culture water from each tube was inoculated into new tubes and further incubated for another 3 days.

Following total community DNA extraction, PCR-DGGE was performed as per Boon et al. (2000). Bacterial primers P338F with GC clamp and P518r for 16SrRNA gene were used for PCR. DGGE was run in the Bio-Rad D Gene System (Hercules, CA, USA) with 8% w/v polyacrylamide gels in 1×TAE, containing a linear chemical gradient ranging from 45-60% denaturant where 100% denaturant contains 7M urea and 40% formamide. PCR products (10µl) were used for separation in denaturing gradient gels. The electrophoresis was run for 16h at 60ºC, at 38V. The gels were stained using SYBR GreenI nucleic acid (FMC BioProducts, Rockland, ME, USA) according to the manufacturer instructions. The stained gel was immediately photographed on a UV transillumination table with a video camera (module Vilbert Lourmat, Marne-la valle, France).

Results and discussion

For samples collected on day 3, after 72h incubation, marine agar plating could not detect the yellow coloured colonies of GR8 although DGGE gel patterns confirmed its presence in the community (Fig. 1). TCBS plating showed the highest *V. harveyi* 002 numbers when chs3 cells were used as substrate ($1.3\pm0.6\times10^8$cfu.ml^{-1}) and the lowest numbers when fks1 ($3.2\pm0.8\times10^7$cfu.ml^{-1}) were used as substrate. Rifampicin marine agar plating showed lower number (10^6cfu.ml^{-1}) of GR10 in WT yeast-based culture than in the others cultures. However both GR10 and LVS2 could not be detected on DGGE gel. The amount of CFU of LVS2 (no selective plating method available) was calculated as the difference between total CFU number and the CFU of the other strains. Based on this method no LVS2 seemed to be present. Marine agar plating showed the 2-5 times higher number of probiotics in mnn9-based culture than in the other groups.

For samples collected on day 6, TCBS plating showed the highest *V. harveyi* 002 numbers in chs3-based cultures (3×10^7cfu.ml^{-1}). Rifampicin marine agar plating (GR10) results showed no significant difference among the different groups (8×10^6cfu.ml^{-1}). Marine agar plate counting showed an increase of probiotics up to 10^7cfu.ml^{-1}. DGGE patterns demonstrated the reproducible presence of the probiotic LVS2 in mnn9 and chs3-based cultures but not in others (Fig. 2). The

DGGE gel also reflects an increase in the biodiversity of probiotic strain in the second subculture, where the all three probiotic strains could be detected when supplied with certain substrates.

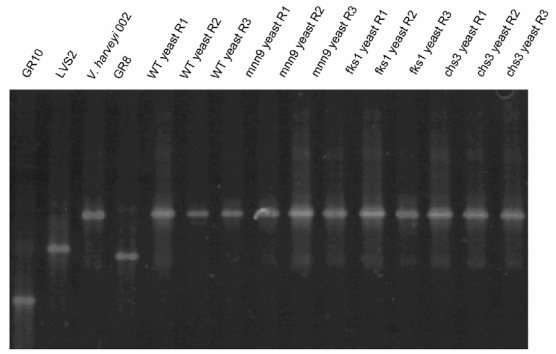

Fig. 1. DGGE analysis of the microbial community containing three probiotic strains and *V. harveyi* 002, when offered different dead yeast cell wall mutants as substrate.

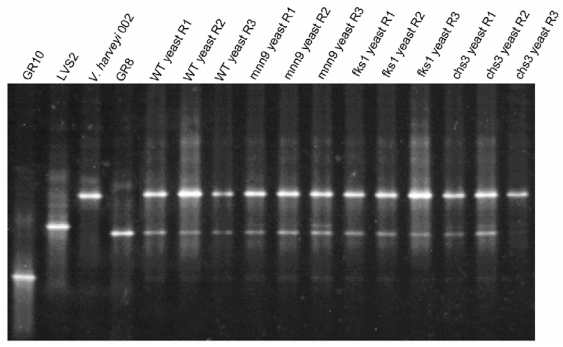

Fig. 2. DGGE analysis of the microbial community containing three probiotic strains and *V. harveyi* 002, when offered different dead yeast cell wall mutants as substrate on day 6 (after 2 × 3 days of culturing). R1, R2, R3: 3 replicates.

To increase the effect of probiotics, there is a need to combine them with prebiotics called synbiotics, allowing for an improved colonisation of the target ecosystem (Bomba et al., 2002). Although we can detect some probiotics by selective culture medium, there is generally no selective plating method available to enumerate each community member and hence quantify the effects of mixed probiotics to one or more pathogens. The molecular-based method of denaturing gradient gel electrophoresis (DGGE) has been introduced in microbial populations and communities studies for many years. DGGE can reveal changes in the bacterial species composition starting from enrichment cultures with different inoculum dilutions (Jackson et al., 1998). In this particular experiment, the DGGE band patterns allowed direct identification of the species. Temmerman et al. (2003) used this culture-independent method to analysis the effect of probiotic products. They found that, compared with culture-dependent analysis, the

DGGE approach had a much higher sensitivity for detection of microbial strains in probiotic products. In our experiments, DGGE technique allowed to visualise the dynamic changes of probiotics by subculturing, as well as the effect of certain yeast cell wall mutants on the microbial community composition.

Conclusions

The DGGE gel patterns of a microbial community with 4 species, demonstrated the three putative probiotic strains were able to compete with a pathogenic vibrio, when dead yeast (often used as food in *Brachionus* cultures) cells were supplied as substrate. The composition of the microbial community could be manipulated by subtle changes (different yeast cell wall mutants) in the substrate for the bacteria. This suggests that yeast cell wall composition can have a prebiotic effect on the microbial community. Agar plating method failed to assess the differences and dynamic changes of probiotics. The results with this experimental microbial community suggest that the establishment of a certain microbial community (e.g., in a *Brachionus* culture) could be favoured (symbiotic effect) by a well chosen feed composition.

Acknowledgements

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INDUSTRIAL SCALE RECIRCULATION SYSTEM FOR HIGH-DENSITY CULTURE OF ROTIFERS (L-STRAIN)

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Introduction

Intensive production of juvenile cod in Norway demands a high amount of rotifers. Conventional batch and semi-continuous cultures are shown to be unstable and unpredictable, and culture techniques must be developed to ensure high productivity and stable quality at necessary quantities. Since the pioneer recirculation system of Suantika et al. (2000), few articles dealing with recirculating rotifers systems have appeared in the literature. A new system has been built by SINTEF Fisheries and Aquaculture and is described in this paper.

Materials and methods

The system consists basically of a production tank (3000 l), a feeding system for rotifers, an external filter for rotifers, a 410-l foam fractionator (protein skimmer, model Helgoland 500, Sander, Germany), and a 2000-l biofilter with 2-3-mm diameter polyurethane balls as filter substrate (Fig. 1). Ozone is produced by an ozonater (model A2000 Sander, Germany) connected to the suction line of the protein skimmer.

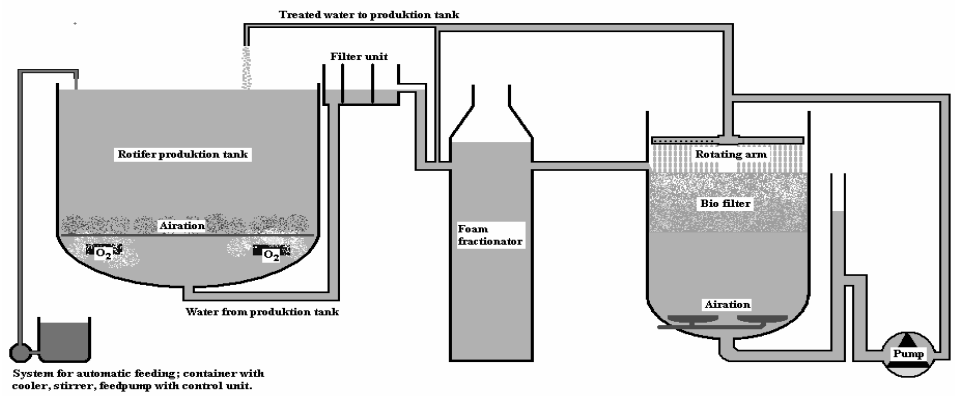

Fig.1. Schematic view of the recirculation system.

Water from the rotifer culture is gravity-fed from the bottom of the production tank to the filter unit (Fig. 2). This filter contains two rotating filter wheels; the first one with a 250-µm mesh allowing rotifers to pass through, while the bigger particles are removed from the water by continuous backwashing. The second wheel filter with a 60-µm mesh hinders rotifers from leaving the system. From the chamber between the two filters wheels, concentrated and rinsed rotifers are brought back to the production tank by air-lift. The recirculation flow rate of the system was of 300%. This means that the whole volume of water (3000 l) was recirculated 3 times in 24h.

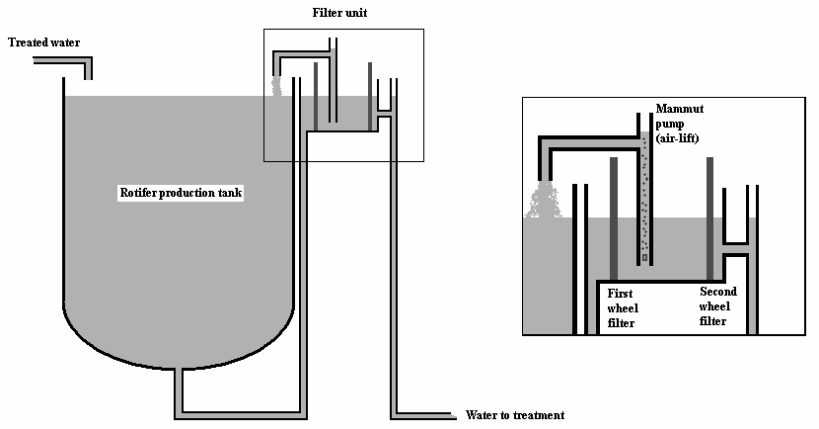

Fig. 2. Schematic view of the external filter unit.

The system has been tested using a rotifer culture of *Brachionus plicatilis* in a feeding experiment with bakers' yeast and Marol E (lipid emulsion, SINTEF, Norway) at a ratio of 20:1, respectively, during 15d in a semi-continuous production system at 25ºC with a daily harvesting of 500 l rotifers. Total ammonium (TAN), nitrites, and nitrates (not shown) were measured in the bio-filter outlet water using a Hack DR 890 colorimeter instrument.

The rotifer lipid quality in the recirculation system was determined according to Rainuzzo et al. (1992). Likewise, these results were compared to a flow-through semi-continuous system with similar feeding and culture conditions

Results and discussion

Some of the production parameters and physical metabolites produced during the feeding trial are shown in Table I. The rotifers fed on bakers' yeast and Marol E exhibited stable nutritional parameters such as docosahexaenoic acid (DHA) content (25-26mg.g^{-1} DW), eicosapentanoic acid (EPA) of 3.8-4.2, and DHA/EPA ratio (6.0-6.3) during the whole production period (Fig. 3). The total lipids (not shown) of about 17% were also stable during the rotifer cultivation.

The stability of the nutritional lipid quality is of great advantage when daily harvesting of rotifers are carried of in the recirculation system.

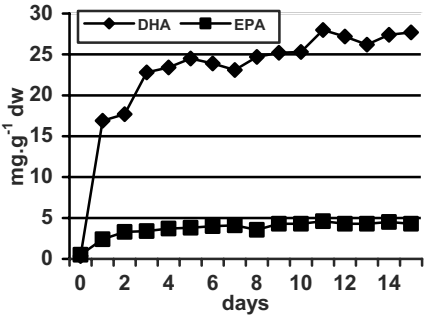

Fig. 3. DHA and EPA contents (mg.g^{-1} DW) in rotifers during 15 days culture.

The contents of DHA, EPA, and n3-HUFA were slightly higher in the recirculation system compared to a traditional flow-through system (Fig. 4).

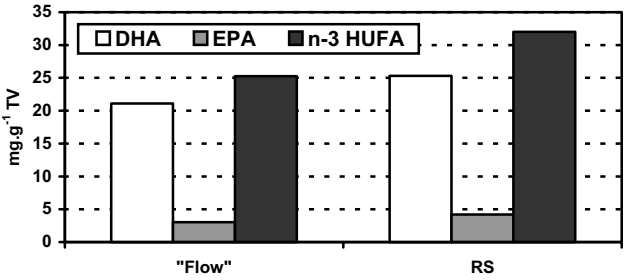

Fig. 4. Contents of DHA, EPA, and n-3 HUFA in two rotifers production systems: "flow-through" (flow) and recirculation (RS)

The daily production of rotifers by the present recirculation system was about 1.25 billions rotifers however this amount maybe increased by increasing the rotifers density. Thus, more food should be added to the system but taking care of the filter unit support the higher amount of food in the rotifers tank. Eventually a better food could result in a higher rotifers density.

Ammonium, nitrites, nitrates, and redox potential were all below toxic levels. The production system has been developed and tested in commercial hatcheries with good results. However, the system at the present has not been tested longer than 15d since by this time the culture started to show signs of lower quality (high amount of suspended organic material in the culture and low pH).
The recirculation system described here has been successfully tested in a commercial cod hatchery in Norway, significantly reducing rotifer production time.

Table I. Overview of some cultivation parameters during 15 days culture in the recirculation system.

Day	Density ind.ml^{-1}	Egg ratio	Yeast kg	Marol E g	NH_3-N mg.l^{-1}	NO_2 mg.l^{-1}	pH
0	420	0.23	2	100	0.10	0.02	7.79
1	674	0.51	3	150	0.13	0.03	6.98
2	1010	0.66	4	200	0.14	0.02	6.13
3	1955	0.40	5	250	0.16	0.02	6.40
4	2488	0.41	6	300	0.18	0.02	6.21
5	2365	0.42	6	300	0.20	0.03	6.04
6	2614	0.34	6	300	0.21	0.02	6.30
7	2589	0.29	6	300	0.22	0.03	6.12
8	2680	0.36	6	300	0.31	0.03	6.10
9	2100	0.38	6	300	0.27	0.02	6.05
10	2454	0.34	6	300	0.25	0.03	6.06
11	2607	0.40	6	300	0.28	0.04	6.10
12	2549	0.32	6	300	0.34	0.06	5.97
13	2410	0.35	6	300	0.26	0.04	6.03
14	2550	0.40	6	300	0.24	0.05	5.95
15	2600	0.33	6	300	0.30	0.05	5.90

Conclusions and recommendations

The system may still be improved and adapted to the different need of commercial hatcheries. The recirculation system should be able to work for more than 15 days in order to keep a constant and stable production of rotifers. Some improvements should be made as for example a new filter unit design which could help to increase the rotifers density and consequently the rotifers production. Several feed qualities and feed regimes should be tested in order to obtain the appropriate rotifers nutritional quality for a given fish larvae cultivation.

Acknowledgements

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DIGESTIVE ENZYME ACTIVITY DURING BLACK SPOT SEABREAM LARVAE AND POST-LARVAE DEVELOPMENT

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Intoduction

Blackspot seabream (*Pagellus bogaraveo*) is a potential candidate species for European aquaculture. Despite recent developments in its production, few data exists on the specific nutritional requirements of this species. The knowledge of larval digestive enzymes activity is a very important step in adapting a diet to fish larval digestive capacity.

Materials and methods

Eggs were obtained from a blackspot seabream broodstock adapted to captivity at IEO facilities, and incubated at 14 ºC in cylindro-conical tanks with running water during 4 days. After hatching, fish larvae were kept in the same tanks until yolk-sac was absorbed (2 days), then transferred to larval tanks (1000 l) where they were maintained until the end of the experiment (60 days post-hatching, dph). Fish larvae were fed with rotifers from 5-30dph, *Artemia* naupli from 31-35dph, enriched *Artemia* metanauplii from 36-50dph (enrichment with *Isochrysis galbana*), and an inert diet (Gemma 0.3mm; Trow, France) from 45-60dph.

A pool of fish larvae was sampled in triplicate at 7, 11, 21, 45, and 55dph for digestive enzymes analysis. These samples were carefully rinsed with distilled water, immediately frozen in liquid nitrogen, and stored at -80ºC until assayed.

Enzymatic activity was determined on the whole body of fish larvae younger than 22dph, and on dissected abdominal portion in older fish larvae. Samples were homogenised with ice-cold distilled water. Trypsin activity was measured using Bapna as the substrate (Tseng et al., 1982). Amylase activity was meas-

ured using starch as the substrate (Métais and Bieth, 1968). Lipase activity was measured using p-nitrophenyl myristate as substrate (Iijima et al., 1998). Alkaline phosphatase activity was measured using p-NPP as the substrate (Bessey et al., 1946). Enzyme activities were calculated as µmol of substrate hydrolysed per minute – i.e., U at 37ºC for AP and 25ºC for trypsin. Enzyme activities were expressed as specific activities, i.e., $U.mg^{-1}$ protein. Amylase activity was expressed as the equivalent enzyme activity, which was required to hydrolyse 1mg of starch in 30min at 37ºC. One unit of lipase activity was defined as 1µmol of p-nitrophenol released per minute at 30ºC. Protein was determined using the Bradford method (Bradford, 1976).

Homogeneity of variance was verified by using Bartlett's test. Enzymatic activity data were compared by one-way ANOVA, followed by Bonferroni multiple range test, when significant differences were found at the α=0.05 level (SPSS, v. 12.0)

Results and discussion

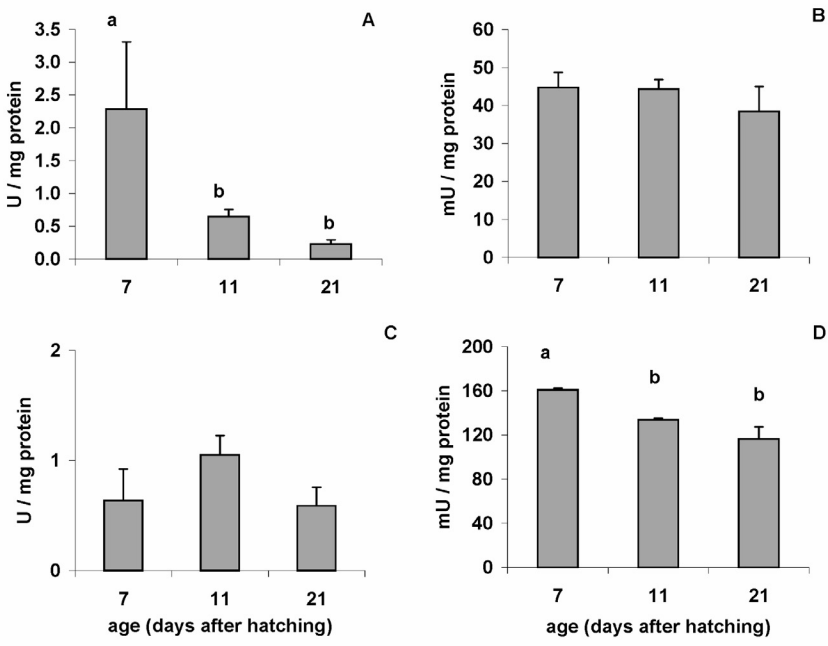

Fig. 1. Digestive enzymes during *Pagellus bogaraveo* larval development. Values are presented as mean±SE (n=3). Different superscripts indicate significant differences A - Amylase; B - Trypsin; C - Lipase; D - Alkaline phosphatase.

The studied digestive enzymes exhibited a similar pattern of variation during the development of the black spot seabream larvae (Fig. 1). Digestive enzymes spe-

cific activity presented a peak of activity at 7dph, exception made for trypsin that appears to have had its peak before 7dph, decreasing thereafter to a stable activity level. This pattern of variation fits in the description previously made for the early life stages of marine fish (Zambonino-Infante and Cahu, 2001), where digestive enzyme development is characterized by a strong variation during the early stages, reaching a peak of activity decreasing thereafter to a rather stable value of activity.

Compared to sea bass and Senegalese sole, black spot seabream showed a similar or higher trypsin specific activity, respectively; whereas amylase specific activity was considerably lower (Ribeiro et al., 1999; Zambonino-Infante and Cahu, 1994). These differences might suggest a low ability of black spot seabream larvae to use carbohydrates in the diet.

No significant differences were observed in trypsin (Fig. 2), amylase, and alkaline phosphatase specific activity of black spot sea bream at 45 and 55dph. An opposite pattern was observed for lipase specific activity that significantly increased from 45-55dph ($P<0.05$). This variation could be related with the percentage of lipids in the diets. An indicative value was calculated, resulting in a difference of 3% more lipids in the inert diet composition than *Artemia* metanauplii (Ribeiro et al. 2002; information from diet manufacturer). This suggests that after a certain stage of development, a slight increase in the lipid content of the diet might stimulate lipase secretion in black spot seabream.

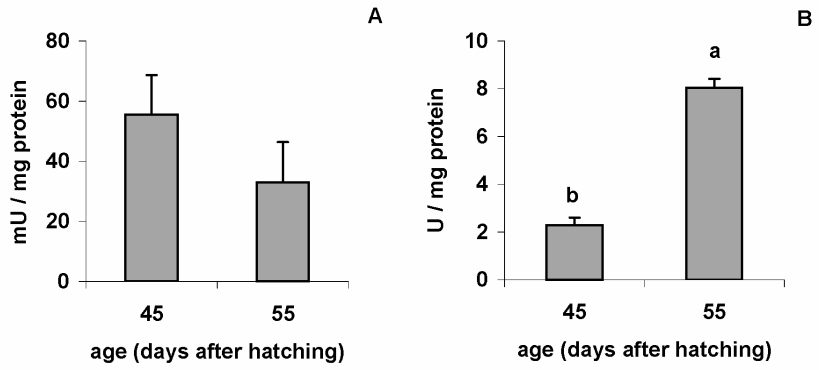

Fig. 2. Trypsin (A) and Lipase (B) specific activity in dissected *Pagellus bogaraveo* post larvae. Values are presented as mean±sem (n=3). Different superscripts indicate significant differences.

Besides the high activity of lipase this species exhibits lipid accumulation after a certain age, independently of the dietary lipid content of the diet (unpublished results), suggesting metabolic changes that allow the fish to convert other dietary components into fat.

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BACTERIAL TRANSLOCATION AND PATHOGENESIS IN THE DIGESTIVE TRACT OF FISH: WHAT ARE THE INDIGENOUS BACTERIA AND PATHOGENS DOING?

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The digestive tract of fish is essentially a muscular tube lined by a mucous membrane of columnar epithelial cells that exhibit regional variation in structure and function. In the last decade, our understanding of translocation of bacteria in the fish gut has increased, and electron microscopy has contributed to this knowledge. This review focuses on translocation of bacteria (transcellular and paracellular), cellular damage (specific attack on tight junctions and desmosomes caused by pathogenic bacteria), and on pathogenesis in the gastrointestinal tract of larvae and fry using light and electron microscopy.

Currently, it is generally accepted that the major routes of infection in fish are through the skin, gills, and gastrointestinal tract. As the gastrointestinal tract is a potential port of entry for pathogens, the use of electron microscopy in studies on microbiota in the fish digestive tract is a valuable method for increasing our understanding of the mechanisms involved in entry of the pathogens. Possible mechanisms involved in translocation of bacteria [autochthonous (indigenous) and pathogens] in the fish gastrointestinal tract are discussed.

Intensive worldwide fish production has increased the risk of infectious diseases. Before an infection can be established, pathogens must penetrate the primary barrier. In order to prevent microbial entry, fish have various protective mechanisms, including production of mucus by goblet cells, the apical acidic microenvironment of the intestinal epithelium, cell turnover, peristalsis, gastric acidity, lysozymes, and antibacterial activity of epidermal mucus. At the same time, pathogenic microorganisms have evolved mechanisms to target skin, gills, or gastrointestinal tract – the three major routes of infection – as points of entry.

Under normal conditions, the mucus secreted by goblet cells functions as a barrier, compromising the ability of bacteria to escape the intestinal lumen. However, various critical conditions may lead to loss of barrier function, enabling both viable and non-viable bacteria and bacterial products (such as endotoxins) to migrate from the intestinal lumen through the epithelial mucosa to infect otherwise sterile tissues. This process is termed translocation. Bacterial translocation is a recommended indicator for probiotic safety assessment since translocation through the gastrointestinal tract is the crucial step in the pathogenesis of intra-abdominal infections.

Although numerous papers have described pathogenesis in fish, fewer investigations have used light and electron microscopy to evaluate intestinal translocation of bacteria in larvae, fry, juvenile, and adult fish, or to detect fish pathogens in the gastrointestinal tract. The present paper aims to provide an overview on translocation of bacteria in the digestive tract of larvae and fry, and the occurrence of pathogens in the gastrointestinal tract. Furthermore, we will present data on cell damage of gut enterocytes caused by pathogenic bacteria in larvae and fry.

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WATER OZONATION IMPROVES SURVIVAL AND REDUCES DISEASE IN LARVAL ROCK LOBSTER (*JASUS EDWARDSII*)

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Introduction

Rock lobsters constitute valuable fisheries worldwide but are fully exploited. Thus, aquaculture is now being considered to meet the high demand for product, and the only way to sustainably farm lobsters is by hatchery-rearing of seedstock from eggs. Kittaka (1994) in Japan reported the first success in raising phyllosoma larvae to metamorphosis. More recently, Matsuda and Takenouchi (in press) reared larger numbers of larvae utilising improved water quality, system design and, more importantly, health. During culture, larvae are vulnerable to infections with opportunistic bacteria, typically *Vibrio* and *Leucothrix*-like spp., as occurs when feeding with *Artemia*. Even with *Artemia* disinfection, the few remnant bacteria quickly establish infective populations in larvae (Tolomei et al., 2004). In Japan, antibiotics were relied on for culture with considerable success but may be unsuitable for long-term food production. This paper presents results on seawater ozonation, which creates oxidative breakdown products, to reduce bacterial contamination and improve larval survival in culture, allowing us in Australia to produce lobster seedstock without antibiotics.

Materials and methods

Newly hatched phyllosoma were cultured in 10-l tubs (4 tubs.treatment^{-1}) at 300 larvae.tub^{-1} (Ritar, 2001). Four treatments of seawater ozonation were compared: 1) No O_3 – UV-disinfected, unozonated water with oxidation reduction potential (ORP) of 300mV; 2) Low O_3 – water in treatment 1 ozonated to an ORP of 400mV; 3) Medium O_3 – water in treatment 1 ozonated to an ORP of 500mV; and 4) High O_3 – water in treatment 1 ozonated to an ORP of 600mV.

ORP values were monitored daily. *Artemia* (Artemia Systems, INVE, Belgium) were hatched and grown to 1.5mm (Ritar et al., 2004) and fed to phyllosoma tubs at 1.5 *Artemia*.ml^{-1}. Larval survival was determined after moults to Stage II on Day 14 and to Stage III on Day 25 after hatch.

For bacterial content, triplicate larval samples were collected from each tub at the start and on Days 5 (Stage I), 12 (Stage II) and 21 (Stage III) after hatch. Samples were homogenised, serially diluted (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) and plated onto ZoBell's or TCBS agar medium to determine total aerobic marine heterotrophic bacteria and *Vibrio* spp., respectively. One-way ANOVA and Tukey-Kramer HSD tests were performed for post-hoc multiple comparisons. The level of significance for all analyses was $P<0.05$.

Results and discussion

Larval survival differed significantly (Fig. 1). The high-O_3 treatment was toxic with no larvae moulting to Stage II. All were trapped in the moult, remaining in an abnormal naupliosoma-like shape, preventing feeding, and death occurring probably by starvation by 18 days post-hatch (dph). Larvae in no O_3 had 68% survival by 14dph but all died by 25dph, probably from *Vibrio* infection. Larvae receiving low or medium ozonation had survivals of 81% and 71% to 14dph, respectively, and of 55% and 64% to 25dph, respectively. Lengths of surviving larvae were not significantly different between treatments (Stage I: 2.02±0.11 mm; Stage II: 3.02±0.20 mm; Stage III: 4.06±0.37 mm).

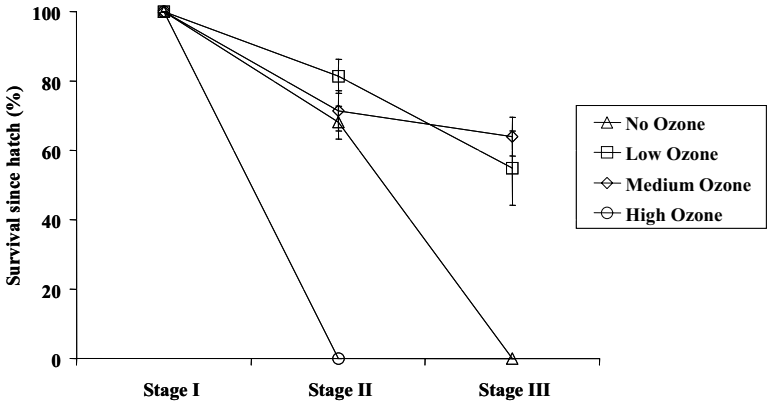

Fig. 1. Phyllosoma survival to Stage III at different ozonation levels.

There were significant differences during culture in *Vibrio* and total bacteria content in healthy phyllosoma receiving no O_3 compared to those receiving ozonated water (Fig. 2). At 5dph, total bacterial numbers were similar for all treatments, while there were no *Vibrios* for the medium and high treatments compared to no O_3 (333) and low O_3 (1667) treatments. On 12dph, *Vibrios* were highest in no O_3 (150), compared to low (0), medium (8), and high (6). On 21dph in no O_3, just prior to the complete mortality of all phyllosoma, all bacteria (>126 000) were *Vibrios*, whereas there were no *Vibrios* in the low treatment and 51 in the medium treatment.

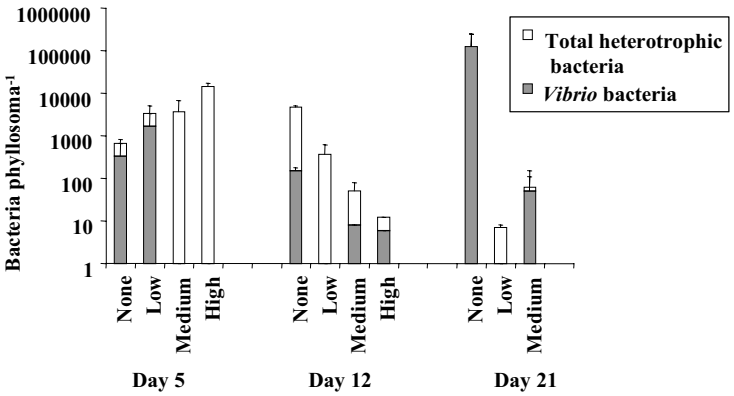

Fig. 2. Total and *Vibrio* bacteria in phyllosoma.

Conclusions

The results show that seawater ozonation reduces bacterial content, particularly of *Vibrio* spp., thereby improving phyllosoma survival during culture. However, excess ozonation appears to cause deformities resulting in mortalities. The use of ozonated seawater assisted us in culturing larvae to metamorphosis of lobster seedstock and appears to have wider application in other crustaceans.

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DOES THE PRESENCE OF MICROALGAE INFLUENCES FISH LARVAE PREY CAPTURE?

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Introduction

Green-water technique has been widely described to improve fish larval growth and survival rates. Microalgae addition to the rearing tanks produces changes not only in the colour of the medium but also in its chemical composition (Naas et al., 1992; 1996). The aim of this study was to analyse the influence of microalgae on the feeding ability of marine fish larvae (*Solea senegalensis* and *Sparus aurata*) during development.

Materials and methods

Both fish species larvae were reared in 200-l tanks one day post-hatching (dph) until the end of the experiment, 23 and 15dph for seabream and sole, respectively. Feeding regime consisted in rotifers (4-23dph) and *Artemia* nauplii (16-23dph) in the case of seabream larvae, whereas sole was fed with rotifers (2-6dph), *Artemia* nauplii (5-10dph) and metanauplii (10-15dph). Rotifers and *Artemia* metanauplii were enriched with DHA Protein Selco (Inve, Belgium) and Easy Selco (Inve, Belgium) + Microfeed (Ewos, Norway), respectively.

Six different treatments were used to study larval fish feeding ability: 1) *Isochrysis galbana*, 65 000 cells.ml^{-1}; 2) *Tetraselmis chuii*, 40 000 cells.ml^{-1} (Cahu et al., 1998); 3) Phytobloom®, 1 000 000 cells.ml^{-1} (microalgae paste - 99% *Nannochloropsis oculata*; Necton, Portugal); 4) clear water; 5) *T. chuii* supernatant (40 000 cells.ml^{-1}, centrifugation 5min 1162×g); and 6) food green stain, 15µl.l^{-1}. Cell density and stain quantity were calculated based on the amount of light diffused in the tanks (µmol electrons.s^{-1}.m^{-2}, Underwater Fluorometer; Walz Diving Pan) using *T. chuii* cell density as reference.

At different development stages 40 unfed fish larvae were transferred (9, 16, and 23dph for seabream; 4, 9, and 14dph for sole) to 18 (6×3) small tanks containing 2 l of seawater in identical conditions as the rearing tanks. Fish larvae were ac-

climatised for 1h, subsequently water volume was adjusted to 3 l with seawater and the respective treatment together with the live prey. Fish larvae were sampled (n=30 each tank; except 9dph seabream larvae where n=20) two hours after feeding, being anaesthetised with 2% 2-phenoxyethanol before they were fixed with 4% buffered formaldehyde.

For gut content analysis, formaldehyde was replaced by 95% ethanol during two hours, and than by 1% potassium hydroxide. The period in this solution changed with the size of fish larvae, ranging from 1-3d. The number of prey in fish larvae gut was quantified using a binocular microscope. Feeding ability was evaluated by the feeding rate (FR), percentage of larvae with preys, and feeding intensity (FI), number of preys in the larva digestive tract (Yin and Blaxter, 1987).

Data on FR was arcsin-transformed followed by a one-way ANOVA, using Bonferroni multiple range test when significant differences were found at the α=0.05 level. FI was compared among treatments using median test (detects distributional differences and shape) followed by a χ^2 square-test. Statistical analysis was done using the statistical software SPSS for Windows (SPSS Inc., USA).

Results and discussion

Fish larvae FR increased with development for seabream and sole, ranging from an average value of 72-99% and from 88-100%, respectively. FR values below 100% were only observed for 4-dph sole. No significant differences on FR was observed between treatments for both species, exception made for seabream at 23dph, where larvae from the clear water treatment exhibited significant lower values of FR ($p<0.05$). The fact that *T. chuii* supernatant and stain treatment exhibited FR values identical to microalgae treatments indicates that both colour and composition of microalgae are important for seabream larvae FR at 23dph.

As expected, fish larvae FI increased with larval development for both species. Sole showed higher values of FI than seabream at the first two ages analysed. The higher number of preys ingested by sole than by seabream is consistent with the growth pattern previously described, where sole exhibits twice the growth rate of seabream during this period of development (Parra and Yúfera, 2001).

Fish larvae feeding ability was significantly influenced during development by the presence and type of microalgae (Table I). At 9dph seabream larvae from Phytobloom® treatment exhibited higher values of FI, although the presence of chemical substances seemed to play a major role in this stage of development, since *T. chuii* supernatant treatment exhibited values similar to *T. chuii* treatment and stain treatment exhibited a higher number of FI below the median. At 16dph Phytobloom® treatment FI values were markedly above median, but at this stage of development seabream feeding ability seemed to be affected by an interaction

between colour and chemical composition, since clear water treatment exhibited a higher number of FI below the median, although supernatant and stain treatments presented similar values of FI and high frequencies above the median. At 23dph, feeding ability was again mainly influenced by chemical composition but at this stage of development by *Tetraselmis chuii,* since *T. chuii* and *T. chuii* supernatant treatments exhibited the higher number of FI above median.

Table I. Fish larvae feeding intensity during development was compared between treatments using the median test (values represent the number of larvae above or below median based on their FI), followed by a χ^2-square test (n=90). Significant differences among treatments were identified by superscript at column age. Treatments used: 1- *Isochrysis galbana*; 2 - *Tetraselmis chuii*; 3 -Phytobloom®; 4 - clear water; 5 - *T. chui* supernatant; 6 - food green stain.

			Treatments					
Species	**Age**	**Median**	**1**	**2**	**3**	**4**	**5**	**6**
Sparus aurata								
	9 dph*	>	23	27	37	23	26	13
		≤	37	33	23	37	34	47
	16 dph*	>	51	48	62	22	42	43
		≤	39	42	28	68	48	47
	23 dph*	>	25	72	47	28	66	30
		≤	65	18	43	62	24	60
Solea senegalensis								
	4 dph*	>	68	73	38	29	20	22
		≤	22	17	52	61	70	68
	9 dph	>	54	41	37	47	39	37
		≤	36	49	53	43	51	53
	14 dph	>	40	41	30	38	37	39
		≤	50	49	60	52	53	51

Sole larvae FI at 4dph was significantly higher in the *I. galbana* and *T. chuii* treatments, suggesting a synergism between colour and chemical factors, since all the other treatments exhibited the higher number of FI values below median. At 9 and 14dph no significant differences were observed in sole FI among treatments, suggesting that the prey itself was more important than the green water effect. This observation might be explained by the fact that sole species are known to rely mainly on chemical cues to feed (De Groot, 1971) and by the fact that Artemia homogenates increased fish larvae ingestion rates (Kolkovski et al., 1997). The fact that sole is under metamorphosis at this stage justifies the higher FI values below the median, since during this process sole decreases their feeding ingestion (Parra and Yúfera, 2001).

Conclusions

Fish larvae feeding ability was influenced by the interaction between colour and chemical substances of microalgae during development. Seabream was more dependent on microalgae addition than sole larvae, which can be related with the type of prey and larval physiology.

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DIGESTIVE PHYSIOLOGY OF MARINE FISH LARVAE: PROCESSING CAPACITY OF PROTEINS, PEPTIDES AND AMINO ACIDS AND HORMONAL CONTROL OF THE DIGESTIVE PROCESS

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The major function of the digestive system of growing larvae is to supply dietary nutrients to the body tissues. Most marine fish larvae, including target species for aquaculture, hatch from small, mostly pelagic eggs, and their digestive tracts are still developing at the onset of exogenous feeding. A fully developed digestive system, including a functional (acid-producing) stomach, is acquired during ontogeny. Although the larval gut is not adult-like at the onset of exogenous feeding, it is capable of supporting high growth rates providing suitable feed is available.

It is technically difficult to quantify the in vivo performance of the digestive system in fish larvae, particularly for the processing of proteins and amino acids. One approach is a method which combines tube-feeding of ^{14}C-labelled nutrients and a CO_2 trap with scintillation-counting of dissected organs or body compartments. This single-meal method provides a useful starting point and a framework for investigating features of gut absorption, oxidation, and retention (assimilation) of nutrients.

Using this method, a ^{14}C-salmon serum protein (^{14}C-SSP) was administered in equal amounts relative to body wet weight to larval and post metamorphosed juvenile Atlantic halibut (*Hippoglossus hippoglossus* L.). The absorption efficiency was lower in larvae than in juveniles (25% versus 59%) which suggests inferior proteolytic capacity in the larval stages. Further studies in the larval stage demonstrated that the absorption efficiency of the intact test-protein was reduced with increasing dose. The ^{14}C-SSP was then hydrolysed to various degrees (intact protein, pepsin-hydrolysed protein and highly hydrolysed protein) and administered to larval Atlantic halibut. The absorption efficiency of the hydrolysed protein was higher than the intact protein and was also constant independent of degree of proteolysis and the administered dose. This supports the notion that absorption efficiency of dietary protein in larvae is limited by proteolysis rather than absorption. The initial absorption rate increased with the de-

gree of hydrolysis. The absorption of partly and highly hydrolysed protein from the gut was 2.2 and 3 times as fast, respectively, as that of intact protein. The results from the trials with intact protein suggests that the gut evacuation rate is a critical factor for the assessment of absorption efficiency. Based on this it is suggested that the intestinal throughput rate is a critical factor in the utilisation of slowly digested and absorbed feed components, such as intact proteins, for fish larvae.

Digestion is a complex process and is regulated by nervous and hormonal systems. Cholecystokinin (CCK) is believed to play a key role in controlling the release of pancreatic enzymes and bile. It is also implicated in control of appetite and satiation. There are clear differences in the distribution pattern of CCK-producing cells and the timing of appearance in the gut among the studied teleost species. In Atlantic halibut and bluefin tuna which have a rotated gut, CCK-producing cells could not be detected until the middle of the larval stage. They were distributed in the anterior part of the midgut. In ayu and Atlantic herring, which have a straight gut, CCK-producing cells were present from hatching and were widely distributed throughout the midgut.

There seems to be a relationship between the distribution pattern of CCK-producing cells and the macroscopic anatomy of the gut. Most likely, CCK-producing cells are strategically located where they optimize the sensing of chemical signals from digested food in order to achieve a good feedback control of the digestive process. The reason underlying the observed late appearance of CCK cells in larvae with a rotated gut should be further investigated

Both in situ and immunohistochemistry shows the presence of CCK in the larval brain. Our recent studies on eviscerated bodies and guts of developing Atlantic halibut larvae have shown that neural CCK in the CNS seems to be the quantitatively dominant form in these early stages.

Food deprivation experiments on herring larvae showed that CCK production in the larvae with yolk-sac were not influenced by one to three days of starvation. However, older larvae (30 days after hatch) with three days of starvation showed a dramatic decrease of the number of CCK-producing cells. Our studies failed to demonstrate a diurnal variation in the CCK content in these stages but there was a correlation of the larval CCK content and feeding status. These results indicate that CCK production is pre-programmed in the early larval stage but gradually influenced by luminal dietary factors in older larvae.

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DEVELOPMENT OF SKELETAL DEFORMITIES IN GILTHEAD SEA BREAM *(SPARUS AURATA)* REARED UNDER DIFFERENT LARVAL CULTURE AND DIETARY CONDITIONS

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Introduction

Fry quantity and quality are the main constraints for the further development and improved productivity of commercial hatcheries. Though standard procedures for larval rearing of some marine species such as gilthead sea bream and sea bass are well established, commercial hatcheries occasionally encounter sudden mortalities and several problems related with fry quality such as skeletal deformities. Hence, hatcheries are forced to discard abnormal sea bream sometimes amounting to 15-50% of production, varying with farm and country and causing significant economical impact. Osteological development of the gilthead sea bream has been described in detail, although only few studies describe the abnormalities from their origin at early stages to their full development and the related events that induce their occurrence.

Thus the aim of this study was to evaluate the contribution of early feeding and type of rearing system on the apparition of 5 different selected skeleton abnormalities common in intensive gilthead sea bream commercial hatcheries.

Material and methods

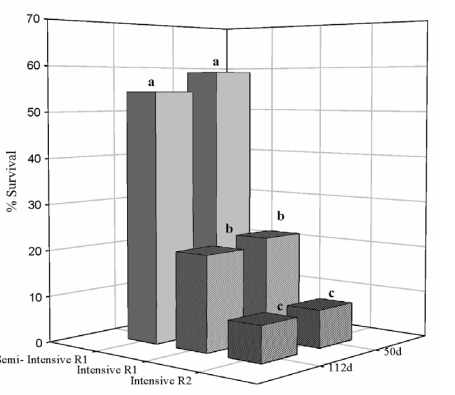
Two different rearing systems were used, based on tank capacity and larval density: a semi-intensive system formed by two cylindroconical 40-m^3 tanks for larval rearing stages, stocked with 7 eggs.l^{-1} and an intensive system formed by two cylindroconical 2-m^3 tanks stocked with 125 eggs.l^{-1}. For dietary treatment, four 2-m^3 cylindroconical fibre glass larval tanks were stocked with 125 eggs.l^{-1} in an intensive larval rearing system. Culture conditions were the same than in experiment I. Two commercial emulsions for enrichment of rotifers were tested: DHA Protein Selco, (Inve Aquaculture, Dendermonde, Belgium) (R_1) and Red Pepper Paste, (Bernaqua bvba, Turnhout, Belgium) (R_2).

For deformity characterisation, six hundred post-larvae (50dah) per treatment were sampled using anaesthetic overdose and the internal and external morphology were individually monitored under stereoscope. In older stages (95dah fry) samples from each treatment were radiographed with soft X-ray to check abnormalities presence. Observed abnormalities were recorded and classified.

Results

Semi-intensive R_1 system significantly improved survival obtained in the intensive one. Larvae fed R_1 rotifers enrichment significantly ($P<0.05$) improved survival when compared to R_2 fed larvae (Fig. 1).

Fig.1. Survival rates per treatment. Different letters on the same age means significant differences between treatments ($P < 0.05$).



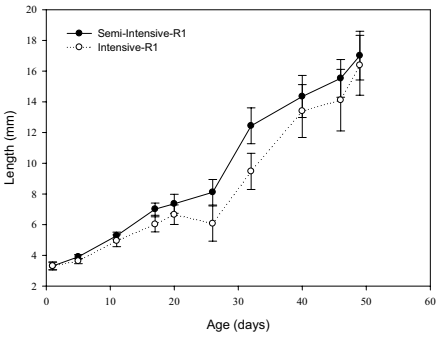


Fig. 2. Total length evolution at larval stages according with the rearing system ($P< 0.05$).

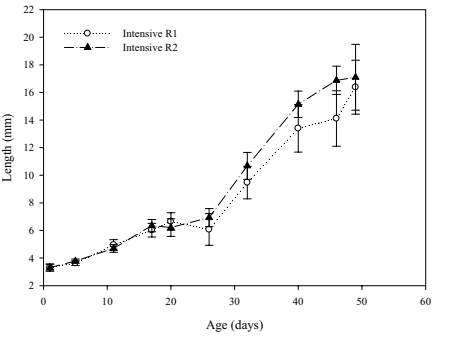


Fig. 3. Total length at larval stages according with the dietary treatment ($P< 0.05$).

Growth in terms of total length were significantly higher from 10 to 45dah in seabream reared in semi-intensive system (Fig. 2). From 20dah to the end of the larval period, larvae fed with R_2 showed significant improved growth in terms of total length (Fig. 3). Some differences were found between R_1 and R_2 rotifers in

fatty acid content, being docosahexaenoic acid (DHA, 22:5n-6) significantly higher in R_2 rotifers, whereas the level of eicosapentaenoic acid (EPA) and arachidonic acid (ARA) were not significantly different between dietary treatments. Of particular interests was the high level of DPA reaching 10 times higher in R_2 (Table I).

Table I. Proximal analysis and FA of enriched rotifers used. n=3 (mean± SD (P<0.05).

	Rotifers R_1	**Rotifers R_2**
% n-3 HUFA	22.6± 0.10[a]	27.5± 1.96 [b]
DHA	14.3± 0.66 [a]	19.7± 2.01 [b]
EPA	5.51± 0.52 [a]	5.04± 2.04 [a]
ARA	1.49± 0.35 [a]	3.9± 2.97 [a]
DPA n-6	0.99± 0.04 [a]	9.8± 5.05 [b]
DHA/EPA	2.6± 0.37 [a]	4.3± 2.16 [a]
DHA/DPA n-6	14.4± 0.08 [a]	2.3± 1.41 [b]

Rearing system did not affect total larvae fatty acid content except at 20dah, where DHA were significantly higher in Semi-intensive system. However, dietary treatment clearly affected total larvae fatty acid content, DHA and DPA level were significantly higher in larvae fed with R_2 reflecting the enrichment products fatty acid (FA) profile (Fig. 4).

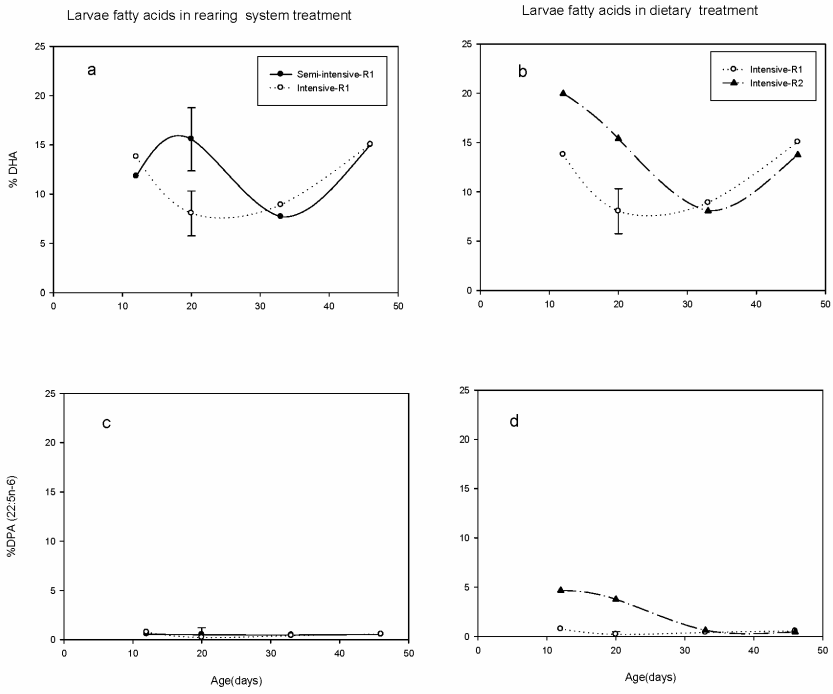


Fig. 4. Results of the analysis of some fatty acids in larvae from system and dietary treatment expressed in area % of total lipids.

After 50dah, log-linear statistical analysis showed that abnormalities frequency depend on the rearing system (χ^2: 78.64; P>0.05). Larvae reared in the intensive system and fed with different diets, showed independence of the abnormalities recorded from the diet used at this stage.

After 95dah another morpho-anatomical characterization was done. There was a slight increase in the deformities in comparison to the 50dah analysis, possible due to the clearer appearance of the deformities. The osteological malformation incidence significantly increased (P<0.05) (χ^2: 239; P>0.05) in the intensive system at this age.

On the other hand, dietary treatment also affected the incidence of opercular deformity and slight lordosis being significantly higher in larvae fed R_1-enriched rotifers (Table II).

Table II. Visual and soft X-ray morpho-anatomical characterization at 50 and 95dah, respectively. Different letters in the same column within the same age denote significant differences (P<0.05).

	Quality Evaluations	Op	Jaw	Fu	Lo-	Lo+	LSK
50d	Semi-Intensive R_1	0.2 ^{a}	0.7 ^{a}	-	-	0.2 ^{a}	0.2 ^{a}
	Intensive R_1	6.2 ^{b}	1.1 ^{ab}	-	-	0.3 ^{ab}	0.2 ^{ab}
	Intensive R_2	5.1 ^{b}	2.3 ^{b}	-	-	1.2 ^{b}	0.7 ^{b}
95d	Semi-Intensive R_1	1.5 ^{a}	1.1 ^{a}	2.4 ^{a}	3.5 ^{a}	1.4 ^{a}	0.0 ^{a}
	Intensive R_1	10 ^{b}	4.2 ^{b}	2.7 ^{ab}	13.9 ^{b}	5.9 ^{b}	0.7 ^{b}
	Intensive R_2	4.2 ^{c}	2.0 ^{b}	4.5 ^{b}	6.5 ^{c}	5.1 ^{b}	1.1 ^{b}

Conclusions

1. Intensive rearing conditions could be acting as chronic stressors, resulting in a stress related mortality and poor growth in this type of system.
2. High amounts of DPA (22:5n-6) in rotifers enrichments seems to have negative effect over larval survival although no effect was found over larval quality.
3. The relationship between deformities and rearing system is mainly due to significantly lower different rates in opercular abnormalities and lordosis in semi-intensive system.
4. The level of DHA in the rotifers phase is suggested as a key factor to diminish the opercular deformities in *Sparus aurata* larval rearing.

Acknowledgements

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COD JUVENILE PRODUCTION – STATUS ON R&D AND COMMERCIAL DEVELOPMENTS

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Atlantic cod (*Gadus morhua*) has been a new candidate for aquaculture for some decades in countries around the North Atlantic Ocean, but until recently commercial activities have been rather absent. Greatest interest in developing a cod farming industry is found in Norway, Canada, and the United Kingdom as well as in Iceland and the USA (Northeast). An established first-feeding method for cod based on cultivated plankton generally includes a sequence with rotifers and algae followed by *Artemia* and co-feeding a dry feed. However, it took more than ten years of dedicated research and development before these efforts now are being translated into up-scaled hatchery protocols.

Cod larvae have a very high growth potential (> 10%·day^{-1}), are aggressive feeders, and can be cannibalistic if food availability is suboptimal. Research has addressed cod larval nutrition in both qualitative and quantitative requirements. Great efforts have been made to establish good protocols for cultivation and enrichment of live feed, which will support the nutritional needs of cod larvae. Also, development of feeds and feeding strategies that would allow early weaning onto inert diets thus reducing the dependency on live feeds has been a primary objective in cod juvenile production research.

Huge variation in survival and a high incidence of deformities have been reported in hatchery-reared cod juveniles. These observations have been ascribed partly to unfavourable environmental conditions. Research has included studies on water quality in broad terms, including amongst others temperature, water replacement, water current, gas saturation, and microbiology, as well as light conditions and general tank management.

This paper will summarise R&D relevant for scaling up the production processes, making cod juvenile production more reproducible with respect to survival and quality. Finally, commercial developments will be presented.

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FEEDING REGIME BASED ON THE SIZE SELECTIVITY OF LARVAE AND FLOW FIELD CONTROL IN REARING TANKS IMPROVES QUALITY OF REARED LARVAE: CASE STUDIES WITH THE SEVEN-BAND GROUPER *EPINEPHELUS SEPTEMFASCIATUS*

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In hatchery production of marine fish, early mortality is one of the biggest concerns. In order to improve the early mortality, many attempts have been done to control the biotic and abiotic environment. Among these environmental factors, we have been focusing on the two environmental factors which have not been investigated but are important for larval rearing. One factor is the preference of larvae feeding on live feeds. Although feeding preference of marine fish larvae have been documented, they have not examined whether the feeding regime based on the larval feeding preference may affect the larval quality. The other factor is the flow field in the rearing tank. Few studies have been conducted on the flow field in the rearing tank, although the flow in a rearing tank is assumed to have the great impact on marine fish larvae. This paper reviews recent findings on establishing a feeding regime based on size selectivity of feeding larvae and flow field control for larval rearing using a hydrodynamics approach in seven-band grouper (*Epinephelus septemfasciatus*). This species was chosen because grouper larvae are known to be difficult for initiating first-feeding and are highly sensitive to physical stress, although it is expected to be the new target species of aquaculture and stock enhancement in Japan.

Three morphotypes from the euryhaline rotifer *Brachionus plicatilis* complex were used in the experiment. The L-type (lorica length 119-241μm), S-type (93-179μm), and SS-type (90-159μm) were fed to the grouper larvae from first-feeding (day 4) until day 20. Food selection among different sizes of rotifers (for 20-μm size classes) was calculated using Chesson's α-index. Larvae ranging from 2.1-3.0mm SL (standard length) preferred SS-type and S-type rotifers

(100-160μm), while larvae ranging from 3.0-4.0mm SL preferred rotifers 120-180μm, and larvae >4.0mm SL preferred rotifers >160μm. Based on this result, we investigated the effect of different feeding regimes on survival, growth, and quality of larvae. Three feeding treatments (SS, L, and SS-L) were prepared. SS tanks were fed SS-type rotifers solely from the first day of feeding until day 20. L tanks were fed L-type rotifer only throughout the experiment, while SS-L tanks were fed SS-type rotifer from day 4 until day 10 (<3.0mm SL), and then fed L-type rotifer from days 11 to 20. After the rearing trial, the survival, growth, and quality of the larvae was evaluated. Larvae from each tank were transferred to another small tank, and survival one hour after transfer was determined. Survival of larvae on day 20 was 24.2%, 7.2%, and 33.6% in SS, L, and SS-L tanks, respectively. SL of larvae on day 20 was as follows: 4.1mm (SS tank), 3.8mm (L tank), and 4.3mm (SS-L tank). Survival one hour after transfer of larvae in the SS-L tank was higher than other feeding treatments. Small-sized rotifers (SS-type, S-type) are necessary during the first-feeding (<3.0mm) while L-type rotifer is essential for larvae bigger than 3.0mm SL. These results also demonstrated that survival, growth, feeding incidence, and quality of larvae can be improved when fish were fed with the feeding regime based on feeding selectivity.

We attempted a series of experiments in which stationary flow was measured in $1m^3$ polyethylene rearing tanks with four aeration rates (1000, 200, $50ml \cdot min^{-1}$, and no aeration). A spherical aerator was set at the bottom center of the tank to generate tank flow. The effects of aeration on the survival of seven-band grouper larvae were examined. Aeration at $200ml \cdot min^{-1}$ produced the highest survival and growth for grouper larvae. Measurements of stationary flow in a rearing tank identical to those used in rearing experiments were made. The results indicated that the stationary flow in the rearing tank was vertical and the horizontal circulation was typical. However, a considerable amount of time was required to measure the flow in the rearing tank. In addition, since the optimum stationary flow varies for fish species and developmental stages, the measurement of the flow for each kind of larvae is impractical in terms of time and cost. Therefore, we developed a computation model for estimating the flow field in the tank. The numerical computational method is a finite differential scheme of the MAC (Marker and Cell) type. The calculation method for estimating the stationary flow field in the rearing tank was simplified. The flow in a rearing tank was calculated two-dimensionally based on experimental results of the flow measurements. The simplified method of calculation was satisfactory for determining the stationary flow and velocity in the rearing tank; the method compared favourably to the results obtained in the experiments. We expanded this approach to the mass-culture scale ($100m^3$) and quantified the optimal flow field, resulting in three times higher survival than the former rearing methods. The results from these studies may be very useful for estimating the stationary flow in a rearing tank and for designing suitable tanks for rearing larvae.

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INTERACTIONS BETWEEN BACTERIA AND SUSPENDED PARTICLES IN INTENSIVE LARVAL CULTURE: A COMPARISON BETWEEN MICROALGAE, ALGAL CONCENTRATE AND CLAY

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Introduction

Enhanced growth and survival of marine fish larvae by use of the green-water technique has mainly been attributed to nutritional effects (Naas et al., 1992; Reitan et al., 1993). Suspended particles in the water may also have a significant impact on the microbial environment (Lind et al., 1997), and therefore be an important factor in the establishment of a primary microflora of larvae. We present a comparison of the effect of live microalgae, algal concentrate, and clay on the bacterial community of the water.

Materials and methods

Five tanks (70 l) were filled with sandfiltered water and kept stagnant with aeration at 12°C for 24h. The water exchange rate was thereafter set at $2\times.d^{-1}$. Three tanks were added either microalgae (*I. galbana*, semi-continous culture, 2mg C. l^{-1}), algal paste (*N. oculata*, Instant Algae, 2mg $C.l^{-1}$), or clay (kaolite, 100mg. l^{-1}). After 30min, all except one tank (negative control) were added 25µg yeast $extract.ml^{-1}$ to simulate the increased substrate levels that are characteristic at several phases during first feeding (e.g., hatching, feeding).

Samples for analysis of total cfu (Marine agar, 15ºC, 14d incubation), fraction of opportunistic bacteria (fast-growers; Salvesen and Vadstein, 2000), *Vibrio* spp. (TCBS agar, 15ºC, 4d incubation), and particle size distribution were taken before and 2, 6, and 10h after substrate addition. Samples for particle analysis (50ml) were filtered (200µm), fixed with acid Lugol solution, and stored at 12ºC in the dark until analysis (4 weeks). Samples were analyzed in a CASY 1 Cell

Counter and analyzer System, model TTC with a 150-µm capillary. The lower detection limit was 2% of the capillary diameter (3µm).

Results and discussion

There was an immediate response in the bacterial community of the relatively low temperature water to the supply of nutrients (Fig. 1). Clear differences between the different water qualities were observed as early as 2h after addition. In nutrient enriched water without addition of particles (= clear water), bacterial levels increased from 3.7×10^3 to 1.3×10^5 cfu.ml^{-1} and continued to increase throughout the experimental period up to a final level of 9.8×10^5 cfu.ml^{-1} after 10h. The increase was less pronounced in all 'green-water' qualities (algae, clay, and paste) which had 1-6× lower bacterial numbers and an average final level of 3.1×10^5 (±0.8) cfu.ml^{-1}.

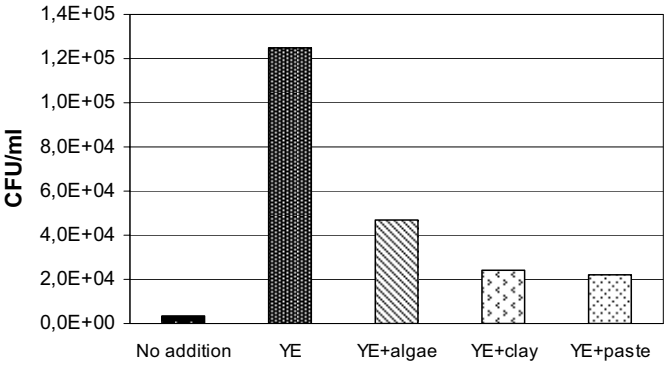

Fig. 1. Bacterial levels 2h after yeast extract (YE) was added to the tanks to stimulate bacterial growth.

The up-shift in nutrients stimulated blooms of opportunistic bacteria in all water qualities, but the use of 'green-water' techniques seemed to slightly delay their proliferation (Fig. 2). Whereas opportunistic bacteria constituted 95% in nutrient enriched clear water 2h after the up-shift, a slightly lower level of 79-87% was observed in 'green water' qualities. In clear water without nutrient addition, the fraction of opportunistic bacteria decreased steadily and levelled off at less than 20%. The *Vibrio* spp. fraction remained at a low level of less than 3% of total cfu in all water qualities, except for the two clear water qualities which both had a peak of 11% between 2-6h after the up-shift (data not shown).

'Green-water' qualities added live microalgae or algal paste had initially 8.2-8.9×10^4 particles.ml^{-1} in the size range 3-20µm (data not shown). Numbers were 3× lower in the clay tank, but total particle numbers are probably underestimated for this water quality because no estimates of particle sizes below 3µm are avail-

able. Whereas the particle level only decreased to a slight extent in 'green-water' qualities with microalgae or clay, algal paste particles sedimented relatively rapidly out of the water column, and after 2h numbers were reduced to 1.8×10^4 particles.ml^{-1}.

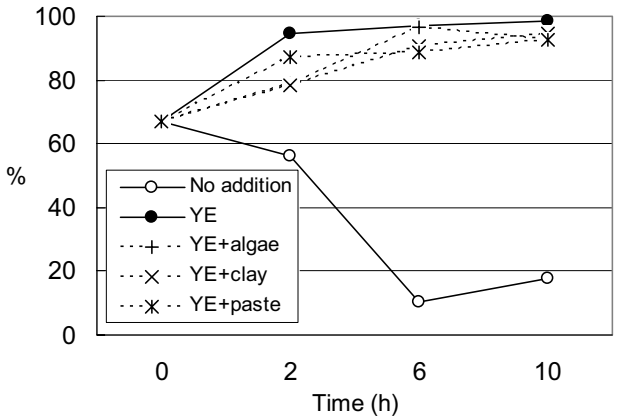

Fig. 2. Changes in the percentage opportunistic bacteria of total CFU in the various bacterial communities.

Conclusions

The three 'green-water' techniques used in this study include inorganic and organic, live and dead particles of different sizes, but all techniques seemed to have a similar effect on the bacterial community of the water, i.e., to reduce bacterial proliferation after nutrient up-shifts. Further studies will reveal if this effect is caused by aggregate formation of the suspended particles with DOC and bacteria that precipitate and/or remain in the water column.

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RELATIONSHIP BETWEEN PERFORMANCE AND LIPIDS IN EGGS FROM FARMED AND WILD ATLANTIC COD (*GADUS MORHUA*) BROODSTOCK

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Introduction

The hypothesis investigated was that potential quality and performance differences between eggs obtained from different cod broodstock would be reflected in differences in lipid and fatty acid composition of the eggs. The study compared total lipid content, lipid class composition, and fatty acid composition of total lipid, as well as lipid-soluble pigment (carotenoid) content in eggs obtained from wild and farmed broodstock populations. These measurements were related to basic performance parameters such as fertilisation rate, symmetry of cell division, and survival at hatching.

Materials and methods

Eggs were obtained from cod broodstock at a commercial hatchery on the west coast of Scotland. Three groups of broodstock, termed farmed (F), wild/fed (WF), and wild (W) were used. The farmed broodstock (F) were hatched in spring 2002 and entirely tank-reared. The wild/fed broodstock (WF) were captured in the Clyde estuary between September 2003 and February 2004 and were fed the same formulated diet as for the farmed broodstock from capture until spawning. At the beginning of April 2004, a third group of cod were obtained from the wild and spawned naturally over a period of two days before spawning ceased. These fish constituted the wild broodstock (W) group and were not fed prior to the cessation of spawning.

Eggs were spawned spontaneously and fertilised in the tank. The percentage of fertilisation was estimated by counting random samples of at least 300 eggs as 3 batches of around 100 eggs per sample point. The symmetry of the first cell divisions was also estimated in these samples. A numerical score ranging from 1 (almost all eggs showing asymmetry) to 5 (no asymmetry) was allocated to each batch of eggs to reduce the degree of subjectivity in the estimation. Survival at

hatching was determined in specific batches. Total lipid was extracted from eggs by homogenising in ice-cold chloroform/methanol (2:1, v/v). Separation of lipid classes was performed by high-performance thin-layer chromatography with quantification by densitometry. Fatty acid methyl esters of total lipid were prepared, purified, separated, identified, and quantified by gas-liquid chromatography. Extraction, separation, and quantification of astaxanthin were performed by HPLC. Differences between broodstocks were analysed by ANOVA with Tukey's post-test using GraphPad Prism (GraphPad Software, San Diego). Differences between mean values were regarded as significant when $P < 0.05$.

Results and discussion

Eggs from farmed broodstock showed significantly lower fertilisation rates and cell symmetry than eggs from wild broodstock (Fig. 1). Survival to hatching rate was also lower in eggs from farmed broodstock compared to wild/fed broodstock although the difference failed to achieve statistical significance due to high inter-batch variation. Quarantine controls prevented batches of eggs from the wild broodstock being incubated.

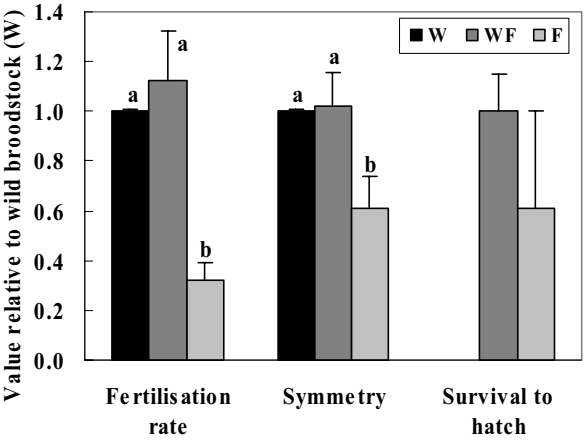

Fig. 1. Fertilisation rate, cell symmetry score, and survival to hatching of eggs from wild (W), wild/fed (WF), and farmed (F) cod broodstock. Results are means ± SD (n = 3 and 7 for W/WF and F, respectively) and are presented relative to the wild broodstock. Different letters indicate significant ($P<0.05$) differences.

There were no differences in total lipid content or the proportions of the major lipid classes between eggs from the different broodstocks (data not shown). However, the eggs showed a gradation in the content of the quantitatively minor phospholipid, phosphatidylinositol, with the level being highest in eggs from W broodstock and lowest in eggs from farmed broodstock, with eggs from the WF broodstock showing an intermediate content. Eggs from the F broodstock were

also characterised by having significantly lower levels of the carotenoid pigment astaxanthin than eggs from WF broodstock with the level in eggs from wild fish being rather variable between batches (Fig. 2).

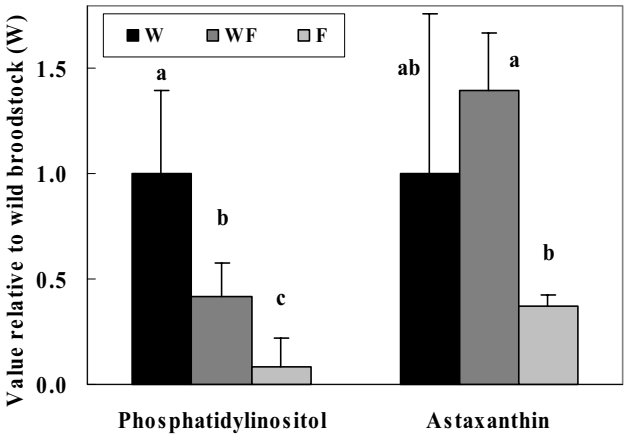

Fig. 2. Content of phosphatidylinositol and astaxanthin in eggs from wild (W), wild/fed (WF) and farmed (F) cod broodstock. Results are means ± SD (n = 3, 11, and 7 for W, WF and F, respectively) and are presented relative to the wild broodstock. Different letters indicate statistically significant (P<0.05) differences.

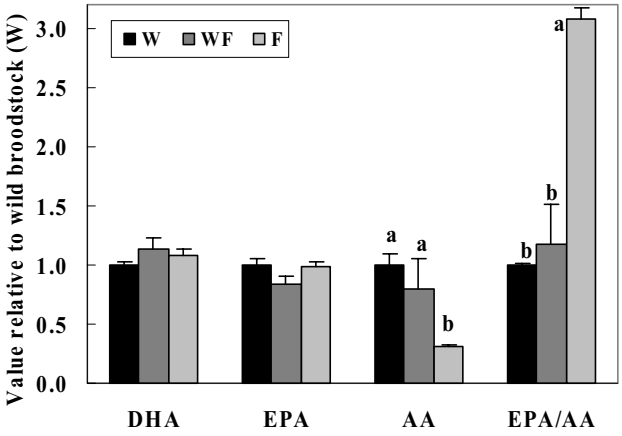

Fig. 3. Contents of EPA, DHA, AA, and the EPA/AA ratio in eggs from wild (W), wild/fed (WF), and farmed (F) cod broodstock. Results are means ± SD (n = 3, 11, and 7 for W, WF, and F, respectively) and are presented relative to the wild broodstock. Different letters indicate significant (P<0.05) differences.

There were no differences between eggs from farmed and wild broodstocks in the proportions of saturated, monounsaturated and total polyunsaturated fatty

acids (not shown). The eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) contents were also similar (Fig. 3). However, eggs from the farmed broodstock had significantly lower levels of arachidonic acid (AA; 20:4n-6), and consequently significantly higher EPA/AA ratios than eggs from wild broodstock (Fig. 3).

Conclusions

The eggs that displayed the best performance with the highest fertilisation rate, best symmetry and highest survival at hatching and, hence, could be regarded as of the higher quality, were characterised by higher levels of AA and phosphatidylinositol, the predominant AA-containing lipid class, and carotenoid pigment. Perhaps surprisingly, and in contrast to the EPA/AA ratio, the DHA/EPA ratio was not associated with egg quality or performance. Therefore, in future studies it will be important to establish the optimum ratio of DHA/EPA/AA in cod eggs. However, the results of the present study suggests that it may not be simply the overall level of AA or the EPA/AA ratio that is crucial, but possibly the level of AA in phosphatidylinositol that is an important determinant of egg quality in cod. In the light of studies (Bell et al., 1997; Pickova et al., 1997; Mazorra et al., 2003), including the present one, showing an important association between AA content and egg quality and performance in marine fish, consideration of AA supplementation to broodstock diets may be required. The inclusion of phospholipid-rich products may also be prudent (Bell et al., 1997).

Acknowledgements

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GENETICALLY-BASED RESISTANCE TO SUMMER MORTALITY IN THE PACIFIC OYSTER (*CRASSOSTREA GIGAS)* AND INITIAL RELATED PHYSIOLOGICAL CHARACTERISTICS

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Pacific oyster summer mortality is a major problem in several countries. It has been reported globally for many years but no specific pathogen was systematically associated to this phenomena. A complex combination of environmental and biological parameters has been hypothesized. A national program "Morest" was initiated in France in 2001 to document this question by a multidisciplinary approach (genetics, physiology, immunology, ecology, ecotoxicology, and pathology). The project is based on field observations in the three main oyster production areas and in laboratory studies to identify the relative importance of the different parameters involved in summer mortality outbreak. Wild spat were compared to hatchery-produced spat (43 full sib families) in different growout sites and experimental controlled conditions.

Results showed that temperatures >19°C caused a Summer Mortality Risk (SMR). At that temperature oysters had a reproductive effort and SMR appeared correlated to increasing trophic conditions. Glycogen or ATP storage decreased when trophic condition and reproductive effort increased and led to a negative scope for growth. In these experimental conditions, a stress was necessary to induce mortalities, and challenges with vibrios previously isolated from moribund oysters demonstrated a higher susceptibility of highly fed oysters compared to others.

However, when the hatchery spat produced in first generation (G1) were tested in the field, 45% of the observed variance in mortality was due to variation among families. A second generation was therefore produced in 2002 by breed-

ing within the best and the worst G1 families. Results confirmed the high heritability of survival for juveniles <1-yr-old. The high-selected line ('R'; resistant progenies) had a higher survival than the low-selected line ('S'; susceptible oysters). Interestingly, no correlation was observed between growth and survival. Such results were corroborated in the third generation and allowed conclusion that a family-based selective program could be engaged for summer mortality resistance in the field at spat stage.

When compared each other, S oysters stayed a longer time without spawning and had a higher mortality rate than R oysters which had a synchronic major spawning, suggesting a different spawning strategy. In experimental high trophic conditions, S oysters had a higher investment in reproduction compared to R ones, but showed partial spawning. However in low trophic condition, their reproductive effort and spawning date were identical. No significant differences between the 2 selected lines were observed either for the glycogen and ATP utilisations during gametogenesis or scope for growth parameters. During the critical period, expression of genes coding for glucose metabolism enzymes (Hexokinase, GS, PGM, PEPCK) were significantly lower for S oysters than R ones, suggesting differences in capability for glucose mobilisation. Similarly haemocyte parameters such as phagocytosis or granulocyte concentration were significantly lower in S progeny than in R ones. Again, hypoxia stress combined with pesticides led to a drastic decrease of phagocytic rate for S oysters compared to R ones. On the contrary, haemocytes from S oysters had a significant higher reactive oxygen intermediate (ROI) production capacity than R ones the previous month of the summer mortality event.

To compare R and S oysters in a more integrative way, a suppression subtractive hybridisation (SSH) library was produced between these oysters during a mortality event. Twelve percent of clones matching with known genes were implicated in energy generation and 16% in immune functions.

An overview of these first results would suggest that difference in reproductive strategy is a key point of the process. The partial spawning strategy for the S oysters could possibly result from high germinal investment at high trophic level compared to R ones. During partial spawning, pathogen transmission is favoured and that could explain the over-expression of ROI production observed in S oysters before a mortality event. First molecular results showed that response of S oysters seemed to be limited during the critical period. This could be either a secondary effect of the infection process of S progeny or a difference in glucose management capability between the two selected lines. A micro-array strategy is developed to better identify the limited number of genes involved in this resistance phenomena as suggested by the observed high heritability of spat survival.

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THE INFLUENCE OF SALINITY, TEMPERATURE, AND DENSITY ON THE MORTALITY OF ADULT *ARTEMIA* DURING TRANSPORTATION

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Introduction

Culture of *Artemia* has successfully operated in the middle gulf of Thailand, mostly in earthen ponds previously used for solar salt production (Tunsutapanich, 1979). Production of *Artemia* biomass is transported throughout the country and supply various aquaculture activities such as ornamental fish, shrimp, and fish hatchery. Imitative transportation of adult *Artemia* in plastic bags was done to determine the optimal salinity, temperature, and maximum density of adult *Artemia* that can be transported during a certain time.

Materials and methods

A. franciscana SFB strain cultured in earthen ponds (29-30°C and 90ppt seawater) were transported in plastic bags (37.5×55cm) and packed in Styrofoam boxes (40×55×25cm). Each bag was equipped with collecting pipe (2cm diameter) for sampling and counting (Fig. 1). Adult *Artemia* are packed in bags and each bag was filled with 3.5 l before filling with pure oxygen. All bags were shaken every 30min to imitate real transportation. Mortality was counted by shaking the *Artemia*-seawater mix before taking a 10-ml sample through the pipe. Non-moving *Artemia* was determined dead and included in mortality. The experiment was done in 3 replicates and divided into 2 parts: 1) salinity and density and 2) temperature and density. Mortality was normalized by arcsine-transformation and analyzed by multiple comparisons, using SPSS v.10 for Windows.

For Part 1 (test of salinity and density), *Artemia* were packed in seawater in plastic bags at 29-30°C with 3 different levels of salinity (20, 50, and 80ppt) and 3 different densities (85.7, 142.9, and 200g.l^{-1}). Mortality was checked after 0, 1, 2, 3, 4, 5, 6, and 7h of the experiment.

For Part 2 (test of temperature and density), *Artemia* were packed in 30ppt seawater with varied different temperatures (4, 15, and 30°C) and densities (85.7, 142.9, and 200g.l^{-1}). Temperature was checked every 15min and adjusted by putting ice cubes and tap water in the boxes. Mortality was check after 0, 2, 5, and 7h of the experiment.

Fig. 1. Plastic bags and collecting pipe used in the experiment.

Results and discussion

In Part 1, after 7h transport, the results showed the highest mortality (77.00%) of *Artemia* at 80ppt and 200g.l^{-1}, whereas the lowest mortality (3.67%) was found at 50ppt and 142.9g.l^{-1}. Mortality after 1, 2, and 3h were significantly different whereas no difference was found after 2, 3, and 4h (Table I). Transporting *Artemia* at high density and salinity causes high mortality especially during 5-7h.

Table I. Mortality of *Artemia* during 7h transport in 3 different salinity and density.

Density (g.l^{-1})	Salinity (ppt)	Mortality at different hour								Average Mortality (%)
		0	1	2	3	4	5	6	7	
85.7	20	3.33	4.67	12.67	8.00	4.67	14.67	4.33	7.67	5.9±4.25^{a}
	50	3.67	3.67	5.00	3.00	7.67	5.67	7.67	7.00	
	80	0.67	1.00	3.67	3.67	3.00	9.67	8.67	8.67	
142.9	20	0.00	2.00	1.33	2.00	5.33	8.00	4.67	11.00	4.4±4.11^{b}
	50	0.33	1.33	2.00	4.00	3.67	4.67	7.00	3.67	
	80	0.33	1.67	5.00	1.33	4.67	8.67	7.67	15.33	
200	20	2.67	5.33	9.67	7.00	5.33	1.33	13.67	7.67	12.1±17.53^{c}
	50	2.00	1.33	7.33	6.67	4.67	3.67	18.00	33.33	
	80	2.67	2.67	8.00	5.67	6.67	16.00	41.33	77.00	
Mean (%)		1.7± 2.09^{a}	2.6± 1.94^{b}	6.1± 4.44^{c}	4.6± 2.98^{c}	5.1± 2.70^{c}	8.0± 5.06^{d}	12.56± 2.73^{e}	19.04± 23.53^{f}	

Means and standard deviation with different superscripts are significantly different ($p<0.05$).

In Part 2, after 7h, the lowest mortality (2.00%) was found in *Artemia* transported at 15°C (85.7g.l^{-1}) and the highest mortality 67.00% found in temperature 30°C (200g.l^{-1}).

Table II. Mortality of *Artemia* during 7h transport in 3 different temperature and density.

Temp (°C)	Density (g.l^{-1})	Mortality at different hour			Average mortality (%)
		2	5	7	
4	85.7	0.7±1.15	1.7±1.15	3.3±3.51	4.0±4.70[a]
	142.9	0.7±1.15	6.7±6.51	3.0±2.00	
	200	2.0±2.65	6.7±5.69	11.3±5.78	
15	85.7	0.3±0.58	2.3±2.52	0.7±1.15	4.9±5.86[a]
	142.9	1.0±1.73	4.3±1.53	5.7±3.51	
	200	2.0±1.73	12.3±10.07	14.0±4.00	
30	85.7	3.3±2.31	13.7±4.73	8.0±6.56	23.0±26.69
	142.9	4.0±1.00	10.0±7.00	52.0±13.11	
	200	2.0±1.73	47.0±28.51	67.0±24.88	
Average mortality (%)		1.8±1.85[a]	11.6±16.5[b]	18.5±24.43[b]	

Means and standard deviation with different superscripts are significantly different ($p<0.05$).

There is no significant difference of mortality of *Artemia* when transported at 4 and 15°C (4.0±4.7 and 4.9±5.86, respectively) while mortality of *Artemia* after transport at 30°C (23.0±26.69) was significantly higher.

Mortality after 5 and 7h transport was not different but significantly higher than transport after 2h (Table II).

Conclusions

The results revealed the influence of salinity, temperature, and transport time on the mortality rate of *Artemia*. Care should be taken when transporting adult *Artemia* at high salinity and density longer than 4h. Lowering temperature from 30°C to 4°C (200g.l^{-1}, 7h) significantly increased (up to 55%) survival during transport. Due to the small difference in mortalities at 4°C and 15°C, it's recommended to transport adult *Artemia* at 15°C rather than 4°C because it's simpler to control and less expensive at 15°C.

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DO PROTEIN HYDROLYSATES IMPROVE SURVIVAL AND GROWTH OF NEWLY HATCHED SPOTTED WOLFFISH, A NON-METAMORPHIC SPECIES?

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Introduction

Spotted wolffish (*Anarhichas minor*) is an aquaculture species extremely well suited for cultivation under cold northern climates such as those found in the coastal areas of Québec, Canada (Le François et al., 2002). At hatch, wolffish are at a relatively advanced stage: they have a twisted and differentiated digestive tract contrary to other aquaculture coldwater marine fish species (cod, halibut, haddock; Falk-Petersen and Hansen, 2001). Despite larval robustness, this species displays highly variable survival at first-feeding that could be attributable to diet, egg quality, or digestive capacities (Falk-Petersen et al., 1999).

If digestive capacities are a limiting factor at first-feeding for this species, providing the young fish a more digestible diet should improve both growth and survival performances. Protein hydrolysates (pre-digested proteins; PH) are known to enhance larval performances of several fish species based on the assumption that an "immature" digestive systems limits nutrient absorption (Hardy, 2000). We propose to investigate the use of three levels of PH to assess the relevance of their addition in the feed of post-hatched spotted wolffish, a non-metamorphic species. In an attempt to generate sub-optimal conditions and increase the potential beneficial effect of using PH, growth rate and survival response was modulated by using different rearing temperatures: 4, 8, and 12ºC. Fish should display impaired growth at 4ºC and a growth boost and higher mortality at 12ºC, compared to 8ºC which is considered the optimal rearing temperature for newly-hatched spotted wolffish.

Materials and methods

The study was carried out at the facilities of the Centre Aquacole Marin (Québec, Canada). Fish were reared at three different temperatures (4, 8, and 12°C) and given three different diets for each temperature in triplicates. Fish were fed in excess every hour (8h00-17h00). The compound diets were formulated and processed at Ifremer, Centre de Brest (France) (Table I). The three experimental diets were isoproteinic, isolipidic, and isoenergetic. They differed only in the molecular form of a part of the protein fraction, control diet containing only native protein, diet H10 and H20 containing 10 and 20% of hydrolysated protein. Fish were sampled and measured regularly over 60d.

Table I. Composition of the experimental feeds.

Ingredients[1] (in %)	Control	H10	H20
Fish meal	74	64	54
Protein hydrolysates[2]	-	10	20
Precooked potato starch	5	5	5
Cod liver oil	3	3	3
Soy lecithin	5	5	5
Vitamin Mixture[3]	8	8	8
Mineral Mixture[4]	4	4	4
Betaine	1	1	1
Astaxanthin	-	55mg.kg^{-1}	-

[1] Fish meal and cod liver oil: from *La Lorientaise* (Lorient, France); soy lecithin: *Ets Louis François* (St Maur des Fossés, France); precooked potato starch (Nutralys): *Roquette* (Lille, France); Astapep: ABK Gaspésie (Matane, QC), Astaxanthin: DSM Nutritional Products (Heerlen, Netherlands).

[2] Asta-Pep, protein hydrolysates from shrimp wastes, total carotenoids 550±10µg.g^{-1}, Proteins 65.8±1.5% dry product, Minerals 6.9±0.2% dry product, Total lipids 20.4±1.5% dry product.

[3] Per kg of vitamin mix: retinyl acetate 1g; cholecalciferol 2.5mg; all-*rac*- α-tocopherol acetate 10g; menadione 1g; thiamin 1g; riboflavine 0.4g; D- calcium pantothenate 2g; pyridoxine HCl 0.3g; cyanocobalamin 1g; niacin 1g; choline chloride 200g; ascorbic acid 20g; folic acid 0.1g; biotine 1g; meso-inositol 30g.

[4] Per kg of mineral mix: KCl 90g; KI 40mg; $CaHPO_4 \cdot 2H_2O$ 500g; NaCl 40g; $CuSO_4 \cdot 5H_2O$ 3g; $ZnSO_4 \cdot 7H_2O$ 4g; $CoSO_4 \cdot 7H_2O$ 20mg; $FeSO_4 \cdot 7H_2O$ 20g; $MnSO_4 \cdot H_2O$ 3g; $CaCO_3$ 215g; $MgSO_4 \cdot 7H_2O$ 124g; NaF 1g.

Results and discussion

Preliminary results indicate that PH additions do not appear to have any effect on growth (Fig. 1) or survival at any temperature ($p>0.05$). However, high variability of the data precludes us from confirming the absence or presence of any effect of PH on wolffish at first-feeding.

SGR increased with increasing temperatures ($p=0.001$; Fig. 2). Growth rates of 1.75, 4.29, and 4.85%.d^{-1} were achieved at 4, 8, and 12°C, respectively (similar to those reported by Hansen and Falk-Petersen, 2001; 2002).

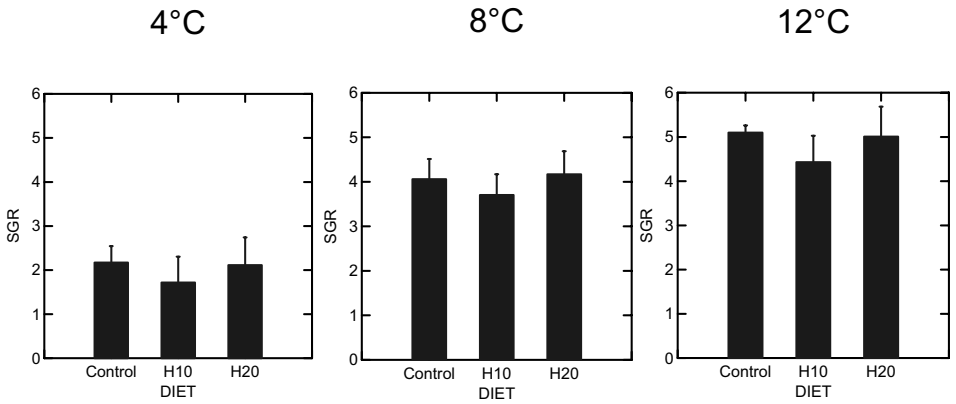
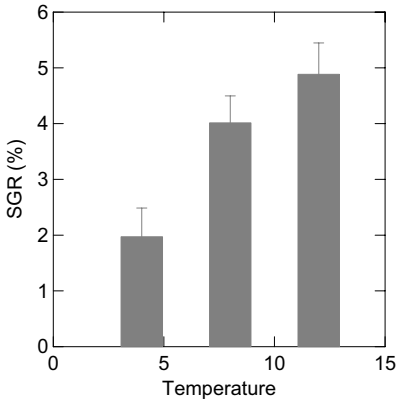


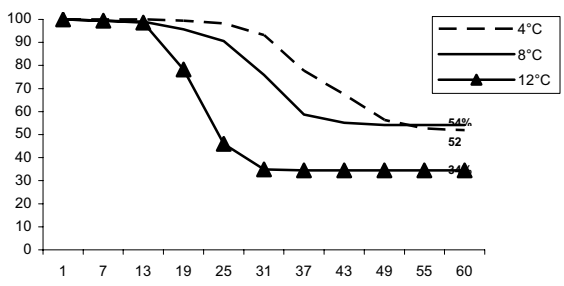
Fig. 1. Specific growth rate of the spotted wolfish from d0 to d60 according to temperature and diet.

Fig. 2. Specific growth rate (SGR) of the wolffish according to temperature.



Survival after 60d (52, 54, and 34% at 4, 8, and 12°C, respectively) was significantly lower at 12°C ($p<0.001$; Fig. 3).

Fig. 3. Survival of the wolfish at different temperatures.



Many species begin exogenous feeding before the onset of fully functional digestive system. Digestion of intact proteins requires the presence of several proteolitic enzymes, synthesized by pancreas, stomach and intestine, which appear

some weeks after hatching in some species (Hardy, 2000). PH added in moderate proportion to the feed enhanced survival and growth of many species at the early stages but have no effects or even depress growth later on in development (Cahu and Zambonino-Infante, 2001). Wolffish hatch with the morphology of a juvenile rather than that of a larvae. Interestingly, Lamarre et al. (2004) suggested that trypsin activity levels at hatch could be used as an indicator of growth and survival in a closely related species (*Anarhichas lupus*). We suggest that precocious ontogeny of the digestive system of wolffish could preclude any significant effect of using PH compared to metamorphic species.

Conclusion

The absence of any significant effect on growth rate and survival following the addition of PH at any temperature is an indication that digestive capacities are not the factor limiting the performances of newly hatched spotted wolfish.

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EARLY GROWTH AND DEVELOPMENT OF THE PACIFIC BLUEFIN TUNA, *THUNNUS ORIENTALIS*

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Introduction

Early growth and development of the Pacific bluefin tuna (PBT), *Thunnus orientalis*, were described morphologically, physiologically, and behaviourally. From these results, the PBT early life strategy and the fingering production technology development adapted to their unique biological traits were discussed.

Materials and methods

Fertilized eggs were obtained from captive broodstock. Larvae and juveniles were fed rotifers, *Artemia* nauplii, live fish larvae, and minced fish meat as they grew.

Results

Growth was rapid and average body length (BL) was 35.74mm at 30 days post-hatch. Growth was especially accelerated from postflexion stage. Notochord flexion occurred between 6-8mm BL. The adult complement of fin ray counts was attained at 10mm BL, when the juvenile melanophore pattern was attained. Relative growth of many body parts showed a phase change from late postflexion to early juvenile stage.

Newly hatched larvae still have mucous cells in their epidermis and fin membranes, and they increased in number with age. During flexion stage, functional jaw teeth were found and blind sac, gastric glands, and pyloric caeca began to form. Pepsin-like enzyme and trypsin-like enzyme activities increased drastically from postflexion.

RNA/DNA ratio of individual fish decreased with the growth from preflexion to early postflexion stage, and thereafter it increased. The ratio ranged from 1-2 during the larval and early juvenile stage. Protein/DNA ratio decreased from

pre-flexion to the transitional stage from preflexion to flexion, and thereafter it increased. The red and white lateral muscle volume and numbers of red and white muscle fibers abruptly increased from 80mm BL.

Discussion

The growth strategy of the PBT apparently is to develop foraging structures and digestive organs before other organs to enable feeding on larger organisms. Under aquaculture conditions, their size preference in rotifer feeding quickly shifted to a larger one. However, in larval rearing, it is difficult to cultivate rotifers larger than 200μm, and on the other hand, *Artemia* nauplii compared to rotifers are too large for later larvae. This size mismatch is an unsolvable problem. Strong piscivory appears at the stage of notochord flexion. The foraging and digestive organs well developed before its regular appearance. In the rearing of the PBT larvae, it is necessary to feed them live fish larvae to mitigate the cannibalism.

The abundant mucous cells in the PBT larvae cause the adhesion of larvae to the water surface and bring a serious mortality in rearing.

Higher growth rate in spite of low RNA/DNA in PBT larvae and juveniles suggests that they make the fast growth possible by an allocation of synthesized protein to growth and to body maintenance at a peculiar rate.

Acceleration of the lateral muscle development started at the juvenile stage when collisions of reared juveniles with tank wall or net mesh occurs frequently in their rearing.

Our future research on the PBT early life history will be aquaculture-oriented where the useful characteristics concerning the muscle, adipose tissue, and immune system will be determined, and selective breeding will be conducted using genetic markers which represent such characteristics.

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EFFECTS OF TURBULENCE AND TURBIDITY ON GROWTH, SURVIVAL, AND FEED INTAKE OF STRIPED TRUMPETER, *LATRIS LINEATA*, LARVAE

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Introduction

Most marine larvae including striped trumpeter, *Latris lineata*, (Pankhurst and Hilder, 1998) are primarily dependant on vision for successful feeding during early larval development (Blaxter, 1986). However, vision in young larvae is often very poor and thus factors critical to a successful predator-prey interaction must be optimal. Two such factors, water turbulence and turbidity, both have the potential to improve predator-prey interactions. Theoretical studies have predicted a dome-shaped relationship between turbulence and larval fish ingestion rates (MacKenzie et al., 1994). Such a relationship is predicted based on the competing elements of increased encounter rates (Rothschild and Osborn, 1988) and decreased pursuit and capture success (MacKenzie and Kiorboe, 2000) associated with increases in turbulence. Algal cell induced turbidity has also had a positive effect on the growth and survival of many larval species and has been shown to increase short term prey intake of striped trumpeter larvae (Cobcroft et al., 2001). The aim of the study was to investigate the effects of turbulence and turbidity on the growth, survival and prey intake of striped trumpeter larvae during rotifer feeding, 6-16 days post-hatching (dph).

Materials and methods

Two separate experiments were conducted using a system comprising of 24, 300-l black hemispherical tanks. Experiment 1 examined the effect of turbulence. Turbulence was provided via aeration from the bottom center of each tank. There were 6 aeration levels, 0, 200, 400, 800, 1600, and 3200ml.min^{-1}, each with 4 replicate tanks. Experiment 2 examined the effect of turbulence and turbidity in an orthogonal design. There were three turbidity treatments; clear

water (0 Nephelometric Turbidity Units - NTU), live algae (*Nannochloropsis oculata* at 3NTU), and concentrated algal paste (*N. oculata* re-suspended at 3NTU, ProAqua), each conducted at 2 aerations levels (200ml.min^{-1} or 200ml.min^{-1} from the experiment onset increasing to 400ml.min^{-1} 10dph).

Larvae were stocked into the experimental system, held at 16ºC, 2 and 4dph in experiments 1 and 2, respectively. Larvae were fed enriched rotifers *Brachionus plicatilis* (Algamac 2000: Aquafarma Bio-Marine, Inc.) from 6dph at a density of 5.ml^{-1} once daily. Rotifers and algae were added to tanks at 0900h when lights gradually came on, aeration turned on and water flow to each tank stopped. At 0100h lights faded off, water flow was turned on at a rate of 112.5 l.h^{-1} and aeration stopped. Thus larvae had aeration only during the photophase. During the scotophase rotifers and algae were stripped from the tanks, allowing for the new algae and rotifers to be added at 0900h.

At two-day intervals rotifer intake was assessed by sampling 20 larvae from each tank after a 2-h feeding period. Sampled larvae were preserved in 10% neutral buffered formalin, measured for standard length and the number of rotifers in the stomach of each larva was counted under a dissecting microscope. Experiments were stopped 14dph (Experiment 1) and 16dph (Experiment 2) at which time all remaining larvae in each tank were counted and 20 larvae from each tank were assessed for standard length and swim bladder inflation.

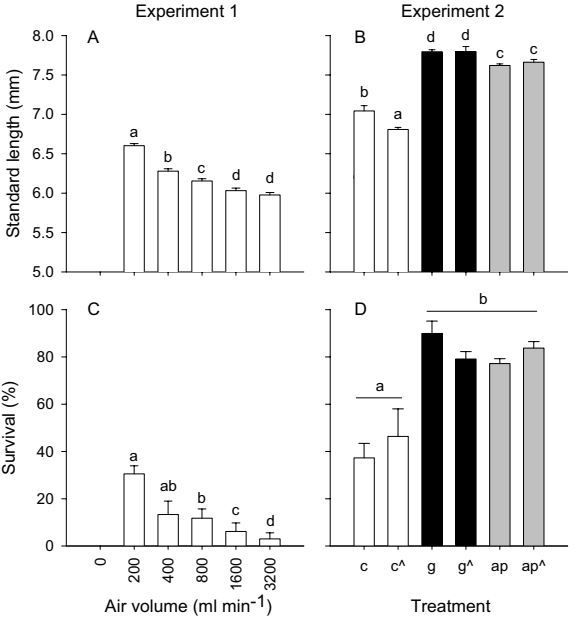

Fig. 1. Growth and survival of larvae in Experiment 1, 14dph (A, C) and Experiment 2, 16dph (B, D). c - clear water, g - live algae, ap - concentrated algal paste. ^ denotes increasing aeration.

Results and discussion

Results of experiment 1 indicate that survival and growth of larvae decreased with increasing levels of aeration (Fig. 1a,c). Zero aeration resulted in 100% mortality, most likely due to larvae becoming trapped in the surface film when trying to capture rotifers rafting at the surface. Swim bladder inflation was also very poor, less than 5% across all treatments, possibly due to the tank water surface not being sufficiently clean. Feed intake 8dph was highest at an aeration of 200ml.min^{-1} and decreased with increasing aeration, with the exception of the zero aeration treatment, which had significantly lower rotifer intake. The trend remained relatively constant 10, 12, and 14dph; however, in general feed intake at the higher aeration levels of 400 and 800ml.min^{-1} improved with larval age. Better prey intake at higher levels of turbulence with age would be expected based on the associated improvements in sensory and locomotor capabilities of the larvae.

Results of experiment 2 indicate that growth and survival of larvae in either type of green water was significantly higher than in clear water. There was no difference in survival between live algae and concentrated algal treatments, however, growth was significantly higher in live algae (Fig. 1b,d). Swim bladder inflation was also significantly higher (>85%) in all green water treatments in comparison to clear water (<70%). Increasing aeration 10dph had no effect on subsequent growth and survival of larvae; however, feed intake by clear water larvae 10dph under increased aeration was suppressed in comparison to all other treatments. Increasing aeration in green water treatments did not result in decreased feed rates, thus green water larvae, despite being the same size as clear water reared larvae 10dph, were more capable of dealing with the increase in aeration in comparison to larvae in clear water. Feed rates of clear water reared larvae were generally significantly lower than those of larvae reared in green water. Decreased feed rates in clear water are likely due to significantly greater proportion of larvae observed “walling” and therefore spending large periods of time not feeding. Also improved feeding in green water is likely due to improved visual encounter of prey in green water (Naas et al., 1992) and improved nutrition and health of rotifers in green water, resulting in larger larvae and therefore higher feeding rates. The growth of larvae in either green water treatment at an aeration level of 200ml.min^{-1} was the highest achieved for striped trumpeter to date and resulted in greater than 70% viable (those alive and with an inflated swim bladder) larvae 16dph.

Conclusions

An aeration level of 200ml.min^{-1} resulted in the best growth and survival in clear water, whilst under static conditions no larvae survived. It is possible that aeration levels between 0 and 200 may result in further improvements to feed intake,

growth and survival. The addition of either live algae or concentrated algal paste resulted in further improvement in growth, survival and swim bladder inflation of striped trumpeter larvae, in comparison to clear water. Given the encouraging results the use of concentrated algal paste may be a suitable alternative to live algal cells.

Acknowledgements

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SUCCESSFUL FEEDING OF DACE, *LEUCISCUS LEUCISCUS* LARVAE USING AN ARTIFICIAL DIET

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Abstract

A feeding trial was conducted to study the effect of different diets on growth and survival for larvae of dace, *Leuciscus leuciscus* under controlled culture conditions. Two days after hatching, the larvae were exposed to different feeding conditions: (i) freshly hatched *Artemia* nauplii, (ii) dry decapsulated *Artemia* cysts, (iii) crumble carp starter, a commercial diet (200-300 µm) used for 5 days and then switched to a larger size of diet (300-500 µm), (iv) *Artemia* nauplii used for 7 days and then switched to crumble carp starter (300-500 µm) (v) *Artemia* nauplii used for 14 days and then switched to crumble carp starter (300-500 µm). After four weeks feeding trial, the highest survival rate was obtained with the larvae receiving decapsulated *Artemia* cysts and *Artemia* nauplii. Direct feeding of the larvae (immediately after yolk resorption) with carp starter resulted in high survival that was comparable to the other treatments. However, the larvae fed on carp starter were significantly lower in length and weight (wet and dry) compared to the larvae receiving *Artemia* nauplii and decapsulated cysts. The results show that dace larvae may directly be fed on carp starter at onset of exogenous feeding.

Introduction

The dace *Leuciscus leuciscus* (L.) is typically a fish that can be found in clear, fairly fast-running streams and rivers, but are occasionally found in lakes and lowland rivers. The dace is common in the whole of Europe except the Balkans, Italy and the Iberian Peninsula**.** Little is known about the larviculture of dace.

Several freshwater fish species such as *Clarias gariepinus*, *Cyprinus carpio* and *Heterobranchus longifilis* can be exclusively reared on artificial diet from the start of exogenous feeding. However, many freshwater fish need live food for several days before switching to artificial diets.

The use of decapsulated cysts has been suggested as an alternative to *Artemia* in the larval rearing of several fish species (Vanhaecke et al. 1990; Shiri Harzevili et al. 2003). Despite the encouraging results, commercial application of decapsulated cysts in aquaculture industry is restricted to only a few shrimp hatcheries.

An experiment was carried out to evaluate different feeds on growth and survival of dace larvae.

Materials and methods

Dace eggs were obtained from broodfish held at the Research Centre (Linkebeek). Fertilized eggs were incubated in 300-ml mini-incubators (10°C) using flow-through system. To accelerate the hatching process, the eggs were transferred to 16°C (48h before hatching). All larvae were hatched after 24h. Two days after hatching, the larvae were exposed to different feeding conditions: (i) freshly hatched *Artemia* nauplii ($4.ml^{-1}$), (ii) decapsulated *Artemia* cysts, (iii) crumble carp starter, a commercial diet (200-300 µm) used for 5 days and then switched to a larger size of diet (300-500 µm) (manufactured by Coppens International), (iv) *Artemia* nauplii used for 7 days and then, switched to crumble carp starter (300-500 µm), (v) *Artemia* nauplii used for 14 days and then, switched to crumble carp starter (300-500 µm). The stocking density was kept at 200 individuals per aquarium (10 $larvae.l^{-1}$). Water temperature ranged between 18 and 20°C. There were three replicates per treatment. Growth parameters (length and wet and dry weight) were measured on days 1, 7, 14, 21, and 28 of the experiment. The initial average total length (mean±S.D.) and wet body weight were 8.89±0.27 mm and 2.55 mg. For the measurements, 10 larvae were randomly collected from each replicate. Survival of the larvae was recorded by counting the fish in the aquaria on d14 and at the end of the experiment.

Larvae were fed with dry diet and decapsulated cysts based on 20% of their body weight for three weeks, then the food reduced to 10% of their body weight.

Results and discussion

At d7, no significant differences in length and dry and wet weight were found among the treatments. (Figs 1, 2, and 3). At d14, the mean size (length, wet and dry weights) of the larvae receiving dry food was significantly ($P<0.05$) lower compared to the larvae fed on *Artemia* and decapsulated cysts (Figs 1, 2, and 3). The dry weight of larvae feeding with decapsulated *Artemia* was significantly ($P<0.05$) higher than the larvae fed on *Artemia* nauplii. On d21, larvae receiving decapsulated *Artemia* had significantly higher growth in terms of length, wet and dry weight in compared to the other treatments. At d28, the mean size (length, wet and dry weights) of the larvae receiving dry food was significantly ($P<0.05$) lower compared to the larvae fed on *Artemia* and decapsulated cysts. Feeding

larvae with carp starter from d8-15 did not produce higher growth compared to the treatments receiving *Artemia* nauplii and decapsulated cysts.

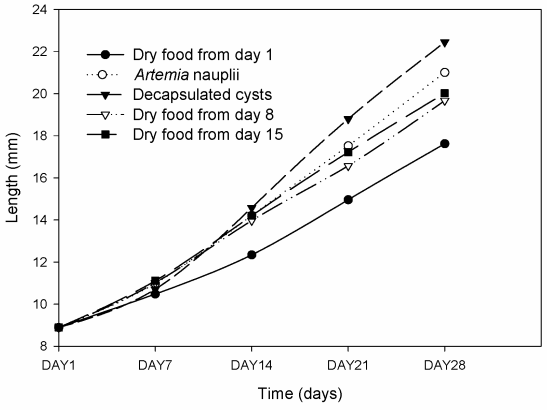

Fig. 1. Length (mm) of dace larvae measured on day 7, 14, 21, and 28 of the experimental course.

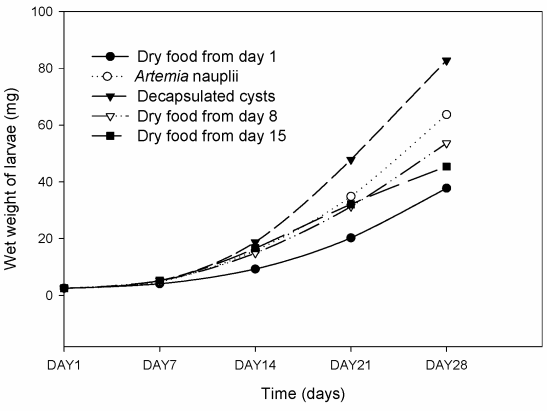

Fig. 2. Wet weight (mg) of larvae measured on day 7, 14, 21, and 28 of the experimental course.

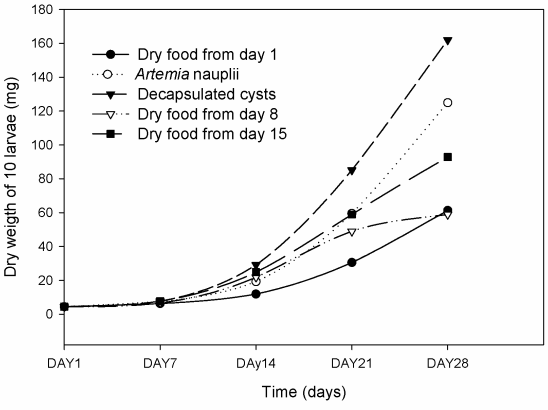

Fig. 3. Dry weight of 10 larvae (mg) measured on day 7, 14, 21, and 28 of the experimental course.

At d14, the survival of larvae fed on *Artemia* nauplii were not significantly different (P>0.05) from those receiving decapsulated cysts (Fig. 4). The larval survival was significantly (P<0.05) lower in treatments receiving carp starter as compared to the other treatments. No significant (P>0.05) difference was found between the larval survival fed from day 1 and those fed from day 8 on dry diet. At the end of the experiment, the highest survival rate was obtained with the larvae receiving decapsulated cysts and those larvae fed on *Artemia* (Fig. 4). Larval survival fed on dry food (from d1) was 89.6 % and this was significantly lower than the treatments receiving decapsulated cysts and *Artemia* nauplii.

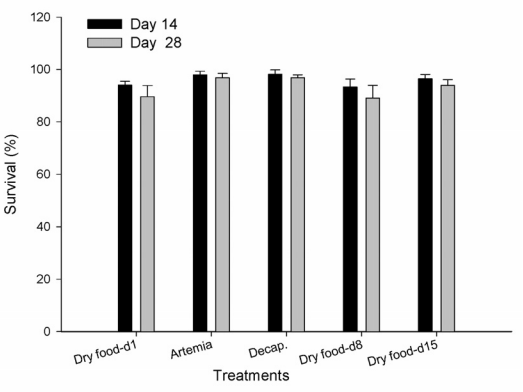

Fig. 4. The survival rate of larvae counted on day 14 and the end of experiment.

Good growth and survival of dace larvae fed on decapsulated cysts indicated that the decapsulated cysts were well accepted and ingested even after 21 days continuous feeding with decapsulated cysts. This is in contradiction to our previous findings (Shiri Harzevili et al., 2003 and Shiri Harzevili et al., 2004) with chub and dace larvae.

Direct feeding of dace larvae with dry diet resulted in a better survival and growth as compared to other cyprinids (chub and ide), although no similar diets were used for ide and chub larvae.

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MARKING OF AQUACULTURE-IMPORTANT *VIBRIO* STRAINS WITH INSERTED GENES CODING FOR DIFFERENT COLOR FLUORESCENT PROTEINS ENABLING SIMULTANEOUS IN VIVO AND IN SITU OBSERVATIONS OF MORE THAN ONE STRAIN INSIDE THE GUT OF *ARTEMIA FRANSCISCANA, LITOPENAEUS VANNAMEI*, AND *SOLEA SENEGALENSIS* LARVAE

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Introduction

The initiation of exogenous feeding in marine fish larvae has been identified as a critical phase in terms of survival and growth, as their digestive tract is still rudimentary and the larvae are exposed internally to environmental microbial communities for the first time. Contact with the bacterial community present in the water column and on potential food items can lead to beneficial or detrimental effects both on nutrition (digestion capacity of feed, rate of assimilation of nutrients) and health (capacity to resist colonization by pathogenic bacteria). The potential contribution to exogenous enzymes by bacteria established in the larval digestive tract and the potential competitive exclusion of pathogenic bacteria, have been important arguments and criteria in the search and development of probiotic bacterial strains. The modification of culture water microflora through the use of probiotic bacteria has been thoroughly demonstrated at the experimental level in fish larviculture, and frequently correlates with increased survival. However, most studies have indirectly proven the effect of a probiotic on experiments done at the population level, whilst the true consequences of bacterial interaction happen at the level of individual larvae.

The present work describes a protocol to genetically modify *Vibrio* strains enabling the individual cells to produce a range of inert and innocuous fluorescent proteins which eliminates the problems of non-specificity and leaching of the fluorescent dyes and satisfies the need for simultaneous comparison of the in vivo and in situ dynamic interaction of both pathogenic and probiotic bacterial

strains inside the larvae gut (Soto-Rodriguez et al., 2003). This technique will prevent leaching of the fluorescent marker, increasing observation resolution, and enable observation of multiple generations as the bacteria themselves are responsible for the production of a fluorescent protein.

Materials and methods

Strains tested in this study come from the Collection of Aquaculture Important Microorganisms (CAIM) from CIAD-Mazatlan, Mexico (www.ciad.mx/ciam) and included 3 known fish *Vibrio* pathogens (*V. anguillarum*, CAIM 763; *V. damselae*, CAIM 24T; *V. ordallii*, CAIM 608T), one known crustacean pathogen (*V. parahaemolyticus*, CAIM 170), and two potential probiotic strains (*V. alginolyticus*, CAIM 36; *V. alginolyticus*, CAIM C7b).

The insertion of genes for GFP (Green Fluorescent Protein) or dsRed (Red Fluorescent Protein or RFP) and kanamycin resistance was attempted for all mentioned *Vibrio* strains by using a mini-Tn5 cassette and inserted in the chromosome by means of the pUT delivery plasmid (Tolker-Nielsen et al., 2000). The delivery plasmid was mobilized from *Escherichia coli* CC118 pir to the recipients using the helper strain *E. coli* HB101(RK600) as previously described (Andersen et al. 1998). The obtained GFP or RFP marked cells were selected on agar plates supplemented with kanamycin (50µg.ml^{-1}) and rifampin (100µg.ml^{-1}). GFP and RFP fluorescence was visualized by a Dark Reader illuminator (Clare Chemical Research, Denver, CO) and by epifluorescence microscopy.

Results and discussion

The natural resistance to kanamycin on several strains proved to be a major constraint to use the present protocol as selection on agar plates supplemented with this antibiotic was inconclusive. Resistance to kanamycin, even low concentrations, was much more common than initially expected and several strains had to be discarded due to this problem. Not all strains were marked with both red and green fluorescent proteins. However, marked strains were easily observed on an epifluorescence microscope confirming the potential of this marking technique to enable simultaneous in vivo and in situ observations of more than one strain inside the gut of fish and crustacean larvae. Transposons with resistance to alternative antibiotics are being investigated, as well the development of simple expression vectors for inserting these fluorescent proteins as plasmids. Preliminary observations using *Artemia* metanauplii, *Litopenaeus vannamei* and *Solea senegalensis* larvae are currently under way.

Acknowledgements

The authors would like to aknowledge the laboratory help by the M.Sc. Roxana Atondo Mexia.

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PROBIOTIC APPROACH FOR HEALTH MANAGEMENT IN JUVENILE PRODUCTION

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Introduction

Marine larvae lack a well-developed specific immune system at hatching, and stress (microbial, chemical, physical, malnutrition) induced by intensive rearing conditions can suppress the innate, non-specific immune defence of larvae during the earliest developmental stages. Opportunistic bacteria present in the water and live feed may therefore proliferate, infect, harm, and kill high numbers of larvae and juveniles. Good hygiene is extremely important, but it is not possible to rely on a strategy that only includes vaccination, disinfection, or sterilization against this problem. The juvenile-farmers will have to follow a probiotic approach to microbial management in water treatment, live feed production, and in first-feeding and weaning, and thereby build up a sound microflora and resistant larvae. A probiotic approach includes the selection and use of bacteria with beneficial effects on the larvae, as well as stimulation of the larval resistance against infections by opportunistic and pathogenic microorganisms.

Screening and use of probiotic bacteria for microbial control of water and live food organisms

Strategies to avoid growth and transfer of opportunistic bacteria into larvae are necessities. A probiotic approach to the problem can be either to create conditions that select for a beneficial microflora or to add bacteria with beneficial effects on health (probiotics).

The screening for bacteria with probiotic properties is performed by use of several criteria, where production of antimicrobial substances and blocking of adhesion of pathogens are among the most important. Impact on non-specific, and

later, specific immune responses is also probably highly important, but for aquaculture species this has so far received less attention than for warm-blooded animals and humans. Introduction of probiotic bacteria can also be expected to contribute with enzymes and micronutrients that are positive for the larvae. Development of good techniques for the evaluation of probiotic candidates and how they affect the larvae are crucial for further screening and implementation of probiotics in marine juvenile production. In vivo testing is necessary and must be used as ultimate evaluation of the potential candidates, but challenge tests with pathogens should be kept to a minimum due to animal welfare. Reliable in vitro tests and good parameters for measure of stress and disease resistance in larvae may reduce the need for large time- and fish-consuming in vivo trials.

Water is normally filtered and often disinfected before use, as a barrier to introduction of pathogenic bacteria and viruses. However, the microflora in water with a very low bacterial density has low stability to perturbations, which induces rapid proliferation of opportunistic species (Salvesen et al., 2001). Recolonization by more non-opportunistic species may be obtained by use of biofilter, in flow-through or recirculating systems. The filter substrate can be colonized by a flora developed over time by the selecting forces present in the conditions and/or by addition of selected bacteria. Establishment of a functional biofilm on the surfaces in the environment for the larvae by probiotic bacteria, has also been proposed (Hjelm et al., 2004).

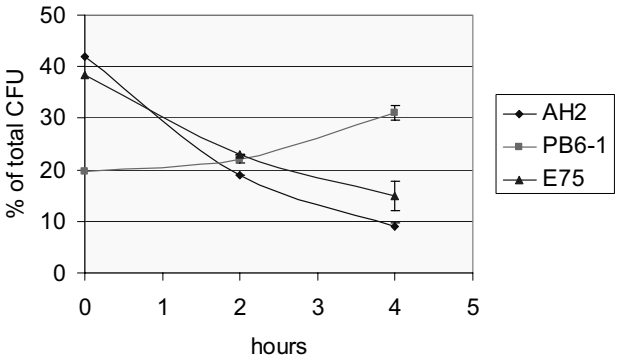

Fig. 1. Stability of three probiotic strains (AH2, PB6-1 and E75) in rotifers (*Brachionus plicatilis*) after feeding to fish larvae. The temperature in the tank was 20°C and the rotifer density 5ind.ml^{-1}.

To reduce the transfer of opportunistic bacteria from live feed cultures to the larvae, the bacterial flora can be exchanged and replaced by probiotics. The probionts can be administered as single strains or as mixtures of selected strains, by incubating the animals in a bacterial suspension for a period equal to the gut filling time (Makridis et al., 2000). When administered as mixtures of several strains, different strains may be ingested at different rates by rotifers and *Ar-*

temia, and have various stability in the animal as they in varying degree will be digested or expelled (Fig. 1).

In the animal gut, competition for nutrients between strains may also take place, but because of the relatively short retention time, this is expected to have less impact on the stability of each strain within the live feed organisms. However, the feeding regime is very important, as well as the choice and handling of bacterial species. The use of probiotics should not introduce extra or time-consuming operations to the farmer. The search and use of probiotics for marine larvae is at research level, but commercial products turn up and are being used.

Improved survival and growth by immunostimulation of larvae and juveniles

Intensive culture conditions may suppress the immune system of larvae, but administration of non-specific immunostimulants to larvae is shown to be a very promising strategy to increase larval survival and growth. Today, most feed producers include non-specific immunostimulants in their product programs for larvae, juveniles, and on-growing stages. These are often glucans derived from yeast cell walls, or algal polysaccharides such as alginates. However, well-documented immunostimulants do not always work as expected because of a lack of knowledge about optimal use. Protocols for use of this concept for larvae and juveniles should therefore be optimized for each species and developmental stage. Further, the exploration of different sources has lead to the discovery of new stimulatory compounds with better properties than commercial stimulants.

Microalgae are commonly used in juvenile production because of good effects on the bacterial flora of the water, improved nutrition of the live feed organisms, and creating better physical conditions for the larvae in the tank. Many different species are used, and some are shown to contain immunomodulatory glucans (Størseth et al., 2005). When this algal glucan was extracted and administered via rotifers to Atlantic cod (*Gadus morhua* L.) larvae, survival nearly doubled. During the stressing period with early weaning to formulated diet, the stimulated larvae continued to grow, whereas the control larvae and larvae stimulated with a commercial yeast glucan stopped growing during this period. The algal glucan also induced less bacterial colonization of the gut, and preliminary examination has shown that the algal glucan stimulates the immune system of cod (Strand et al., 2004). It is not known whether the changes in the bacterial gut flora were caused by increased colonization resistance against specific bacteria in the gut. The gut mucosa contains cells with an important role in non-specific defense, and that can probably be modulated by feed-administered immunostimulants. Other mechanisms from feeding with glucan could be selective stimulation of the growth of beneficial bacterial strains in the gut, as described for use of prebiotics.

Artemia and rotifers can be used for delivery to larvae. In a recent commercial-scale trial run, increased protection against vibriosis was obtained by periodically administration of an immunostimulatory HighM-alginate to Atlantic halibut (*Hippoglossus hippoglossus*) larvae and juveniles via *Artemia* (Skjermo and Bergh, 2004). This demonstrated how non-specific immunostimulants can be used during larval stages to prepare for stress in the juvenile stage. Because long-term supply of immunostimulants is assumed to over-stimulate or exhaust the immune system, the stimulants should be fed in shorter periods. Changing of the cultivation protocols in the hatcheries can induce extra work and may be experienced negatively by the farmers. The costs from running the treatments must therefore be paid back in terms of reproducible production of a higher number of high quality juveniles.

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DOSE DEPENDENT PROTECTION OF *ARTEMIA* NAUPLII AGAINST *VIBRIO CAMPBELLII* BY ISOGENIC YEAST CELLS DIFFERING IN β-GLUCAN CONTENT

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Introduction

Microbial diseases are a major threat to the sustainability of aquaculture, being responsible for massive mortalities occurring especially in the early life stages of aquatic organisms. In these larval stages, most organisms, including vertebrates, cannot rely on an acquired immune system to combat disease, but have to rely on the innate immune system. Since larviculture still suffers from unpredictable survival rates, there is a growing interest in a better understanding of the innate immune response of these organisms. The use of specific biological compounds (immunostimulants), such as β-glucans, to enhance the immune response of target organisms, has been advocated as an excellent preventive tool to control pathogens in aquaculture (Anderson, 1992). Although many studies with immunostimulants have been performed, the validity of some conclusions with respect to the benefit of immunostimulation has recently been questioned by Smith et al. (2003). They claimed that most of the experiments published so far present poor experimental design, lack of statistical analysis and reproducibility. Therefore, they called for the design of standardized trials under controlled rearing conditions, which should provide unequivocal evidences of the beneficial effects of immunostimulants in reducing invertebrate susceptibility to disease or infection. In addition, such standardized tests should provide the necessary material to understand, at the biochemical level, the mode of action of immunostimulants.

The brine shrimp *Artemia* produces cysts that can be easily disinfected using the decapsulation technique. When these axenic cysts are hatched in aseptic conditions, it is possible to set-up gnotobiotic *Artemia* nauplii cultures, allowing highly reproducible survival and growth rates when they are fed with axenically grown yeast cells or microalgae (Marques et al., 2004a,b). This set-up was recently extended with a challenge test, using a *Vibrio proteolyticus* strain and a *Vibrio campbellii* strain (Marques et al., 2005).

The present study investigates the effect of two yeast strains of *Saccharomyces cerevisiae*, namely a wild type and its isogenic mnn9 mutant, which display different levels of cell wall bound β-glucans (Marques et al., 2004b) in gnotobiotic *Artemia* challenge tests, and looks particularly at the amount of yeast cells necessary to obtain a protective effect.

Materials and methods

Experiments were performed with *A. franciscana* cysts from the Great Salk Lake, Utah, USA. Bacteria-free cysts and nauplii were obtained, following Marques et al. (2004b). Two different axenic live strains of the bakers' yeast *S. cerevisiae* were provided to *Artemia*: the wild type strain (WT) and its mnn9 isogenic yeast mutant. Yeast cultures were performed according to Marques et al. (2004b), using Yeast Extract Peptone Dextrose medium (YEPD) to culture the WT yeast and Yeast Nitrogen Based medium (YNB) to culture the mnn9 yeast. WT was harvested in the stationary growth phase, while the mnn9 yeast was harvested in the exponential growth phase. These culture conditions result typically in a cell wall composition of 1.2% chitin, 47.3% mannose, and 51.5 % glucans in the WT yeast, while the mnn9 yeast mutant contains 8.7% chitin, 16.3% mannose and 75.0% glucans (Marques et al., 2004a).

In a first experiment, 20 axenic Instar II nauplii were fed (for 5 days) with WT and mnn9 yeast and challenged on d3 with *V. campbellii*, according to the procedure described by Marques et al. (2005). In a similar experimental set-up, WT and mnn9 yeasts were only provided to *Artemia* in small amounts (10% of the yeast ash free dry weight, AFDW, provided in first experiment) together with an autoclaved bacterium LVS 3 (constituting 90% of the total AFDW offered) (unpublished data). In this way the nauplli received the same amount of feed (in terms of AFDW) relative to the first experiment. Nauplii survival was measured at the end of the experiments.

In a subsequent experiment, 20 axenic Instar II nauplii were cultured in sterile Falcon tubes filled with 30ml of FASW (4 replicates per treatment), fed with WT or mnn9 yeast for 8h only (priming). The nauplii were subsequently washed in order to remove all remaining yeast cells from the culture medium and fed with autoclaved LVS 3 at a density of 10^7 cells.ml^{-1}.d during 2 days. At the same time the nauplii were challenged with a virulent *V. campbellii* pathogen (strain LMG21363 - VC) at a density of 5×10^6 cells.ml^{-1}. At the end of the experiment the nauplii survival was measured.

Results and discussion

Gnotobiotically grown *Artemia* nauplii fed only WT yeast or its isogenic mnn9 deletion mutant react totally different in a challenge test with *V. campbellii*.

When challenged at d3, WT-fed *Artemia* did not survive, while mnn9-fed *Artemia* performed as good as unchallenged *Artemia* (Marques et al., 2005). In a further test, the amount of yeast fed was reduced to 10% of the original level, while the remainder of the feed was offered as a dead Gram-negative bacterium LVS 3, in order to verify if *Artemia* would still be protected against *V. campbellii*. As in the previous test, the WT yeast was not able to protect the *Artemia* nauplii, while the mnn9 yeast provided total protection (unpublished data). This experiment indicated that a small amount of mnn9 yeast cells was sufficient to protect *Artemia* against pathogenic bacteria.

Therefore, it was verified whether primed *Artemia* nauplii; i.e., animals receiving a short-term exposure to WT or mnn9 yeast cells (during 8h), would be sufficient for their protection against pathogenic bacteria (Table 1). After the priming, nauplii only received dead bacteria (LVS 3) as feed, which was known not to protect *Artemia* against *V. campbellii* (see Table I).

Table I. *Artemia* naupliar survival in a challenge test after priming with isogenic yeast cells (the same letter in superscript indicates values not statistically different, $p>0.05$.)

Treatments	Priming (0-8h)	Feed (8-48h)	Challenge (8h)	Survival (%)	
				Day 2	Day 3
No feed	-	-	-	26 ± 6^{d}	0^{c}
LVS 3	-	LVS 3	-	86 ± 3^{a}	25 ± 4^{b}
LVS 3+live VC	-	LVS 3	VC	24 ± 3^{d}	0^{c}
WT+LVS 3	WT	LVS 3	-	88 ± 3^{a}	41 ± 6^{ab}
WT+LVS 3+live VC	WT	LVS 3	VC	56 ± 5^{c}	0^{c}
Mnn9+LVS 3	mnn9	LVS 3	-	90 ± 4^{a}	61 ± 10^{a}
Mnn9+LVS 3+liveVC	mnn9	LVS 3	VC	75 ± 4^{b}	4 ± 3^{c}

In the absence of a challenge, a short-term exposure to yeast cells had a strong beneficial effect on survival, lasting till day 3, while nauplii challenged immediately after the priming displayed some beneficial effect of the priming on d2, irrespective of the yeast genetic background. The priming effect was stronger with the mnn9 yeast than with WT cells. Yet, on d3, no nauplii survived in the LVS 3 control treatment and in the WT priming experiment, while in the mnn9 experiment a very small amount of individuals survived. Then latter result indicates a very large drop in survival between d2-3.

In the present paper we showed that *Artemia* is protected against a *V. campbellii* challenge when fed a yeast strain (mnn9) with elevated glucan concentration in the cell wall. When only 10% of this glucan-enriched yeast cell is provided, the nauplii are still protected against pathogenic bacteria. After a short-term exposure to the mnn9 yeast, only temporary protection is observed, lasting until d2 but not until d3. Although an clear causal link between the increased protection by mnn9 and the increased glucan in its cell wall has not yet been established,

the data certainly warrants some further such research. Priming studies of Moret and Siva-Jothy (2003) with the mealworm beetle, *Tenebrio molitor*, revealed a long-term protection (50d) against the pathogenic fungus *Metarhizium anisopliae* after priming the innate immune system with lipopolysaccharides (LPS). Although the compounds used in both experiments are different, both LPS and glucans are immunostimulants for which the innate immune system has specific receptors (Janeway and Medzhitov, 2002), resulting in a general antimicrobial response (Beutler, 2004). The mnn9 yeast cells are able to protect gnotobiotic *Artemia* in challenge test, although in this particular priming experiment short-lived protection was obtained. Hence, it appears the priming of the innate immune system is either compound-specific or species-dependent.

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CULTIVATION OF BLUE KING CRAB LARVAE, *PARALITHODES PLATYPUS*: EFFECTS OF DIET, TEMPERATURE, AND DENSITY

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Introduction

The blue king crab (*Paralithodes platypus*) is a commercially valuable crustacean that occurs in isolated populations in the Bering Sea, Gulf of Alaska, and southeast Alaska, as well as the western Pacific Ocean near Japan and Russia (Hoffman, 1968). Abundance of blue king crab populations near St. Matthew Island and the Pribilof Islands in the Bering Sea declined precipitously in the late 1990s and these areas have been closed to fishing since 1999 and 2002, respectively (NPFMC, 2002). Little is known about the early life history of blue king crab; in order to study young crab in the laboratory we investigated the best conditions for cultivation of the larvae from hatching to the first juvenile crab stage (C1). We tested the effects of diet, temperature, and rearing density on larval survival. Diets tested included no feeding, *Artemia* nauplii enriched with the diatom *Thalassiosira nordenskioeldii*, newly-hatched unenriched *Artemia* nauplii with *T. nordenskioeldii* added to the culture water, and a control diet of *Artemia* nauplii enriched with frozen *Isochrysis* paste. All diets were tested at 6°C and a larval density of 10 zoeae.l^{-1}. The *Isochrysis* diet was also tested at 3°C and 9°C and at densities of 20 and 40 zoeae.l^{-1}.

Materials and methods

The experiment consisted of 8 treatments grouped by diet, temperature, and density. Four treatments consisted of larvae fed four different diets at 6°C: 1) larvae receiving no food (UNFED); 2) larvae receiving *Artemia* nauplii enriched with *Isochrysis* Instant Algae® paste (Brine Shrimp Direct, USA) (ISO6), considered the "standard" diet; 3) larvae receiving *Artemia* nauplii enriched with *T. nordenskioeldii* (THAL); and 4) larvae receiving newly hatched unenriched *Artemia* nauplii plus culture water supplemented with *T. nordenskioeldii* (A+THAL). Two treatments consisted of the *Isochrysis* diet with beakers held at 3°C (ISO3) and 9°C (ISO9). Each of these 6 treatments was comprised of six replicates of 10 zoeae per beaker. The final two treatments were fed the *Isochrysis* diet at 6°C,

but consisted of beakers with 20 (DENS20) or 40 zoeae.l^{-1} (DENS40). All treatments were maintained on a 12:12 L:D cycle at approximately 70lux.

Experiments were conducted by placing larvae inside a 150mm length of 75mm diameter PVC tube, with 675µm polyethylene netting glued to the bottom. Each tube was set into a 1-l glass beaker filled with 800ml of filtered and UV-sterilized seawater. Larvae (in tubes) were transferred to clean beakers with fresh seawater daily prior to feeding. All beakers were fed daily with approximately 1750 *Artemia* nauplii per beaker (i.e., 2.2 nauplii.ml^{-1}). Feeding was terminated when all zoeae in a beaker had molted to the glaucothoe stage. Numbers of surviving larvae were counted once each week in all beakers. Final survival was determined when all surviving crabs had molted to stage C1, and each treatment was considered completed when larvae in all six beakers finished molting to C1.

Proportional survival data were subjected to angular transformation. Homogeneity of variances was tested using Levene's test prior to use of ANOVA. Survival to stage C1 was compared between treatments by ANOVA, and post-hoc multiple comparisons of each treatment versus the ISO6 diet were conducted with Dunnett's test. ANOVA was also used to compare raw numbers of surviving crab in each of the three density treatments that used the same diet and temperature (ISO6, DENS20, and DENS40).

Results and discussion

Survival varied widely and significantly among treatments. The highest survival from hatching to stage C1 was obtained with the A+THAL diet and was significantly greater than in any other treatment (Table I). At the other end of the spectrum, all UNFED larvae died within 21 days (Fig.1); this was the only treatment with survival significantly lower than the control diet (ISO6) (Table I). Survival in all other treatments was not significantly different from the control diet. Temperature had little effect, as mean survival was similar between the ISO3, ISO6, and ISO9 treatments (Table I). At first look, density also appeared to have little effect, as proportional survival among the three density treatments was not significantly different, though it was greater in the DENS20 treatment than in the ISO6 or DENS40 treatments. However, the actual numbers of surviving larvae in the DENS20 and DENS40 treatments (77 and 66, respectively) were similar (Table I). Numbers of crab larvae in the DENS40 treatment declined abruptly during week four to levels similar to those in the DENS20 treatment, and followed a similar trend thereafter. Surviving C1 crab were significantly different among the three density treatments, but post-hoc tests showed they were similar between the DENS20 and DENS40 treatments, and between the latter and the ISO6 treatment. These results imply that the experimental conditions of diet, *Ar-*

temia concentration, and temperature used could support an upper limit of about 13 zoeae per 800ml beaker, or about 16.2 zoeae.l^{-1}.

Table I. Survival of blue king crab (*P. platypus*) larvae from hatching to stage C1. Values are numbers of crab surviving in each replicate beaker, and sum, mean, and SD of total survival. Mean and SD of percent survival are also shown for each treatment. Best survival was on the A+THAL diet with 10 larvae.l^{-1} at 6°C. Groups that were significantly different from the control diet (ISO6) by Dunnett's test are designated by *.

	Beaker						Numbers			Percent	
Treatment	**1**	**2**	**3**	**4**	**5**	**6**	**Sum**	**Mean**	**SD**	**Mean**	**SD**
THAL	7	0	4	8	5	7	31	5.2	2.9	51.7%	29.3%
A+THAL*	8	10	10	9	10	8	55	9.2	1.0	91.7%	9.8%
UNFED*	0	0	0	0	0	0	0	0.0	0.0	0.0%	0.0%
DENS20	12	4	14	12	19	16	77	12.8	5.1	64.2%	25.4%
DENS40	5	10	3	13	13	22	66	11.0	6.8	27.5%	17.0%
ISO3	9	5	8	3	8	1	34	5.7	3.2	57.9%	30.9%
ISO6	10	2	9	6	0	2	29	4.8	4.1	48.3%	41.2%
ISO9	5	7	2	9	2	0	25	4.2	3.4	41.7%	34.3%

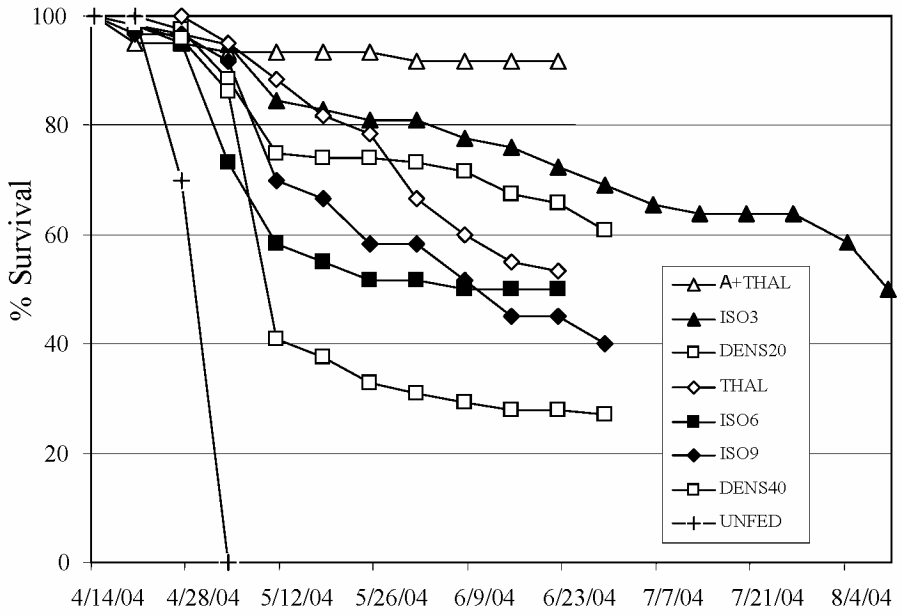

Fig. 1. Percent survival of blue king crab (*P. platypus*) larvae at weekly intervals, from hatching to stage C1, under different culture conditions. All points are means of 6 replicates.

Conclusions

Extremely high survival (91.7%) of blue king crab larvae from hatching to C1 was obtained on a diet of newly-hatched unenriched *Artemia* nauplii and culture water supplemented with *T. nordenskioeldii* and at a temperature of 6°C. Much lower survival occurred when *Artemia* were enriched with *T. nordenskioeldii* prior to feeding them to crab zoeae. Unfed zoeae died within a few weeks, demonstrating that these zoeae are not lecithotrophic like those of the golden king crab *Lithodes aequispinus*. Based on these results, we infer that crab larvae obtain nutrients directly from the diatoms that they do not obtain when the diatoms are first consumed by *Artemia*. Nutritionally, phytoplankton can supply vitamins, protein, carbohydrates, fatty acids and pigments that may not be present in unenriched *Artemia*. The presence of diatoms in the culture water may also provide other benefits such as removal of nitrogenous wastes, the addition of oxygen and helping to balance pH. Survival on the standard diet (*Artemia* enriched with frozen *Isochrysis* paste) decreased with increasing temperature, from 3°C to 9°C, though not by a significant amount. However, development time from hatching to stage C1 was identical (mean 74 days) at 6°C and 9°C, yet it was lengthened considerably (105 d) at 3°C. Thus, increased survival at 3°C is offset by much longer development time, with associated labor costs and higher risk of failure.

Our experimental conditions were adequate to produce numbers of larvae (several hundred) useful for laboratory experimentation, and could be repeated at this level. Ultimately, these techniques could be adapted for use to produce larger quantities of small crab for research or enhancement of natural stocks.

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ENVIRONMENTAL INFLUENCES ON THE DEVELOPMENT OF LORDOSIS AND MUSCULO-SKELETAL TISSUES IN SEA BASS (*DICENTRARCHUS LABRAX*): THE ORCIS PROJECT

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The aim of this EU ORCIS project was to optimise the rearing conditions of larval and juvenile sea bass (*Dicentrarchus labrax*) such that the incidence of lordosis was significantly reduced and co-ordinated musculo-skeletal growth was improved. This was achieved through an integrated, multidisciplinary approach where an understanding of the underlying science was addressed in consort with the aquaculture industry.

The vertebral deformity lordosis is a significant problem in sea bass. Skeletal abnormalities in reared fish exert disadvantages upon performance, welfare, market image, and consumer acceptability and, consequently, on fish commercial value, productivity, and efficiency of the aquaculture industry. The application of appropriate rearing conditions solved the problem of lordosis induction by swimbladder non-inflation. However, lordosis continued developing in reared sea bass with functional swimbladders reaching frequencies of 25-70%. Other studies have implicated a range of factors which might influence lordosis induction in fish with functional swimbladders. These factors include dietary deficiencies, notochordal distortions in early development, pollutants, myopathies, and high swimming activity due to high current velocity.

The increasing contribution of sea bass to the aquaculture industry with decreasing financial return, due in part to lordosis, stresses the importance of improving production efficiency. The aim of this project was to evaluate the influence of environmental and nutritional factors on the incidence of lordosis and on the development and function of the musculo-skeletal system. The project aimed to understand the reasons for lordosis and the optimum rearing conditions for its elimination and for improved musculo-skeletal growth. The findings from this project will lead to benefits for the consumer, producer, and animal.

The specific objectives of this project were (1) to assess the influence of rearing temperature and current velocities and their interactions on lordosis incidence and (2) to assess, for the same factors and groups of fish, the influence on musculo-skeletal development and growth at tissue, cell, protein, and molecular level, as an informed basis for reducing the problem of lordosis and for optimising musculo-skeletal growth.

Sea bass were reared at 15°C and 20°C at egg and larval (first feeding, notochord flexion, and metamorphosis) stages. At metamorphosis, fish reared at 15°C and 20°C were kept separately and placed in tanks (18.5°C) for two weeks, before swim currents (0, 25, 50, and 75% of the species relative critical swimming speed, RU_{crit}) were applied in the phase of 20-45mm mean TL.

Larval and juvenile samples were analysed at the tissue, cell, protein, and molecular levels (using a range of techniques including histology, RT-PCR, electron microscopy, and biomechanical modelling) to determine the effects of temperature and current velocities on musculo-skeletal development and growth. Lordosis development was documented using x-ray analysis and histology.

Overall, this project has highlighted some interesting results. It has shown that the occurrence of lordosis is exacerbated at 20°C compared to 15°C and that higher current velocities (particularly at 15°C) also have a role to play in the production of this deformity. Additionally, temperature has been shown to affect myogenesis (by altering myogenic factor differentiation and insulin-like growth factor (IGF) expression), vertebrae, and myomere allometry and, thus, possibly causing a “buckling” effect. Swim speeds were also implicated in these biological alterations. Ultimately, high susceptibility to lordosis of larvae and juveniles reared in extreme environmental (higher temperatures and currents) conditions need to be addressed, as they have a large impact on the production rate of sea bass (in terms of survival and selling aesthetics), which in turn affects economies and employment.

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REVIEW AND CURRENT TRENDS IN HATCHERY TECHNIQUES FOR CHINESE MITTEN CRAB *ERIOCHEIR SINENSIS*

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Introduction

Aquaculture production in China is dominated by freshwater finfish, mollusks, and, more recently, marine finfish. Production of crustaceans, mainly *Penaeus chinensis*, increased up to 1991 and then fell due to disease-related problems and sub-optimal management. The decline in shrimp production was offset by a subsequent increase in Chinese mitten crab *Eriocheir sinensis* production, which already entered the top twenty of farmed species in developing countries.

A breakthrough in mass propagation of *E. sinensis* larvae was made in the 1970s and during the following two decades, substantial improvements in hatchery techniques resulted in a big increase in larvae production. The objectives of this paper are to provide historical background on the development of mitten crab hatchery techniques in China; to summarize the different hatchery techniques currently used in terms of water management, nutrition, and zootechnical aspects; and to identify problem areas and formulate recommendations for future research.

E. sinensis is native in East Asia, with a natural distribution along the eastern coast of China. Its distribution is centered on the Yangtze River basin. Three populations are described according to the river systems where they are living: the Liao River, Yangtze River, and Ou River populations, which belong to the same species, but display slight genetic and biochemical polymorphism due to the long term geographic isolation (Li and Zhou, 1999; Zhao and Li, 1999). Among the different populations, the Yangtze population has a higher commercial value in terms of taste and maximum size attained.

In the beginning of the 20th century, E. sinensis established itself in the Rhine River in Germany via ballast water of ships voyaging between the east coast of China and Europe (Cohen and Carlton, 1997). After that, E. sinensis spread rap-

idly throughout northern and western Europe. Its present estimated distribution ranges from Finland, through Sweden, Russia, Poland, Germany, the Czech Republic, the Netherlands, Belgium and England to France. The crab has also been reported from North America. It is therefore considered as an invasive species in continental Europe and America.

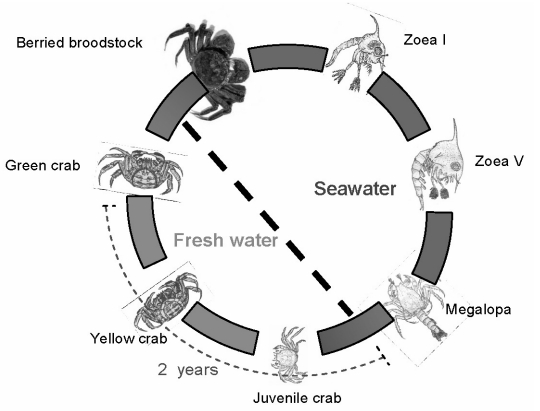

Fig. 1. Life cycle of *Eriocheir sinensis*.

As catadromous species, *E. sinensis* spends most of its life in fresh water, but migrates seawards to breed. During their second to third year, "green crabs" migrate downstream, attaining sexual maturity in the tidal estuaries. November to January is the mating season. After mating the females continue migrating seawards, carrying fertilized eggs, over-winter in the deeper water and return to brackish water in spring to release their eggs. The larval development includes several molts: zoea I to zoea V and megalopa stage. The juveniles gradually move upstream into fresh water to complete the life cycle (Fig. 1).

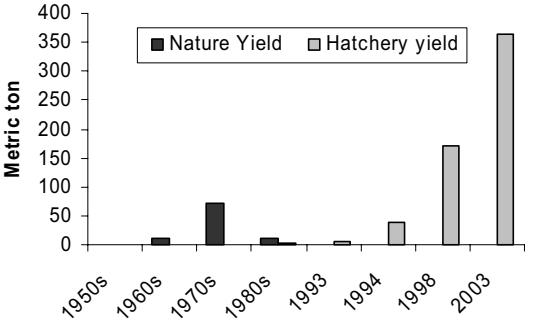

Fig. 2. Natural and hatchery yield of *Eriocheir sinensis* megalopa in China.

The natural yield of *E. sinensis* megalopa had been abundant for hundreds of years, but reduced dramatically in the 1950s and earlier 1960s (Fig. 2). In the late 1960s and 1970s, the Chinese government encouraged people to collect juvenile crabs from the river estuaries and release them into rivers and inland freshwater lakes to increase natural resources. Until the early 1980s, mitten crab

production relied on the provision of wild postlarvae. In the late 1980s, the natural production dropped to 10t, and even less than 300kg in 1997 due to overfishing of broodstock, industrial pollution, agricultural fertilizers, and irrigation projects. The decreasing natural recruitment warranted the development of controlled seed production to stock ponds. Through the development of controlled breeding techniques, the hatchery production increased from the late 1980s, and reached 400t of megalopae in 2002. It is estimated that hatchery production currently constitutes more than 98% of total megalopa production in China.

Materials and methods

Both wild and mature farmed crabs are used as broodstock. Wild berried females are captured from river estuaries in spring; these crabs normally produce large quantities of good quality eggs, but are in short supply for hatchery purposes and spawning is less synchronous. Mature, non-berried females are captured in spring, and then induced to mate and spawn. Since these broodstock have consumed a lot of energy in order to survive winter, they have fewer nutrients accumulated in the hepatopancreas and gonads, and mortality is usually higher.

Healthy male and female crabs are selected from culture ponds in autumn, fed with rich diets and then induced to mate and spawn. The broodstock are maintained in outdoor earthen ponds, indoor concrete tanks or basements over winter, with a stocking density varying from $4.m^{-2}$ in outdoor ponds to $10.m^{-2}$ indoor; crabs are fed with fresh trash fish, sand worms and clams. In this way, good egg production (both quality and quantity) can be obtained and larval production can be easily managed. The latter technique is currently most frequently applied in the local hatcheries.

Larval rearing can be carried out in natural seawater or diluted brine, well water (Li, 1996), or artificial seawater (Zhao, 1980). Although the latter is more expensive, it offers the possibility to operate mitten crab larviculture in inland areas. Artificial seawater moreover has the advantage that it prevents contamination with pathogens and that the chemical composition of the water can be adjusted according to the requirements of the crab larvae (Zang, 1998).

There are two major techniques nowadays currently used in China: (1) indoor intensive larviculture: with temperature control, aeration, and supply of live food (algae, rotifers, *Artemia* nauplii), frozen rotifers and copepods, egg yolk, or micro-encapsulated artificial diets. The zoea I density can be 0.2-0.5 million.m^{-3}, and megalopa production can be 150-500g megalopa.m^{-3}. (2) Outdoor extensive larviculture: production of algae, rotifers and copepods in the ponds is stimulated before stocking zoea I larvae into the ponds. No temperature control, aeration, or supplementary feeding are exercised. The zoea I density is less than 0.1 million.m^{-3}; megalopa yield is not stable.

Future research

Present techniques for mitten crab larviculture are largely based on shrimp hatchery technology and experience; no specific research has been performed. Along with the remarkable increase in crab production, some prominent issues arose such as the low and variable larval survival rate and quality, the smaller adult size attained, and disease outbreaks. In order to improve larval rearing techniques for *E. sinensis*, the following needs to be investigated and optimized:

- Population genetics for broodstock selection to establish a genetic marker for the best performing strain or population
- Broodstock nutrition because of the migratory behavior and euryhaline character of mitten crab, especially the PUFA requirements (n-3/n-6, DHA, EPA) seem to be of interest. Other factors such as phospholipid, vitamin C, and carotenoid pigments should also be considered.
- Larval nutrition: feeding strategy, fatty acids nutrition (HUFA), vitamin requirements, and use of artificial diets need to be studied.
- Microbial control in larval rearing systems: immunostimulants and probionts should be studied to make larval cultures less susceptive to disease.
- Larviculture zootechniques: Optimizing various zootechnical parameters in batch culture, such as larval rearing density, interaction between larval rearing density and live food density, effect of water management on water quality parameters, should be studied and improved. In addition, a recirculating system should be evaluated as an alternative to the batch culture system.

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EFFECTS OF DIETARY PHOSPHOLIPID LEVELS ON REPRODUCTIVE PERFORMANCE OF CHINESE MITTEN CRAB (*ERIOCHEIR SINENSIS*) BROODSTOCK

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Introduction

In recent years, mitten crab has become a commercially important species in China. Farming techniques have largely been developed through trial and error, with relatively little scientific support.

Reproductive performance and larval quality are of primary importance for the success of the hatchery phase of any species. It is well known that crustaceans are incapable or have a limited ability to biosynthesize phospholipids (PL) de novo (Teshima and Kanazawa, 1979; Shieh, 1969). The aim of this study was to investigate the effects of dietary PL levels on broodstock reproductive performance and larval quality of *E. sinensis.*

Materials and methods

5 × 40 female crabs (BW 95-120g, GSI 1-2%) were selected in September, engraved with an identification number, and distributed into 5 indoor concrete tanks at a density of 5.m^{-2}. The crabs were cultured in fresh water at ambient temperature and fed with semi-purified compound diets containing 0.3, 1.5, 2.7 and 3.9% PL over winter; trash fish (*Chaeturichthys stigmatia*) was fed to the control. After six months of feeding, the rearing water salinity was gradually increased to 20ppt, and male crabs were introduced into the culture tanks (female:male 3:1). After mating, spawning occurred within approximately 20 days.

The crabs were first sampled after two months of feeding: the gonadosomatic (GSI) and hepatosomatic (HSI) index of 10 crabs from each treatment was determined. A second sampling was done after spawning (six months feeding): relative fecundity, total egg mass, and egg diameter were determined. The percentage of crabs spawning in each treatment was determined as the percentage

of spawners per number of survivals upon mating. Egg samples were subsequently hatched in vitro and the hatching rate and total larval production was determined.

Significance of difference among treatments was determined using one-way ANOVA. Tukey's multiple range test was applied to detect significant differences between means ($p<0.05$). Percentage data were square root and then arcsin transformed prior to analysis.

Results and discussion

During their ovarian development, brood crabs first accumulate plenty of energy and nutrients in the hepatopancreas from exogenous feeds, and later transfer these to the ovaries via the haemolymph and thus the developing embryo (Harrison, 1990). After two months feeding, the HSI of the crabs had decreased from 10% to about 6%; no significant differences in HSI were observed between the treatments with different dietary PL levels. On the other hand, the GSI increased dramatically from 1.8% to 8-10%. 2.7% dietary PL resulted in a significantly higher GSI than 0.3% PL, indicating that PL may facilitate transport of lipids to the gonads, resulting in higher GSI (Fig. 1).

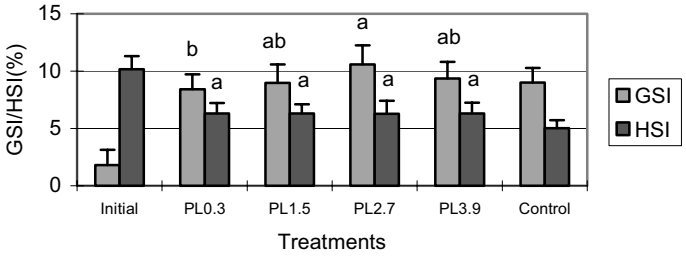

Fig. 1. GSI and HSI of *E. sinensis* after being fed with semi-purified compound diets containing different phospholipid levels for 2 months.

The percentage of crabs spawning was higher (80-90%) when receiving compound diets than when fed trash fish (control, 60%; Fig. 2) in which 1.5% PL and 3.9% PL treatments resulted in faster spawning (majority of the crabs spawned within 10 days after spawning). The 2.7% PL treatment performed slightly worse due to higher mortality in this period.

Other egg and larval quality criteria were not significantly different between the treatments (Tables I and II). Fecundity seemed slightly higher in treatments 2.7% PL and 3.9% PL. Total egg mass weight and egg diameter were lowest for the control. From Table I it is clear that eggs could be hatched in vitro (at a salin-

ity of 20ppt and 22°C). Egg hatching rate for females fed compound diets was about 50%, whereas only 10% for the control. The total number of zoeae produced per crab was however highest in the control.

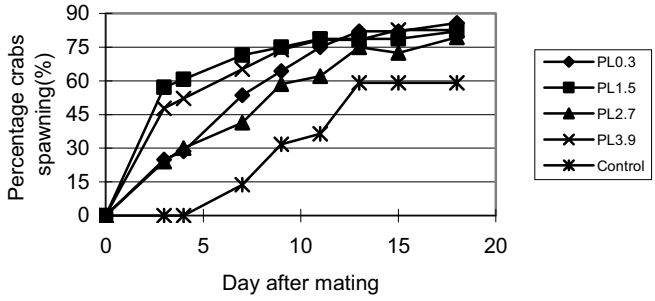

Fig. 2. Spawning percentage of *E. sinensis* fed with semi-purified compound diets containing different phospholipid levels.

Several studies investigated the response of broodstock of crustaceans to dietary PL. Results are however not always clear (Alava et al.; 1993; Cahu et al., 1994; Cavalli et al, 2000). In the current study, a dietary PL level of 2.7% resulted in the highest GSI after 2 months feeding. There were also indications that crabs fed diets containing higher PL levels (PL1.5 and PL3.9) spawned earlier, but results were somewhat contradictory. Although with increasing dietary PL level, also egg mass weight, fecundity and relative fecundity were slightly higher, no significant differences were obtained due to the high variation among the replicates (see Table I). Similarly, there were no significant differences between treatments in total weight, total number and individual DW of zoea I, although higher PL levels on average performed better than lower PL levels (see Table II). How reproductive performance and egg and larval quality correlates with PL composition of the hepatopancreas, gonads, eggs and larvae will be demonstrated after chemical analysis.

Table I. Total egg mass weight, fecundity, relative fecundity and egg diameter of E. sinensis fed with semi-purified compound diets containing different phospholipid levels.

Treatment (# of samples)	**Egg mass Weight (g)**	**Fecundity ($\times 10^4$ eggs. female^{-1})**	**Relative fecundity (eggs.g^{-1} female)**	**Egg diameter (μm)**
PL0.3 (10)	12.55 ± 1.88 [a]	33.67 ± 8.32 [a]	2861 ± 586 [a]	350.81 ± 13.60 [a]
PL1.5 (10)	14.24 ± 1.89 [a]	33.94 ± 4.89 [a]	2907 ± 464 [a]	342.56 ± 8.71 [a]
PL2.7 (9)	14.59 ± 1.86 [a]	39.88 ± 9.18 [a]	3339 ± 431 [a]	344.53 ± 3.79 [a]
PL3.9 (10)	14.70 ± 1.76 [a]	41.64 ± 8.36 [a]	3576 ± 614 [a]	341.77 ± 14.52 [a]
Control (8)	12.15 ± 4.26	40.90 ± 16.77	3397 ± 950	331.33 ± 10.05

Table II. Average egg hatching rate and total number, total wet weight and average individual dry body weight of zoea I produced from *E. sinensis* broodstock fed with semi-purified compound diets containing different phospholipid levels

Treatment (# of samples)	Average hatching rate (%)	Total number of zoea I ($\times 10^5$.female^{-1})	Total wet weight of zoea I (g.female^{-1})	Individual dry body weight of zoea I (μg)
PL0.3 (8)	53.63 ± 11.73 [a]	3.53 ± 1.55 [a]	44.75 ± 15.68 [a]	13.77 ± 1.95 [a]
PL1.5 (6)	54.83 ± 12.50 [a]	4.12 ± 0.34 [a]	44.67 ± 5.58 [a]	13.88 ± 1.22 [a]
PL2.7 (7)	51.40 ± 11.78 [a]	3.93 ± 1.03 [a]	47.71 ± 12.55 [a]	14.50 ± 1.24 [a]
PL3.9 (6)	50.29 ± 16.16 [a]	3.65 ± 0.65 [a]	45.33 ± 12.01 [a]	15.03 ± 0.76 [a]
Control (6)	9.69 ± 3.25	5.41 ± 1.47	66.67 ± 7.37	13.57 ± 0.71

Conclusions

Feeding increasing levels of dietary PL did not significantly affect the reproductive performance and larval quality of *Eriocheir sinensis*. The PL requirement of *E. sinensis* broodstock, may be satisfied in commercial feeds through the inclusion of ingredients containing some endogenous PL.

Acknowledgements

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NON-LETHAL HEAT SHOCK PROTECTS *ARTEMIA FRANCISCANA* AGAINST VIRULENT VIBRIOS

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Introduction

The present study examines sub-lethal heat shock (HS) response in *Artemia franciscana* larvae with the aim to refine HS protocols for *Artemia* nauplii exposed to pathogenic bacteria. Gnotobiotically grown *Artemia* (Marques et al., 2004) were exposed to several HS conditions and parameters such as HS temperature, duration of HS and recovery period were optimized. Subsequently nauplii were challenged with *Vibrio campbellii* (PN9801), a luminous *Vibrio* strain described by Soto-Rodriguez et al. (2003) as a strong pathogen for *A. franciscana* nauplii.

Materials and methods

In the first experiment, HS intensity was tested by exposing axenically-hatched *Artemia* nauplii (Instar II; Sorgeloos et. al., 1986) to a series of different HS temperatures from 28-32°C, 37°C, and 40°C for 30min, with a subsequent recovery period of 6h and 24h. Non-heat-shocked nauplii were used as a control in all experiments. The challenge test performed in this experiment was modified from Marques et al. (2005); i.e., the bacterial suspension containing pathogenic *V. campbellii* were added at an inoculation density of 10^7cfu.ml^{-1} and incubated at 28°C for 24h with constant agitation and lighting. All nauplii used in this study were not fed throughout the experiment. This protocol and the associate gnotobiotic culture system allowed the nauplii to ingest the pathogen as soon as their mouth opened. *Artemia* survival was determined after 24h of exposure by counting live nauplii. The best HS temperature (with the highest *Artemia* larval survival) was selected to perform the following experiments.

Experiments 2 and 3 involved testing of recovery periods and required time of HS exposure leading to the protective effects of *Artemia* larvae towards infection. Axenically hatched *Artemia* nauplii were given a sub-lethal HS of 37°C (best temperature adapted from previous experiment) for 30min and recovery

periods tested were 2h, 6h, 12h, and 24h at incubation temperature of 28°C between heat treatments and challenge test. The ideal recovery period was determined for use in experiment 3. Duration of HS was investigated at various HS time of 15, 30, 45, and 60min, respectively. The animals were given a 6h recovery period (best recovery period adapted from experiment 2 and subsequently challenged with 10^7cfu.ml^{-1} of *V. campbellii*. *Artemia* survivals were determined after 24h of challenge test. All experiments were performed twice to verify the reproducibility of results and each treatment was tested in three replicates.

Artemia larval survivals were determined after 24h of challenge test in all experiments by counting the number of swimming animals. Live nauplii were collected and fixed in Lugol's solution for easy counting. Values of larval survival (%) were arcsin-transformed to satisfy normal distribution and homocedasticity. Differences on HS and non-HS larval survival in *Artemia* challenge test were investigated by performing analysis of variances (ANOVA) using statistical analysis software SPSS® version 11.5 for Windows®.

Results and discussion

Artemia nauplii that were given sub-lethal heat treatments performed better in the challenge test as shown in Table 1. Survival of nauplii was significantly higher in all treatments of HS32, HS37, and HS40, as compared to the controls (non-HS *Artemia*) with subsequent recovery period of 6h. However, high mortality and no significant differences were observed in the challenge test when nauplii were given a sub-lethal HS with 24-h recovery period. Based on the results, it was also revealed that a sub-lethal HS from 28°C to 37°C for 30min gave the best survival rates and was thus chosen for use in experiment 2 and 3.

Table 1. Experiment 1 average survival (mean ± SD) of *Artemia* nauplii after 24h challenge test using 10^7cfu.ml^{-1} of *Vibrio campbellii* in relation to different heat shock (HS) temperatures for 30min exposure with subsequent 6h and 24h recovery period. A and B are duplicates, and different superscripts are significantly different ($p>0.05$).

HS treatments (°C)	A		B	
	Survival (%)		Survival (%)	
	6h recovery	24h recovery	6h recovery	24h recovery
CTRL 28	36.00 ± 4.00^a	14.00 ± 5.29^a	38.00 ± 6.00^a	16.67 ± 6.11^a
HS 32	64.67 ± 2.31^b	17.33 ± 4.16^a	62.67 ± 4.62^b	18.00 ± 5.29^a
HS 37	70.67 ± 7.02^b	28.00 ± 8.72^a	71.33 ± 2.31^b	24.00 ± 5.29^a
HS 40	68.00 ± 8.00^b	26.67 ± 10.07^a	63.33 ± 1.15^b	21.33 ± 7.57^a

Artemia nauplii that were given a recovery period of 2h and 6h after heat treatment showed significantly higher survival rates in the challenge test as compared to the controls (Table 2). Survival rates were observed to decline in relation to

prolonged recovery periods in both HS and the controls treatments. Conditions of 6h recovery period (with bigger differences in survivals between HS and CTRL) were chosen for use in experiment 3.

Table II. Experiment 2 average survival (mean±SD) of *Artemia* nauplii after 24h challenge test using 10^7cfu.ml^{-1} of *Vibrio campbellii* in relation to different recovery periods with sub-lethal HS temperature of 37°C for 30min. A and B are duplicates, and different superscripts are significantly different (p>0.05).

Recovery period (h)	A		B	
	Survival (%)		Survival (%)	
	CTRL	HS	CTRL	HS
2h	33.33 ± 3.06^{a}	64.67 ± 5.03^{a}	37.33 ± 2.31^{a}	60.00 ± 2.00^{a}
6h	29.33 ± 4.62^{a}	62.67 ± 6.43^{a}	30.00 ± 6.00^{a}	65.33 ± 6.11^{a}
12h	24.67 ± 4.16^{a}	45.33 ± 5.05^{b}	25.33 ± 5.03^{a}	42.67 ± 2.31^{b}
24h	9.33 ± 4.16^{b}	30.00 ± 2.00^{c}	12.67 ± 1.15^{b}	18.00 ± 7.21^{c}

Significant differences in challenge survival were observed between HS and CTRL nauplii in both experiments (Table 3). However, data revealed that the duration of HS does not have a big impact although a minimum of 15min HS is required to induce immunopotential effects. The HS duration of 30min (with the highest survival rates) was chosen as control treatment for the next experiment.

Table III. Experiment 3-average survival (mean ± SD) of *Artemia* nauplii after 24h challenge test using 10^7 cfu.ml^{-1} of *Vibrio campbellii* in relation to different heat shock duration with sub-lethal heat shock temperature of 37°C and 6h recovery period. A and B are duplicates, and different superscripts are significantly different (p>0.05).

HS duration (min)	A	B
	Survival (%)	Survival (%)
CTRL	36.00±4.00^{c}	32.67±3.06^{c}
15 mins	66.67±10.07^{b}	64.00±4.00^{b}
30 mins	76.67±6.43^{a}	74.00±5.29^{a}
45 mins	76.00±4.00^{ab}	70.67±7.57^{ab}
60 mins	74.00±8.72^{ab}	65.33±5.03^{ab}

Pioneering work towards the study of heat shock proteins (HSPs) in *Artemia* by Miller and McLennan (1988) and later on by Frankenberg et al. (2000), Clegg et al. (2000) and MacRae (2003) demonstrated that HS proteins could be induced in *Artemia* larvae by application of sub-lethal heat treatment. Based on these results, it is postulated that intracellular HSP production induced during sub-lethal HS boost protections of *Artemia* against deleterious bacteria. The *Artemia* gnotobiotic experimental system allowed careful control of the experimental system and hence, investigate the factor that are important to boost the protection i.e. type of Vibrios, strains of *Artemia* and the influence of nutritional factor.

Further study on investigating in depth the immunopotentiating role of non-lethal HS and/or HSPs in *Artemia* would be performed.

Conclusion

Heat-shocked *Artemia* were less susceptible and more resistant to pathogenic *Vibrio* compared to non-heat shocked individuals, as survival rates were significantly higher after challenge test. A sublethal hs of 37°C for 30 minutes with a subsequent recovery period of 6 hours at 28°C would be ideal to boost the protective effects of the *A. franciscana* nauplii.

Acknowledgements

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CHANGES IN ATLANTIC COD (*GADUS MORHUA*) SPERM QUALITY WITH TIME

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Introduction

The knowledge of sperm biology remains limited in cod, despite the development of its aquaculture. Ageing of sperm has been reported in many fish species and it affects sperm quality as the reproductive period progresses. This paper aims at settling tools used to study sperm biology in cod and at investigating changes in sperm quality in relation with time.

Materials and methods

Hatchery-reared cod (mean weight±SD: 2.6±0.6kg) were maintained in 15-m^3 indoor tanks and subjected to the natural variation of photoperiod and to a chilled cycle of temperature (7-14°C). Sperm were sampled by stripping ripe males during the spawning period of the females (December 20 - March 20). Faeces and urine were carefully discarded. Sperm concentration was determined after dilution (2µl:1998µl distilled water) both by counting on Malassez cells and by absorbance (Uvikon 923) at 260nm. The percentage of motile spermatozoa was estimated using a 2-step procedure: (1) 2.5µl sperm in 50µl of a non-activating medium (1/3 seawater and 2/3 freshwater) and (2) 2.5µl diluted sperm in 80µl of an activating medium (seawater+2% BSA). In order to determine the minimum spermatozoa:egg ratio required to obtain maximum fertilisation rates, 2 egg batches and 3 milt collections were used after stripping. Triplicate samples of 1ml ova were inseminated in 30-ml beakers with increasing sperm:egg ratios (from 2000:100 000) and 0.5ml of seawater at 8°C was added. After 5 min, the beakers were filled with seawater. Fertilisation rates were assessed after 6 hours as the number of 4-cell stage eggs × total number of $eggs^{-1}$.

In order to estimate individual (magnetic markers inserted in cod) changes in sperm quality with time, males were sampled at the beginning, middle, and end

of the female spawning period. Sperm quality was estimated by measuring spermatocrit (% packed cells after centrifugation at 10 000rpm for 10 min), sperm concentration, percentage of motile cells, short-term storage capacity of undiluted sperm at 4°C for 48h, and composition of seminal fluid after centrifugation at 8000rpm for 10 min: pH (Tacussel, Minisis 8000), osmotic pressure (Roebling), protein (Lowry), and ion concentrations (Synchron EL-ise).

Data were expressed as mean±SD. After angular transformation, data were compared using one- or two-way ANOVA. A repeated design was used to compare changes in sperm quality with time. Significant differences were determined using a Tukey a posteriori test.

Results and discussion

A highly significant correlation (r^2=0.993) was observed between sperm concentration estimated on Malassez cells and absorbance (Fig. 1). This result provides a simple and rapid technique to assess sperm concentration at a 1:1000 dilution. A significant decrease (P<0.05) of sperm motility was observed after 168h storage at 4°C. This decrease was significantly reduced in sperm samples diluted (1:9) in the non-activating solution (Fig. 2). 10 000-50 000 spermatozoa were required per egg in order to obtain maximum fertilisation rate. This requirement is between those for turbot (*Psetta maxima*: 6000) and seabass (*Dicentrarchus labrax*: 66 000) (Fauvel et al., 1999; Suquet et al., 2000). In order to test the quality of sperm samples, a discriminating sperm:egg ratio of 5000:10 000 must be used in cod.

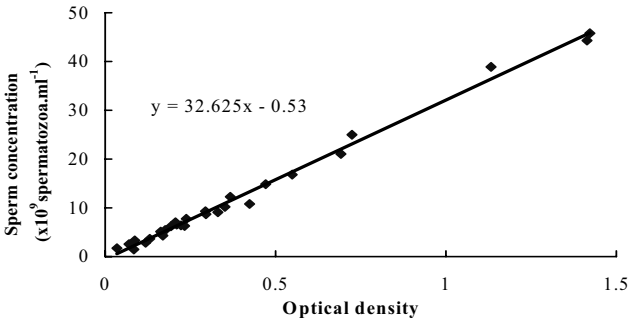

Fig.1. Correlation between optical density observed at 260 nm and spermatozoa concentration assessed using Malassez cells.

Significant changes were observed in storage capacity at 4°C with time, osmotic pressure, pH, and protein, Na, Cl, and Ca concentrations of the seminal fluid, but not in spermatocrit, sperm motility, and CO_2 contents (Table I). Sperm concen-

tration increased and then decreased in opposition to Rakitin et al (1999), who observed a continuous increase as the season progressed.

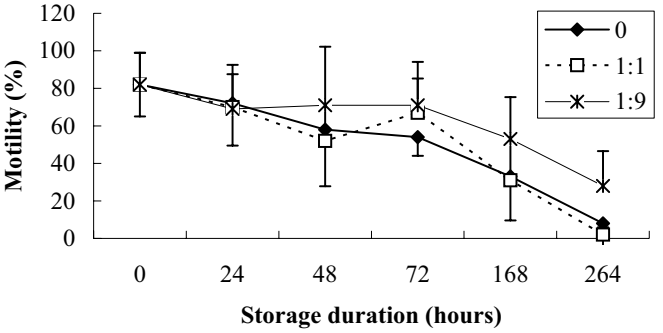

Fig. 2. Changes in percentage of motile spermatozoa in relation with storage duration at 4°C and sperm dilution (n=6 males).

Table I. Changes in seminal fluid quality with time (Treatments with different letters are significantly different; n=10 males).

Parameter	Beginning	Middle	End	Statistics
Spermatocrit (%)	35.5±9.4	42.0±11.0	40.4±8.9	NS
Sperm concentration ($\times 10^9$spermatozoa.ml^{-1})	4.49±1.47 ^{a}	8.67±1.99 ^{b}	5.43±1.60 ^{a}	P<0.001
Sperm motility (%)	43±19	52±25	41±15	NS
Storage (% motile, 48h)	64±22 ^{a}	56±39 ^{a}	23±35 ^{b}	P<0.05
Osmotic pressure	368.2±8.9^a	384.1±11.1^b	362.9±15^a	P<0.01
pH	7.87±0.17^a	8.05±0.26^b	8.41±0.21^c	P<0.001
Protein (mg.ml^{-1})	1.37±0.59^a	1.15±0.50^{ab}	0.94±0.42^b	P<0.05
Na (mmol.l^{-1})	183.1±15.1^a	196.7±10.1^b	186.7±11.9 ^{b}	P<0.05
Cl (mmol.l^{-1})	175.0±6.4^{ab}	179.3±6.1^a	171.0±7.2^b	P<0.05
CO_2 (mmol.l^{-1})	8.6±1.7	9.5±1.4	9.8±2.0	NS
Ca (mmol.l^{-1})	2.14±0.41^a	2.81±0.45^b	2.74±0.61^b	P<0.001

In conclusion, changes in cod sperm quality were observed with time.

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MINERAL REQUIREMENTS IN THE YOUNG SWIMMING CRAB *PORTUNUS PELAGICUS*

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Introduction

Swimming crab (*Portunus pelagicus*, Linnaeus, 1758) is recognised as potential species for commercial culture. The culture techniques have been developed in Thailand, including research on feed and its nutritional requirements to promote commercial culture of swimming crabs. This study was aimed to investigate suitable levels of mineral mixture in crab diet for standardizing complete feeds.

Materials and methods

Mineral complex based on Davis and Lawrence (1997) was supplemented in the basal diet at 0, 1, 2, 3, and 4% in experimental diets namely diet 1, 2, 3, 4, and 5, respectively. The composition of the mineral mixture was in the ratio of KH_2PO_4 (1.0): $CaHPO_4.2H_2O$ (1.0): $NaH_2PO_4.2H_2O$ (1.5): KCl 0.5. The measured values (AOAC, 1990) of protein and lipid in basal diet were 33-34% and 13-15%, respectively. The young swimming crabs *(P. pelagicus*) at initial weight of 0.04 grams were cultured for 10 weeks in five 300-l fibreglass tanks which were each divided into 20 compartments to provide 20 replications. Crabs were stocked individually in each compartment. Rearing water was exchanged daily by pumping water every 15min via PVC pipe along the bottom of each culture tank with a sand substrate. Aeration was supplied via 4 air stones per tank. Crabs were fed experimental diets at satiation level 3× a day at 9:00am, 1:00pm, and 5:00pm. Molting of crab was recorded daily and individual weights of crabs were determined weekly to determine survival rate, growth, and molting frequency. At termination, crabs were freeze-dried and subjected to proximate analysis. Results were analyzed by a one-way ANOVA (Sokal and Rohlf, 1981) and significant differences determined by a Tukey multiple comparison test.

Results and discussion

The dietary mineral mixture significantly affected growth of young crabs from week 9 onward ($p<0.05$). Diet 5 resulted in the highest weight gain (23 636%), followed by diet 4 (23 193%), diet 3 (22 835%), diet 2 (18 137%), and diet 1 (14 525%). Similar trends were observed in shell width and length. This increased weight gain, shell length, and width along with increasing level of mineral mixture in diets showed the important role of dietary minerals for crab growth. Growth of crustaceans occurs through molting, and its regulation is involved in metabolism and water-mineral balance. In the present study, the best growth of crab was in the highest dietary mineral mixture, revealing the better balance of minerals for molting process. In addition, crabs fed 4% mineral mixture had significantly higher protein and lipid levels in their tissue: 34% and 5%, respectively ($p<0.05$).

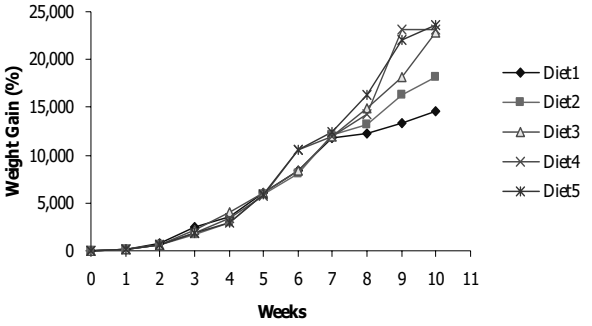

Fig. 1. The effect of mineral mixture supplemented in diet on weight gain (%) of young swimming crab *(Portunus pelagicus)*.

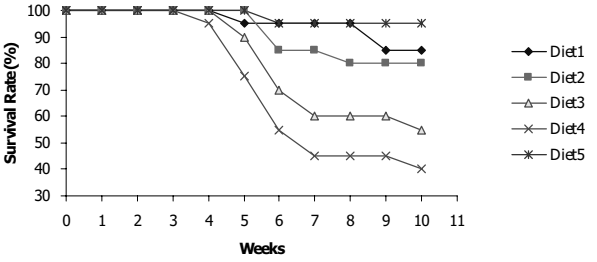

Fig. 2. The effect of mineral mixture supplemented in diet on survival rate (%) of young swimming crab *(Portunus pelagicus)*.

Crab fed diets 3 and 4, which are larger than those fed diets 1 and 2, died during molting. Although crabs fed diets 1 and 2 were able to molt, their growth was lowest, perhaps due to the limited mineral in their diet. The higher mortality in crabs from diets 3 and 4 suggests higher energy requirements and a higher risk to larger crabs for molting, since this process requires both energy (Sheen and Wu, 1999) and minerals.

Table I. Results of the growth, survival rate and molting frequency of young swimming crab fed with 5 different levels of dietary mineral mix for 10 weeks. Values are the average of three replicates. (mean±SD)

Diets	Mineral mixed (%)	Mean body weight (g.crab^{-1})		Weight gain (%)	Survival rate (%)	Width (cm)	Length (cm)	Total Molting (times)
		Initial	10 weeks					
1	0	0.044 ±0.005	6.382±1.515^{b}	14525.29±2724.17^{b}	85	4.423±0.351^{b}	2.276±0.183^{b}	3.30±1.22^{a}
2	1	0.044 ±0.005	7.841±2.215^{b}	18137.50±4720.99^{b}	80	4.764±0.478^{b}	2.431±0.220^{b}	3.40±1.79^{a}
3	2	0.044 ±0.005	10.317±1.859^{a}	22835.91±4511.12^{ab}	55	5.247±0.408^{a}	2.632±0.186^{ab}	3.20±1.61^{a}
4	3	0.044 ±0.005	10.308±1.717^{a}	23193.13±5223.75^{ab}	40	5.335±0.340^{a}	2.681±0.123^{a}	3.15±1.35^{a}
5	4	0.044 ±0.005	10.466±2.133^{a}	23636.58±4690.53^{a}	95	5.306±0.419^{a}	2.672±0.198^{a}	3.85±1.27^{a}
	ANOVA							
P			<0.001	<0.001		<0.001	<0.001	0.208
F			3.749	1.88*10^{7}		0.167	0.037	1.501

Table II. Mineral profile (mg.g^{-1}) of experimental diets and young swimming crabs fed with mineral mixture supplemented diets for 10 weeks.

Sample		Na	Mg	K	P	Ca	Cr	Fe	Mn	Co	Cu	Zn	Se	Cd
Diet	1	8.19	3.95	4.42	2.68	19.3	1.67	28.9	67.2	0.20	7.10	39.9	N.D.	0.43
	2	8.84	3.48	4.68	2.67	18.3	1.57	28.0	62.7	0.19	6.67	41.4	N.D	0.39
	3	9.63	2.26	5.78	1.98	21.4	1.71	25.6	61.0	0.17	6.64	60.4	N.D	0.40
	4	10.5	3.76	6.53	3.73	22.2	1.27	26.5	58.8	0.22	6.59	39.6	N.D	0.38
	5	11.2	3.76	7.73	4.18	22.3	1.69	24.0	60.9	0.19	6.64	39.6	N.D	0.40
Crabs	1	15.5	15.6	2.78	1.40	216	1.57	7.24	43.5	0.31	15.3	27.0	N.D.	0.28
	2	17.8	12.1	2.97	1.16	211	1.28	1.30	41.8	0.29	17.1	36.4	N.D	0.34
	3	15.9	10.8	2.94	1.25	208	1.08	2.34	45.5	0.38	16.6	33.3	N.D	0.32
	4	16.9	10.7	3.06	1.26	186	0.78	0.69	26.7	0.28	45.1	46.1	N.D	0.36
	5	14.0	10.7	4.74	1.56	205	0.84	0.90	43.6	0.34	14.4	34.2	N.D	0.29

Catacutan et al. (2003) noted that mud crab, *Scylla serrata*, lost minerals during molting, seen by a high capacity to digest crude ash in feed. Analysis showed that the major mineral deposited in whole crabs was Ca, which ranged from 186-216mg.g^{-1}. The minerals in culture water may not be sufficient for molting and growth, therefore young *P. pelagicus* require a dietary mineral mixture to compensate for minerals lost during molting and required for shell formation. From the overall results, diet 5 with minerals mixed in diet up to 4% contributed to the best growth performance, survival, and molting.

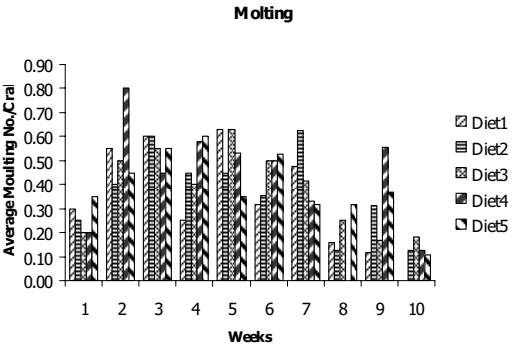

Fig. 3. The effect of mineral mixture in the diet on molting frequency of young swimming crab *(Portunus pelagicus)*.

Conclusions

This study helps understand the role of minerals in young crabs for molting success, molting frequency, and, consequently, growth and survival rate. In addition, the best level of dietary mineral mix for young crab is 4%.

Acknowledgements

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PERSPECTIVES OF LARVAL FEEDING AND NUTRITION: TOOLS AND CONCEPTS

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Larval feeding and nutrition were the major bottlenecks to the mass production of marine fish until the 1960s when rotifers (*Brachionus plicatilis*) and the brine shrimp (*Artemia salina*) were introduced. These zooplankters were relatively easy to culture and enrich with essential nutrients for marine fish larvae.

Watanabe's seminal studies in the 1970s laid the foundations and the initial tools for marine larval nutritional research, particularly in the area of live food enrichment. As a result of marine teleosts' inability to synthesize DHA and EPA from shorter chain precursors, these fatty acids had to be supplemented in the live food enrichment. This led to dramatic improvements in larval growth, survival, neural development, and resistance to handling stress. The importance of dietary supplementation with arachidonic acid was demonstrated only recently. This n-6 HUFA was shown to be associated with improved growth and survival, apparently by modulating the stress response as well as larval osmoregulatory capacity.

The free amino acid (FAA) pool in tissues provides the essential building blocks for the synthesis of structural and functional proteins. In the developing egg and larvae of marine fish, the role of FAA appears multifaceted and changing. They serve as osmotic metabolites during oocyte hydration, which leads to egg buoyancy. During egg development, FAAs provide an energy substrate in the first hours after fertilization. In our studies we demonstrated that the inclusion of FAAs – such as glycine, alanine, and arginine – in microdiets (MD) stimulate appetite and improve their ingestion.

Lately we demonstrated that live feed ingestion is associated with the release of gastrointestinal hormones such as bombesin and cholecystokinin (CCK). In first-feeding herring larvae, it is likely that FAAs signal a cascade of endocrine events that control their digestion. When these larvae were tube-fed with selected FAAs together with the soluble protein BSA, CCK synthesis and digestive tryptic activity increased five-fold!

The interest in maximizing larval performance through increasing the rate of metamorphosis was tested by Lam and his group who utilized thyroid and cortisol treatments, which stimulated an increase in the digestive enzyme response. Similarly we showed that supplementing MD with digestive enzymes improved the larval digestive process. These processes can be presently identified as well as better understood and quantified using various new molecular tools. These include expression vectors such as the mRNA of various digestive enzymes whose magnitude could be detected and measured. Furthermore, these expression vectors can help localize the digestive processes by using in situ hybridization methods.

MD research hasn't yet produced an artificial diet for larval marine fish for complete replacement of live feeds. Based on new molecular tools and a deeper understanding of the mechanisms involved in appetite stimulation, digestion, and absorption, very promising strides are presently made around the globe toward the complete replacement of live feed by MD.

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GENETIC EFFECT OF SELECTIVE DOMESTICATION PRESSURES ON PACIFIC OYSTER LARVAL STAGE

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The Pacific oyster, *Crassostrea gigas*, is the most economically important bivalve produced worldwide. Most production relies on collection of spat from the wild. However, hatcheries and nurseries play a growing role in the production of spat. These impose specific artificial rearing conditions, especially at early stages of development. The larval stage is relatively poorly known, particularly in a domestication context. We aimed to examine the consequences of the involuntary domestication process resulting from the high variance of reproductive success at the larval stage, the keystone of the life cycle. We studied two types of specific selective processes that are common in hatcheries: the effect of discarding the smallest larvae on genetic diversity and the effect of temperature (20 versus 26°C; i.e., wild versus hatchery conditions).

In order to monitor the effect of the selection of fast growing larvae by culling, growth variability and genetic diversity were studied in a larval population bred from a factorial cross. We used a mixed-family approach to reduce potentially confounding environmental bias. The retrospective assignment of individuals to family groups was performed using a set of three multiplexed microsatellite markers. Two different rearing batches were raised in parallel. For the first, the smallest larvae were progressively discarded by selective sieving in three replicated 50-l tanks. For the second, in three other tanks, no selective sieving was performed. The intensity of selective sieving was adjusted so as to discard 50% of the larvae in a progressive manner over the whole rearing period. When the larvae reached the pediveliger stage, ready-to-settle larvae were sampled for genetic analysis.

To study the effect of temperature, we used a similar mixed-family approach. The progeny from a factorial cross was divided between three replicated 50-l tanks maintained at 26°C and three others at 20°C. Maximum size variability

was maintained for this experiment (i.e., no culling). Individual growth measurements were performed at days 22 and 30 after fertilization for larvae genetically assigned to their family in both temperature conditions. We also recorded individual growth of genotyped juvenile oysters (80 days after fertilization).

At the phenotypic level, relative survival and settlement success were higher for sieved batches of larvae. Culling appears to be a time-saving procedure associated with better relative survival of larger larvae. At the genetic level, we observed different parental contributions in the ready-to-settle cohorts (early versus late). This is consistent with previous results and confirms the existence of significant genetic variability for early developmental traits in the Pacific oyster. However, because of these differential contributions, discarding the smallest larvae can also lead to a significant loss of diversity at the larval stage. The early-settled cohorts exhibited lower values of effective population size than those settled late, which would be discarded in the case of the selective sieving.

Temperature exerted a phenotypic and genetic effect at larval and spat stages. Genetic variability for growth was expressed earlier during development in the batches raised at 26°C than those at 20°C. Temperature also interacted with spat growth. In the 26°C treatment, juvenile size was similar between early and late settled cohorts but at 20°C, size in the early settled cohorts remained higher. This partly supports previous studies where a positive correlation was observed between larvae and spat growth.

These results show that our mixed-family approach, combined with microsatellite-based family assignment, is a powerful tool for the study of genetics of bivalve larvae. They also demonstrate that genetic effects of intensive rearing conditions are significant and should be taken into account in hatchery practices.

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BRINE SHRIMP *ARTEMIA* PERFORMANCE FOR AMELIORATING LARVAL SURVIVAL RATE AND METAMORPHOSIS TIME OF SWIMMING CRAB (*PORTUNUS PELAGICUS*)

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Introduction

In Vietnam currently, there have been successful firstly for commercial aquaculture of the swimming crab (*Portunus pelagicus*) from artificial seedlings at the risked shrimp farm sections. Of the key techniques for raising hatchery larvae acquiring the high qualify seedlings determined was the feed regime for larval stages. *Artemia* nauplii that cultured biomass by some of the shrimp formulated feeds as Dry Algae, Lansy, and Frippak has played an important nutritional role for ameliorating and sustaining survival rate and metamorphosis time of the experimented larvae fed on those. It was proved that the outcomes of brine shrimp *Artemia* performance seemed to be a clue of the created technological process that being applied effectively for hatchery swimming crab seedlings in 7 provinces.

Materials and methods

Berried females of *P. pelagicus* that were collected off Nha Trang, Khanh Hoa province on the central seawaters Vietnam had weight of 100-300g.individual^{-1} and their egg color in bright. They were kept in the incubation tanks for 4 or 7 days, thereafter newly hatching larvae were transferred into the open-seawater rearing tank system of volume 1m^3.tank^{-1} with stocking densities of 120.l^{-1}.

Water for hatchery experiments was from natural seawater source that pumped up and stored in the treatment tank for 7-10 days before filtered through the sand-filter system. Variance of the environmental parameters in the tank systems was tested everyday.

Artemia nauplii for feeding larvae were cultured by different feeds including fresh algae, juice of minced prawn and cockle meat for biomass cultured group 1(G1), and Dry Algae, Lansy, and Frippak for biomass cultured group 2(G2).

The processed feed (PF) was made from the blended meat of prawn, mackerel, cockle and chicken eggs, and then steamed up well-cooked. There were two treatments of feed regimes for reared larvae set up into 5 replicates for each. At the stage zoae 1 and 2: all of them fed on Dry Algae, Lansy, Frippak with 3g.tank^{-1} 5 times daily and newly hatched *Artemia* nauplii at 4-5nauplii.ml^{-1} once daily. At the stage zoae 3 and 4 : the first treatment larvae fed on PF with 5g.tank^{-1} 3 times daily and G1 of 5 cultured days with 3nauplii.ml^{-1} once daily; the second treatment fed on PF with 5g.tank^{-1} 3 times daily and G2 of 5 cultured days with 3nauplii.ml^{-1} once daily. At the stage Megalopae and Crablet : the first fed on PF with 10g.tank^{-1} 3 times daily and G1 of 12-14 cultured days with 3nauplii.ml^{-1} once daily; the second fed on PF with 10g.tank^{-1} 3 times daily and G2 of 12-14 cultured days with 3nauplii.ml^{-1} once daily.

The obtained data were analyzed by bio-statistic method and T-test and carried out using 2001's computer Excel software

Results and discussion

It was different significantly ($P<0.05$) in survival rate, metamorphosis duration of larval stages and larval health between two experiment conditions after the larval stage 2. During 40 experimental days, the environmental main parameters in rearing tank systems were tested and controlled, it was completely suitable for larval growth (Table I). For the first treatment, larvae of almost the rearing tanks had low survival rate due to they were affected diseases that caused by bacteria as *Vibrio, Pseudomonas* and fungi as *Lagenidium* (Table II) led to elongation of 35 metamorphosis days to crablet. While the second, their survival rate from zoae 1 to crablet 1 was averagely 22% and the whole of metamorphosis time was 22 days (Fig. 1).

Table I. Some of the environmental elements determined at the rearing tank system during the experimental time of 40 days.

Water temperature (^oC)	**S‰**	**Dissolved oxygen (mg.l^{-1})**	**pH**	**NH_3 (mg.l^{-1})**	**H_2S (mg.l^{-1})**	**Comments**
25.72 ± 0.31	31.6 ± 1.14	6.74 ± 0.18	8.10 ± 0.10	Mini-track	Mini-track	Hatchery standard

Table II. Some of the disease symptoms identified on larvae in the first experimental treatment tanks.

	Larval stages						Comments
	Zoae1	**Zoae2**	**Zoae3**	**Zoae4**	**Megalopae**	**Crablet**	
Symptoms	no samples	no samples	weak, starved, and illuminated	weak, starved, and illuminated	weak, incomplete molt, and mass mortality	-	survival rate 2-4%
Metamorphosis duration (days)	3	3	4	7	10	8	too long
Causative agents	*Vibrio*	*Vibrio*	*Vibrio*	*Vibrio*	*Pseudomonas* and *Lagenidium*	-	genus names only

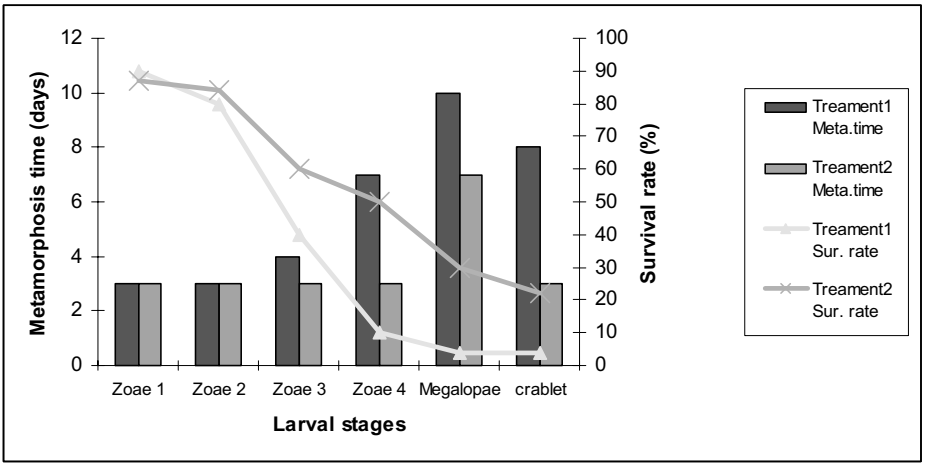

Fig. 1. Differences ($P<0.05$) between the two experimental treatments in metamorphosis time and survival rate from stage zoae 3 to crablet.

Although *Artemia* nauplii that cultured by different feeds were not analysed on nourishing composition, it was different extremely for the experimental larvae fed on G1 or G2. In fact, disease control of fresh algae in culture conditions *Artemia* nauplii is not easy and the kinds of formulated feeds of shrimp larvae do not match in order to utilize for swimming crab larvae, even some of the other crustacean species. Replacement of fresh algae to dry ones and conversion of the quality industrial feeds into brine shrimp *Artemia* for rearing others are of efficient applications.

Conclusions

Brine shrimp *Artemia* has played the prerequisite role for converting the formulated feeds into feeding components for culture swimming crab larvae in order to harvest high qualify seedlings with economic costs. It was based on to establish

the technological process of artificial seed production reaching to 20-25 000 crab juveniles.m^{-3} of the culture tank during 22-24 of reared days.

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IMMUNE RESPONSES AND DISEASE RESISTANCE AGAINST *AEROMONAS HYDROPHILA* IN ROHU (*LABEO ROHITA* HAM.) FINGERLINGS FED ON *MENTHA PIPERITA* (L.) EXTRACT MIXED DIET

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Introduction

The use of immunostimulants has been considered as a prophylactic measure in aquaculture. Plant extracts as immunostimulants have shown advantages as eco-friendly, easier to apply, and less expensive. The present study focused on the immune responses and disease resistance against *Aeromonas hydrophila* in rohu (*Labeo rohita* Ham.) fingerlings fed on *Mentha piperita* (L.) extract mixed diet.

Materials and methods

M. piperita (L.) plant (50g) was crushed and soaked overnight in mixture of 100ml ethanol and 100ml distilled water. The solution was centrifuged to get crude extract free from debris. Treatment diets were prepared by mixing normal diet with the crude extract at doses of 10, 20, and 30ml.100g^{-1} feed, named T_1, T_2, and T_3. Rohu fingerlings were randomly divided into different groups in duplicate (10 fish each). The control group was fed a normal diet, and different diets were fed in separate tanks.

After three weeks of treatment, blood was collected from all fish groups for testing non-specific immune responses. Neutrophil activity of the fish phagocytes followed Nitroblue Tetrazolium (NBT) test (Secombes, 1990) based on optical density (OD) value of the formazan blue solution formed by presence of oxygen species, produced by fish phagocytes, and NBT dye. The OD value was read in the ELISA reader (Labsystems Multiskan MS, Genesis Version: 3.03, Full Mode) at 620nm. Plasma bactericidal activity followed Rainger and Rowley (1993) with bacterial colony forming units (cfu).ml^{-1} counted by the pour plate method on Triptone Soya Broth (TSA). The bactericidal activity of the plasma was expressed as %cfu in the treatment group to that in the control group.

Then the fish were immunized intraperitoneally (ip) with formalin-killed *A. hydrophila* (10^6 cells.fish^{-1}). Agglutination test followed Roberson et al (1990). The antibody titres were expressed as the reciprocal of the highest plasma dilution giving positive agglutination. Subsequently, after two weeks of immunization, all the groups were challenged ip with live *A. hydrophila* (10^6 cells.fish^{-1}). After challenge, all the fish groups were fed on the same normal diet. Mortality of the fish was recorded daily and the tests were repeated after one week of challenge.

Validity was determined by the Student's t-test. $P<0.05$ was considered statistically significant.

Results and discussion

The results showed that there was significant increase in neutrophil activity of the fish phagocytes in the treated groups compared to that in the control group after three weeks of treatment (Table I). It is generally accepted that fish phagocytes, after stimulation by specific agents, are able to generate superoxide anion (O_2^-) and its reactive derivatives, which are considered to be toxic for fish bacterial pathogens (Hardie et al., 1996). The results suggested that *M. piperita* extract on its own was able to enhance the neutrophil activity.

Table I. Neutrophil activity of fish phagocytes and plasma bactericidal activity of experimental rohu fingerlings. Treatments within the column having the same letter(s) are not significantly different. Treatments within the row having the same number(s) are not significantly different ($P<0.05$). N=normal saline.

Groups	After 3 wks of treatment	After 1 wk of immunization	After 2 wks of immunization	After 1 wk of challenge
Neutrophil activity (OD values)				
Control	$1.03^{a} \pm 0.11_{1}$	$1.16^{a} \pm 0.13_{1}$	$1.59^{a} \pm 0.05_{2}$	$1.19^{a} \pm 0.60_{1}$
T_1	$1.54^{b} \pm 0.23_{1}$	$2.27^{b} \pm 0.60_{2}$	$2.38^{b} \pm 0.36_{2}$	$1.29^{a} \pm 0.76_{1}$
T_2	$1.67^{b} \pm 0.11_{1}$	$2.31^{b} \pm 0.46_{2}$	$2.64^{b} \pm 0.46_{2}$	$1.65^{a} \pm 0.36_{1}$
T_3	$1.48^{b} \pm 0.27_{1}$	$2.19^{b} \pm 0.22_{2}$	$2.17^{b} \pm 0.13_{2}$	$1.38^{a} \pm 0.47_{1}$
Plasma bactericidal activity (% bacterial survival)				
N*	100	100	100	100
Control	$71.07^{a} \pm 2.67_{1}$	$66.32^{a} \pm 2.73_{1}$	$61.48^{a} \pm 2.38_{2}$	$61.36^{a} \pm 3.90_{2}$
T_1	$69.70^{a} \pm 6.31_{1}$	$64.49^{ab} \pm 7.98_{1}$	$57.06^{ab} \pm 2.19_{2}$	$55.77^{ab} \pm 1.96_{2}$
T_2	$66.40^{ab} \pm 0.51_{1}$	$61.64^{b} \pm 6.48_{2}$	$56.24^{b} \pm 3.87_{2}$	$53.71^{b} \pm 3.43_{2}$
T_3	$66.07^{ab} \pm 5.09_{1}$	$58.18^{b} \pm 2.45_{2}$	$55.68^{b} \pm 4.19_{2}$	$51.93^{b} \pm 4.33_{2}$

The complement system known as enzyme cascade system, found mainly in plasma and tissue fluids of fish, plays an important role in bactericidal mechanisms (Harrell et al., 1976). In the present study, the significant higher plasma bactericidal activity in T_2 and T_3 groups after immunization suggests that there was existence of a synergistic effect on the plasma bactericidal activity between *M. piperita* extract and the formalin-killed bacteria in the treated fish (Table I).

Specific antibody response – agglutination test

After one week of immunization, there was higher antibody titre to *A. hydrophila* in all the treated groups compared to that in the control group. However, there was no difference in plasma antibody titre to *A. hydrophila* among the treated groups (Table II).

The higher antibody titre to *A. hydrophila* in the treated groups compared to that in the control group suggested that *M. piperita* extract enhanced specific antibody response to *A. hydrophila* antigen in the plant extract treated fish. However, an effective dose and period at which treated fish show highest antibody response remains to be determined through further study.

Table II. Antibody titre against experimental *A. hydrophila* infection of rohu (*Labeo rohita* Ham.) fingerlings. N = normal saline-injected group.

Groups	After 1 week of immunization		After 2 weeks of immunization		After 1 week of challenge	
	Antibody titre	Log_2 antibody titre	Antibody titre	Log_2 antibody titre	Antibody titre	Log_2 antibody titre
N	-	-	-	-	-	-
Control	1:16	4	1:16	4	1:32	5
T_1	1:32	5	1:64	6	1:64	6
T_2	1:32	5	1:64	6	1:64	6
T_3	1:32	5	1:64	6	1:128	7

Challenge study

The result showed that the relative percent survival (RPS) in the control group was lowest compared to those in all the treated groups (Table III).

Table III. Cumulative mortality and relative percent survival (RPS) of rohu (*Labeo rohita* Ham.) fingerlings after challenge with live Aeromonas hydrophila. N = normal saline-injected group.

Groups	Initial stock	Daily mortality of fish after challenge							% mortality	% RPS
		1	2	3	4	5	.....	14		
N	10 × 2	8	6	0	0	0	0	0	70	-
Control	10 × 2	4	6	0	0	0	0	0	50	28.57
T_1	10 × 2	2	4	0	2	0	0	0	40	42.86
T_2	10 × 2	2	2	0	4	0	0	0	40	42.86
T_3	10 × 2	2	2	0	2	0	0	0	30	57.14

Enhanced nonspecific immune responses and disease resistance to furunculosis were found in rainbow trout *Oncorhynchus mykiss* fed a β-glucan product and *S. cervisiae* (Siwicki et al., 1994).Practically, oral application of immunostimulants

appears to be the route of choice in aquaculture. However the metabolism mechanisms of immunostimulants in the gastrointestinal tract are not known. The higher relative percent survival to *A. hydrophila* in the treated groups compared to that in the control group indicated that the *M. piperita* extract enhanced disease resistance to *A. hydrophila* infection in the treated fish.

Conclusion

M. piperita (L) plant extract enhanced neutrophil activity of phagocytes, specific antibody response, and disease resistance against *A. hydrophila* in the treated fish. However, the plant extract showed positive effect on plasma bactericidal activity in the treated fish only after immunization at the doses of 20 and 30ml.100g^{-1} feed.

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EFFECTS OF MICROBIAL COMMUNITIES IN AXENIC CULTURES OF *BRACHIONUS PLICATILIS* SENSU STRICTU

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Introduction

Rotifers (*Brachionus* spp.) have been found to be valuable and indispensable food organisms in the industrial larviculture of fish and crustaceans throughout the world (Lubzens et al., 1997; Lee and Ostrowski, 2001; Liao et al., 2001). In recent years, research has focused on the bioencapsulation of rotifers and other live food organisms with selected bacteria, which can favour the growth and survival of the predating fish larvae. Axenic rotifers were used as a tool for studying the role of specific bacterial strains or microbial communities, in both nutritional and probiotic aspects. Recently, several studies have been directed toward obtaining axenic rotifer cultures either from disinfected resting eggs (Douillet, 1998) or from disinfected parthenogenetic eggs (Martinez-Diaz et al., 2003) by using antibiotic mixtures or different kinds of disinfectants.

The aim of this study is to evaluate the effect of different microbial communities on the rotifer growth performance, using axenic rotifers hatched from disinfected amictic eggs.

Materials and methods

Clone 10 of *Brachionus plicatilis* sensu strictu was used in the study. It was obtained from CIAD (Centro de Investigación en Alimentación y Desarrollo, Mazatlan Unit for Aquaculture) in Mexico.

To feed the rotifers, an axenic inoculum of *Chlorella* sp., strain CCAP 211/76, was obtained from the Culture Collection of Algae and Protozoa (Dunstaffnage Marine Laboratory, Dunbeg) in Scotland. Axenic *Chlorella* was grown in closed 500-ml bottles provided with aeration. The culture was maintained at 19°C, light intensity 4000-5000lux, using a standard Walne medium supplemented with vitamins and 0.22-µm-filtered and autoclaved regular seawater (FASW), which was diluted with tap water to have a salinity of 25g.l^{-1}.

The wild-type strain of baker's yeast (*Saccharomyces cerevisiae*) and its isogenic mutant strain mnn9 were obtained from EUROSCARF (Institute of Microbiology, University of Frankfurt) in Germany. Axenic yeast cultures were grown in sterile Erlenmeyer's on a shaker at 150rpm and 30°C. The culture medium used was YEPD (Yeast Extract Peptone Dextrose) medium, containing yeast extract (Sigma, 1% w/v), peptone bacteriological grade (Sigma, 1% w/v) and D-glucose (Sigma, 2% w/v). This medium was prepared in 25g.l^{-1} FASW.

Two types of microbial communities (MC) were used in the study, isolated either from normal-performing or from crashed rotifer cultures, and utilized under two forms: fresh-isolated or preserved at -80°C and regrown on Marine Agar (MA) before use.

Rotifers (clone 10), hatched from axenic amictic eggs, were used in all experiments. Disinfection of amictic eggs was done using 100ppm of glutaraldehyde with 2h exposure time at 28°C. In total, twelve experiments were conducted (Table I).

Table I. Outline of the experiments on the effect of microbial communities. MC: Live and freshly-collected microbial community. MCR: Live microbial community, which was preserved at -80°C and regrown on MA. Experiments presented in the same row were carried out at the same time.

Axenic – No bacteria	**Xenic 1 – MC from clone 10 normal culture**	**Xenic 2**
Exp. 1.1	Exp. 1.5	MC from L1 crashed culture (Exp. 1.9)
Exp. 1.2	Exp. 1.6	MCR from L1 crashed culture (Exp. 1.10)
Exp. 1.3	Exp. 1.7	MCR from L3 crashed culture (Exp. 1.11)
Exp. 1.4	Exp. 1.8	MCR from clone 10 crashed culture (Exp. 1.12)

Results

Table II represents the population growth rates over five days for each food type. Comparison is made between synchronous experiments with and without addition of MCs (Table III). No significant stimulation of the growth rate by both types of MCs was found ($p>0.05$) in the treatments where *Chlorella* was used as food source, except in experiments 1.1, 1.5, and 1.9. In contrast, differences were seen when the two yeast strains were used as food. Growth rates were significantly improved ($p<0.001$) when MCs from normal-performing cultures were added. The behaviour of MCs from crashed cultures was more variable, since they were collected from the cultures of different rotifer strains, and were utilized under two different forms: freshly-isolated or preserved at -80°C and regrown afterwards on MA. When comparing by origin of preserved MCs, only the addition of MC from L3 crashed culture showed a significant improvement ($p<0.05$) in growth rate (exp. 1.3 and 1.11). MC from L1 crashed culture was

used in two forms. The fresh-isolated MC could stimulate the growth performance significantly ($p<0.001$, exp. 1.1 and 1.9), while no stimulation was found ($p>0.05$) when that MC was preserved and regrown before use (exp. 1.2 and 1.10).

Table II. Growth rate over 5 days (mean ± SD, n = 4) of *Brachionus plicatilis* sensu strictu (clone 10) hatched from disinfected amictic eggs and fed three types of food. MC: Freshly-collected microbial community. MCR: Microbial community which was preserved at -80°C and regrown on MA.

Exp.	Bacterial treatment	Food type		
		Chlorella	**Wild-type yeast**	**mnn9 yeast mutant**
1.1	No bacteria	0.64 ± 0.07	0.18 ± 0.06	0.36 ± 0.05
1.2	No bacteria	0.55 ± 0.05	0.36 ± 0.07	0.32 ± 0.05
1.3	No bacteria	0.58 ± 0.02	0.34 ± 0.03	0.31 ± 0.04
1.4	No bacteria	0.60 ± 0.03	0.35 ± 0.07	0.33 ± 0.09
1.5	MC from clone 10 normal culture	0.83 ± 0.04	0.63 ± 0.01	0.64 ± 0.07
1.6	MC from clone 10 normal culture	0.62 ± 0.02	0.54 ± 0.01	0.58 ± 0.01
1.7	MC from clone 10 normal culture	0.59 ± 0.05	0.55 ± 0.01	0.58 ± 0.02
1.8	MC from clone 10 normal culture	0.67 ± 0.05	0.55 ± 0.02	0.58 ± 0.01
1.9	MC from L1 crashed culture	0.79 ± 0.02	0.56 ± 0.03	0.71 ± 0.07
1.10	MCR from L1 crashed culture	0.60 ± 0.05	0.45 ± 0.08	0.40 ± 0.03
1.11	MCR from L3 crashed culture	0.62 ± 0.02	0.44 ± 0.04	0.40 ± 0.04
1.12	MCR from clone 10 crashed culture	0.59 ± 0.02	0.44 ± 0.05	0.42 ± 0.04

Table III. Comparison in growth rate over 5 days of *Brachionus plicatilis* sensu strictu (clone 10) in the presence of microbial communities (Tukey test). Values indicate significance (p-value).

Pair of experiments	Food type		
	Chlorella	**Wild-type yeast**	**mnn9 yeast mutant**
Exp. 1.1 and exp. 1.5	0.000	0.000	0.000
Exp. 1.2 and exp. 1.6	0.671	0.000	0.000
Exp. 1.3 and exp. 1.7	0.999	0.000	0.000
Exp. 1.4 and exp. 1.8	0.645	0.000	0.000
Exp. 1.1 and exp. 1.9	0.002	0.000	0.000
Exp. 1.2 and exp. 1.10	0.866	0.173	0.324
Exp. 1.3 and exp. 1.11	0.585	0.006	0.011
Exp. 1.4 and exp. 1.12	1.000	0.319	0.285

Conclusions

It was proven by the study that the presence of an "endogenous microbiota" is essential for the growth of rotifers, especially when low quality food (yeast) is offered. Such an effect could be obtained with a microbial community originating from a normal or a crashed culture, but not with a MCR, namely MC regrown in the lab on rich medium. The results further suggest that the MC iso-

lated from one particular crashed rotifer culture was probably not responsible for the crash, as no negative effects were observed by adding this MC to axenic rotifers. This of course does not exclude the possibility that crashes in other rotifer cultures are due to the presence of certain microorganisms.

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IN VITRO DIGESTIBILITY OF WATER SOLUBLE AND WATER INSOLUBLE PROTEIN FRACTIONS OF SOME COMMONLY USED FISH LARVAL FEEDS AND FEED INGREDIENTS

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Introduction

Fish larvae have high dietary protein requirements due to high growth potential and extensive combustion of amino acids (AA) in their energy metabolism. Protein digestibility is crucial to dietary protein utilisation efficiency and knowledge in specific digestibility of feed ingredients is important in formulation of optimal diets for larvae in aquaculture. Despite of its importance, little is known about the specific digestibility of protein sources used in compound diets for first feeding larvae. The specific digestibility of protein sources in adult fish and other animal models has only limited relevance to larvae due to the special digestive physiology of common fish larvae, especially the absence of stomach and gastric digestion.

Live feeds, which provide the best results with regards to larval survival and growth in aquaculture, contain high fractions of soluble nitrogen (e.g., Carvalho et al., 2003). Carvalho et al. (2004) showed that substituting dietary insoluble protein (native casein) with soluble protein (sodium caseinate or hydrolysed native casein) increased survival and growth in start feeding carp larvae fed with compound diets. This indicates that larvae have a particular dietary requirement for soluble protein.

Soluble proteins may be more exposed for attack from intestinal proteases and are probably more efficiently taken up by pinocytosis. Pinocytosis and intracellular digestion is suggested to be quantitatively important for protein digestion and uptake in stomach-less fish larvae (Watanabe, 1981; Govoni et al., 1986). Protein solubility may be a key factor in the efficiency of uptake by this route because aggregations of insoluble proteins may exceed the practical size limit for efficient cellular uptake by pinocytosis. Insoluble proteins thus have to be solubilised (e.g., by proteolytic cleavage) before they can efficiently be taken up

by pinocytosis, while soluble intact proteins will exist as single molecules within a size range that can be efficiently engulfed and taken up.

We wanted to study the specific digestibility of the water soluble and water insoluble fractions of live feeds and some protein sources that are commonly used or have a potential for use in larval compound diets. As a first approach we wanted to study the relative digestibility by using an in vitro method.

Materials and methods

Frozen samples of *Artemia* (nauplii), *Calanus* (copepod stage), and some common protein sources used in compound larval diets were homogenised in a phosphate buffer (pH 8.0) at 4°C. The insoluble fraction was separated from the soluble fraction by centrifugation and crude protein (N × 6.25) was measured in both fractions using a Leco FP-528 nitrogen analyser, Leco, Sweden. Insoluble fractions were resuspended in Eppendorf tubes, 20mg of crude protein in 1ml of phosphate buffer (pH 8.0). Soluble fractions were diluted to contain the same crude protein concentrations and sub samples were transferred to Eppendorf tubes. A mixture of trypsin (type IX, *bovine* pancreas), chymotrypsin (type II, bovine pancreas) and bacterial protease (type XIV, *streptomyces griseus*), all obtained from Sigma, USA, were added to each tube (100µl) to final concentrations of 73, 145, and 64mg.ml^{-1}, respectively. Digestion was performed at room temperature (22°C). The in vitro digestion was terminated by addition of 250µl of 40% trichloroacetic acid (TCA) at 0, 1, and 12 h after addition of the proteases. Four parallel tubes were sampled at each sampling time. All tubes were centrifuged and the crude protein content in the supernatant was measured and considered to represent digested protein while TCA precipitating protein was considered to be undigested.

Results and discussion

The distribution of crude protein into water soluble and insoluble fractions is showed in Table I. The results are in accordance with Carvalho et al. (2004) with regards to the high fraction of soluble nitrogen in live feeds. As could be expected, a high fraction of soluble nitrogen was also found in the trypsin-digested cod filet (65%), approximately twice the level found in fresh frozen cod filet (34%). The different marine meal products analysed in the present experiment all contained lower fractions of soluble nitrogen. The fraction of soluble nitrogen in such products will depend upon the raw materials and procedures used for the production.

The in vitro digestibility after 12h measured as increase in TCA soluble nitrogen showed that all water soluble fractions except for the water soluble fraction of fresh frozen cod filet were digested equally well as a reference soluble protein

(sodium caseinate, about 95%). Compared to the soluble fractions the insoluble fractions were less digestible. The live feeds and the fish meal insoluble fractions were digested to about 70% while the roe meal and trypsin-digested cod filet were digested to a lesser extent. From the fresh frozen cod filet both fractions were digested to about 80%. A water-insoluble reference protein (native casein) was digested equally well as the soluble reference protein (95%).

Table I. Distribution of crude protein into water soluble and insoluble fractions

Sample	Water-soluble fraction (%)	Insoluble fraction (%)
Artemia (nauplii)	67	33
Calanus (copepod stage)	54	46
Squid meal	11	89
Fish meal (Rieber, Norway)	17	83
Roe meal	11	89
Trypsin-digested cod filet	65	35
Fresh frozen cod fillet	34	66

The in vitro digestibility analysis presented above cannot directly be related to the digestibility of the same fractions in larvae as the digestion in larvae will rely on a complicated series of factors such as feed ingestion rate, intestinal flow rate and the larval ability to sustain secretion of proteases. There is, however, a chance that the relative in vitro digestibility will reflect the relative digestibility in larvae. With regards to this, roe meal should be avoided as protein source in larval diets due to poor digestibility. The relative differences in digestibility between soluble and insoluble fractions were not pronounced and equal digestibility of the water soluble and the water insoluble reference proteins were found after 12h. After 1h a difference was found, however, indicating a more rapid digestion of the water soluble reference protein. Based on the suggested importance of pinocytosis on dietary protein uptake in larvae, the relative differences in digestibility between the insoluble and the soluble protein fractions can be expected to be greater in larvae than in the in vitro system, although this remains to be seen.

Carvalho et al. (2004) found improved larval performance when insoluble native casein was substituted with soluble sodium caseinate as the only protein source in an experimental compound diet. When 75% of the native casein was substituted with hydrolysed casein, on the other hand, a strong negative effect on larval survival was found. Also other authors have found negative effects on larval performance when high levels of hydrolysed protein were supplemented to the diet. Contrarily, Tonheim et al. (2005) showed that hydrolysed protein were absorbed faster and more efficient than intact protein in tube fed larvae, and failed to find any shift in the larval AA metabolism that could explain the inferior effect of hydrolysed protein on larval performance. One possible explanation to the negative effect from hydrolysed protein is dietary protein leakage leading to

total protein contents below larval requirements in the ingested diet. This will indeed be detrimental to the larval performance. Intact soluble protein will also be leaking from the feed but at slower rates due to the larger molecular weights. All the water soluble fractions studied in the present experiment contained large initial fractions of TCA soluble nitrogen (40-100%). This nitrogen represents free amino acids (FAA) and small peptides that will potentially be extensively lost to the water by leakage if used as ingredients in compound micro feeds. In live feeds, on the other hand, soluble components are protected by bio membranes and tissue concentrations are under homeostatic control.

Successful formulation of compound larval diets will probably rely on a balanced compromise between digestibility and leaching. Concerning dietary protein requirement studies on fish larvae based on compound experimental diets, more focus should be put on nutrient leakage and protein digestibility to improve interpretation of data.

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EFFECT OF DIETARY LIPID LEVELS ON LIPASE ACTIVITY OF SPOTTED SAND BASS *PARALABRAX MACULATOFASCIATUS* JUVENILES

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Introduction

Dietary lipids play an important role as a source of essential fatty acids for carnivorous fish and are high-energy nutrients that can be utilized to partially substitute protein in aquaculture feeds. The study of lipase activity in fish has increased recently. Using biochemical and molecular approaches, several different lipases such phospholipase A2, pancreatic lipase, and bile-salt activated lipase (BAL) of a variety of species have been reported (Izquierdo et al., 2000). In carnivorous marine teleosts, it is generally thought that BAL plays an important role in lipid digestion (Gjellesvick et al., 1992). However, some results suggest that fish also contain a mammalian type pancreatic lipase (Iijima et al., 1998). In order to contribute to the knowledge on lipid digestion we study lipase enzyme activity of spotted sand bass juveniles fed diets containing different dietary levels of lipids (5, 10, and 15%), using biochemical and molecular approaches.

Materials and methods

Spotted sand bass juveniles (0.38±0.06g) were randomly distributed (42 fish.tank^{-1}) in nine 140-l tanks and reared according to Alvarez-González et al. (2001). The formulation of the diets was the same as described in Carrasco-Chávez (2004), where the percentages of total lipids were 5, 10, and 15% of lipids in diet. Dietary treatments were evaluated, in triplicate, during 42d.

Pancreatic section of 10 juvenile fish per tank were sampled and lyophilized until the measurements were done. Samples were homogenized in cold 50mM

Tris-HCl buffer, pH 7.5. Supernatants obtained after centrifugation (16 000×g for 15min at 5°C) were stored at -20°C until used for enzyme analysis. Lipase activity was measured according to Versaw et al. (1989) at two times, 14 and 42d of diets feeding. This assay is based on the hydrolysis of the colorless ester ß-naphthyl caprylate to yield colored ß-napthol at 37ºC and 30min, which is measured spectrophotometrically at 540nm. Lipase activity is expressed as $U.mg^{-1}$ of protein. Protein was determined according to Bradford (1976) using bovine serum albumin (1 $mg.ml^{-1}$) as a standard.

TRIzol® reagent (Gibco BRL) was used for total RNA extraction from pancreatic segment. Then, 5µg of total RNA were reverse-transcribed to cDNA with the Ready To Go T-Primed First-Strand Kit (Pharmacia Biotech). The expression of pancreatic triglyceride lipase from *P. maculatofasciatus* was studied by real time PCR using Gene Expression assays from the Assays-by-DesignSM (Applied Biosystems) consisting of mix of unlabeled PCR primers and TaqMan® MGB probes (FAM™ dye-labelled). The TaqMan® probes were designed taking into account the sequence that coded for triglyceride lipase of *P. maculatofasciatus* (EMBL AJ418039).

The Eukaryotic 18S rRNA (Applied Biosystems) was used as endogenous control for normalising mRNA levels of the target gene. For the triglyceride lipase, the forward primer was 5´CAGTAATCCCCTCATCATCATCACT-3´, and the reverse primer was 5´CACCCAGCCCTCCATCAT-3´. Thermal cycling and fluorescence detection were conducted using the 7000 Sequence Detection Systems (Applied Biosystems). Thermal Cycler conditions were as follows: Initial Setup 2min at 50°C followed by 10min at 95°C by only one cycle; denature 15s at 95°C followed by 1min at 60°C for anneal/extend by 40 cycles.

Results are given as mean ± S.D (n = 10). Data were compared by one-way ANOVA followed by Neuman Keul's multiple-range test when significant differences were found ($P < 0.05$).

Results and discussion

Lipase activity pattern and lipase expression levels from *P. maculatofasciatus* juveniles were obtained from the two sampling dates and the three different dietary lipids levels (Figs. 1 and 2).

The high triglyceride lipase expression level observed along the experiment with the diet containing 15% lipids, is in accordance to the results of growth (not shown), were specific growth rate and standard length were the highest in the spotted sand bass fed 15% lipids. However, the lipase expression levels were not consistently correlated to lipase activity value, and we can hypothesize that a post-transcriptional regulation could be involved in fish fed the diet containing

5% lipids (i.e., hormonal mechanism) where highest activity values were observed.

The pancreatic enzyme pattern was also positive influenced, especially with those larvae fed 5% lipids (data not shown). At the end of the experiment, no differences were observed between dietary treatments in weight gain, condition factor, feed intake, lipid efficiency ratio and whole fish composition (Carrasco-Chávez, 2004) (data not shown). Future experiments are needed to elucidate and control the factors affecting gene expression and lipase activity in spotted sand bass juveniles using high levels of lipids.

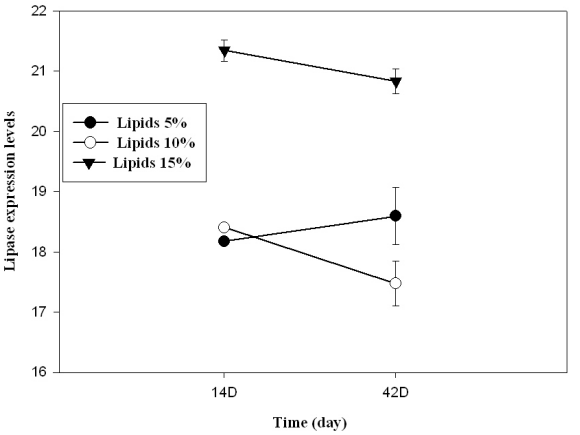

Fig. 1. Lipase expression levels related to eukaryotic 18s rRNA of spotted sand bass fed with different concentration of lipids. Means ± SD (n=3).

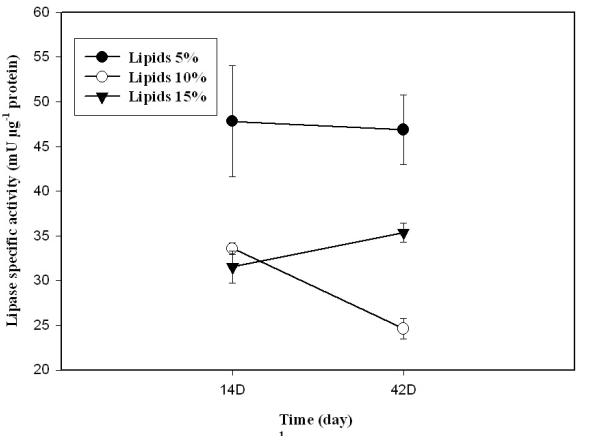

Fig. 2. Lipase specific activities (mU μg^{-1} protein) of spotted san bass fed diets containing different lipids levels. Means ± SD (n=3).

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EFFECT OF MANIPULATION OF NITROGEN IN A DIATOM CULTURE MEDIUM ON THE IMPROVEMENT OF PRODUCTION OF ABALONE POST-LARVAE

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Introduction

Benthic diatoms form a main source of food for post-metamorphic abalone until they reach a size of 8mm in shell length (Kawamura, 1996; Kawamura et al., 1998). The nature and amount of this food available is a critical factor in the early survival of abalone, with the food consumption of the early juveniles increasing rapidly with growth (Roberts et al., 1999). Hatcheries might improve growth and survival of the post-larvae by isolation and culture of diatom strains which are comparatively more digestible by the post-larvae, and thus may considerably improve their production (Kawamura et al., 1998). The objective of the present study was to determine the effect of nitrogen manipulation on the culture media of different diatom strains in determining their influence on the growth and survival of two species of abalone including *Haliotis rufescens* (red abalone) and *H. iris* ("paua").

Materials and methods

Experiments with the red abalone, *H. rufescens*, were carried out at the Marine Invertebrate Hatchery of the Universidad Austral de Chile (MIH-UACH). Trials included: 1) Comparisons of the nutritional value of *Navicula incerta* cultivated with different levels of nitrogen; and 2) Comparisons of the effects of nitrogen on the nutritional quality of indigenous diatoms and *N. incerta*. These trials included the evaluation of eight strains of diatoms identified as *N. incerta* H, *N. incerta* N, strain 95H, strain 95N, strain 92H, strain 92N, strain 122H, and strain 122N. The strains of local diatoms were identified by numbers as they are still in the process of being identified. The key "N" refers to algae culture medium having a normal nitrogen content, and "H" to the medium with a high nitrogen content.

Trials with *H. iris* were carried out at the Cawthron Institute in New Zealand. These included: 3) Comparisons of the effects of modification of nitrogen level in the culture medium on nutritional value of native diatoms. The native diatom species included *Cocconeis* sp., *Nitzchia sp*. (needle form), and *N. ovalis* ; and 4) The effects of daily addition of cultured diatoms with normal and high levels of nitrogen to the feeding test systems were observed. These trials included constant renewal of diatoms cultured on test plates, and required daily counting and replacement of each diet.

Post-larvae of *H. rufescens* were produced in the MIH-UACH, located at Puerto Montt, Chile, while those of *H. iris* were produced at Abalone New Zealand Ltd., located in southern New Zealand.

Results

The experiment feeding red abalone post-larvae with both *Navicula incerta* N and H showed a significant effect of the level of nitrogen in which the diatoms were grown, with better growth (21.9µm.day^{-1}) of the post-larvae on *N. incerta* H (P=0.006). Survival was also higher with this diet, reaching 67%.

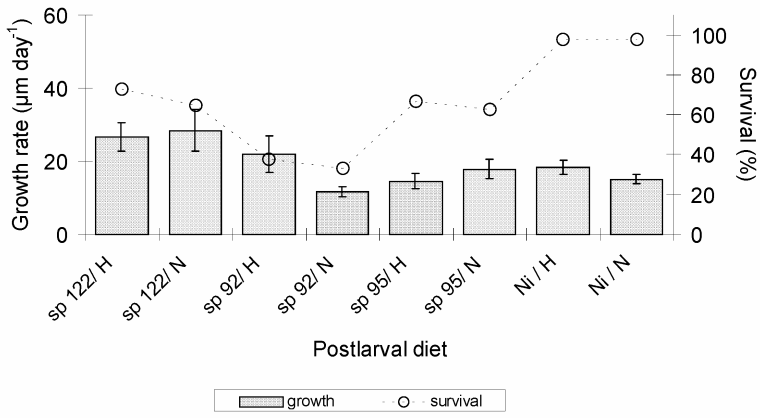

Fig. 1. Growth rate and survival of abalone postlarvae, *H. rufescens,* fed on diatom films of *N. incerta*, strain 95, strain 92 and strain 122 of high and normal nitrogen level. H= high nitrogen level, N= normal nitrogen level.

The microalgal diet affected the growth of the red abalone post-larvae, but this effect was not associated with the diatom culture media (Fig.1). A significant effect was observed between the species of diatom and the growth of the post-larvae (P =0.0309) but there was no effect of the level of N of the culture medium on the diatom. The highest growth rates (26.5µm.day^{-1}) were observed in post-larvae fed with diatom strain 122H, followed by that of post-larvae fed with strain 92. The least effective diets were diatom strain 95 and *N. incerta*. Survival

rates above 75% were obtained in abalone fed with diatoms *N. incerta* at all combinations, strain 92N and strain 122H. Lowest survival was obtained with strain 92H.

The microalgal diet affected the growth of the post-larvae of New Zealand paua, (P=0.002), but this effect was not associated with the diatom culture media (Fig. 2). Growth rates of the abalone post-larvae ranged from 30-50µm.day^{-1}. The lowest growth rate of post-larvae was obtained with *N. ovalis* H, intermediate values were observed on post-larvae fed on both types of *Cocconeis* sp. and *Nitzchia* sp. (needle form) N, and best growth was obtained with *N. ovalis* N and *Nitzchia* sp. (needle form) H. The survival rates were above 80%.

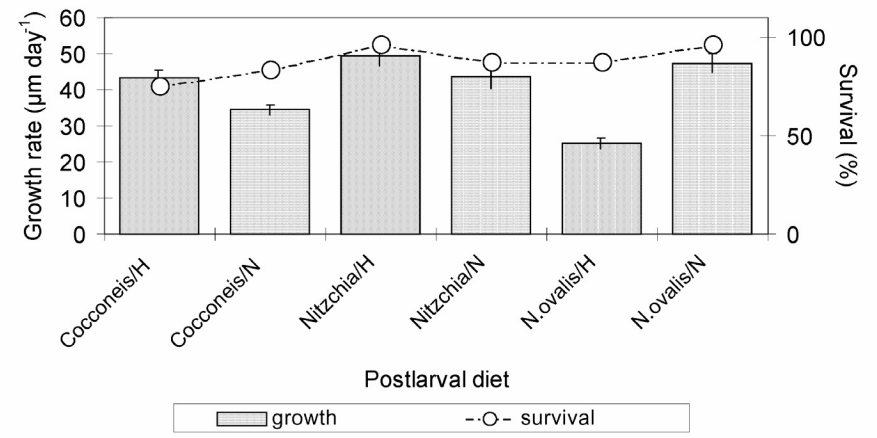

Fig. 2. Growth rate and survival of paua post-larvae, *H. iris,* fed on diatom films of *Cocconeis* sp., *Nitzchia* sp. (needle form) and *N. ovalis* of high and normal nitrogen level. H= high nitrogen level, N= normal nitrogen level.

In the diatom-addition experiment, growth rates of the post-larvae were very low. A significant interaction was observed between the diet species and N-level upon the growth rate, with *Nitzchia* sp. (needle form) N and *N. ovalis* N providing the best results, although the values were less than 20µm.day^{-1}; survival values were also very low compared with results obtained using diatom films.

Discussion

The results showed that the increase in nitrogen could improve the nutritional quality of some diatoms used in feeding postlarval abalone. The best example of this was *N. incerta*, and to a lesser degree the Chilean strains 122. There was, in general, no clear increase in protein content as a result of raising the nitrogen content of the microalgal culture medium, as occurs with planktonic species traditionally used in hatchery-cultured bivalve larvae (Uriarte et al., 2004), and thus may depend on the diatom species studied.

The finding that the better growth of the post-larvae in Experiment 1 was associated with a greater protein content in *N. incerta*, while there was no correlation between values for total protein when comparing the effects of various diatom species (Experiments 2 and 3) showed that the protein level was not the only factor which produced accelerated growth in abalone post-larvae.

Experiment 4, which explored the value of adding diatom cultures to plates holding early juvenile abalone in order to improve the food offering, showed that not all species of diatoms tested functioned equally in this endeavour. It also showed that treatment with elevated N levels could even lower the nutritional value of these diatoms. Both types of *Nitzschia* grown at normal N levels provided the best diets, with survival values greater than 50% and 13µm.diameter^{-1} of growth. Therefore, the addition of species such as these were key factors in the feeding of juvenile abalone when the food offering on the plates was unable to support the grazing pressure of these herbivores.

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EFFECTS OF DIETARY HUFA AND TEMPERATURE ON SEA BASS LARVAE (*DICENTRARCHUS LABRAX)* DEVELOPMENT

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Introduction

In marine fish, important morphological and functional changes occurred during larval stage and could be affected by nutritional and environmental factors (Zambonino Infante and Cahu, 2001). This raises the question whether specific diets combined with water temperature could promote the development of some metabolic functions, in particular the enzymatic pathways involved in high unsaturated fatty acid (HUFA) production.

Materials and methods

Larvae were reared in 40 l tanks (density: 60 larvae l^{-1}) supplied with running water. Oxygen and temperature were regularly controlled. Four replicated groups were fed microparticulated diets from mouth opening, day 6 post-hatching (Cahu et al., 2003). Two diets with a low (LH) or high (HH) HUFA content were tested: 0.8 and 2.2% EPA+DHA (20:5n-3+22:6n-3) for LH and HH respectively. Larvae were reared at 16 or 22°C and the experiment lasted 45 days. The four experimental conditions were 16LH, 16HH, 22LH, and 22HH.

Final mean survival rate was estimated using the ratio initial/final number of larvae in each tank. Thirty larvae were collected in each tank at day 39 and day 45 for wet weight (formalin preserved) and dry weight (105°C, 24H) measurements. Fatty acid analysis was performed on frozen larvae. Total lipids were extracted according to Folch et al. (1957), with 23:0 added as internal standard for quantification. Neutral and polar lipids were separated according to Juaneda and Rocquelin (1985), on a sub sample. Fatty acids methyl esters (FAME) were prepared with sulfuric acid in methanol (with toluene for total lipids) and separated by gas chromatography (Perkin Elmer autosystem GC, equipped with 30m × 22mm BPX 70 0.25 capillary column).

For each treatment, 30 larvae were collected at day 39 and day 45 to measure the expression of delta-6 desaturase (Δ6D; access number: AJ715505). ARN were extracted by trisol, treated by DNase and reverse-transcripted (iScript^{TM}cDNA Synthesis Kit Bio-Rad Laboratories Inc.). The quantification of Δ6D transcripts was performed by real-time PCR, using SYBR green. The relative expression ratio of the gene was calculated using REST$^{©}$ software (www.wzw.tum.de/gene-quantification/). This ratio for a considered gene is based on the PCR efficiency (E) and the C_T of a sample versus the control, and expressed in comparison to the reference gene (GAPDH), according to Pfaffl's mathematical model (Pfaffl, 2001).

Results are expressed as mean±SE. Temperature (t) and diet (d) effects and their interaction (t*d) were analysed by factorial ANOVA using Statistica 6. Previous analysis, data were normalised by a natural logarithmic transformation (weight) or by arcsin square root transformation (survival and fatty acid percentages).

Results and discussion

Survival rate was significantly higher at 16°C than at 22°C ($p<0.001$; Table I). Larvae weight was significantly affected both by temperature and diet ($p<0.01$; Table I) which resulted from joined feeding and metabolic adjustments as generally observed in fish (Jobling, 1997).

Table I. Survival rate (%; mean±se; n=4) and individual wet and dry weight (mg; mean±SE; n=4) per treatment at day 45.*: significant differences ($p<0.05$) with temperature (t), diet (d) and interaction (t*d).

	Treatment				Statistical results		
	16LH	**16HH**	**22LH**	**22HH**	**t**	**d**	**t*d**
Survival (%)	50±6	54±5	22±1	25±2	*		
Wet weight (mg)	15.0±1.7	18.1±0.4	84.4±4.4	94.8±10.7	*	*	
Dry weight (mg)	3.2±0.2	4.0±0.1	20.6±1.2	21.0±2.3	*	*	

As it is generally observed, fish fatty acids (FA) composition at day 45 reflects that of diet (Table II). A higher DHA (22:6n-3) content was found in larvae than in their diets (×2 for LH; ×1.5 for HH). DHA is known to be selectively retained, but may be also produced from n-3 precursors. A significant increase of 18:3n-6 content in LH larvae revealed a desaturation from 18:2n-6 not related to diet composition. Larvae 18:3n-6 content was very low. Several significant differences were also observed according to temperature, with some interactions. The polar lipids DHA content remained lower in LH larvae (around 15% of FAME) than in HH larvae (around 24%). It has to be pointed out that DHA values lower than 20% in PL indicate a deficiency in sea bass juveniles (Skalli and Robin, 2004).

Table II. FA composition of diet and larvae at day 45 (mean±SE; n=4): total FAME content (mg larvae^{-1}), selected FA in total lipid (TL) and in polar lipids (PL) expressed in % of total FAME. *: significant differences ($p<0.05$) with temperature (t), diet (d) and interaction (t*d; values having the same letter are not significantly different).

	Diet		**Treatment**				**Statistical results**		
	LH	**HH**	**16LH**	**16HH**	**22LH**	**22HH**	**t**	**d**	**t*d**
Total FAME			0.5±0.0	0.6±0.1	3.0±0.2	3.0±0.4	*		
TL									
16:0	18.1	18.8	17.6±0.5	19.1±0.2	17.7±0.2	19.0±0.2		*	
18:1n-9	12.9	10.4	11.3±0.1	9.1±0.1	12.6±0.1	10.1±0.2	*	*	
18:2n-6	44.1	31.0	38.8±0.2	26.3±0.2	38.6±0.1	25.8±0.4		*	
18:3n-6	0.1	0.1	0.3±0.0^{a}	0.1±0.0^{b}	0.5±0.0^{c}	0.2±0.0^{b}	*	*	*
20:3n-6	0.0	0.1	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0			
20:4n-6	0.3	0.6	0.5±0.0	1.0±0.0	0.5±0.0	1.0±0.0		*	
18:3n-3	4.2	3.0	3.1±0.2	2.1±0.0	2.8±0.0	2.1±0.1		*	
20:5n-3	2.3	5.5	3.3±0.0	6.4±0.0	3.1±0.0	6.1±0.1	*	*	
22:6n-3	3.7	9.7	7.6±0.2	15.3±0.2	7.5±0.1	15.0±0.5		*	
PL									
20:4n-6			0.8±0.0	1.5±0.0	0.9±0.0	1.6±0.0	*	*	
20:5n-3			5.0±0.1^{a}	9.1±0.1^{b}	5.1±0.1^{a}	8.5±0.1^{c}	*	*	*
22:6n-3			13.2±0.4^{a}	24.1±0.7^{b}	15.3±0.5^{c}	24.2±0.5^{b}	*	*	*

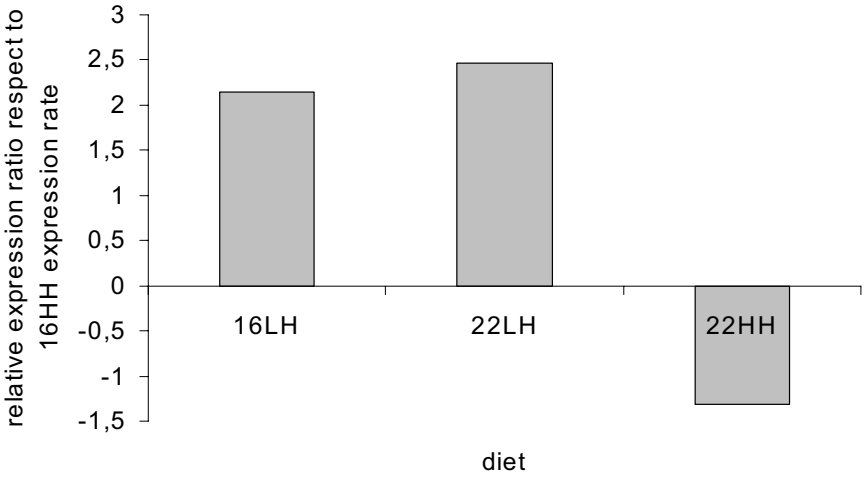

Fig. 1. Relative expression ratio of Δ6D as a function of diet and temperature, 16HH being the reference group.

At day 39, the relative expression of Δ6D was higher in LH larvae than in HH ones (Fig. 1). This suggests that low HUFA diets could stimulate the transcription of Δ6D gene. This stimulation of the first step of desaturation elongation pathway could allow synthesis of FA needed to compensate low dietary HUFA.

Conclusions

These results demonstrated the possibility to modulate Δ6D transcription and activate the HUFA synthesis pathways in marine fish larvae fed with a low HUFA microparticulated diet from first feeding until day 45. Activation of this pathway had a possible but unclear impact on larvae HUFA composition, except for 18:3n-6 content.

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WATER QUALITY IN JUVENILE COD CULTURES

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Introduction

Water quality in juvenile marine fish rearing may affect survival, growth, and quality of the fish. Flow rate controls both oxygen supply and microbial growth by removal of metabolites and organic waste. Water treatment may affect microbial composition, either by killing potential fish pathogens or by selection for slow-growing and more harmless bacteria. Finding the optimal conditions of flow rate and water treatment may enhance juvenile production, and improve fish welfare. The present work therefore presents results for cod during early juvenile stages, reared with three different water treatments, each with four different flow rates.

Materials and methods

A 36-tank experiment was conducted using juvenile cod to assess the impact of water quality on cod juvenile welfare parameters. Each tank was equipped with a separate water inlet with a high-grade flow meter. The tanks were organized in three rigs that received three different water qualities, each rig containing twelve tank units. The water used was from 160m depth in the fjord and sand-filtered. The different water qualities were achieved by treatment in a recirculation system, by UV-sterilization (TMC P8-440W UV-unit), and no treatment as a control. All water was adjusted to 12°C and aerated before use. The recirculation unit was a TMC 5000 Marine System (Tropical Marine Centre, London) used in conjunction with a Sander A1000 Ozoniser controlled by a Burkert ORP 8206 transmitter with a PFGK-gel-2A/A redox sensor. The redox potential was kept between 320-340mV, and pure oxygen was used to both facilitate ozone production and maintain oxygen levels at the lowest flow rate in the recirculation unit. Approximately 5% of the water in the recirculation rig was removed daily during tending of the tanks, and recirculation rate was therefore ~95%. Within each treatment, four different flow regimes were applied in triplicate tanks. The water exchange rate was 10, 20, 40, and 70 times the tank volume (32 l) per day. Each

tank was stocked with 200 weaned cod juveniles at a mean weight of 0.048g. The fish were fed a commercial dry feed (Aglonorse, EWOS) in a 24 h cycle with belt feeders. Light intensity was adjusted to 20µW/cm^2 at the surface of each tank (Osram Biolux 72 light tubes). Fish mortality was registered daily in all tanks, and growth was determined at the end of the experiment, which lasted 37 days. Water samples were collected once a week for determination of ammonia and bacteria (total and species). Total bacteria were determined by a FacsCalibur flow cytometer (Becton and Dickinson, Franklin Lakes, New Jersey, USA) with an air-cooled laser providing 15mW at 488nm with standard filter setup. The water samples were fixed in 4% phosphate-buffered formaldehyde and stained with "SYBR Green I" before analysis. A survey of the bacterial population in water samples was done by means of extraction of total community DNA, PCR-amplification of 16S rDNA, and separation of bands on a Denaturating Gradient Gel Electrophoresis, according to the methods of Sandaa et al. (2003). Hydrography (temperature, salinity, oxygen, pH, and redox) was also determined once a week with an YSI 556 MPS. Two-way ANOVA statistics were used to test significant differences of water treatment and water exchange rate.

Results and discussion

Growth rates and survival were clearly affected by water quality. Specific growth rates (SGR) among individual tanks ranged between 6.8 and 9.8% per day. Increased water exchange rate resulted in higher SGR in all water treatments ($p=0.0001$). SGR was also affected by water treatment ($p=0.012$), being highest in UV-treated water and lowest in recirculated water. This effect was most pronounced at the lower water exchange rates.

Survival in individual tanks ranged between 39.5-91.5% and was significantly affected by water treatment ($p=0.00001$), and highest survival was observed among the tanks receiving recirculated water. Independent of water treatment, survival was also significantly higher with increasing water exchange rate ($p=0.00008$). Mortality was determined from daily collection of dead fish, and total numbers of survivors. Unaccounted mortality (difference between observed mortality from collected fish and final numbers of surviving fish) may be interpreted as cannibalism. This mortality was independent of water exchange rate but significantly lower in the recirculated water ($p=0.0006$). In contrast, the observed mortality (countable mortality) was not dependent on water treatment, but increased significantly with decreasing water exchange rate ($p=0.0002$). In this sense, the two types of mortality seemed to be differentially affected by water quality. The higher survival in recirculated water was most likely caused by low cannibalism in this water treatment. This is intriguing and may raise the questions of what mechanisms lead to cannibalistic behaviour, and what characteristics of the recirculated water that reduce this behaviour? At present, no explana-

tion could be given, but it seems obvious to start the search among olfactory or chemical stimuli.

Although growth rate was lowest in recirculated water, the superior survival in this water quality gave the highest biomass yield. Biomass at experiment termination was significantly dependent on both water quality ($p=0.00001$) and flow rate ($p<0.000001$). Lowest biomass was found in untreated water and at low flow rate. Water flow counted for most of the variation in biomass, which among individual tanks varied between 1.8 and 7.5$g.l^{-1}$ at experiment termination. However, differences were least between the 40 and 70 times water exchange rates.

Attempts to relate survival and growth rate to water quality parameters showed a significant positive correlation between average oxygen saturation throughout the experiment and SGR ($R^2=0.46$, $p=0.008$), as well as with survival ($R^2=0.48$, $p=0.006$). Average oxygen levels were lowest at low flow rates, and varied between 65.2-94.9% saturation among individual tanks. However, levels down to 46.4% were measured during the last week of the experiment, without significant increase in mortality. Further, no overall correlation was found between growth rate and survival. This indicates that growth has not been density-dependent and limited by food availability.

Total ammonium concentration among individual tanks varied between 3.6-48.0μM, being highest in tanks with low flow rate and in the recirculation rig. Growth rate was negatively correlated with average concentration of total ammonium throughout the experiment ($R^2=0.34$, $p=0.03$). In contrast, overall survival was not correlated with ammonium concentration, although such correlations were strong within each water treatment rig. Lack of overall correlation was a result of generally higher ammonium levels in the recirculation unit. Thus, since survival was considerably better in the recirculation unit, the ammonium concentrations may not have been directly affecting survival. Along with high ammonium concentration, a considerable drop in pH was observed (pH ranged from 7.15-8.07). In particular this was the case for the recirculation system, indicating accumulation of CO_2 in the system. Ammonia is the toxic form of ammonium in water, and the formation is largely dependent on pH. Low pH will shift the equilibrium towards the non-toxic ammonium, which was the case at the low flow rates and in the recirculation unit.

It is not possible to separate the effect of low oxygen and high ammonium on growth. Long-term exposure to both these conditions separately may reduce growth, but in our experiment low oxygen and high ammonium co-occurred.

Samples of total bacteria were grouped according to high DNA content, low DNA content, small-size bacteria (probably including virus), cyanobacteria, and "fat bacteria" (surface structure with high degree of light reflection). The latter

group counted for most of the variance between samples over time within a tank, with peaks completely outnumbering the other groups. Total bacterial numbers were between 1.7×10^5 and 2.7×10^7, with lowest numbers in the UV treated tanks. The recirculation units and the untreated tanks had on average ca 4.1 and 1.5 times more bacteria than the UV treatment, respectively. The bacteria groups with low and high DNA content was dominating in all tanks, but cyanobacteria were more abundant in the recirculation treatment while small-sized bacteria occurred at higher fractions in the UV and untreated rigs. Further analysis of bacterial species and genera is in progress.

Conclusions

Both water quality and exchange rate had a significant effect on early juvenile growth rate and survival in Atlantic cod. At densities of ca 6 post-weaned juveniles per litre, water exchange should be more than 20 times the tank volume per day. However, a flow rate beyond 40 times of the tank volume per day does not seem to further enhance growth and survival. Recirculation significantly reduced unexplained mortality (cannibalism), and did not increase mortality caused by other factors (e.g., high ammonium concentrations). Although nitrification occurs very slowly at low temperature, it was possible to maintain the levels of ammonia at acceptable levels in the recirculation unit. Compared to the other treatments, the recirculation unit showed distinct differences regarding bacterial numbers and group composition.

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CURRENT STATUS OF LARVICULTURE IN PERU

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Introduction

Scarce publications exist on the state of larviculture in Peru. In this paper we report the level of the current status of seeds production in *Argopecten purpuratus, Litopenaeus vannamei, Macrobrachium rosenbergii, Colossoma macropomum, Piaractus brachypomus*, and the prospective of this key activity in the development of the Peruvian aquaculture.

Marine bivalve hatcheries

The production of *A. purpuratus* has increased in the last 10 years, and its trend of annual growth is about 30% (Table I).

Table I. Peruvian aquaculture production by species during the last ten years (t)

Species	1996	1997	1998	1999	2000	2001	2002	2003	2004[1]	2005[2]
L. vannamei	5 258	6 080	3 462	4 312	615	731	2 592	3 328	4 369	1 247
M. rosenbergii	31	23	45	16	13	6	7	7	2	--
A. purpuratus	1 027	465	1 905	2 640	3 916	3 914	5 701	6 670	9 651	643
P. brachypomus	1	0	2	5	26	20	36	9	2	--
C. macropomum	8	10	58	57	14	19	54	203[3]	21	--

[1] Preliminary values; [2] First three months; [3] Includes the values Department of Loreto

In Peru there are 4 laboratories that produce scallop seeds producing a total capacity of 8 million seeds per batch of production. However, this does not manage to cover the annual demand of both principal producing companies (Table II), being the deficit covered by seeds caught naturally.

In the past years the technological advance in the production of scallop has allowed an improvement in its production, reaching at present a demand of 120 million seeds annually. The efforts of this sector are concentrated in the innovation of the production processes, management systems and sanitary control, accompanied by the genetic improvement.

Marine and freshwater crustacean hatcheries

L. vannamei

After the epidemic of "white spot", which dramatically effected production from 2000 (Table I), the shrimp industry has recovered rapidly towards 2004 to similar levels of production in 1999, for intensive implementation of a culturing technology called "bioseguro" (Berger et al., 2005).

Nowadays only one hatchery exists, being its level of current production 50 million post-larvae a month. It works on the basis of nauplii imported from Ecuador. The cost of these post-larvae is of US$2.2 per thousand (Table II).

The principal limitation in the production is the lack of genetically selected broodstock, which forces a dependence on importing nauplii or post-larvae to be able to cover the requirement of 1200 million seeds annually.

The recovery of the shrimp industry through the use of the culture system type greenhouse ("bioseguro") has allowed an increase in the current demand of post-larvae, being around 1200 million annually. An increase is projected from 8-9 times the current demand, since one expects to reach the 1000 hectares of intensive culture (70-100 post-larvae.m^{-2}) in the next 10 years. There is no major area of expansion for the semi-intensive systems.

The projection of the increase in larval demand makes it necessary for the implementation and installation of larval production centers of closed cycle and the incorporation of genetic selection programs, guided to the improvement in the yields of production and quality of the larvae, as well as disease resistance.

M. rosenbergii

Unlike the white shrimp, the Malaysian prawn is a product for the internal market, not for exportation. The annual production of the Malaysian prawn has fallen 6 times from 1998-2003 (Table I), due to fundamentally a "loss of prestige" of the product for the lack of care in the post-crop, reaching the consumer damaged (chelae and broken shell, etc).

Though the technology of seeds production has been implemented successfully, and basically it is not a problem, in the last years some laboratories have closed their doors due to the fall in the demand. The rest of the hatcheries are working below their installed capacity (Table II). For these reasons the price of the larvae (US$20 for one thousand) is kept high to cover the operational costs.

In this crustacean it becomes necessary to notably improve the management of the post-crop, the processing and transformation of the final product with a major added value, in order to reintroduce on the market, which demand is about 130t monthly, with a trend to grow in the next 10 years.

The previously mentioned would allow the hatcheries to begin operation to full capacity and to lower the price of the post-larvae. In this context, it becomes necessary the renovation of the genetic stock of broodstock, which has not been realized since the prawn from Israel and Panama were introduced in 1983, to support the sustainable growth of this industry which recovery would be based almost exclusively on an internal captive market.

Table II. Installed infrastructure, current, and future market of Peruvian hatcheries per species.

Species	Number of hatcheries	Actual level per batch of seed production ($\times 10^6$)	Cost of seed (US$$\times 10^3$)	Annual seed demand ($\times 10^6$)	Projected seed demand to 2015 ($\times 10^6$)
L. vannamei	1	50	2.2	720-1200	9600-10 800
M. rosenbergii	5	0.5-0.7	20	1	10
A. purpuratus	4	8	5-10	120	1200
P. brachypomus	7	0.5	70-100	4	8
C. macropomum	7	0.5	70-100	4	8

Amazonian fish hatcheries

The statistics reveal a fluctuation in the production of *P. brachypomus* and *C. macropomum* reported (Table I), among other reasons, to the irregular supply of fingerlings and to the difficulty of the collection of statistical information for a lack of roads and governmental budget.

Six of the 7 laboratories are of investigation and governmental promotion of aquaculture. Though the potential demand comes closer to 4 million of fingerlings, the production reaches only 500 000 seeds (Guerra, 2000). The high cost of the thousand of fingerlings (US$70-100) makes some producers of Madre de Dios Department (Peruvian Amazon locality not included in this analysis) to buy in Brazil for the value of US$45 per thousand (Huanqui, pers. comm.).

Due to the seasonal shortage of fish in the Amazon (increase of the water level of the rivers) it becomes necessary to implement investigations to manage the reproductive cycle (manipulation of exogenic factors of the vitellogenesis and spermiogenesis).

Likewise, it is necessary to improve the supply of fingerling, raising the survival through an improvement in the zootechnical process of production.

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DEFORMITIES IN LARVAL GILTHEAD SEABREAM (*SPARUS AURATA*): A QUALITATIVE AND QUANTITATIVE ANALYSIS USING GEOMETRIC MORPHOMETRICS

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Gilthead seabream (*Sparus aurata*), one of the many cultivated species in the Mediterranean, has become an important research topic over the years. The focus has mainly been with respect to aquacultural aspects, and the implementation of techniques to improve growth. However, more fundamental approaches may prove to be very useful for understanding the biology of cultivated species, as well as to expose the nature behind development and growth problems experienced in cultivation facilities. One of the many problems that commercial farms are confronted with is deformities that arise during development, with a frequent retardation of growth as a consequence.

Studying the pattern behind deformities and related growth disturbances is frequently based on biometric analysis, relying on point-to-point measurements of several structures and parts of the body plan. Such an approach is useful for studying size changes – thus growth – but is insufficient in describing aspects of shape (especially size-independent shape), e.g., in the case of deformities. For that reason, geometric morphometrics allow a more complete description of shape, and thus a more robust and fine analysis of shape variation in growing and metamorphosing fishes.

A fast recognition of abnormal shapes of fishes, due to arising deformities, is of utmost importance for fish farmers in order to minimize costs of nutrition with respect to growth benefits. For that reason, we performed a geometric morphometric analysis on the external morphology of a growth series of *S. aurata*, with the goal to localize when and where one of the most prominent abnormalities of this species takes place: deoperculation. This may also allow a fast recog-

nition protocol for detecting early opercular deformation in fish farms. Additionally, to understand the anatomical basis of the opercular deformation, we performed a similar morphometric analysis on the skeletal morphology of the head, opercular system, and pectoral girdle in *S. aurata*, in order to localize the detailed pattern behind deoperculation.

The method applied is landmark-based geometric morphometrics, using the thin plate splines method (TPS). For the external morphology, a total of 17 landmarks were used to describe the body outline, the head, the pectoral fin, the abdominal region, and the tail region. This was done on a total of 310 specimens bred in captivity and 5 specimens obtained from the wild. The age of the specimens ranged from 50 to 69 days post-hatch, with a standard length between 11.5 and 22.0mm. For the analysis of the skeletal deformations, a total of 42 specimens were used, on which 26 landmarks were defined. Those specimens were all of the same age (110 days post-hatch) and were selected for normal and deoperculated features. After standardization for size, position, and rotation (applying generalized procrustes analysis), a principal component analysis (PCA) was performed on the obtained scores for the shape variables (partial warp scores). A regression analysis between the shape changes and body size (expressed as standard length) was performed, to investigate underlying allometric growth changes in body and skeletal morphology.

The PCA analysis of the external morphology showed that the most important shape changes involve an overall increase in head height, especially at the posterior margin of the skull. The abdominal part also heightened with increasing PC1-values, coupled to a more subtle lengthening. The tail region, however, remained fairly constant and even shortened a bit. The pectoral fin shifts to the ventral side with increasing PC1-values. The regression analysis showed a close relationship between those shape changes and an increasing body size. Apparently, the external shape changes in this sample merely showed normal allometric growth patterns, where no striking abnormalities were detected. This analysis also revealed the striking fact that in the wild-type specimens, the observed shape features of the bred specimens only appear at about a size of 17.8mm (instead of 13.3mm in bred specimens).

The analysis of the skeletal shape changes, on the other hand, did show very clear results with respect to opercular deformations: (1) shape differences between the selected operculated and deoperculated specimens are striking; (2) the deformations involved only the posterior margin of the opercular bone, whereas the surrounding skeletal elements hardly change; (3) the within-group variation seemed not to be size related; and (4) a striking difference may exist between the left and right side (a perfectly normal right side may coexist with a complete deformed left side).

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EFFECTS OF ESSENTIAL FATTY ACIDS ON LARVAL DEVELOPMENT OF SENEGAL SOLE (*SOLEA SENEGALENSIS*)

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Introduction

The Senegal sole is a flatfish found along the Mediterranean and South Atlantic coast, and is a prime candidate for aquaculture in Spain and Portugal. The rearing methods for Senegal sole have been well documented but little is known about the larval nutritional requirements. Like all flatfish, Senegal sole undergo metamorphosis, with a transition from an upright swimming behaviour to the "flat" morphology. During this time the eye migrates from one to the other side and provision of adequate nutrition is particularly critical (Dhert et al., 1990). Thus, this study was designed to investigate the role of dietary fatty acids on growth, survival, eye migration and final pigmentation of Senegal sole larvae using a dose-response design.

Materials and methods

Commercially available oils rich in the select fatty acids – Neuromins (Martek) rich in DHA, Vevodar (DSM Food Specialties) rich in ARA, and EPA500TG (Croda) rich in EPA – were used. Mixtures with olive oil were designed to produce emulsions with "nil" (-N), "low" (-L), "medium" (-M), and "high" (-H) levels of each fatty acid (Table I). Oil mixtures were emulsified using soy lecithin and equal amounts of water using an Ultra-turrax T25 at high speed. Emulsions were kept in plastic syringes in upright position at 4ºC until use. *Artemia* nauplii were enriched at 100 nauplii.ml^{-1} for 18h at 26ºC with 0.6g.l^{-1} of the emulsions. For ARA and DHA requirement studies (Experiments 1 and 2), EG strain (INVE) of *Artemia* was used whereas for the EPA requirement study (Experiment 3), an Argentinean strain of *Artemia* with low initial levels of EPA (MAYRO Cantarini, Argentina) was selected.

Table I. Formulation of the experimental emulsions. *Include soy lecithin, α-tocopherol. Canola and soybean blend oil was also used in ARA and DHA emulsions in graded quantities. TFA: Total fatty acids

Formulation	**ARA**				**DHA**				**EPA**		
(mg.g^{-1})	**-L**	**-M**	**-H**	**-N**	**-L**	**-M**	**-H**	**-N**	**-L**	**-M**	**-H**
Neuromins	500	500	500	0	252	504	757	198	132	66	0
Vevodar	0	150	300	50	50	50	50	81	54	26	0
Croda	-	-	-	-	-	-	-	0	303	606	910
Olive oil	355	222	90	860	573	287	0	631	421	212	0
Others*	145	128	110	90	125	159	193	90	90	90	90
DHA (% TFA)	20.2	19.1	19.2	0.0	4.4	7.7	14.7	8.5	8.8	9.1	9.4
EPA (% TFA)	0.2	0.4	0.7	1.7	1.6	1.7	1.7	0.0	19.4	40.0	62.1
ARA (% TFA)	0.2	7.9	14.8	1.3	1.6	1.5	1.4	3.9	4.0	4.1	4.4

Senegal sole larvae were obtained from CIFPA "El toruño" (Cádiz, Spain). Larvae were distributed (50 larvae.l^{-1}) into 35-l, 150-µm mesh baskets distributed amongst four, 1500-l holding tanks in 3 consecutive experiments. The tanks were connected to a recirculation unit already described (Carbó et al., 2003). Temperature, salinity, and photoperiod were the same for all the experiments: 19±1ºC, 34ppt, and 18h light:6h dark, respectively. *Artemia* ration was adjusted according to the body weight of the larvae in all the experiments in order to reduce the amounts of unenriched nauplii in the tanks. Standard length and dry weight were measured at 1, 4, 6, 10, 12, 15, 20, 30, and 35dph. Eye migration was assessed according to Cañavate and Fernandez-Diaz (1999). Survival rate and final pigmentation success were determined at the end of each experiment. Triplicate samples of the larvae were also taken at the end for lipid and fatty acid analysis of the tissues (heads, guts, and carcasses) although the data are not presented here.

Results and discussion

Table II shows the results in terms of growth (length and dry weight, DW), survival and pigmentation success of the 3 experiments.

Experiment 1 - ARA

Survival was not affected by any diet with an average survival of 50%. Growth was not significantly different among the treatments although eye migration was delayed in ARA-H larvae at days 15 and 20 (data not shown, Villalta et al., 2005). Significant differences were found in pigmentation rate with ARA-M and ARA-H fed larvae showing a higher degree of albinism. A significant, negative linear regression was found between the ARA content and pigmentation rate (Pigmentation = 149.23-3.12ARA, P=0.0012, r^2=0.706). Thus, a clear relation-

ship can be established between the dietary content of ARA, the accumulation of ARA in larval tissues and the pigmentation rate in Senegal sole larvae.

Table II. Results in growth (length and dry weight, DW), survival, pigmentation, and fatty acid content of the larvae at the end of the experiments. * Length and DW data of EPA experiment correspond to 30-dph larvae. Fatty acid composition of the larvae are under analysis.

	ARA			**DHA**				**EPA***			
	-L	**-M**	**-H**	**-N**	**-L**	**-M**	**-H**	**-N**	**-L**	**-M**	**-H**
Final length, mm	11.8	11.9	12.2	10.6^{bc}	10.2^{ab}	10.9^{c}	9.8^{a}	8.5^{ab}	8.3^{a}	8.6^{ab}	9.0^{b}
Final DW, mg	4.5	5.1	4.8	3.0	2.7	3.1	2.4	1.9	1.9	2.0	2.3
% Survival	44.4	58.9	54.9	77.0	85.2	72.2	89.8	50.6^{b}	40.2^{b}	57.7^{b}	34.1^{a}
% Pigmentation	99.7^{c}	38.9^{b}	15.8^{a}	100	100	100	100	95.6^{b}	96.5^{b}	99.4^{a}	99.9^{a}
DHA (% TFA)	8.7	8.6	7.7	1.5	4.5	10.5	14.1				
EPA (% TFA)	2.6	2.2	1.7	0.8	0.9	1.4	1.5				
ARA (% TFA)	1.3	5.2	7.6	2.2	1.5	2.6	2.5				

Experiment 2 – DHA

No significant differences were obtained in growth and survival of sole larvae. The larvae fed DHA-N *Artemia* performed just as well as those fed DHA enriched nauplii, indicating a low or negligible requirement for this fatty acid. The low requirement for DHA observed might explain the ease of culture of this species on most types of *Artemia* and the high survival rate. To explain this low requirement we hypothesized (Villalta et al., 2005, in press) that during the first 10 days of larval pelagic life the requirements of n-3 PUFA and DHA might be different to those found after metamorphosis. Before metamorphosis in the wild the main prey item are copepods rich in DHA while after metamorphosis juveniles feed on polychaetes rich in EPA. Fish fed DHA-deficient *Artemia* nauplii might have preferentially conserved the high DHA present in the newly hatched larvae. On the other hand the slightly lower growth in length observed in DHA-H fish is probably a consequence of the reduction in lipid reserves and oleic acid present in the emulsion used for "fuel".

Experiment 3 – EPA

A slightly but significantly higher growth in length was observed in the larvae fed EPA-H enriched nauplii that can be explained for the significantly lower survival observed in this group of fish. No significant differences were observed in final DW. Pigmentation was significantly affected by the diet. Fish from EPA-N and -L groups showed a higher degree of albinism than those of -M and -H groups that were almost 100% normal.

Low survival of EPA-H fish can be explained considering that the oils rich in DHA and oleic were not included in the diet. Albinism might be explained considering that both ARA and EPA are involved in the synthesis of eicosanoids, and eicosanoids have been already indicated (Estévez et al., 1999) to be involved in pigmentation of flatfish.

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EFFECTS OF TEMPERATURE, SALINITY, AND FOOD CONDITIONS ON GROWTH AND REPRODUCTION OF A BRACKISHWATER CLADOCERAN *MOINA MONGOLICA* DADAY (*MOINIDAE*)

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Introduction

Moina mongolica (Daday) is one of few halophilic Old World cladocerans in the genus *Moina*, and has been found in brackish water lakes in China. He et al. (1988a) reported that optimal temperature and salinity of *M. mongolica* was 25°C and 5-40g/l, respectively. However, Wang and He (2001) found the animal grew better at 30°C and 10g/l. It is therefore necessary to re-evaluate the growth and reproduction potential of *M. mongolica* at a range of temperature, salinity, and food conditions because this brackishwater species has been adapted in seawater for 15 years.

Methods and materials

M. mongolica used in this study were collected in Xiaochi lake, China, and individually reared in test tubes containing 15ml culture medium with *Chlorella* sp. (cell dimension 3-4μm) and baker's yeast (Meishan Incorp) as food. Photoperiod was set at 14h L: 10h D.

Three experiments were performed. In experiment I, four temperatures (20, 25, 30, and 33°C) were used each with 12 replicates. The salinity was 32g/l and food was *Chlorella* at 3.0×10^9 cells.l^{-1} in all culture vessels. In experiment II, five salinities (5, 10, 20, 30, and 40g/l) were used each with 12 replicates. The temperature was 25°C and food was *Chlorella* at 3.0×10^9 cells.l^{-1}. In experiment III, three foods: *Chlorella* at 3.0×10^9 cells.l^{-1} (C), yeast at 30mg.l^{-1} (Y), and *Chlorella* at 1.5×10^9 cells.l^{-1} plus yeast at 15mg.l^{-1} (C+Y) were used each with 10 replicates. The temperature was 25°C and salinity 10g/l.

At the start of experiment I, one neonate was placed into each test tube containing 15ml medium. During the experiment, the body length of each *M. mongolica* was measured daily. Newly-born neonates and dead individuals were recorded daily and removed from culture vessels. The procedures of experiments II and III were the same as experiment I.

Data were tested using one-way ANOVA. Regression analyses were used to examine the relationships between body length and developmental time. $P<0.05$ was regarded significant in difference.

Results

At each temperature, the body length (L: mm) could be predicted from the age (T: d) using the equation: $L=a+b\log_{10}T$, where a was 0.569, 0.582, 0.638, and 0.717 at 20, 25, 30 and 33°C, and b was 0.692, 0.693, 0.778, and 0.335, respecitvely. The body size at maturity was 1.26±0.01mm at 20°C, 1.07±0.04mm at 25°C, 1.10±0.02mm at 30°C, and 0.97±0.02mm at 33°C. The maximum size observed was 1.63, 1.54, 1.32, and 1.03mm at 20, 25, 30, and 33°C, respectively.

Table I. Demography of *M. mongolica* reared under various temperatures (mean±SE, n=12). Different letters in the same row indicate significant difference ($P<0.05$).

	20°C	**25°C**	**30°C**	**33°C**
Longevity (d)	23.0±2.3^{a}	13.8±2.1^{b}	5.7±0.4^{c}	5.3±0.4^{c}
Age at maturity (d)	8.3±0.1^{a}	5.3±0.1^{b}	4.3±0.1^{c}	4.6±0.2^{c}
No. days between broods	5.7±0.6^{a}	3.7±0.2^{b}	1.8±0.2^{c}	2.0± 0.1^{c}
No. of brood per female	5.9±0.8^{a}	5.2±0.9^{ab}	3.0±0.5^{bc}	1.4±0.3^{c}
No. of young per brood size	10.2±0.5^{a}	9.2±0.6^{ab}	7.5±0.4^{b}	4.1±0.6^{c}
No. of young per female	62.9±9.2^{a}	48.2±8.4^{a}	21.3±3.3^{b}	5.3±0.8^{b}
No. of young per day of a female	2.6±0.4^{a}	3.2±0.4^{a}	3.6±0.5^{a}	1.0±0.1^{b}

The longevity, age at maturity and the brood intervals of *M. mongolica* at 20 and 25°C was greater than that of animals reared at 30 and 33°C (Table I). The survival rate after first brood at 20, 25, 30, and 33°C was 92%, 75%, 42%, and 17%, respectively. The number of broods per female and at 20 and 25°C was greater than that at 33°C. The number of young per female at 20 and 25°C was greater than that at 30 and 33°C. The number of young per brood and number of young per day of a female at 20, 25, and 30°C was greater than that at 33°C.

At each salinity, the relationship between body length (L mm) and developmental age (T: d) followed the equation: $L=a+b\log_{10}T$, where a was 0.738, 0.718, 0.727, 0.749, and 0.733 at 5, 10, 20, 30, and 40g/l, and b was 0.670, 0.701, 0.615, 0.594, and 0.601, respectively. The maximum body size at 5, 10, 20, 30, and 40g/l was 1.65, 1.70, 1.63, 1.51, and 1.53mm.

Table II. Demography of *M. mongolica* reared under various salinities (mean±SE, n=12). Different letters in the same row indicate significant difference ($P < 0.05$).

	5g/l	10g/l	20g/l	30g/l	40g/l
Longevity (d)	17.5±1.5	17.9±1.8	14.9±1.8	14.8±1.3	12.0±1.7
Age at maturity (d)	5.2±0.2	5.0±0.0	5.4±0.2	5.3±0.1	5.0±0.0
Brood intervals (d)	2.5±0.1	2.4±0.1	2.4±0.1	2.2±0.1	2.0±0.0
No. of brood per female	6.4±0.6	7.8±0.8	5.8±0.9	5.9±0.8	5. 0±0.6
No. of young per brood size	11.4±0.5	11.3±0.6	11.0±0.6	9.8±0.5	9.8±0.4
No. of young per female	70.5±14.1^{ab}	86.7±31.8^{a}	62.5±34.0^{ab}	60.1±35.0^{ab}	49.0±22.4^{b}
No. of young per day of a female	4.3±0.3	5.0±0.6	4.2±0.4	3.9±0.5	4.3±0.5

There were no significant differences in longevity, age at maturity, brood intervals, number of brood per female, number of young per brood and number of young per day of a female among the individuals reared in 5, 10, 20, 30, and 40g/l, but the number of young per female in 10g/l was greater than that in 40g/l (Table II).

In each food treatment, the relationship between body length (L: mm) and developmental age (T: d) followed the equation: $L=a+b\log_{10}T$, where a was 0.781 at C , 0.723 at C+Y, and 0.695 at Y, and b was 0.577 at C, 0.534 at Y, and 0.710 at C+Y.

M. mongolica fed Y matured later than those fed either C or C+Y. The number of brood per female was higher in the animals fed Y than those fed C, but was not significantly different between animals fed Y and C+Y. The number of young per female and number of young per day of a female was lower in animals fed Y than those of animals fed either C or C+Y (Table III).

Table III. Demography of *M. mongolica* fed *Chlorella* (C), yeast (Y), and Chlorella+yeast (C+Y) (mean±SE, n=10). Different letters in the same row indicate significant difference ($P < 0.05$).

	C	Y	C + Y
Longevity (d)	21.8±2.3	16.9±3.3	22.2±1.9
Age at maturity (d)	5.8±0.1^{a}	7.7±0.6^{b}	5.4±0.2^{a}
brood intervals (d)	1.1±0.1	1.6±0.4	1.2±0.1
No. of brood per female	7.0±0.9^{a}	3.2±0.8^{b}	5.1±0.6^{ab}
No. of young per brood size	7.3±0.5	9.0±2.7	9.3±0.5
No. of young per female	49.0±5.7^{a}	14.0±4.7^{b}	45.5±4.2^{a}
No. of young per day of a female	2.2±0.2^{a}	0.9±0.2^{b}	2.2±0.2^{a}

Discussion

In the present study, the daily production of neonates per female was not significantly different among 20, 25, and 30°C, but sharply reduced at 33°C, suggesting *M. mongolica* have a high rate of reproduction from 25-30°C. Results of the present study supported the conclusion that *M. mongolica* had high intrinsic rate of increase at 30°C (Wang and He, 2001). The lower demographic parameters of *M. mongolica* observed at 30°C may contribute to the abnormally high mortality at this temperature (He et al., 1988a).

He et al. (1988a) found salinity did not significantly affect growth and reproduction of *M. mongolica* in a range from 5-40g/l. The present study confirmed that demographic parameters of *M. mongolica* were not significantly different across 5-30g/l, suggesting this species can be cultured in a broad range of salinity. Achuthankutty et al. (2000) reported that the optimal salinity for feeding, survival, growth and neonate production in *Diaphanosoma celebensis* was 5-17g/l. In comparison, *M. mongolica* has a much better ability to adapt to salinity variations.

M. mongolica fed yeast alone had declined age at maturity and number of young per brood compared with animals fed *Chlorella* (He et al., 1988b). In the present study, the mixture of 1.5×10^9 *Chlorella*.l^{-1} and 15mg.l^{-1} yeast yielded comparable longevity, number of broods per female and number of young per day of a female as *Chlorella* was a sole food, suggesting *Chlorella* could be replaced by yeast supplement without causing negative impacts on the growth and reproduction of *M. mongolica*.

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INFLUENCE OF EMULSION PREPARATION METHOD ON ENRICHMENT OF *ARTEMIA* NAUPLII

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Introduction

Nauplii of the brine shrimp, *Artemia*, are widely used as live food organism for feeding marine fish and shrimp larvae. The nauplii do however not fully cover nutritional requirements of marine fish and shrimp larvae. With the objective to enhance and standardize the nutritional value of the nauplii, several artificial 'enrichment' techniques have been developed. Most enrichment studies have focused on increasing the levels of n-3 highly unsaturated fatty acids (HUFA) in the nauplii. There however exists an important variability in obtained enrichment values, even when using *Artemia* of the same batch under standard enrichment procedures. One of the reasons for the variability might be related to the particle size distribution of the emulsions, which might affect the ingestion efficiency of the filter-feeding *Artemia* (Navarro et al., 1999; Gelabert, 2001).

In the present study, we examined the particle size distribution, the stability, and the creaming properties of an experimental high-HUFA emulsion, prepared by three different methods (hand-shaking, high-shear blending or microfluidization). A 24-h enrichment trial compared incorporated HUFA levels in the *Artemia* nauplii.

Materials and Methods

The experimental lipid emulsions ICES 30/0.6/C (International Council for the Exploration of the Sea, ICES) contained approximately 30% of total n-3 HUFA (% dry matter) with a DHA/EPA ratio of 0.73. The emulsion consisted of 62% lipid, 30% water (% wet weight), emulsifiers, antioxidants, and liposoluble vitamins.

Three experimental preparation methods were used for diluting the emulsion (1.25g per 30ml artificial seawater, prepared with Instant Ocean) prior to addition to the enrichment bottle, i.e., either high-shear blending at 8000rpm using an Ultra-Turrax TV45 (Janke&Kunkel), microfluidization at 1120bar using a Microfluidizer M-110S (Microfluidics), or classical hand-shaking. The time of blending or shaking was 5min. Particle sizes were measured immediately after preparation, after 24 and 48h and 1wk in a refrigerator (±4°C) by laser diffraction, using a Mastersizer S (Malvern), equipped with a 300RF lens and a MS-17 wet sample dispersion unit and by Foton Correlation Spectroscopy.

A. franciscana cysts (INVE Aquaculture N.V., Belgium) from Great Salt Lake (Utah, USA) were used in the enrichment trials. The cysts ($2g.l^{-1}$) were incubated in filtered seawater (0.45µm cartridge filter) at 28°C under continuous aeration and light. After hatching, nauplii (more than 90% instar I) were separated from the cyst shells and transferred to 10-l PVC tanks (in triplicate) in 28°C seawater with continuous aeration. The freshly prepared lipid emulsions were added ($0.3g.l^{-1}$) at the beginning of enrichment (t=0h) and after 12h (t=12h). Nauplii were harvested after 24h, rinsed with tap water and stored at -20°C for fatty acid analysis. The fatty acid composition of the *Artemia* nauplii was analyzed by a direct transmethylation method according to Lepage and Roy (1984). Data represent means of duplicate analyses. They were further analyzed by one-way ANOVA followed by Tukey's HSD test ($P<0.05$).

Results

Fig. 1 shows the particle size distributions of the emulsion prepared either by hand-shaking, high-shear blending or microfluidization. Particle size distribution in the hand-shaken emulsion averaged 1.5µm. High-shear blending rendered the distribution of the particles approximately 3-fold smaller (0.2-2µm, averaging ~0.5µm). Microfluidization resulted in even much smaller particles with mass median particle size ~0.2µm (0.1-0.6). Particle size distribution was stable over the 1-week cold storage period (no statistical differences between particle size distribution at T0, after 24 and 48 hours, and after 1 week).

Table 1 shows the fatty acid composition ($mg.g^{-1}$ dry weight) of *A. franciscana* nauplii enriched for 24 hours with ICES 30/0.6/C, prepared by the three different methods. Statistical analysis indicated a significantly lower incorporation of all major fatty acid groups in the nauplii enriched with the microfluidized emulsion compared to the hand-shaken and high-shear blended emulsion.

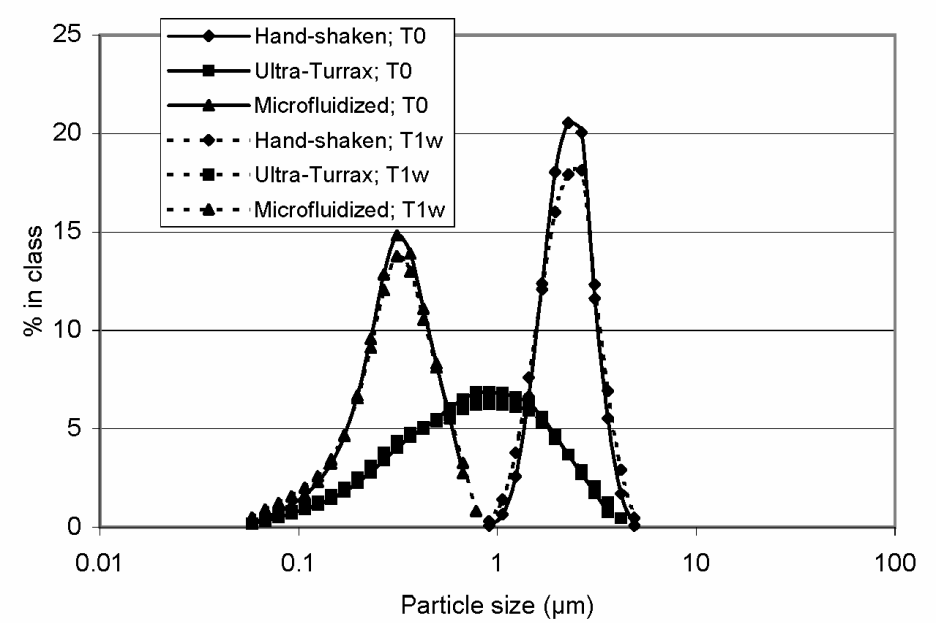

Fig. 1. Particle size distribution (at T0 and after 1 week cold storage) of ICES 30/0.6/C emulsion prepared through hand-shaking, high-shear blending (Ultra-Turrax), or microfluidization.

Table 1. Fatty acid profile (mg.g^{-1} DW) of *Artemia* nauplii enriched for 24h with ICES 30/0.6/C emulsion prepared by hand-shaking, high-shear blending, and microfluidization.

	Hand-shaking	**High-shear**	**Microfluid.**
Saturates	37.1 ± 1.8 [a]	39.6 ± 1.5 [a]	32.8 ± 1.3 [b]
Mono-unsaturates	68.8 ± 2.4 [a]	75.2 ± 2.4 [a]	53.9 ± 1.5 [b]
Sum (n-3) ≥20:3(n-3)	57.1 ± 6.8 [a]	61.8 ± 9.2 [a]	23.6 ± 2.1 [b]
Sum (n-6) ≥18:2(n-6)	17.5 ± 0.6 [a]	19.5 ± 1.2 [a]	12.8 ± 0.4 [b]
Total FAME	226.0 ± 7.7 [a]	224.8 ± 10.6 [a]	166.1 ± 3.0 [b]

Discussion

The high variation in HUFA levels of *Artemia* nauplii after enrichment with lipid emulsions has been emphasized by several authors. Despite numerous standardization efforts, important discrepancies between enrichment levels in the *Artemia* nauplii are still noted (Han et al., 2000). The size distribution of the lipid droplets in the emulsion is one of the factors that may influence the efficacy of ingestion by the nauplii. During their naupliar stages, following the opening of the alimentary tract at the instar II stage (about 8h after hatching), *Artemia* are believed to be passive filter-feeders and thus to be unable to select the food particles. Their ingestion therefore depends on the size (<50µm) and the availability of the particles in the water column (Sorgeloos et al., 2001). In general, particles between

0.1µm and 5µm seem to be rapidly ingested by the nauplii, as indicated by the few studies which document the size of the artificial feed particles used. Liposomes with a mean size of 2.6 µm were taken up easily by Instar II nauplii (Ozkizilcik and Chu, 1994). The same finding was reported with the smaller (0.2-0.4µm) liposomes in the study of Hontoria et al. (1994).

In the present study, the levels of total n-3 PUFA incorporated by the nauplii enriched with hand-shaken and high-shear blended emulsions were 57-61mg.g^{-1} *Artemia* dry weight. These levels are in line with previous data (Han et al., 2000). The much lower levels incorporated in nauplii enriched with the microfluidized emulsion suggest that lipid emulsion particles in the range of 0.2µm are less efficiently taken up than particles of 0.5-1.5µm (high-shear blended and hand-shaken), which partially contradicts the study of Hontoria et al. (1994). The main problems when studying uptake in *Artemia* using artificial feed particles are particle agglomeration, clumping, settling and degradation by bacteria. In the present study we showed however that particle size distribution of the microfluidized emulsion was stable over 1 week and that the emulsion was very resistant to creaming. This indicates the good stability of the emulsions and the absence of agglomeration or bacterial development. In summary, the present data show that the emulsion preparation method affects the particle size distribution, but all preparation methods left particle size distribution stable during 1-week storage at 4°C. Particle size distribution in its turn did however affect enrichment efficiency of *Artemia* nauplii, with particles of 0.2µm being less efficiently taken up. Creaming properties had no effect on enrichment efficiency.

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THE CULTURE OF MARINE SHRIMP WITHOUT *ARTEMIA*: AN ELUSIVE GOAL?

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Introduction

In a survey conducted by Juarez and Fegan (2001), hatchery technicians declared research on nutritional requirements and the development of cost-effective feeds for larval rearing of marine shrimp top priority. Primarily, it is the wish of every hatchery technician to replace live food with nutritionally balanced artificial feeds, thus simplifying larviculture systems and optimizing growth and survival of the shrimp larvae. Live *Artemia* nauplii suffer from inconsistent supply and quality as they are obtained from cysts collected in the natural environment. The use of artificial diets can help to reduce and stabilize costs, provide consistent nutrition and predictable output. These diets are now successfully used to partially replace live *Artemia* in commercial shrimp hatcheries, but at what percentage can they actually be replaced and how far away is the ultimate goal of total replacement?

Artemia consumption by marine shrimp

Some hatcheries want to start feeding Instar 1 *Artemia* nauplii as early as possible, because it is known that shrimp benefit from a zooplankton diet in the zoea stages. However, shrimp larvae at that stage are poor hunters and the use of live *Artemia* results in an inefficient food uptake. Therefore, heat-killed or frozen *Artemia* Instar 1 can be fed to late zoea stages and mysis to ease capture. In Vietnam, umbrella stage *Artemia* – harvested from the *Artemia* hatching tank after only 12h – are sometimes fed to *P. monodon* from mysis 1 to postlarvae 2 (R. Bijnens, INVE ASIA Ltd., Vietnam Rep. Office, pers. comm.). Umbrella-stage *Artemia* have higher energy content, are smaller, and are easier to capture than *Artemia* nauplii.

Mysis stages start exhibiting increased raptorial carnivorous feeding. In most shrimp hatcheries, consumption of *Artemia* nauplii starts at zoea3/mysis 1 and increases progressively with larval stage. *Artemia* is distributed at rates of 1-5 nauplii.ml^{-1}.d in the mysis stages. In the postlarval stages, the consumption of *Artemia* increases and feeding regimes range from 1-40 nauplii.ml^{-1}.d (approximately 10-1000 nauplii.postlarva^{-1}.d). As enzyme activity increases with postlarval development, live food replacement levels can also increase.

Replacement of *Artemia* with artificial diets

In spite of the wide use and broad range of commercial artificial diets, there is no evidence that they have been used successfully in commercial marine shrimp or freshwater prawn hatcheries to totally replace live food during culture of larval and early postlarval stages. In the eighties, the groups of David Jones (UK) and Akio Kanazawa (Japan) succeeded in the total replacement of live food for penaeid shrimp in zoea and mysis stages provided a single dose of live algae was given at the first-feeding zoea 1 stage (see Table I). Ever since, similar results were only achieved with commercial FRIPPAK® diets (INVE AQUACULTURE nv, Belgium) for larvae and postlarvae as reviewed by Wouters and Van Horenbeeck (2003), yet again under controlled medium-scale tank conditions. Commercial-scale trials and feed-back from customers indicate that the economical benefit of using *Artemia* replacement levels above 65% with these artificial diets is not justifying the increased risk of culture failure. In late postlarval stages, however, total *Artemia* replacement is a possible and common practice.

The difficulties inherent in providing a complete nutritional package in a sufficiently small particle to be ingested and digested by the small larvae of marine shrimp are well documented. Loss of nutrients from such diets can be rapid and results in the loss of nutritional value and fouling of the culture medium. On the other hand, provision of a sufficiently impermeable coat to prevent leaching may result in poor digestibility and availability of the nutrients to the developing larvae (Wouters and Fegan, 2004). Furthermore, the developed artificial diets should be neutrally buoyant in the water column to allow easy capture. There is no report of recent scientific progress that would allow a major breakthrough towards successful total *Artemia* replacement, but it can be expected that *Artemia* replacement levels will gradually increase thanks to improved diet formulation and technical advances in the aquafeed industry.

Table I. Overview of results reported with artificial diets used for partial or total *Artemia* replacement in shrimp larviculture.

Species	Diet	% *Artemia* replacement	Larval stages	Result compared to *Artemia* control	References
Penaeus monodon	Crumbled experimental microbound diet	100	Z-PL	Similar survival but lower growth	Kanazawa 1982
P. monodon	Crumbled experimental microbound diet	100	Z-PL	Similar survival and growth	Kanazawa 1985
P. monodon	Microencapsulated diet FRIPPAK®	100	Z-PL	Similar survival and growth	Jones et al. 1989
Litopenaeus vannamei	Microencapsulated diet	70-100	Z-PL	80% survival compared to 90% survival in live food control (commercial scale)	Jones et al. 1997
L. vannamei	Crumbled microbound diets Microfeast	25, 50, 75, 100	M-PL	Decreased growth rates at 50, 75 and 100% and decreased survival at 100%	Samocha et al. 1999
L. setiferus	Crumbled experimental microbound diets	40, 60, 100	Z-M	Decreased survival, growth, development and stress resistance (but similar survival at 40 and 60% in the presence of algae)	Gallardo et al. 2002
P. monodon	Microencapsulated diet FRIPPAK® Fresh	100	Z-PL	Increased survival, growth and development (one single dose of live algae in Zoea1)	Wouters et al. 2003
P. monodon	Crumbled microbound diet FRIPPAK® Flake	40, 100	PL	Lower survival, similar (100%) or improved (40%) growth	Wouters et al. 2003
L. vannamei	Crumbled microbound diet FRIPPAK® RW+	100	PL	Similar survival and growth in trial 1, lower survival and higher growth in trial 2 (98% survival in *Artemia* Shellfree control)	Wouters et al. 2003
Farfantepenaeus aztecus	Liquid feeds Epifeed™ and Liqualife™	50, 100	M-PL	Decreased survival (except Liqualife™ at 50%), growth and stress resistance	Robinson et al. 2005
F. aztecus	Microbound diets Zeigler™ E-Z Larvae, Zeigler™ Z-Plus and E-Z Artemia	50, 100	M-PL	Decreased survival, growth and stress resistance	Robinson et al. 2005

(*) References used in this table can be found in Jones et al. (1993) and Wouters and Van Horenbeeck (2003).

Conclusion

Live *Artemia* can only be replaced totally in shrimp larviculture in fully controlled laboratory facilities, and partially (up to 65%) with high quality artificial diets in commercial hatcheries. However, because of the lower cost of artificial diets compared to *Artemia*, a partial replacement can result in considerable savings.

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A COMPARATIVE STUDY OF BIOCHEMICAL COMPOSITION IN POND-REARED AND LAKE-STOCKED ADULT *ERIOCHEIR SINENSIS*

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Introduction

Chinese mitten crab is very important aquaculture species in china. There are two main kinds of the crab culture methods in China: pond-reared and lake-stocked, while there were no reports on comparative study of gonads development and tissue biochemical composition of two original mitten crab. So this study was done to compare morphological characteristics, tissue indexes (gonadosomatic index (GSI), hepatosomatic index (HSI) and muscle content), proximate and fatty acid composition of key tissues (gonad, hepatopancreas, and muscle) of pond-reared and lake-stocked *E. sinensis* broodstock.

Materials and methods

Lake-stocked adult Chinese mitten crabs were caught by fishermen in Jingxingyue Lake, Hubei province, PR China, on November 28, 2002. The crabs of this lake depend on natural food (such as zoobenthos, aquatic plants, algae, etc.) in the lake. Pond-reared crabs were obtained from Shanghai crab farm on December 3, 2002. The Pond-reared Chinese mitten crabs were fed a commercial diet (protein 38.4%, lipid 4.5%, ash 15.6%) once a day for two months prior to sampling. All the selected crab had mature gonads and similar body weight according to sex (male: BW132-158g; female: BW100-115g).

All the crabs used for this experiment were dissected; gonad, hepatopancreas, and all somatic muscle of each crab were obtained and weighted. Moisture and crude protein were analyzed following AOAC (1985). Total lipids were extracted according to the method described by Folch et al. (1957). Fatty acid (FA) composition was analytically verified by flame ionization detection(FID) after injecting the sample into Agilent6890 gas chromatograph fitted with an HP-5.5% Phenyl Methyl Siloam capillary column (30.0m×25mm, Agilent 19091J-

413, USA). Peaks were identified by comparing retention times with known authentic standards.

Homogeneity of variance was tested with the Levene statistic, and when necessary, arcsine-square root or logarithmic transformation was performed prior to analysis. Statistical analyses were conducted using one- and two-way analysis of variance (ANOVA/MANOVA) with Duncan's multiple range test used for post-hoc comparison (Sokal and Rohlf, 1995). Probabilities of $p<0.05$ were considered significantly different.

Results and discussion

Table I. Body length, body width, body wet weight, GSI, HSI, and Muscle content of pond-reared and lake-stocked adult *Eriocheir sinensis* used in this experiment. Values in the same column that do not share the same letter are statistically significantly different ($p<0.05$).

Origin (sample amount)	Body Length (cm)	Body Width (cm)	Body Wet Weight (g)	GSI (%)	HSI (%)
Pond Female (15)	5.53±0.12^{a}	6.12±0.14^{a}	103.10±6.50^{b}	8.63±0.94^{b}	6.67±0.79^{c}
Lake Female (10)	5.77±0.07^{b}	6.25±0.09^{b}	104.96±4.09^{b}	12.01±1.98^{c}	5.41±1.23^{b}
Pond Male (15)	5.84±0.06^{b}	6.46±0.08^{c}	140.92±4.15^{a}	3.65±0.96^{a}	4.05±0.60^{a}
Lake Male (10)	6.04±0.14^{c}	6.64±0.21^{d}	140.23±8.73^{a}	2.91± 0.70^{a}	5.36±0.85^{b}

The result showed that the HSI of pond-reared crabs was significantly higher, but the GSI was significantly lower in pond-reared female (Table I). Significantly difference was found on proximate of key tissues of different origin mitten crab (Table II).

Table II. Proximate composition (% of wet weight) in hepatopancreas, mature gonads, and muscle of pond-reared and lake-stocked adult *Eriocheir sinensis*.

Proximate	Pond Female	Lake Female	Pond Male	Lake Male
Hepatopancreas				
Moisture	39.24±2.41 ^{a}	45.60±3.45 ^{b}	60.77±4.92 ^{c}	57.49±4.79 ^{c}
Protein	11.34±0.10 ^{a}	16.45±0.10 ^{c}	10.53±0.20 ^{a}	13.91±0.11 ^{b}
Total lipid	33.43±3.27 ^{a}	19.08±2.77 ^{b}	15.74±1.61 ^{bc}	11.98±2.71 ^{c}
Ash	1.51±0.06 ^{a}	1.98±0.11 ^{c}	2.20±0.06 ^{d}	1.78±0.11 ^{b}
Gonads				
Moisture	47.87±1.50 ^{a}	43.86±3.34 ^{a}	71.19±1.60 ^{b}	71.74±0.58 ^{b}
Protein	30.74±0.49 ^{b}	34.66±0.21 ^{c}	18.71±0.25 ^{a}	18.71±0.21 ^{a}
Total lipid	18.75±1.13 ^{a}	17.70±1.27 ^{a}	1.75±0.42 ^{b}	1.68±0.17 ^{b}
Ash	2.12±0.05 ^{ab}	2.42±0.11 ^{b}	2.70±0.12 ^{a}	2.53±0.10 ^{b}

There were significant differences in proportions of 18:1n9, 18:1n7, 20:5n3, 22:6n3, ΣHUFA, and n3/n6 of hepatopancreas between pond-reared female and lake-stocked female (Table III). The content of principle fatty acids of the testis

and ovary in the pond-reared mitten crab were similar to those of the lake-stocked crab except for 18:1n9 and 20:5n3 (Table IV). In both lake or pond mitten crab, male crab has significant higher proportions of 20:5n3 and 22:6n3 in the hepatopancreas and gonads while the lipid content in those tissues is lower than female crab (Tables II, III, IV).

Table III. Principal fatty acids composition of hepatopancreas from pond-reared and laked-stocked adult *Eriocheir sinensis* (area % of total fatty acids). Values represent means±SD (n=3).

Fatty acids	Pond female	Lake female	Pond male	Lake male
C16:0	18.1±0.0	20.1±2.2	18.5±1.3	17.5±1.9
C16:1n7	10.6±1.0 ^{a}	12.7±3.2 ^{b}	8.0±2.40 ^{a}	11.4±1.3 ^{ab}
C18:1n9	36.1±4.7 ^{c}	22.8±4.6 ^{a}	31.8±0.2 ^{bc}	29.7±0.8 ^{b}
C18:2n6	8.5±2.6 ^{a}	9.6±1.2 ^{a}	0.7±0.1 ^{b}	1.0±0.3 ^{b}
C20:5n3	2.4±1.1 ^{a}	3.6±0.4 ^{ab}	4.7±1.1 ^{b}	7.0±2.4 ^{c}
C20:4n6	1.1±0.8 ^{a}	2.4±0.3 ^{ab}	5.5±0.3 ^{c}	3.5±1.7 ^{b}
C22:6n3	1.5±0.1 ^{a}	6.7±3.5 ^{b}	8.9±1.1 ^{bc}	12.5±2.9 ^{c}
ΣPUFA (≥ 18:2n)	17.8±3.1 ^{a}	27.1±4.7 ^{bc}	22.5±0.8 ^{b}	27.9±1.1 ^{c}
n3/n6	0.4±0.2 ^{a}	0.8±0.2 ^{b}	1.9±0.1 ^{c}	2.7±0.0 ^{d}
ΣHUFA (≥ 20:3n)	7.7±0.1 ^{a}	15.2±4.4 ^{b}	21.0±0.9 ^{c}	25.9±1.6 ^{d}

During the ovarian development of Chinese mitten crab, the HSI and hepatic lipid content (HLC) decreased significantly while the GSI (3.24-12.14%) and the lipid content of ovary (9.85-17.78%) increased dramatically (Cheng et al., 1998; 2000). In the present study, pond-reared female had a lower GSI and a higher HSI and HLC than lake-stocked female. This may be partly due to less HUFA content of hepatopancreas in pond-reared female than those of lake female. Although the commercial diet used in the experiment had a lower HUFA content, especially in EPA, AA, and DHA, the ovary of pond crab had similar HUFAs level with lake crab. This could be explained by preferential accumulation of those fatty acids in reproductive tissues (Mourente, 1994; Wu, 2004).

Table IV. Principal fatty acids composition of gnoads from pond-reared and laked-stocked adult *Eriocheir sinensis* (area % of total fatty acids).

Fatty acids	Pond female	Lake female	Pond male	Lake male
C16:0	13.2±0.6 ^{b}	13.6±0.5 ^{bc}	15.1±1.1 ^{c}	12.4±0.8 ^{ad}
C16:1n7	11.5±4.3 ^{bc}	16.7±3.4 ^{c}	6.6±1.2 ^{ab}	5.0±0.4 ^{a}
C18:1n9	29.5±1.3 ^{c}	25.5±1.9 ^{b}	23.1±2.0 ^{b}	19.6±1.4 ^{a}
C18:2n6	12.4±2.9 ^{c}	7.4±2.0 ^{b}	3.0±0.7 ^{a}	3.5±0.6 ^{a}
C20:5n3	4.6±0.5 ^{a}	6.5±1.0 ^{b}	10.7±0.3 ^{c}	15.8±1.1 ^{d}
C20:4n6	5.5±1.3 ^{a}	5.1±0.6 ^{a}	9.6±0.2 ^{b}	8.2±1.2 ^{b}
C22:6n3	4.3±1.8 ^{a}	6.7±3.5 ^{ab}	8.5±2.7 ^{b}	6.9±1.6 ^{ab}
ΣPUFA(≥ 18:2n)	29.3±3.9 ^{a}	27.8±1.3 ^{a}	34.9±2.9 ^{b}	38.9±2.0 ^{b}
n3/n6	0.6±0.2 ^{a}	1.1±0.3 ^{b}	1.4±0.1 ^{c}	1.6±0.1 ^{c}
ΣHUFA(≥ 20:3n)	15.9±2.7 ^{a}	19.7±2.3 ^{a}	30.8±2.5 ^{b}	35.4±1.8 ^{c}

Conclusions

Our results clearly show that the adult lake-stocked crab have a higher GSI and higher HUFA level compared to pond-reared crab, suggesting that that the quality of lake-stocked crabs as broodstock would be better than that of pond-reared crabs. The reproductive performance and larval quality of the two crab types should be further studied.

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PROTOPLAST OF *PORPHYRA* AS A LIVE FOOD SUBSTITUTE FOR ZOOPLANKTONS AND SHELLFISH SPAT: A PRELIMINARY REPORT

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Introduction

Purple lavers *Porphyra* spp. are known to be one of the most nutritious macroalgae (red algae) and its processed products are well known as the wrapping food material for a famous Japanese dish, sushi-rolls. *Porphyra* contains very high amount of various kinds of minerals and vitamins like vitamin A, and good source of digestible protein (ca. 40% in dry basis). In addition to that it is notable that *Porphyra* contains very high amount of taurine, an important amino acid for aquatic animals. Recently the efficient biochemical technology for mass-producing *Porphyra* protoplast using purified polysaccharases isolated from bacteria was developed. Protoplasts are cells that have had their cell wall completely or partially removed using either mechanical or enzymatic means. As protoplasts have no cell walls they are easily digestible when ingested by animals as foods. In the present experiment we investigated preliminarily the availability of this *Porphyra* protoplast as a live food substitute for culturing microscopic living food organisms and bivalve spats.

Materials and methods

Porphyra protoplast was prepared in the laboratory as follows. As the cell wall of this alga is composed of three kinds of polysaccharides (β-1, 4-mannan, β-1, 3-xylan, and porphyran), three enzymes (β-1, 4-mannanase, β-1, 3-xylanase, and agarase) were produced from some kind of bacteria which have been isolated from marine environments. Suitable conditions for preparing a large amount of protoplasts from *Porphyra* were determined in advance; i.e., pH of reaction mixture, the concentration of each enzyme, and time and temperature of reaction mixture, and so on. After getting protoplast, it was freeze-dried so its nutrient

qualities were retained. Freeze-dried *Porphyra* protoplast was ground into powder form manually by mortar and fed to the test animals. The particle size of protoplast product was varied from several to several-ten μm depending on the level of enzymatic reaction process.

The rearing experiments for live food zooplanktons were carried out in 1-l glass culture-bottles containing freshwater or seawater with mild aeration. Test zooplanktons were inoculated in glass bottles and offered diets once a day (*Porphyra* protoplast or natural phytoplankton: *Chlorella* or *Chaetoceros*). At the end of the culture trial, the environmental parameters were monitored on pH, DO, and NH_4^+-N. As test animal marine rotifer *Brachionus plicatilis*, freshwater cladoceran *Moina macrocopa*, and wild marine copepod *Tigriopus japonicus* were supplied for the experiment. Test for bivalve spats were conducted as follows. Manila clams spat *Ruditapes philippinarum* was obtained from hatchery. Spats (mean length ca. 5 mm) were randomly distributed in small stainless-steel mesh cages with 20 numbers per cage with six replicate of each dietary treatment. Two diets (protoplast or condensed natural diatom *Chaetoceros gracilis*) were fed to the clams four times a day (08:00, 12:00, 16:00, and 20:00) at ad libitum in a flow-through system. Also adult clams were reared in the same condition. Before feeding protoplast was mixed with sea water using mixer and offered to clam in liquid form. The dietary performance was evaluated in the change in population growth for live foods and the growth of shell size, body composition, and survival for clams.

Results and discussion

Proximate compositions of unprocessed dried *Porphyra* and protoplast are shown in Table I. After enzymatic process of *Porphyra* its protein and lipid contents increased although crude ash content decreased due to cell wall polysaccharide decomposition.

Table I. Proximate composition of *Porphyra* protoplast (%, Mean ± SD, n=3).

	Dried *Porphyra*	***Porphyra* Protoplast**
Moisture	8.4±0.5	5.9±0.1
Crude Protein	29.1±0.2	36.7±0.7
Crude Lipid	0.1±0.0	2.9±0.2
Crude Ash	10.9±0.1	3.5±0.1

Clam spats: A review of preliminary trial data on length increase indicated that the clam fed on natural microalgae diets had higher increment in size in comparison to the clam fed on protoplast diet. Nevertheless, overall mortality was low (97.5-99.2%) and independent of the experimental treatments. Also, no significant variation was observed in proximate carcass composition after feeding trial for adult clams (Table II).

Live foods: *Porphyra* protoplast was suitable for culturing rotifer, freshwater Cladocera, and marine Copepoda, and they could be cultured with the sole use of the ground protoplast for several days stably. However, natural microalga-feeding group outperformed the protoplast group under the present experimental conditions. Nevertheless, there were no significant differences in both treatments in terms of the influence on environmental parameters.

Table II. Proximate composition of Manila clam fed on *Porphyra* protoplast and *Chaetoceros gracilis* (%, Mean ± SD, n=3).

	Initial Values	***Porphyra* Protoplast**	***Chaetoceros***
Moisture	81.5±0.9	81.4±0.3	80.2±0.5
Crude Protein	6.8±0.2	7.1±0.1	7.2±0.2
Crude Lipid	0.8±0.1	0.9±0.1	0.9±0.1
Crude Ash	2.1±0.1	2.1±0.1	1.9±0.1

Conclusion

According to the experimental results obtained in the preliminary culture trials, *Porphyra* protoplast proved to be a good candidate food substitute for culturing aquatic animals like zooplanktons and bivalve spat. To enhance the dietary value of *Porphyra* protoplast, we should study further about what kind of nutrients must be supplemented to meet the nutritional requirement of these aquatic animals and the feeding techniques suitable for *Porphyra* protoplast diet from now.

Acknowledgement

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DIETARY PROTEIN REQUIREMENT OF LARGE YELLOW CROAKER (*PSEUDOSCIAENA CROCEA*) DURING EARLY STAGES

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A feeding trial was conducted to investigate the dietary protein requirement for large yellow croaker (*Pseudosciaena crocea*) from 12-42 days after hatching (dah). Five approximately isoenergetic microbound diets were formulated with crude protein levels ranging from 45-65% in 5% increments (Table I). Frozen copepods, contained 54.49±0.24% crude protein, 5.96±0.24% crude fat and 27.21±0.11% ash, was used as control. Each of the experimental diets was fed to triplicate groups of 3500 larvae with individually initial wet body weight of 1.76±0.09mg (mean ± SD) in 180-l white plastic tank at 24±1°C. Both growth gain and survival were significantly increased with increasing dietary crude protein levels up to 55% and then decreased at 60% and 65%. Frozen copepods resulted in midterm final growth and survival (Figs. 1 and 2). Fish fed high protein diets obtained higher protein and ash content. Whereas, fish fed high protein diets had lower lipid content than those fed low protein diets (Table II). Polynomial model was used to analyze the relationships between dietary protein levels and specific growth rate (SGR), and showed that optimum and maximum dietary crude protein levels were 54.84% and 60.22%, respectively (Fig. 3). The amylase specific activity increased with the increasing dietary carbohydrate level during fish development. Moreover, fish fed the high carbohydrate diets observed slower decline of amylase activity than those fed the low carbohydrate diets during development. Fish fed the copepods had low amylase activity similar to those fed 60% and 65% crude protein diets (Table III). Trypsin activity was not affected by the dietary protein content before 42 DAH. At 42 DAH, fish fed the diets upon 55% protein level as well as the copepods had significantly higher ($P<0.05$) trypsin specific activity than those fed the low protein diets (Table IV). The study suggested a later onset of trypsin than amylase in regulation of synthesis.

Table I. Formulation (g.100g^{-1} dry diet) and chemical composition of the formulated diets (% dry matter except moisture, mean ± SD).

Treatment	D_1	D_2	D_3	D_4	D_5
Ingredients					
White fish meal	36	39.5	43	46.5	50
Casein hydrolysate	18.25	21.25	24.25	27.25	30.25
Mussel meal	2	2	2	2	2
Squid meal	5	5	5	5	5
α-starch (precooked)	26.1	19.85	13.6	7.35	1.1
Sodium alginate	2	2	2	2	2
Soy lecithin	5	4.95	4.9	4.85	4.8
Cod liver oil	3.0	2.8	2.6	2.4	2.2
Vitamin premix	1	1	1	1	1
Mineral premix	1	1	1	1	1
Betaine	0.1	0.1	0.1	0.1	0.1
Glycine	0.05	0.05	0.05	0.05	0.05
Choline chloride	0.2	0.2	0.2	0.2	0.2
Immunostimulants	0.25	0.25	0.25	0.25	0.25
Antioxidant	0.05	0.05	0.05	0.05	0.05
Chemical composition					
Dry matter	93.08 ± 0.06	93.16 ± 0.05	92.06 ± 0.01	94.72 ± 0.02	94.37 ± 0.06
Crude protein	47.05 ± 0.19	52.01 ± 0.18	57.08 ± 0.05	62.23 ± 0.20	67.47 ± 0.28
Crude lipid	12.92 ± 0.68	12.46 ± 0.59	12.64 ± 0.48	13.17 ± 0.52	12.97 ± 0.66
Ash	10.50 ± 0.10	10.97 ± 0.04	11.71 ± 0.12	13.11 ± 0.01	13.38 ± 0.09
Gross energy (MJ.kg^{-1})	16.79 ± 0.49	16.65 ± 0.21	16.66 ± 0.57	16.60 ± 0.41	16.53 ± 0.55

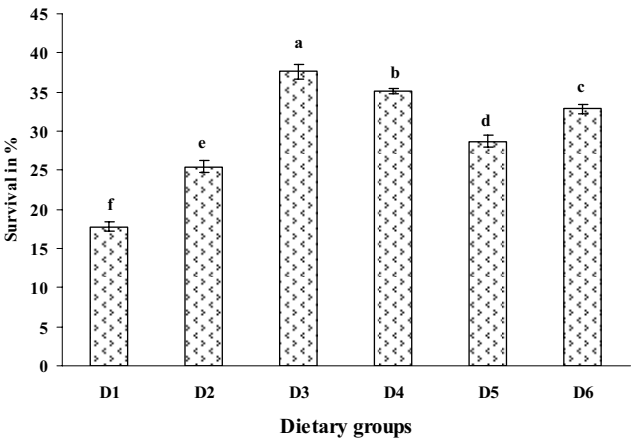

Fig. 1. Survival (%) at 42 DAH of large yellow croaker fed the experimental diets. Means ± SD (n = 3) with different superscript letters are significantly different ($P<0.05$).

Table II. Whole-body chemical composition (% dry matter) of 42-day-old fish fed experimental diets. Different superscripts within a row denote significant differences (P<0.05).

Diets	D_1	D_2	D_3	D_4	D_5	Control
Crude protein	65.72 ± 0.19^{c}	65.69 ± 0.24^{c}	66.77 ± 0.27^{a}	66.49 ± 0.29^{ab}	66.15 ± 0.18^{b}	65.20 ± 0.23^{d}
Crude lipid	10.86 ± 0.54^{a}	10.34 ± 0.33^{ab}	9.23 ± 0.50^{c}	9.57 ± 0.46^{bc}	9.64 ± 0.27^{bc}	8.28 ± 0.35^{d}
Ash	18.83 ± 0.25^{d}	19.60 ± 0.55^{c}	20.11 ± 0.49^{bc}	19.66 ± 0.36^{bc}	20.41 ± 0.44^{b}	24.21 ± 0.34^{a}

Table III. Amylase specific activities (U.mg protein^{-1}) in the pancreatic segments of fish fed the experimental diets at 12, 22, 32, and 42dah. Different superscripts within a row denote significant differences (P<0.05).

Diets	D_1	D_2	D_3	D_4	D_5	Control
12dah	1.26±0.25	1.26±0.25	1.26±0.25	1.26±0.25	1.26±0.25	1.26±0.25
22dah	1.68 ± 0.22^{a}	1.39 ± 0.35^{ab}	1.28 ± 0.68^{ab}	0.80 ± 0.44^{bc}	0.36 ± 0.26^{c}	0.67 ± 0.16^{bc}
32dah	1.42 ± 0.38^{a}	1.11 ± 0.29^{ab}	0.93 ± 0.17^{ab}	0.99 ± 0.33^{ab}	0.67 ± 0.28^{b}	0.84 ± 0.16^{b}
42dah	1.34 ± 0.24^{a}	1.03 ± 0.15^{ab}	0.93 ± 0.17^{b}	0.77 ± 0.19^{bc}	0.41 ± 0.19^{c}	0.53 ± 0.22^{c}

Table IV. Trypsin specific activities (mU.mg protein^{-1}) in the pancreatic segments of fish fed the experimental diets at 12, 22, 32 and 42 DAH. Different superscripts within a row denote significant differences (P<0.05).

Diets	D_1	D_2	D_3	D_4	D_5	Control
12dah	60.49±13.36	60.49±13.36	60.49±13.36	60.49±13.36	60.49±13.36	60.49±13.36
22dah	37.94±23.85	31.92±9.12	40.56±6.83	33.78±8.44	29.02±4.22	50.83±2.23
32dah	37.11±12.78	30.38±5.42	47.69±9.68	36.52±5.60	48.49±10.20	38.91±9.59
42dah	55.86 ± 10.41^{b}	66.63 ± 14.74^{b}	86.82 ± 5.26^{a}	87.57 ± 13.59^{a}	102.42 ± 10.56^{a}	96.37 ± 6.16^{a}

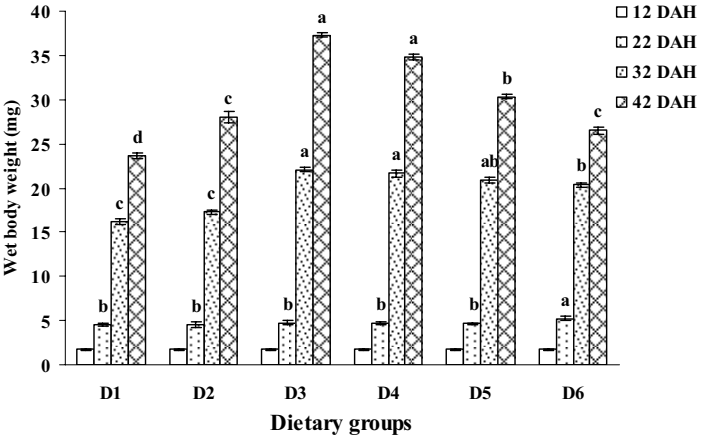

Fig. 2. Wet body weight (mg) at 12, 22, 32 and 42 DAH of large yellow croaker fed the experimental diets. Means ± SD (n = 3) with different superscript letters for the same day are significantly different (P<0.05).

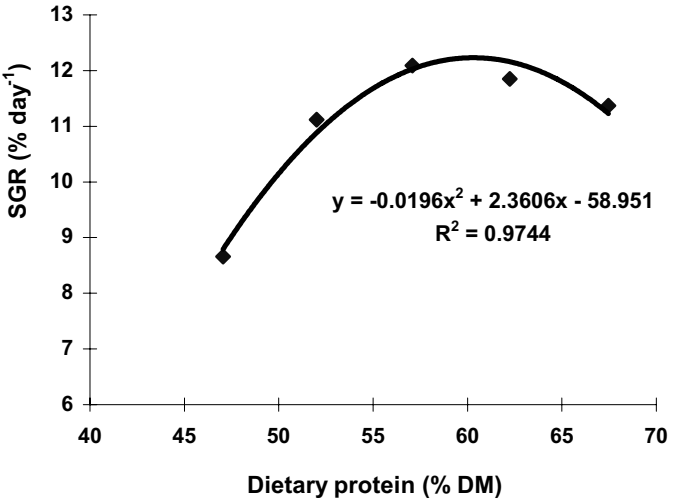

Fig. 3. The effect of dietary protein level on SGR (mean ± SD) of large yellow croaker. The optimum and maximum dietary protein requirements of this fish are 54.84% and 60.22%, respectively.

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THE ONSET OF FEEDING AND OF GUT FUNCTIONALITY IN MARINE FISH LARVAE

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The commencement of exogenous feeding is a crucial moment in developing fish larvae. The understanding of the early ontogeny of digestive functionality as well as of the nutritional and environmental requisites for properly triggering the corresponding series of hormonal and digestive processes occurring in this sensitive period is of primary importance in designing prepared feeds for first-feeding larvae. The start of feeding can be considered the period from which ingestion is possible to when larval growth is detected, usually when the moment for irreversible starvation has been surpassed. The main characteristic of this phase is that the source of matter and energy necessary to continue the larval development changes from the yolk reserves to the ingested food.

Before mouth opening, the yolk is utilised for embryo development with an efficiency characteristic for each species. There are other factors affecting the yolk conversion efficiency, such as the yolk amount at fertilization (depending on the maternal conditions) and temperature. All these circumstances determine the potential existence of a mixed endo-/exogenous feeding period, the resistance to starvation, and, in general, the readiness of larvae to the start the exotrophic life.

The opening of the mouth and the anus marks the moment for the first ingestion. The larva drinks water, starting the bacterioflora colonisation, and some gut content, probably cellular debris, may be observed. From this moment, the mucosa of the digestive tract is in contact with external environment, and together with the annexes glands, plays other roles beyond the strictly digestive functions, such as endocrine, immunology, and osmoregulation.

At the time of the mouth opening, anatomically the digestive tract is a straight tube in which is already possible to distinguish the oesophagus, with a squamous stratified epithelium, and the intestine, with a monostratified columnar epithelium. Anterior, medium, and posterior intestinal sections can be identified in the following days, with the apperance of a primordial pyloric sphincter and a con-

striction corresponding to the future ileo-rectal valve. The loop of the growing intestine to accommodate in the visceral cavity appears in just few hours or days.

This simple digestive tract is functional from the first moment. Lipid absorption in midgut and protein pynocitosis in hindgut may be observed a few hours after start of feeding. Annexed glands (liver, pancreas, and gall bladder) are also functional at the opening of the mouth. The activity of pancreatic enzymes has been measured from first-feeding and in some cases during the yolk phase. Accordingly, mRNA expression of trypsin, amylase, and salt-dependent bile lipase precursors have been detected before the mouth opening in some species. In the intestine, enzymes of the enterocyte cytosol as well as digestive regulatory peptides and hormones have also been detected at this early stage.

At the mouth opening the eyes become pigmented allowing the detection and catch of prey. Nevertheless, not all larvae start feeding at same time. Disparity in feeding ability can be due to differences in anatomical characteristics, prey encounter opportunity, and aggressiveness. This lack of synchronization varies among species, which can last from a few hours to several days for reaching 90-100% feeding incidence. Larvae are able to ingest prey with similar size of the mouth gape, but they selected smaller prey (25-50% of the mouth gape). During the first days of feeding the ingested mass may be above the own larval weight. There are noticeable inter-specific differences in ingestion rate and assimilation efficiency. In addition, the assimilated energy is used in growth and in metabolic processes with a pattern that varies in each species. All these differences are related with the priorities of development to survive in a particular habitat.

During the egg phase and yolk-sac period the organogenesis is regulated by hormones of maternal origin. This is obviously a costly process in term of energy. Consequently, the vitelline reserves are completely or almost exhausted at the time of first-feeding. After the opening of the mouth, the organogenesis has to continue using the elementary digestive system and the hormones produced by the larval genome. Quick growth and differentiation of the digestive tract is necessary during the following days to reinforce digestion and absorption. Any failure or limitation in the uptake of the required nutrients and energy during this short period implies incomplete or malformed organs preventing the subsequent growth and survival.

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DIETARY MODULATION OF SOME DIGESTIVE ENZYMES AND METABOLIC PROCESSES IN DEVELOPING MARINE FISH: APPLICATIONS TO DIET FORMULATION

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Larval development is highly influenced by the nature and level of some nutrients incorporated in the diets since larvae exhibited specific digestive features that suppose particular dietary supplies. Consequently, their ability to assimilate the required nutrients will depend on their capacity to modulate the expression of their digestive enzymes in response to the composition of the diet. This review intends to take stock of the data concerning dietary modulation of enzyme expression in fish larvae and their applications in terms of recommendations for larvae feed formulation.

Proteolytic enzymes are mainly located in the pancreas (trypsin, chymotrypsin, elastase, etc.) and intestine (membranous and cytosolic enzymes). Pancreatic enzymes have been extensively studied and all enzymes examined are generally expressed at first feeding, their expression increasing with age. The secretion of pancreatic enzymes in the intestinal lumen increases during the larval development. This process is controlled by the level cholecystokinin in the larvae which is indirectly and positively regulated by the dietary protein level and chain length. Trypsin activity is also positively correlated with dietary protein level when trypsin transcription is not before an advanced developmental stage in sea bass larvae. Maximum expression of trypsin was generally obtained with diets containing 50-60% proteins. Furthermore, trypsin transcription is influenced by the protein nature, fish meal being a poor inductor of this transcription.

Intestinal cytosolic enzymes are peptidases located in the enterocyte cytosol; these enzymes are highly expressed in immature enterocyte during the three first weeks of life in temperate species larvae and later in coldwater species. The incorporation of protein hydrolysate in the diet stimulates the activities of these cytosolic peptidases and consequently facilitates the assimilation of amino acids by the young larvae. When the maturation of the enterocyte occurs, the activity of these cytosolic enzymes decreases with the development of enzymes located in the brush border membrane. These maturational changes are facilitated when

the protein fraction of the diet is constituted by a moderate level (around 20% of the dry matter) of protein hydrolysate, and this facilitation is always associated with an improvement in larval survival.

Polyamines, in particular spermine and spermidine, are known for their positive role on the maturational process of the gastrointestinal tract in mammals and birds. Similar effects can be obtained in marine fish larvae with diets incorporating 0.33% spermine or diets containing live yeasts that still remain alive in the larval gut and secrete spermine.

Lipase and phospholipase A2 are secreted by pancreas in response to the presence in the lumen of their substrates, triglycerides, and phospholipids, respectively. The expression of these two enzymes is mainly regulated at the transcriptional level. The response of pancreatic lipase to dietary triglyceride level is not linear in sea bass larvae, showing a threshold, around 20% triglycerides in the diet, above which the maximum lipase activity and messenger level are reached. On the contrary, the response of phospholipase A2 to dietary phospholipid level is gradual and exhibited a great modulation range in expression. These differences between lipase and phospholipase A2 expression strongly suggest that fish larvae is better prepared to digest phospholipids rather than triglycerides.

Diets incorporating inadequate sources of lipids or high levels of triglycerides always induce an accumulation of lipid droplets in the enterocytes of the anterior intestine even though this accumulation cannot be considered pathological. This accumulation likely reflects a limited capacity for assembly and secretion of triglyceride-rich lipoproteins; this hypothesis is strengthened by the fact that the microsomal triglyceride transfer protein (MTP) of teleosts, required for assembly and secretion of triglycerides-rich lipoproteins, exhibited a transfert activity 4 times lower in intestine than that of mammals. Consequently, the lipid fraction of diets intended for marine fish larvae should incorporate a significant fraction of phospholipids instead of triglycerides. The incorporation of 12% phospholipids in diets resulted in 18 times and 3 times increase in growth and survival, respectively, compared to an isolipidic diet containing only 3% phospholipids.

Ongoing research has shown that some nutrients act on specific nuclear receptors, RAR, RXR (for Vit. A), PPAR (for polyunsaturated fatty acids), and VDR (for Vit. D). These receptors function by forming obligate heterodimers with RXR, involved in nearly all processes associated with development, emphasizing the pivotal role in the signalling network played by the retinoid pathway. The expression of these receptors is modulated by the dietary level of their specific ligands, and these modulations strongly impact on other signalling pathways determining the morphological and functional development of marine fish larvae. Cross-talks between these different signalling pathways make a global approach necessary to better define nutritional requirements of developing fish.

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DEVELOPMENT OF MICROBOUND DIETS FOR CRAB LARVAE

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The farming of commercially important crab species, such as mud crabs (*Scylla* spp.) and the blue swimmer crab (*Portunus pelagicus*) for both hard-shell and soft-shell crab production is an emerging aquaculture industry sector. To support sustainable growth of this new area of aquaculture, the development of formulated foods for hatchery culture and as a vehicle for studying larval nutrition has been investigated in this laboratory. Adopting techniques used with fish, ^{14}C-labelled rotifers were incorporated as an ingredient of microbound diet (MBD) particles fed to crab larvae. By measuring the ^{14}C content of larvae after feeding on ^{14}C-labelled MBD, ingestion of MBD was determined quantitatively. This technique was the basis for a series of experiments conducted with larvae of both *S. serrata* and *P. pelagicus.* It was shown that, in contrast to fish, dead crab larvae held in water containing ^{14}C-labelled MBD had significantly higher ^{14}C content than background, even after thorough washing. On this basis, an important adaptation of this technique for use with crab larvae was to use dead crab larvae as a control for ingestion experiments.

Our results showed that MBD were readily accepted and ingested by all larval stages of *S. serrata* and *P. pelagicus*. Ingestion of MBD increased with larval development, particularly at megalopal stage. The increase in MBD ingestion from zoea-I to megalopa was 90 times for *S. serrata* and 16 times for *P. pelagicus*. The feeding time required to achieve maximal ingestion increased for later zoeal stages for *S. serrata* while there was no significant difference within a 0.5 to 4h feeding period for all larval stages of *P. pelagicus* except zoea III. This suggests possible differences in the mode of feeding for zoeal larvae of the two species.

Experiments to determine diet particle size preference revealed ontogenetic changes in MBD size preference for both crab species. When various size ranges of MBD were offered, significantly higher ingestion rates of larger particles were recorded for zoea-V and megalopae of *S. serrata* and for zoea-IV and megalopae of *P. pelagicus*. Experiments to determine optimal ration for preferred particle size ranges showed that for *S. serrata*, increasing MBD ration

from the standard equivalent live feed ration did not significantly enhance ingestion while reducing ration to 25% resulted in a significant decrease in ingestion for all zoeal stages tested (zoea-I, III, and V). However, there was no significant reduction in the ingestion rate of megalopa even when ration was reduced to 12.5%. In contrast, zoea-III and IV larvae of *P. pelagicus* showed no significant reduction in ingestion of MBD when ration was reduced to 25%. Interestingly, at zoea-II, a 200% ration is recommended because it resulted in a significant increase in larval ingestion. Similar to *S. serrata,* significant reduction in ingestion by *P. pelagicus* megalopae only occurred when ration was reduced to 12.5%. Based on these results, the optimal size ranges as well as ration for each larval stage of the two crab species were recommended.

The influence of the binder used for MBD preparation on larval ingestion of MBD was also examined for *S. serrata* larvae. There was no significant difference in ingestion at all larval stages tested when MBD were bound with five different binders: agar, alginate, carrageenan, gelation, and zein. However, zein-bound MBD had the lowest leaching rate of all 5 binders.

More recently, research has investigated the potential of MBD as a complete replacement for *Artemia* fed to megalopae and for co-feeding for zoeae of *S. serrata*. The results showed that under both communal and individual rearing conditions, survival and development of *S. serrata* megalopae fed MBD alone were similar to that of megalopae fed *Artemia*. There was also indication that co-feeding MBD with *Artemia* may enhance megalopal survival and development. Rearing trials with zoea-III larvae showed that a 50:50 (MBD:*Artemia*) co-feeding regime resulted in the highest survival (66%) to zoea-IV although the difference to those fed 100% *Artemia* (50%) was not statistically significant. It was also shown that a few zoea-III larvae successfully moulted to zoea-IV when fed MBD only, and those larvae that perished survived substantially longer than unfed controls. Further rearing experiments were conducted to determine whether replacing the live food component of MBD (dried rotifers) with common protein sources, such as fish and squid meal, affected survival and development of *S. serrata* megalopae. No significant differences were found. These results suggest great potential for developing a formulated MBD for total replacement of *Artemia* for mud crab megalopae. Further research in our laboratory will focus on further assessment of factors affecting ingestion, digestion, and nutrient assimilation from MBD, using MBD as a tool to study the nutritional requirements of crab larvae, and optimising the physical properties of MBD for crab larvae.

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