Don’t believe what your eyes are telling you. All they show is limitation.
Look with your understanding. Find out what you already know and you will see the way to fly.

~ Richard Bach – Jonathan Livingston Seagull ~

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ECOLOGICAL INTERACTIONS BETWEEN TICKS, HOSTS AND FOREST TYPES AND THE IMPACT ON LYME BORRELIOSIS RISK

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Contents

List of abbreviations and symbols

Abbreviations

DON	density of nymphs
DOL	density of larvae
NIP	nymphal infection prevalence
DIN	density of infected nymphs (product of DON and NIP)
AIC	Akaike Information Criterion
AICc	AIC corrected for small sample size
SE	standard error
SD	standard deviation
GLM	generalized linear model
LMER	linear mixed-effect model
SR	species richness
s.l.	sensu lato
s.s.	sensu stricto
df	degrees of freedom
SL	shrub layer
manyglm	multivariate generalized linear model
TBEV	tick-borne encephalitis virus
ANOVA	analysis of variance
BBN	Bayesian belief network
VR	variance reduction

Symbols

eH	exponent of Shannon diversity index
n	sample size
w	Akaike weight
p	significance of statistical test
$\chi 2$	chi-squared value
ρ	correlation coefficient
Δ	difference


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1 Introduction

1.1 Biodiversity and human health

The loss and gain of species, genes and biological traits are part of Earth's evolutionary processes, but ecosystems worldwide are now losing biodiversity at accelerating rates (Cardinale et al. 2012). Current species extinction rates are estimated to be a thousand times higher than natural background rates of extinction, and future rates are likely to be ten thousand times higher (De Vos et al. 2015). This loss of biodiversity is mainly caused by habitat loss and fragmentation, pollution, overexploitation, invasive species and climate change, which are all anthropogenic in nature (MEA 2005). Following the Convention on Biological Diversity in 1992, in which the need for conserving biological diversity was stressed, interest grew in understanding the impact of biodiversity loss on ecosystem functioning and services (Cardinale et al. 2012). Recently, research focusing on the ecosystem service of disease control has linked biodiversity loss with increased infectious disease risk (Naeem et al. 2012; Johnson et al. 2013).

Many infectious diseases in humans are zoonotic and vector-borne i.e. the source of the pathogens is a non-human, vertebrate 'host' and the pathogens are transmitted to humans via the bite of an arthropod, called the 'vector' (Taylor et al. 2001; Jones et al. 2008). The vector feeds on the host and so carries the pathogen from one host to another. In order for the transmission cycle of the pathogen to be sustained, the vector must be able to transmit the pathogen to the host during feeding and the host must be susceptible to infection and transmit the infection to the next vector (Randolph and Craine 1995; Gubler 2009). The persistence of the pathogen thus depends on the contact rate between vectors and infected hosts and the transmission potential of the hosts. Host species often differ in their potential to feed and propagate vectors and to transmit pathogens to the vectors. Some species transmit a pathogen efficiently to vectors ('competent' hosts or 'reservoirs'), and other rarely or not ('dilution' hosts) (Matuschka et al. 1992; LoGiudice et al. 2003).

In an effort to elucidate the relationship between biodiversity and vector-borne disease risk, the 'dilution effect hypothesis' has been formulated (Norman et al. 1999; Ostfeld and Keesing 2000). A dilution effect occurs when a higher diversity of host species lowers the disease risk of one specific pathogen by decreasing the relative abundance of competent hosts over dilution hosts. A lower relative abundance of competent hosts decreases the contact probability between the vector and the competent hosts and makes a pathogen less abundant and less likely to persist than in the presence of highly competent host species alone (Keesing et al. 2006; Ogden and Tsao 2009; Wood and Lafferty 2013). A change in the host community composition might also affect the relationships between host species, potentially influencing the behaviour of competent hosts so that contact with the vector and subsequent pathogen transmission becomes less likely (Ostfeld and Keesing 2000; Keesing et al. 2006).

The generality of such a dilution effect, however, remains incompletely understood. The dilution effect relies on the assumption that environments with a low diversity of host are dominated by competent host species (Ostfeld and Keesing 2000; LoGiudice et al. 2003). Yet, adding species to a host community could also increase the disease risk, e.g. by increasing the amount of competent hosts relative to dilution hosts, or by increasing the abundance of vectors due to increased feeding opportunities (Wood and Lafferty 2013). An increasing amount of studies argue that the dilution effect is a local phenomenon driven by the transmission dynamics of the individual pathogens, vectors and host species, rather than by host species diversity itself (Ogden and Tsao 2009; Salkeld et al. 2013; Wood and Lafferty 2013).

1.2 The public and animal health concerns of ticks and tick-borne diseases

Ticks are major vectors of infectious disease agents to humans and domesticated animals, and the importance of tick-borne diseases is rising worldwide (Dantas-Torres et al. 2012; Hartemink and Takken 2016). Ticks constitute a diverse group of arthropods with at least 898 recognized species. In Europe, most cases of human tick-borne disease are related to ixodid tick species (Acari: Ixodidae). *Ixodes ricinus* (Linnaeus, 1758) is a widely spread tick species in Europe and can transmit a variety of viruses, bacteria and parasites to humans and domesticated animals (Randolph 2009; Heyman et al. 2010; Medlock et al. 2013). It is a host generalist that ascends the vegetation to wait for a passing host (called 'questing') to consume a blood meal. It may readily bite humans, thereby occasionally transmitting

pathogens (Gray 1998). Contrary to this exophilic behaviour, other ixodid species are more endophilic: they prefer dark, humid places and are usually found in the nests of their host species (Gern et al. 1997). *Ixodes hexagonus* (Leach, 1815) is such an endophilic tick that feeds primarily on hedgehogs, but has been found to infest other vertebrate species as well (Ogden et al. 2000; Sréter et al. 2003). Despite their nest-dwelling behaviour and specialization on hedgehogs, *I. hexagonus* is known to occasionally bite humans and transmit pathogens (Wormser and Wormser 2016), although less frequently than *I. ricinus*. *Ixodes ricinus* and *I. hexagonus* have been reported to feed on the same hosts (simultaneously) and are known to transmit a variety of tick-borne pathogens (Gern et al. 1997; Sréter et al. 2003; Skuballa et al. 2010; Skuballa et al. 2012).

The list of pathogens transmitted by ixodid ticks is growing (Rizzoli et al. 2014). The most frequently reported tick-borne infection in Europe and North America is Lyme borreliosis. The disease caused non-specific, influenza-like symptoms and most commonly manifests as erythema migrans, a slowly expanding skin lesion. The infection can spread to other tissues and organs, causing more severe manifestations that can involve a patient's skin, nervous system, joints or heart (Stanek et al. 2012). Lyme borreliosis is caused by some bacteria belonging to the *Borrelia burgdorferi* sensu lato (s.l.) species complex (Stanek et al. 2012). The *B. burgdorferi* s.l. complex (hereafter called '*Borrelia'*) consists of several genetically and ecologically distinct species ('genospecies'). At least five *Borrelia* genospecies have been shown to be pathogenic to humans, namely *B. burgdorferi* sensu stricto (s.s.), *B. afzelii*, *B. garinii*, *B. spielmanii* and *B. bavariensis* (see Stanek et al. 2012 and references therein). Each of these genospecies is generally associated with a distinct clinical manifestation (Coipan et al. 2016). Current knowledge is that *B. afzelii* is predominantly involved in dermal infections (erythema migrans and acrodermatitis chronica atrophicans), *B. burgdorferi* s.s. usually leads to articular manifestations, and *B. garinii* and *B. bavariensis* are associated with neurological disorders (Balmelli and Piffaretti 1995; Coipan et al. 2016). *Borrelia spielmanii* infection in humans is rare and has only been found in patients with erythema migrans (Maraspin et al. 2006). Other genospecies, e.g. *B. valaisiana*, have been rarely or not (yet) detected in patients, or are not considered to be important pathogens (see Stanek et al. (2012) and references therein).

Lyme borreliosis is a disease of high medical and economic impact. The societal cost of tick bites and Lyme borreliosis for the Netherlands in 2010 constituted approximately €19.3

million, including patient cost, healthcare cost and production loss (van den Wijngaard et al. 2017). The geographic range of *I. ricinus* and Lyme borreliosis in Europe is expanding, also to (sub)urban areas (Rizzoli et al. 2014; Medlock and Leach 2015), hence increasing the potential human health risk. Over the past decade, the incidence of Lyme borreliosis and tick bites has been reported to increase in some European countries, but not in others (see Hubálek 2009 and references therein). In the Netherlands, the incidence of general practitioner consultations for tick bites and diagnosis of erythema migrans has continuously increased between 1994 and 2009, but a recent survey showed a first sign of stabilization of erythema migrans diagnoses and a decreased incidence for tick bite consultations (Hofhuis et al. 2016). The incidence of Lyme borreliosis and tick bites in Belgium, on the contrary, appears to have been stable for at least over a decade. Vanthomme et al. (2012) and Bleyenheuft et al. (2015) observed no increasing trend in tick bites or Lyme borreliosis cases between 2003 and 2012. In 2013 and 2014, *Borrelia* infection in patients appeared to increase, most likely due to increased awareness by the public and health practitioners. Afterwards, the prevalence of *Borrelia* infection in humans in 2015 was again comparable with that of before 2013 (epidemio.wiv-isp.be).

Besides the *Borrelia* genospecies, other established pathogens circulate in the same ixodid ticks and vertebrate hosts, for example tick-borne encephalitis virus, *Anaplasma phagocytophilum*, *Rickettsia helvetica*, *Borrelia miyamotoi* and "*Candidatus* Neoehrlichia mikurensis" (Skuballa et al. 2010; Jahfari et al. 2012; Sprong et al. 2012; Wagemakers et al. 2015). These pathogens can cause non-characteristic, influenza-like symptoms in humans and are often confused with Lyme borreliosis (see Table 1.1 for more information on the different tick-borne micro-organisms). The infections they cause are often self-limiting, but in immunocompromised patients, they can cause severe clinical manifestations (Welinder-Olsson et al. 2010; Hovius et al. 2014; Silaghi et al. 2016). In the framework of human health and biodiversity loss, it is therefore important to gain more insight into the transmission dynamics and ecology of tick-borne diseases.

Table 1.1 The different micro-organisms mentioned in this thesis that circulate in ixodid ticks. Information is shown regarding the mode of transmission, the host species known (or presumed) to carry the micro-organism, the estimated prevalence of the micro-organism in questing *I. ricinus* ticks in Belgium or neighbouring countries and what disease symptoms it causes if the micro-organism has been detected in humans (pathogenic). *Anaplasma phagocytophilum* ecotype III and IV have not been detected in questing *I. ricinus* but are possibly transmitted by other ixodid tick species. Tick-borne encephalitis virus has not been detected yet in humans in Belgium. The data in this table has been collected from other studies and review papers (see Sprong et al. 2009; Jahfari et al. 2012; Bingsohn et al. 2013; Jahfari et al. 2014; Coipan 2016; Jahfari et al. 2017a; Wagemakers et al. 2017 and the research cited within these publications).

Micro-organism	Transmission mode	Host species	Prevalence (%)	Pathogenic	Disease symptoms
***Borrelia burgdorferi* s.l.**	transstadial		10		
B. afzelii		rodents, (squirrel, hedgehog)		√	non-specific, influenza-like, dermal infections
B. garinii		birds		√	non-specific, influenza-like, neurological disorders
***B. burgdorferi* s.s.**		(squirrel)		√	non-specific, influenza-like, arthritis
B. valaisiana		birds			
B. spielmanii		(dormice)		√	non-specific, influenza-like, dermal infections
B. bavariensis		rodents		√	non-specific, influenza-like, neurological disorders
B. turdi		birds			
Borrelia miyamotoi	transstadial, transovarial	rodents, birds	0-4	√	non-specific, relapsing fever, influenza-like
Anaplasma phagocytophilum	transstadial, transovarial		2.6		
ecotype I		cattle, red deer, dog, hedgehog		√	non-specific, influenza-like
ecotype II		roe deer			
ecotype III		rodents			
ecotype IV		birds			
Rickettsia helvetica	transstadial, transovarial	rodents, roe deer	4-16	√	non-specific, influenza-like
"*Candidatus* Neoehrlichia mikurensis"	transstadial	rodents	7	√	non-specific, influenza-like
tick-borne encephalitis virus (TBEV)	transstadial, transovarial	rodents	0-5	√	non-specific, influenza-like, neurological disorders

1.2.1 Lyme borreliosis ecology

In Western Europe, *I. ricinus* is the tick vector that most frequently transmits *Borrelia* to humans (Piesman and Gern 2004) although some *Borrelia* genospecies have also been detected in *I. hexagonus* too (Skuballa et al. 2007; Skuballa et al. 2012).

The life cycle of *I. ricinus* consists of three mobile, parasitic life stages: larva, nymph and adult. Larvae are smaller than 1 mm and have only three pairs of legs, while nymphs and adults have four pairs of legs (Hillyard 1996). Nymphs are 1.2 to 1.5 mm large and are generally light brown. Adult males are 2.4 to 2.8 mm large and have a black shield (scutum) that covers their back. The female is larger than the male (3.0 to 3.6 mm) and its back is only partly covered by the shield, so that it appears to be lighter in colour than the male (Hillyard 1996). The duration of the life cycle of *I. ricinus* may vary between two and six years and depends on several abiotic and biotic variables such as temperature, humidity and availability of hosts, but is usually completed in three years (Gray 1998; Randolph et al. 2002). Each life stage needs one blood meal from a vertebrate host (Fig. 1.1). Adult males usually do not feed but rather climb on hosts to mate with females, who feed on a host to be able to lay eggs (Anderson and Magnarelli 2008). An infected host can transmit *Borrelia* to feeding ticks via its blood. The tick then passes on the infection from life stage to life stage (i.e. transstadial transmission) (Gray 1998). An infected tick, in its turn, can infect an uninfected host. The contribution of transovarial transmission of *Borrelia* (i.e. transmission from infected female to the egg) to the maintenance of *Borrelia* in enzootic cycle is considered to be negligible (Richter et al. 2012; Rollend et al. 2013). Even though transovarial transmission of *Borrelia* in ticks seems to be inefficient, larvae in nature are occasionally infected and *Borrelia* is rarely found in questing larvae (Tappe et al. 2014; van Duijvendijk 2016). This can be due to partial feeding of the larvae and premature detachment from the host, or to occasional successful transovarial transmission. The life stage that is most often responsible for transmitting *Borrelia* to humans is the nymph (Barbour and Fish 1993), because larvae are usually free of infection and nymphs are often 10 fold more abundant in the vegetation than adult females (Perret et al. 2000). The nymph is thus the most interesting life stage to examine in the framework of public health risk.

Ixodes ricinus predominantly occurs in forests, due to the sheltered and humid microclimate and availability of suitable vertebrate hosts for their blood meals (Gray et al. 1998; Lindstrom and Jaenson 2003). They are sensitive to desiccation while questing and periodically return to the moist conditions at the base of the vegetation or the litter layer to restore their water balance (Needham and Teel 1991; Randolph and Storey 1999). In general, *I. ricinus* is inactive during the winter and begins questing when the temperature reaches to at least 7 °C for about 5 days (Perret et al. 2000). Nymphs are usually most active in late spring, often around May. However, the seasonal pattern of the density of questing *I. ricinus* nymphs seems to be variable and depending on local variations in abiotic conditions such as temperature and relative humidity (Tälleklint and Jaenson 1996; Perret et al. 2000; Randolph et al. 2002). Larvae usually quest close to the ground and can make contact with hosts of any size but usually feed on small hosts; nymphs quest slightly higher in the vegetation and miss most of the small hosts; adults quest higher than nymphs and generally feed and mate on large hosts (Mejlon and Jaenson 1997). Although *I. ricinus* is a host generalist that can parasitize a large range of host species, a recent European meta-analysis has established that the majority of *I. ricinus* individuals feed on only a few host species, i.e. small rodents, thrushes and roe deer (Hofmeester et al. 2016), species that are generally abundant and widely spread in most European regions. In many regions and habitats, larvae mainly feed on small rodents such as mice and voles. Nymphs mainly feed on thrushes, but also on smaller birds and rodents. Large ungulates are by far the most important feeding host for adult ticks in Europe and are important in the maintenance and reproduction of *I. ricinus* populations (Gray 1998; Ruiz-Fons and Gilbert 2010; Hofmeester et al. 2016). The role of some host species, for example small rodents and birds (see e.g. Hanincová et al. (2003a), Hanincová et al. (2003b) and Heylen et al. (2014)), in the transmission dynamics of *Borrelia* genospecies in Europe has been intensively studied. For many widespread host species, such as hedgehogs, martens and squirrels, the exact contribution in the ecology of Lyme borreliosis has not yet been determined (Hofmeester et al. 2016).

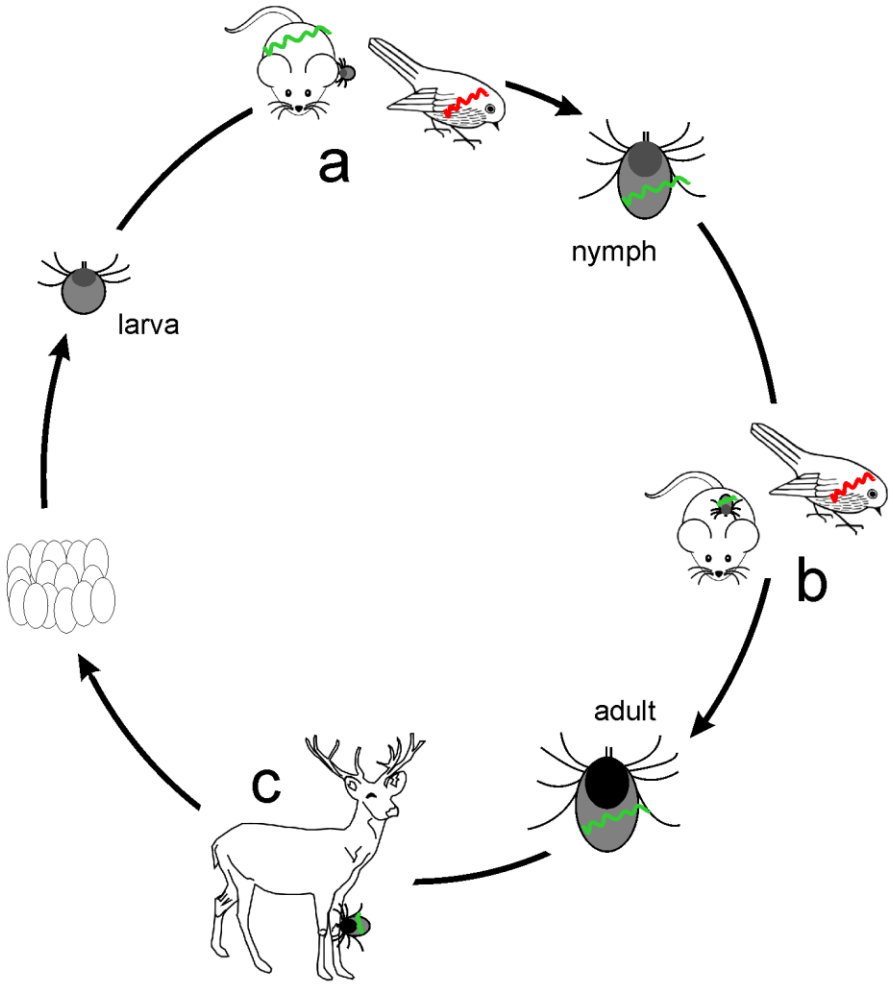

Fig. 1.1: The life cycle of a (female) *Ixodes ricinus* tick for the *Borrelia* transmission cycle in which mice and deer act as host species (one of many possible scenarios; the host species chosen by the different tick life stages and the hosts' infection status can differ from the ones presented here). The green spiral represent the *Borrelia* genospecies *B. afzelii*, which is associated with mice. The red spiral represents the bird-associated genospecies *B. garinii* or *B. valaisiana*. The larva hatches free from infection and will most commonly feed on a small rodent, such as a mouse. An infected mouse can transmit *B. afzelii* to the larva (a). The infected larva will then moult into an infected nymph. The infected nymph could consume its next blood meal on a bird, or it may again feed on a mouse. If the mouse is uninfected, it receives *B. afzelii* infection from the infected nymph (b). The infected nymph will then moult into an infected adult. The adult will most likely feed on a large ungulate, which does not transmit *Borrelia* (c).

Different host species, or ranges of hosts, are associated with different *Borrelia* genospecies. Many studies have already established the association between small rodents and *B. afzelii* (Humair et al. 1999; Hanincová et al. 2003a) and between birds and *B. garinii* and *B. valaisiana* (Hanincová et al. 2003b; Heylen et al. 2014). Some host species, such as the Eurasian red squirrel (*Sciurus vulgaris* Linnaeus, 1758) and the European hedgehog (*Erinaceus europaeus* Linnaeus, 1758) have been suggested to transmit *B. afzelii* as well (Skuballa et al. 2012; Pisanu et al. 2014). Large ungulates such as roe deer are considered incompetent to transmit any of the *Borrelia* genospecies (Fig. 1.1c) (Jaenson and Tälleklint 1992; Matuschka et al. 1993). The genospecies-host association seems to be attributed to the host's immune system, which may kill certain *Borrelia* genospecies that are transmitted via the tick to the host's blood, but does not succeed to eliminate others (Kurtenbach et al. 2002).

1.2.2 Dilution effect for Lyme borreliosis

The dilution effect hypothesis has been postulated, tested and elaborately discussed for the case of Lyme borreliosis in North American ecosystems (Norman et al. 1999; Ostfeld and Keesing 2000). In the forests of North America, the white-footed mouse (*Peromyscus leucopus* Rafinesque 1818) is by far the most competent host species and at the same time also the most common feeding host. LoGiudice et al. (2003) demonstrated that when host diversity increased, and other host species than the competent and ubiquitous white-footed mouse were added to the host community, *Borrelia* infection prevalence in ticks declined. Because these other host species were relatively inefficient in transmitting *Borrelia*, a higher host diversity decreased Lyme borreliosis risk. The ecology of Lyme borreliosis in North America differs from that in Europe. In North America, *B. burgdorferi* s.s. is the main *Borrelia* genospecies, which is not associated with a particular range of hosts in this region (Kurtenbach et al. 2006; Stanek et al. 2012). Furthermore, the host community of larval ticks in North America consists mainly of one competent host and several dilution hosts. In contrast, in Europe there are multiple pathogenic *Borrelia* genospecies associated with specific ranges of hosts, and these host communities probably consist of multiple competent reservoirs for *Borrelia* (Tälleklint and Jaenson 1994; Craine et al. 1995; Ostfeld and Keesing 2000). Therefore, because the impact of hosts densities on different *Borrelia* genospecies differs, the occurrence of a dilution effect for Lyme borreliosis in Europe has been questioned (Begon 2008) but has received little attention so far. In a study of Dutch

heathlands and adjacent forests, Tijsse-Klasen et al. (2010) did not find evidence of a dilution effect of sand lizards (*Lacerta agilis* Linnaeus, 1758), which are incompetent for most common *Borrelia* genospecies. The prevalence of *Borrelia* genospecies in ticks was not lower in the presence than in the absence of sand lizards. The study, however, did not investigate the host community composition in different sites of the same habitat. Instead, they compared ticks from heathland (sand lizard habitat) with ticks from adjacent forests (habitat unsuitable for sand lizards). Hofmeester et al. (2017) indirectly investigated the dilution effect in forest sites in the Netherlands by examining the possible indirect effect of the presence of predators in a host community on the density of reservoir-competent hosts and the tick burden on reservoir-competent hosts. The results from this study suggest that predators can lower the number of ticks feeding on reservoir-competent hosts, which implies that the host community composition can influence the density of infected nymphs. The effect of the whole host community of ticks on the density of infected ticks, however, was not examined. Investigating the impact of different host communities for ticks on Lyme borreliosis risk is crucial to evaluate the human health risk (Wood et al. 2014) seeing the ongoing biodiversity loss in European forests (MEA 2005), and the growing public health concern for Lyme borreliosis.

1.3 The influence of forest characteristics on Lyme borreliosis risk

The densities of *I. ricinus* and the prevalence of *Borrelia* in ticks strongly vary from one forest location to another (Gray et al. 1998; Jaenson et al. 2009). This variability is mainly determined by drivers related to the ticks' ability to maintain optimal water balance and the presence of suitable hosts (Gray et al. 1998). Forest management can influence some of these drivers by e.g. changing the structure of the forest or the composition of the species in the tree, shrub and herb layer.

1.3.1 Forest conversion in Europe

The production of wood has long been the focus of forest management in Europe. At the end of the 18th century, following a period of over-exploitation of the (semi-) natural forest ecosystems, fast-growing conifer species were planted throughout Europe, far beyond the limits of their natural ranges, to meet the demand for wood. Today, the objectives of forest management are increasingly oriented towards maintaining multifunctional ecosystems, embracing all the forests' goods and services (Farrel et al. 2000). Mixed and structurally

diverse forests are supposed to be better at delivering a whole suite of ecosystem services compared to homogeneous forests (van der Plas et al. 2016). Therefore, in many regions in Western Europe, forests are being converted from homogeneous conifer plantations to more natural, structure-rich mixed forests, dominated by indigenous broadleaved tree species (Olsthoorn et al. 1999; Spiecker et al. 2004), hereafter called 'forest conversion'. Compared to homogeneous forests, mixed forests tend to support a higher biological diversity (Lust et al. 1998; Felton et al. 2010) and be more resistant against biotic and abiotic disturbances such as insect attacks and wind damage (Farrel et al. 2000; Löf et al. 2004; Jactel and Brockerhoff 2007; Knoke et al. 2008). Moreover, mixed broadleaved forests are more attractive to visitors and have a higher recreation value compared to homogeneous coniferous forests (Bostedt and Mattsson 1995; Elsasser et al. 2010).

1.3.2 The effect of forest conversion on the dilution effect

In order to explain patterns in Lyme borreliosis risk, an increasing number of studies in Europe have focused on the influence of forest composition and structure (Lauterbach et al. 2013; De Keukeleire et al. 2015; Vourc'h et al. 2016). Estrada-Peña et al. (2011) investigated the geographic distribution pattern of the prevalence of *Borrelia* genospecies in *I. ricinus* ticks throughout Europe and found that the distribution pattern of genospecies showed a clear link with temperature gradients and vegetation features. A large-scale study in forests from southern France to central Sweden and Estonia showed that macroclimate only explained a small fraction of the variation in tick abundance and *Borrelia* prevalence in ticks between most European regions. Properties of macro- and microhabitat, which buffer macroclimate, had a considerable impact on tick abundance (Ehrmann et al. in press; Ehrmann et al. 2017). The abundance of ticks, therefore, strongly depends on habitat properties and on how humans manage forests. Tack (2013) investigated the effect of forest conversion on the abundance of *I. ricinus* in Belgium and found that densities of all life stages were higher in oak than in pine stands, and that tick densities increased with increasing shrub cover. Other studies also found higher densities of ticks in mixed and broadleaved forests compared to coniferous forests (Jaenson et al. 2009; James et al. 2013). The European practice of forest conversion thus appears to increase the density of ticks. However, the risk for human exposure to Lyme borreliosis depends on both the density of host-seeking infected ticks (hereafter called 'acarological risk') and the human-tick contact rate (Jaenson et al. 2009).

Both tick abundance and the prevalence of *Borrelia* in ticks are related to the host community composition (LoGiudice et al. 2003; Randolph 2004; Mannelli et al. 2012; Sprong et al. 2012; Hofmeester 2016), which is influenced by habitat characteristics. Changes in forest structure can improve habitat suitability for both ticks and important tick hosts such as small mammals and roe deer and are likely to be among the most crucial factors affecting tick-borne disease risk (Rizzoli et al. 2009; Tack et al. 2012a; James et al. 2013). Structure-rich mixed forests are considered to contain a higher host species diversity than homogeneous coniferous forests (Kennedy and Southwood 1984; Laiolo 2002; Carnus et al. 2006; Alexander et al. 2006; Brockerhoff et al. 2008; Du Bus de Warnaffe and Deconchat 2008). Tack (2013) found that the densities of roe deer were higher in structure-rich oak stands than in structure-poor pine stands. Forest conversion is thus expected to increase the density and diversity of host species, which will increase the density of ticks. Nevertheless, despite the higher tick density, the higher host diversity in converted forests could lower the *Borrelia* infection prevalence in ticks through a dilution effect, and so reduce the acarological risk for Lyme borreliosis. The effect of forest conversion on the infection prevalence of the different *Borrelia* genospecies in ticks has not been thoroughly investigated yet.

The large-scale forest conversions in Western Europe provide a unique opportunity to test the dilution effect hypothesis in Europe, in a complex setting of multiple pathogenic *Borrelia* genospecies and multiple host community compositions.

1.4 Objectives and outline of the thesis

The overall goal of this thesis was to gain more insight in the ecology of Lyme borreliosis and the relationships between the different components in the transmission cycle: the forest composition, the host community, the ticks and pathogens. The specific objectives of this thesis were:

1. relating forest composition to the diversity of hosts for ticks,
2. characterising the role of some poorly studied host species in the transmission cycle of Lyme borreliosis,
3. assessing the relationship between the host community composition and Lyme borreliosis risk in a spatiotemporal framework,
4. testing the dilution effect hypothesis in a setting of different forest compositions in Europe.

It is not clear yet in what manner the host community composition influences Lyme borreliosis risk in Europe. According to the dilution effect hypothesis, an increased host diversity could decrease the density of infected ticks. The type of habitat is expected to have a large influence on the occurrence and density of both ticks and host species. Research investigating the effect of the host community composition on the density of ticks infected with the distinct *Borrelia* genospecies in different environments is lacking.

In Belgium, conversion of structure-poor pine forests to structure-rich, semi-natural forests dominated by broadleaved tree species occurs on a large scale in the Kempen, a region with a high risk of Lyme borreliosis and a high recreation pressure (Verheyen et al. 2007; Linard et al. 2007; Vanthomme et al. 2012). Forests in this region mainly consist of even-aged homogeneous stands of Scots pine (*Pinus sylvestris* L.), and to a smaller extent Corsican pine (*P. nigra* Arnold subsp. *laricio* (Poiret) Maire) interspersed with more diverse, structure-rich broadleaved forests composed of pedunculate oak (*Quercus robur* L.), the exotic Northern red oak (*Q. rubra* L.), common beech (*Fagus sylvatica* L.), silver birch (*Betula pendula* Roth) and downy birch (*B. pubescens* Ehrh.).

Previous research on the impact of forest conversion on Lyme borreliosis risk by Tack (2013) was conducted in several forest sites in the Kempen, in northern Belgium. Most attention was given to two study sites located approximately 30 km apart (Fig. 1.2). Postel (hereafter called ‘site P’) is located in the north, near the border with the Netherlands.

Averbode-Hertberg (hereafter called ‘site AH’) consists of two forests close to each other, located in the municipalities Herselt and Tessenderlo, separated by 1 to 2 km of agricultural land and pasture. Site P and site AH are similar in terms of topography, soil, composition of the understory vegetation, and historical land use.

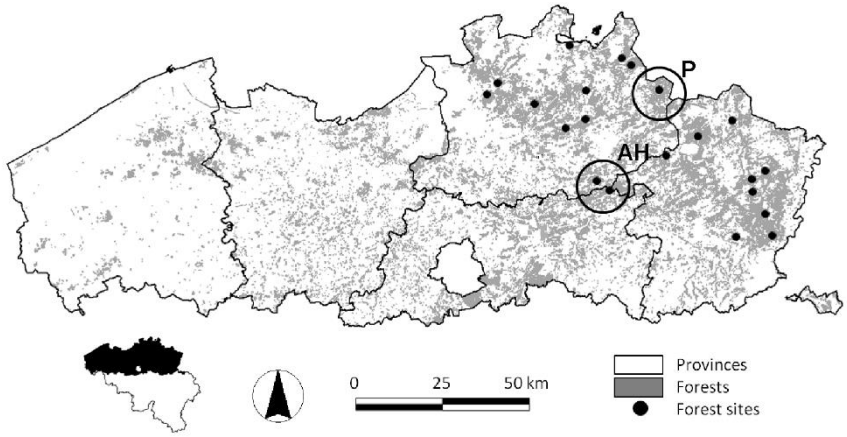

Fig. 1.2: Map of Flanders (northern Belgium) showing the area covered by forest, and the location of the forest sites that are investigated in this thesis. The two main study sites P and AH are depicted as two open circles. Map adapted from Tack (2013).

Tack (2013) investigated forest stands of four different forest types (Fig. 1.3). The stands are dominated by either oak (mainly pedunculate oak) or pine (Scots or Corsican pine), hereafter called ‘oak stands’ and ‘pine stands’. The stands have a well-established shrub layer covering more than 50% of the ground (‘with shrub layer’) or less than 25% of the ground (‘without a shrub layer’). These four forest types represent different steps in the process of forest conversion, with structure-poor pine stands as the first stage in the process, structure-rich oak stands as the intended result and structure-rich pine stands and structure-poor oak stands as intermediate stages. This thesis focusses on the same study area and uses a subset of the forest stands used in the study by Tack (2013).

The thesis consists of three parts (Fig. 1.4).

The first part (Chapter 2 and Chapter 3) focuses on the variation in Lyme borreliosis risk among different forest types. As nymphs are most often responsible for transmitting *Borrelia* to humans, we mainly focus on this life stage. Chapter 2 describes a large-scale field study of Lyme borreliosis risk in 93 stands of different forest types in 20 sites. We related the density of infected nymphs and the prevalence of the different *Borrelia* genospecies to forest type and discuss the results in the light of the dilution effect hypothesis.

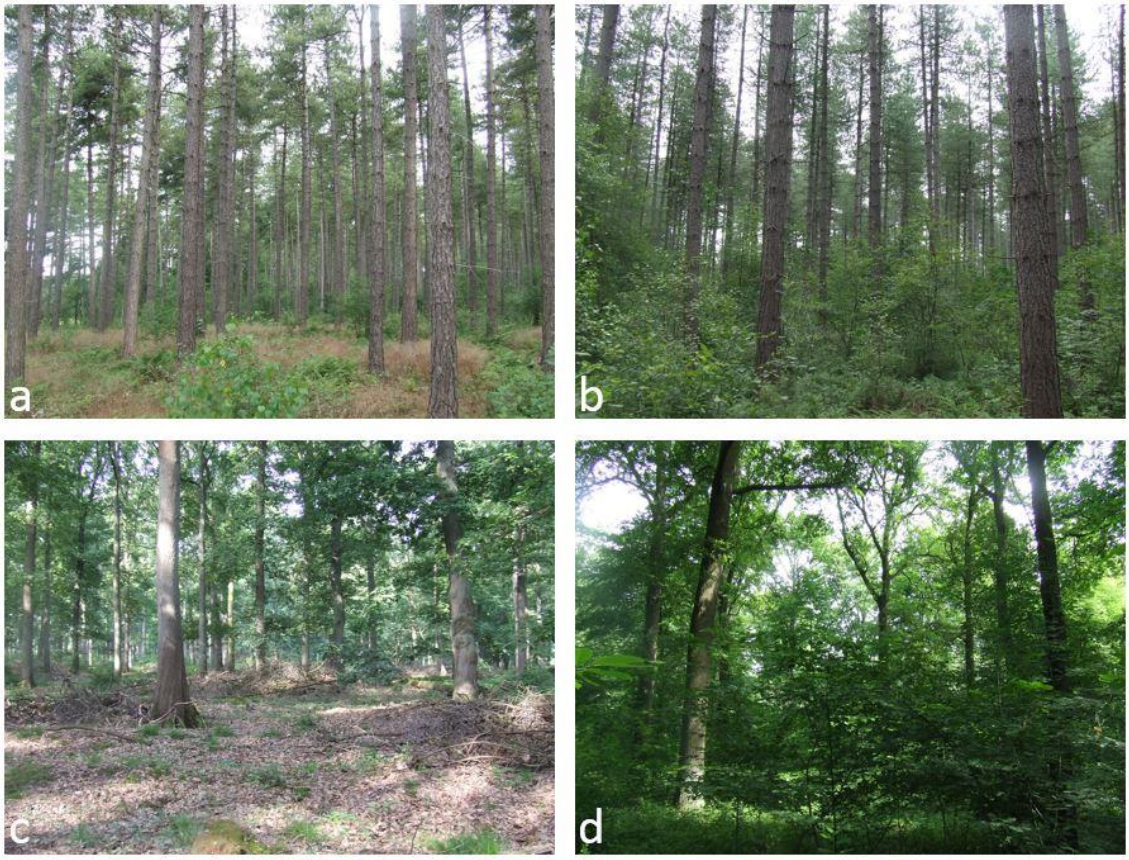

Fig. 1.3: The four different forest types used in this thesis: pine stands without a shrub layer (a), pine stands with a substantial shrub layer (b), oak stands without a shrub layer (c) and oak stands with a substantial shrub layer (d).

For Chapter 3 we used data on nymphs collected in 2009, 2010, 2013 and 2014 in two study sites to describe the spatiotemporal variation in disease risk. We focussed on the rodent-associated tick-borne pathogens *Borrelia afzelii* and *B. miyamotoi* since the spatial and temporal population dynamics of small rodents are expected to be important in explaining tick-borne disease risk. We discuss the possible role of climate, mast years of different tree species and the host community composition in the risk for tick-borne diseases, and stress the need to elucidate the contribution of the different hosts to the transmission cycle of *Borrelia*.

The second part consists of two case studies (Chapter 4 and Chapter 5) in which we investigated the role of two poorly studied host species in the transmission cycle of Lyme borreliosis and other tick-borne diseases. In Chapter 4, we report the results of a study in which we collected larvae, nymphs and adults of *Ixodes ricinus* and *I. hexagonus* from 54 hedgehogs and screened the ticks for presence of *Borrelia* genospecies, *B. miyamotoi*, *Anaplasma phagocytophilum*, *Rickettsia helvetica* and "*Candidatus* Neoehrlichia mikurensis". Chapter 5 deals with the infection prevalence of these tick-borne pathogens in spleen and liver samples from 45 squirrels.

In the third part (Chapter 6), we combined the acquired knowledge from the previous two parts and tested the dilution effect hypothesis for Lyme borreliosis. We used empirical data and a Bayesian belief network as complementary tools to investigate the impact of the proportion of dilution hosts in the host community on the density of ticks infected with *B. afzelii* and to identify the key drivers of the density of infected ticks.

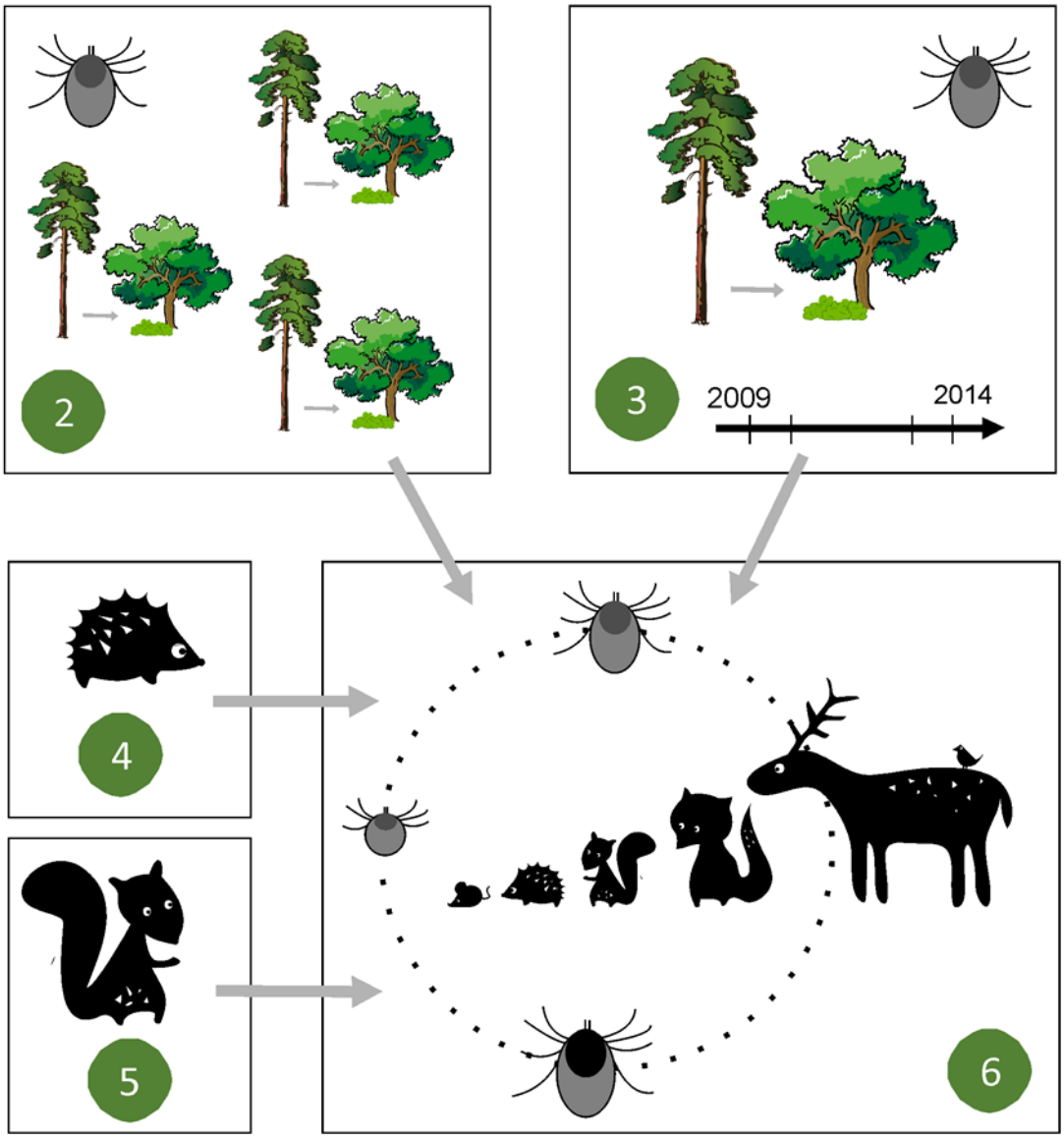

Fig. 1.4: Schematic overview of the thesis. The numbers represent the chapters (2-6). Chapters 2 and 3 investigate the spatiotemporal variability in Lyme borreliosis risk between different forest types that represent forest conversion from structure-poor pine stands to structure-rich oak stands. Chapters 4 and 5 focus on the role of two poorly characterized host species in the transmission cycle of tick-borne pathogens, and Chapter 6 focuses on the effect of host community compositions on Lyme borreliosis risk.


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2 Diversifying forest communities may change Lyme disease risk: extra dimension to the dilution effect in Europe

After: Ruyts S.C., Ampoorter E., Coipan E.C., Baeten L., Heylen D., Sprong H., Matthysen E., Verheyen K. (2016). Diversifying forest communities may change Lyme disease risk: extra dimension to the dilution effect in Europe. Parasitology, 143(10), 1310-1319 (IF 2.713)

2.1 Abstract

According to the dilution effect hypothesis, formulated in North America, nymphal infection prevalence (NIP) with the Lyme borreliosis causing pathogen *Borrelia burgdorferi* sensu lato (‘*Borrelia’*) decreases with increasing host diversity since host species differ in transmission potential. We analysed *Borrelia* infection in nymphs from 94 forest stands of four forest types in the Kempen, which are part of a diversification gradient with a supposedly related increasing host diversity: from pine stands without to oak stands with a shrub layer. We expected changing tree species and forest structure to increase host diversity and decrease NIP. In contrast with the dilution effect hypothesis, NIP did not differ between different forest types. Genospecies diversity however, and presumably also host diversity, was higher in oak than in pine stands. Infected nymphs tended to harbour *Borrelia afzelii* infection more often in pine stands while *B. garinii* and *B. burgdorferi* s.s. infection appeared to be more prevalent in oak stands. This has important health consequences, since the latter two cause more severe disease manifestations. We show that the dilution effect hypothesis must be nuanced for Europe and should consider the response of multiple pathogenic genospecies.

2.2 Introduction

Forest conversion appears to lead to elevated tick densities (Tack et al. 2012a; Tack et al. 2013). This is partially due to the higher shrub cover in more structurally diverse, converted stands, which leads to more suitable abiotic conditions for the ticks. Such a forest conversion will also induce changes in the host community (Brockerhoff et al. 2008; Du Bus de Warnaffe and Deconchat 2008; Tack et al. 2012a). Structure-rich mixed forests, the intended result of forest conversion, are considered to contain a higher host species diversity than homogeneous non-indigenous pine plantations (Kennedy and Southwood 1984; Laiolo 2002; Carnus et al. 2006; Alexander et al. 2006; Brockerhoff et al. 2008; Du Bus de Warnaffe and Deconchat 2008). According to the dilution effect hypothesis, this may dilute the prevalence of *Borrelia burgdorferi* sensu lato ('*Borrelia*') in the ticks. Furthermore, an increase in host diversity may lead to an increase in genospecies diversity, which, due to the association between genospecies and clinical manifestations, can change Lyme borreliosis risk.

By using the different *Borrelia* genospecies as distinct pathogens while testing the dilution effect in Europe, this study contributes to the knowledge gap on the interaction between the *Borrelia* genospecies, the tick *Ixodes ricinus* and hosts. In addition, most studies testing the dilution effect use indirect measures of human disease risk such as nymphal infection prevalence or tick abundance, while we use the density of infected questing nymphs, which better predicts risk (Begon 2008; Ogden and Tsao 2009). Here we investigated forest stands that differed in dominant tree species and presence of shrub cover, leading to four different forest types with supposedly increasing host diversification degree: from stands without a shrub layer dominated by pine trees to stands with a substantial shrub layer dominated by oak. Pine stands with a shrub layer and oak stands without a shrub layer are intermediate in diversification degree. We expect that changing dominant tree species and forest structure will lead to diversified forest communities, which entails increased host diversity, and changes Lyme borreliosis risk. We hypothesize that forest community diversification changes the density of infected nymphs by (i) increasing the abundance of nymphal ticks and (ii) decreasing the nymphal infection prevalence of *Borrelia* through a dilution effect. Furthermore, we expect the *Borrelia* genospecies community composition to change with forest community diversification, with nymphs from diversified forests housing a more diverse genospecies community.

2.3 Material and methods

2.3.1 Study area

For this study, we primarily used the same stands as Tack *et al*. (2012a). Each stand belongs to one of four forest types and represent a gradient of forest community diversification from low diversified pine stands without a shrub layer to highly diversified oak stands with a well-established shrub layer. The pine stands with a shrub layer and the oak stands without a shrub layer are less straightforward to rank mutually according to forest community diversity, but we can reasonably assume they are both intermediate between the high and low diversity forest types. A total of 93 stands were selected in 20 different forest sites: 20 pine stands without shrub layer, 32 pine stands with shrub layer, 19 oak stands without shrub layer and 22 oak stands with shrub layer. We attempted to select at least one stand of each type in every forest site. Due to the strong association between host species and tree species, the host community may differ between the two oak species. In our study, we selected pedunculate oak stands, as well as stands of non-indigenous red oak, but investigated their (indirect) effect on the ticks and *Borrelia* infection later during analysis. Forest stands were on average 1 ha large, ranging from 0.5 ha to 4 ha, and were distributed between 51°16'N, 4°29'E in the Northwestern part of the Kempen and 50°54'N, 5°39'E in the south-east.

2.3.2 Data collection

Each forest stand was visited once when the vegetation was dry between June and September 2013. To minimize the influence of time of day on the sampling of ticks, we visited the forest stands on each sampling occasion in a random order. Questing *I. ricinus* ticks were sampled by sweeping a white flannel blanket (1 m x 1 m) attached to a wooden pole over the herbaceous vegetation ('flagging'). Six 25 m parallel transects were performed in the centre in a representative part of each stand. The sampled area was calculated as the product of distance flagged (6 x 25 m = 150 m) and width of blanket (1 m); nymphs per 100 m^2 was used to as our measure of relative nymphal population density. The composition of the herbaceous layer was comparable between the different stands so we think that it did not influence the density estimates by impeding the sampling technique (Tack et al. 2012a). For that reason, also, sampling in dense shrubbery and bracken fern (*Pteridium aquilinum* (L.) Kuhn) was avoided. Entire-blanket sampling is an established method for ixodid tick collection (Falco and Fish 1992). In this thesis, we use it to compare

densities of ticks between sites, and we do not aim to infer actual densities. With this sampling technique, we caught all life stages of *I. ricinus*, but only collected the nymphs for further analysis. To distinguish between life stages in the field, we relied on morphological characteristics (see Chapter 1). The tick species was determined in the lab based on Hillyard (1996). After each transect, nymphs were removed from the blanket with forceps and stored in vials containing 70% ethanol. Nymphs from the different sampling occasions were pooled per forest stand. Due to limited resources, we only analysed a subset of the collected nymphs. We performed a power analysis in R version 3.2.0 (R Core Team 2017) to estimate the number of nymphs we needed to analyse to have a good chance of detecting any statistical significant effect. Based on this, we randomly selected 35 individuals from each pool. For one stand, only 34 individuals were analysed due to a low number of nymphs caught. DNA extraction from each of these ticks was done by alkaline lysis in ammonium hydroxide, as described previously (Schouls et al. 1999). For the detection of *Borrelia* a duplex qPCR, targeting ospA and flaB genes, was used. For the sequences of primers and probes, we refer to Appendix 2.1. The qPCR was performed using the iQ Multiplex Powermix PCR reagent kit (Bio-Rad Laboratories, Hercules, USA), in a LightCycler 480 Real-Time PCR System (F. Hoffmann-La Roche, Basel, Switzerland). The reaction mix consisted of iQ multiplex Powermix, 100 nM of the B-FlaB-Rc and B-FlaB-Rt primers, 200 nM of the B-FlaB-F, 400 nM of the B-OspA_modF and B-OspA_borAS primers, 100 nM of the B-OspAmodPatto probe, 200 nM of the B-FlaB-Patto probe, and 3 µl of template DNA in a final volume of 20 µl. Cycling conditions included an initial activation of the iTaq DNA polymerase at 95 °C for 5 min, followed by 60 cycles of a 5 s denaturation at 95 °C, followed by a 35 s annealing-extension step at 60 °C (ramp rate 2.2 °C/s and a single point measurement at 60 °C) and a cooling cycle of 37 °C for 20 s. Analysis was performed using the second derivative calculations for crossing point values. For each run, positive and negative controls and blank samples were included. For confirmation of presence of *Borrelia* DNA, the positive samples of the qPCR were further submitted to PCR targeting the variable 5S-23S intergenic spacer region (IGS). The PCR was performed according to the protocol described in Coipan et al. (2013). Identification of *Borrelia* genospecies was done based on the DNA sequence of IGS. This method is able to detect all known genospecies of *Borrelia*, as shown in (Coipan et al. 2013a). PCR products were sequenced using an ABI PRISM BigDye Terminator Cycle sequencing Ready Reaction kit (Perkin Elmer, Applied Biosystems). Sequences were confirmed by sequencing both strands (Sanger et al. 1977). Storage and analysis of the IGS sequences

were performed in BioNumerics version 7.0 (Applied Math, Belgium). *Borrelia* genospecies were assigned based on sequence identity with reference DNA sequences from GenBank (http://www.ncbi.nlm.nih.gov). The sensitivity of the PCR-based detection of *Borrelia* in ticks and the DNA sequencing is described in Heylen et al. (2013). We did not take into account the infection intensity of the *Borrelia* genospecies per tick.

2.3.3 Data analysis

All analyses were conducted in R version 3.2.0 (R Core Team 2017). Graphs were made with the package *ggplot2* (Wickham 2009). We defined nymphal infection prevalence (NIP) as the proportion of nymphs infected with *Borrelia* and the density of nymphs (DON) as the number of nymphs caught per 100 m². The density of infected nymphs (DIN) is the product of NIP and DON. First, we fitted effects for tree species ('pine' or 'oak'), the presence of a shrub layer ('yes' or 'no') and the interaction between tree species and the presence of a shrub layer on DON, NIP and DIN using generalized linear models (*glm*). DON and DIN resembled overdispersed count data, so we applied a negative binomial error distribution using the package *MASS* (Venables and Ripley 2002). For NIP, we used a *glm* with binomial error distribution for proportional data. Significances in all model fits were assessed through analysis of deviance with Chi-square test. We checked for heterogeneity of the residuals following the approach described in Zuur *et al*. (2009).

Second, to examine the impact of the different types of forest composition and structure on the *Borrelia* genospecies community compositions, we fitted a multivariate generalized linear model (*manyglm*) to our data (Warton et al. 2012) as implemented in the R package *mvabund* (Wang et al. 2012). The fitted model assumed a binomial distribution of the prevalence (proportion) data. Predictor variables included tree species, the presence of a shrub layer and the interaction between tree species and presence of a shrub layer. An analysis of deviance for multivariate *glm* fits with likelihood ratio test was employed to determine which variables contributed significantly to the differences between the community compositions. Afterwards, we considered the *Borrelia* genospecies as distinct pathogens, and analysed the effect of the predictor variables on the prevalence of the individual genospecies the same way as described above for the response variable NIP with a binomial *glm*.

Finally, we calculated the diversity of each genospecies community (exponent of Shannon index or equivalent species numbers) with the function *diversity* in the R package *vegan* (Oksanen et al. 2015) and tested for the effect of the forest type variables with a generalized linear model.

To test the impact of the ecologically relevant difference between the stands of indigenous pedunculate oak and non-indigenous red oak, we conducted all above-mentioned analyses on the whole dataset of 93 stands and afterwards on a limited dataset of the oak stands. The outcomes of the analysis on the dataset of the red oak stands were then compared to those of the pedunculate oak stands. No difference was found for any response variable, at the level of *Borrelia* or the genospecies, between the indigenous pedunculate oak stands and the non-indigenous red oak stands, so we considered the category "oak" as robust.

2.4 Results

In the 93 forest stands, a total of 9554 *I. ricinus* nymphs were caught, with a mean density (± SE) of 40.5 nymphs (± 3.6) per 100 m². We examined 3254 nymphs for *Borrelia* spirochetes, and 508 were infected. This corresponds with a mean nymphal infection prevalence (NIP) of 15.6% (± 0.8) over all forest stands. Average density of infected nymphs was 5.9 nymphs (± 0.6) per 100 m². *Borrelia*-positive nymphs were found in all forest stands examined. We identified six different genospecies in 462 (91%) infected nymphs, namely *Borrelia afzelii*, *B. garinii*, *B. burgdorferi* s.s., *B. valaisiana*, *B. spielmanii* and *B. bavariensis*. For the other 46 infected nymphs, genospecies could not be defined because of the lower sensitivity of the conventional PCR in comparison with the qPCR. Only one genospecies was identified in each infected nymph. From the six detected genospecies, *B. afzelii* was the most prevalent genospecies, occurring in 10.6% of the nymphs (± 0.7) or 73.8% (±2.8) of the infected nymphs. The mean prevalence of *B. garinii* and *B. burgdorferi* s.s. in nymphs was 1.5% (± 0.3) and 1.2% (± 0.2), respectively. The mean prevalences of the other genospecies were lower than 1%.

The generalized linear models showed a significant effect of tree species on DON ($p = 0.02$) and a marginally significant effect of the presence of a shrub layer ($p=0.05$) with lowest densities in pine stands without a shrub layer (Fig. 2.1). The interaction between tree species and the presence of a shrub layer had a marginally significant effect on DIN ($p = 0.09$). We found no significant effect of any predictor variable on NIP (Fig. 2.1).

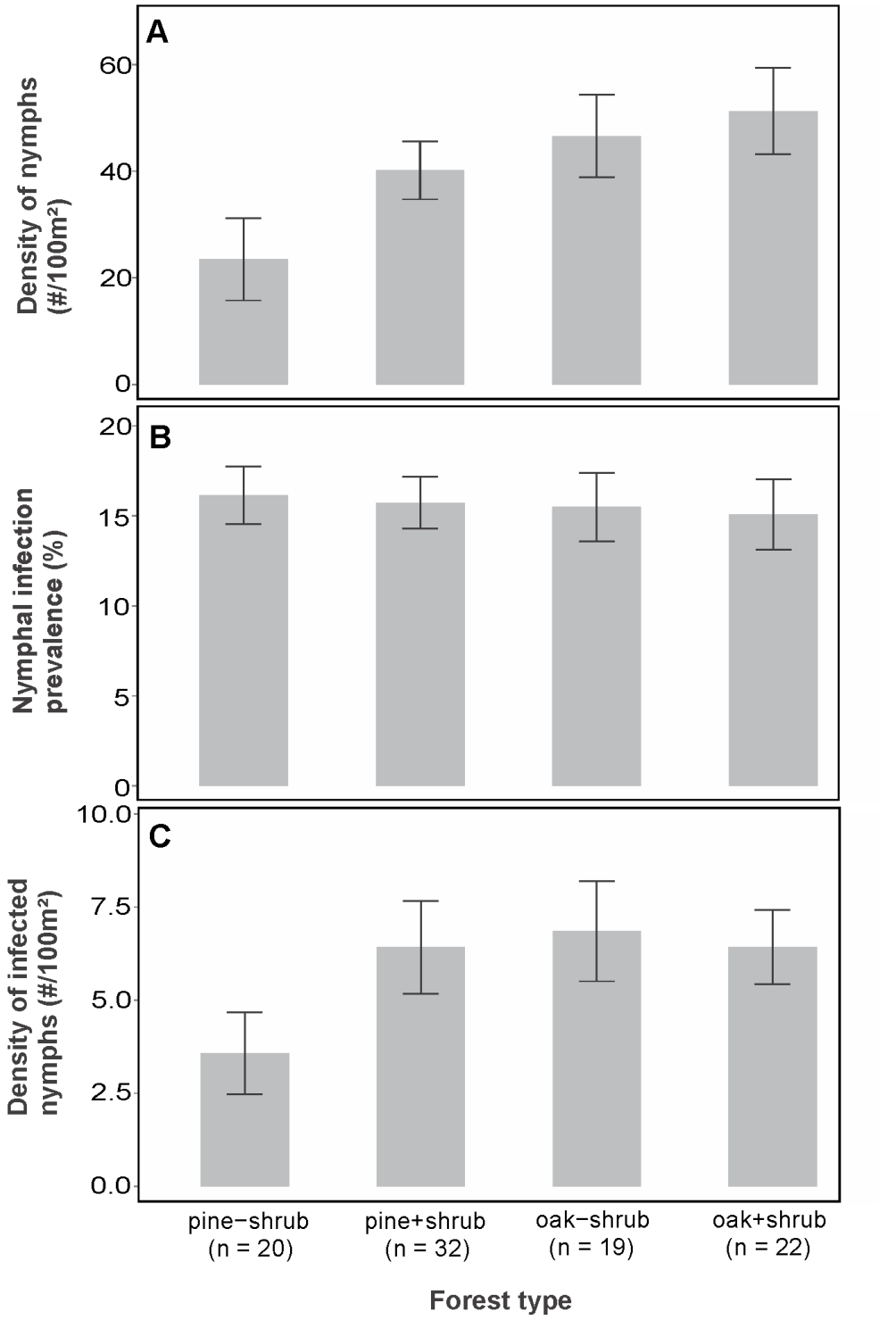

Fig. 2.1 Density of nymphs (a), nymphal infection prevalence (b) and density of infected nymphs (c) in the different forest types (mean ± SE). n: number of stands used per forest type. Note the difference in scaling of the y-axis and the units used.

The community analysis of *Borrelia* genospecies revealed a significant effect of tree species on the diversity of *Borrelia* genospecies communities (Fig. 2.2, Appendix 2.2), with more diverse communities in nymphs from oak stands ($t = -2.21$, $p = 0.03$). The effect of tree species on the genospecies community composition was marginally significant (Dev = 4.44, $p = 0.07$).

When we considered the *Borrelia* genospecies as separate pathogens in the generalized linear models, we could not detect a significant effect of the forest type variables on the nymphal infection prevalence of the genospecies. Nevertheless, we can clearly see in the genospecies community composition (Fig. 2.2) a trend towards a higher prevalence of *B. afzelii* in nymphs from pine compared to oak stands, and for *B. garinii* and *B. burgdorferi* s.s. a trend towards a higher prevalence in oak compared to pine stands. The genospecies community in the infected nymphs appeared to be dominated by *B. afzelii* in all forest types but it was more pronounced in the pine stands (83.9% (95% confidence interval [71.4, 91.6]) of the infected nymphs) compared to the oak stands (62.4% [46.9, 75.8]) (Fig 2.2, Appendix 2.2). *B. garinii* and *B. burgdorferi* s.s. occurred in 6.7% [2.4, 17.6] and 4.9% [1.4, 15.3] of the infected nymphs from the pine stands compared to 12.5% [5.4, 26.5] and 16% [7.6, 30.5] in the oak stands, respectively. The share of *B. valaisiana*, *B. spielmanii* and *B. bavariensis* in the genospecies community in nymphs was relatively low, namely 1% [0.1, 13.3], 3% [0.6, 13.3] and 0.4% [4.8e-5, 2.4] in pine, and 5.7% [1.6, 18.5], 2.9% [0.5, 15.6] and 0.3% [1.9e-5, 3.9] in oak stands, respectively.

2.5 Discussion

This study tests the dilution effect hypothesis for Lyme borreliosis in Europe while focusing on the different *Borrelia* genospecies as distinct pathogens, which, to our knowledge, has hardly been performed. Even though we did not test the dilution effect directly, since we did not empirically quantify the host communities, the association between planted forests and biodiversity has been covered sufficiently in literature to make reasonable assessments about the host diversity in the examined forest types (Laiolo 2002; Carnus et al. 2006; Brockerhoff et al. 2008; Du Bus de Warnaffe and Deconchat 2008). We assumed the host diversity to be higher in the oak stands with a substantial shrub layer than in the pine stands without a shrub layer. Further research that empirically quantifies the host communities, however, is needed to confirm this statement. We show that the dilution effect must be nuanced for Europe and should consider the response of multiple pathogenic genospecies.

The density of infected nymphs and associated human health risk will not necessarily decline with increasing biodiversity or host diversity, but will instead largely depend on the species assemblages of the host community. The different *Borrelia* genospecies can thus best be considered as separate pathogens, each with a separate possible dilution effect.

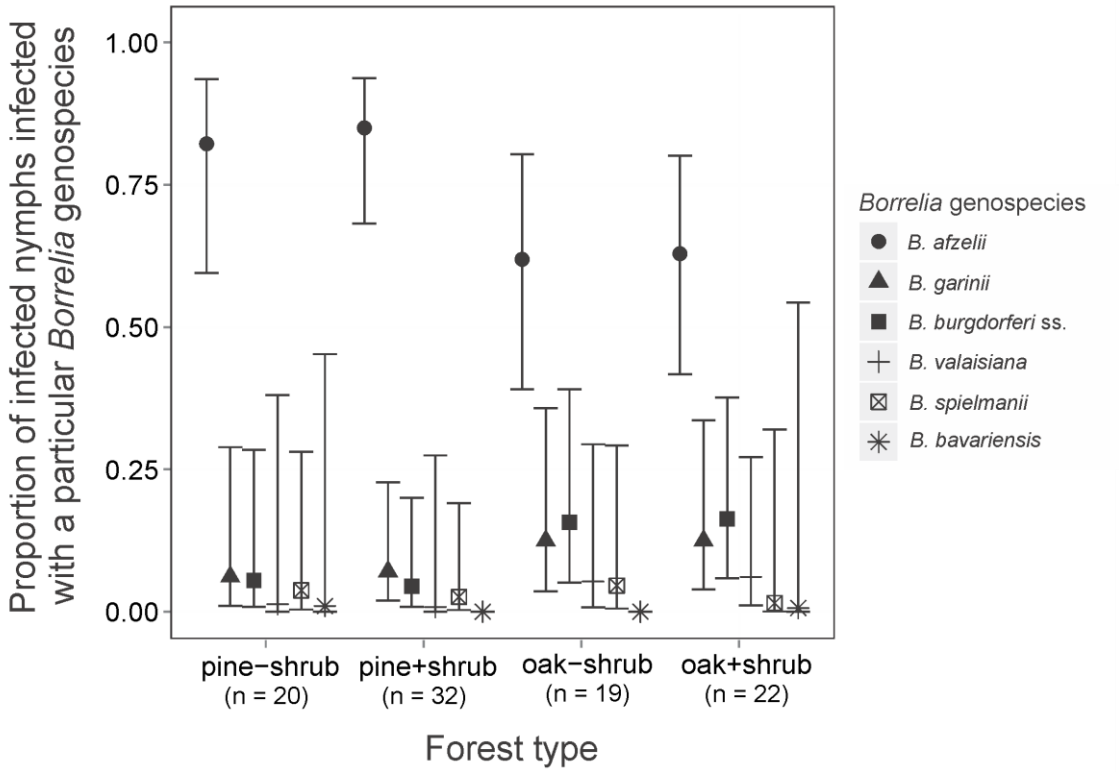

Fig. 2.2 The community composition of *Borrelia* genospecies in field collected nymphs (mean proportion + 95% confidence interval) in pine and oak stands, with or without shrub layer. n: number of stands used per forest type.

Highest densities of nymphs were found in oak stands and lowest in pine stands. Hence, our results show that changing forest composition could lead to increased tick abundances, which confirms the findings of former studies (Tack et al. 2012b; Tack et al. 2013). This could be due to the fact that more host species are present in oak forests, as suggested by Laiolo *et al.* (2004) and Tack *et al.* (2012b). We found an average nymphal infection prevalence of 15.6%, which is higher than the 10.1% mean nymphal infection prevalence usually found in Europe (Rauter and Hartung, 2005). This finding, together with the fact that the forests in the Kempen are readily visited for outdoor activities, suggests that it is

an area of potentially high Lyme borreliosis risk, as already proposed by Linard *et al.* (2007) and Vanthomme *et al.* (2012). The nymphal infection prevalence did not differ between forest types. The lack of a decline in nymphal infection prevalence does not allow us to confirm the dilution effect hypothesis. This might indicate that even though host diversity is likely higher in oak forests, the relative proportion of non-competent hosts is probably not substantially large enough to reduce infection prevalence, e.g. because more competent than non-competent species are added to the host community with increasing host diversity (a possible scenario recognized by Ostfeld and Keesing, 2000b; Wood and Lafferty, 2013). We could also not detect a significant effect of tree species or the presence of a shrub layer on the density of infected nymphs. According to Begon (2008) and Schmidt and Ostfeld (2001), a dilution effect only applies to situations where tick bites are wasted on less competent reservoir hosts and when the compensatory increase in vector abundance, caused by the increase in host diversity, is limited. Regardless of whether the host species in question are competent reservoirs or not, an increase in host abundance, and thus feeding opportunities for the ticks, will increase tick abundance (Ogden and Tsao 2009; Randolph and Dobson 2012). This indeed appears to be the case in our study, with higher densities of nymphs in oak forests.

The most common genospecies found in questing nymphs in Europe are *B. afzelii* and *B. garinii* but the prevalence of the different genospecies varies between regions (Rauter and Hartung 2005). In our study region, we found that *B. afzelii* and *B. garinii* constituted 74.5% and 9.3% of the genospecies communities, respectively. Since *B. afzelii* is associated with small rodents, and the majority of infected nymphs was infected with this genospecies, we can assume that rodents such as mice and voles are important feeding hosts for *I. ricinus* larvae and important transmission hosts of *Borrelia* infection. *B. garinii* (and also *B. valaisiana*) is associated with passerine birds (Heylen et al. 2013b), and birds appear to be less important reservoir hosts in the *Borrelia* transmission cycle of *I. ricinus* ticks than rodents, since the infection prevalence of questing nymphs with *B. garinii* and *B. valaisiana* is much lower than that with *B. afzelii*. This lower prevalence of infection can be due to the fact that passerine birds are less important feeding hosts for the larvae of *I. ricinus* or that they are less efficient at transmitting *Borrelia* infection, or both (Brunner et al. 2008). A recent meta-analysis demonstrated that birds are generally more important feeding hosts for nymphs than for larvae (unpublished results, although this strongly depends on bird species, see Comstedt *et al.*, 2006; Marsot *et al.*, 2012) so that questing larvae are less likely

to obtain infection with *B. garinii* than questing nymphs. In the nymphs in our study area, we also found an unusually high prevalence of *B. burgdorferi* s.s., namely 9.8% (Rauter and Hartung 2005; Burri et al. 2007; Bingsohn et al. 2013). This is the highest reported prevalence of this genospecies in questing nymphs in Western Europe as far as we know. *Borrelia burgdorferi* s.s. is most probably associated with Eurasian red squirrel (*Sciurus vulgaris*) but because this association is not yet strictly determined (Humair and Gern, 1998; Pisanu et al., 2014, but see Kurtenbach et al. 2002), it is plausible that another host species, or range of hosts, is responsible for the transmission of *B. burgdorferi* s.s. to the ticks in our study area. It is however not yet clear which one. The predictions based on the occurrence or abundance of the different host species, however, are based on speculations since we do not possess data on the host species community. Further research should empirically determine the host community composition in different habitats.

The genospecies community composition in questing nymphs did not significantly differ between forest types, but tree species had a marginally significant effect on the community composition. In pine stands, larvae seem to have fed more often on small rodents, which transmit *B. afzelii*. In oak stands, larvae seem to have fed more on other types of hosts, such as birds, which transmit *B. garinii* and *B. valaisiana* (Humair et al. 1999; Hanincová et al. 2003a; Hanincová et al. 2003b; Heylen et al. 2014). There was a clear effect of tree species on the diversity of the genospecies communities, with more diverse communities in oak stands compared to pine stands. This increase in *Borrelia* genospecies diversity in the ticks from diversified forest communities can pose serious health risks. *Borrelia burgdorferi* s.s. and *B. garinii* are most often associated with Lyme arthritis and neuroborreliosis, respectively, while an infection with *B. afzelii* commonly causes skin manifestations such as erythema migrans or acrodermatitis chronica atrophicans (Strle and Stanek 2009). These conditions can be regarded as less severe, compared to damage of joints or the nervous system caused by *B. burgdorferi* s.s. and *B. garinii*. This indicates that human health risk can increase with increasing host diversity, when the contribution to the genospecies community of the more ‘dangerous’ *Borrelia* genospecies increases.

Compared to the average home range of some host species, the forest stands we investigated are of relatively small size (Verkem et al. 2003) and often imbedded in a mosaic of stands of a different forest type. First, tick abundance is not only determined by the availability of hosts, but also by abiotic factors such as humidity. Therefore, the density of tick can be

related to forest type. Second, while ranging, a host species may cross different unsuitable habitats and spend some of its time there. As ticks are distributed while being attached to their host, it is plausible that nymphs that have fed as a larva on a particular host species are occasionally found in a habitat that is not suitable for that host. Thus ticks, and so also typical host specific *Borrelia* genospecies, can occur in a forest type that is not the favourable habitat of the host. Hosts will spend, however, more of their time in their preferred habitat, so that the chance that a tick drops off in that habitat type instead of in an unfavourable habitat type is higher. Moreover, despite the small size of forest stands, Tack (2013) found that populations of bank vole and wood mouse differed between small neighbouring stands of different forest types, with different populations being composed of different individuals. Most larvae will thus probably be distributed by their host to a forest stand that is of a favourable forest type for the host, and so the association between *Borrelia* genospecies and forest type remains reasonable. We think, however, that our estimates of *Borrelia* genospecies prevalence and diversity are only conservative estimates. In a more homogeneous landscape, with larger stands of the same forest type, the pattern in *Borrelia* genospecies occurrence we see now would be even clearer and the differences between the forest types even larger.

2.6 Conclusions

From our results we may conclude that the public health risk associated with Lyme borreliosis in Europe will not only depend on the nymphal infection prevalence of *Borrelia* or on density of infected nymphs, but also on the prevalences of the distinct genospecies. Even if a more diverse host community causes a dilution effect to occur and the density of infected nymphs declines, disease risk can increase if the prevalence of a more 'dangerous' genospecies increases. As already suggested by Kurtenbach et al. (2006), and mentioned above, the interaction between *Borrelia*, ticks and hosts in Europe appears to be much more complex than the situation observed in North America, because of the existence of multiple pathogenic and specialist *Borrelia* genospecies in Europe. We can now confirm the contrast between these two regions and suggest that increasing host diversity, or adding species to the host community, can increase or decrease the prevalence of individual genospecies, depending on the response of the associated host species. Most likely in Europe the prevalence of *B. afzelii* will decline with increasing forest community diversification due to a dilution effect on small rodents, while the prevalence of the more 'dangerous'

pathogens *B. garinii* and *B. burgdorferi* s.s. will increase. It is then still possible for a dilution effect to occur in Europe on the level of *Borrelia*, when hosts that are added to the host community are less efficient in transmitting *Borrelia* to ticks than hosts already occurring in a species poor community (Ostfeld and Keesing 2000; LoGiudice et al. 2003), which decreases the chance that a tick feeds on a competent reservoir species. This scenario is similar to the trend we observed in our study. It is therefore not increasing biodiversity in itself that will decrease Lyme borreliosis risk. The identity of the hosts that are added to the host community and their interactions with each other and the ticks will determine if a dilution effect will occur (Ostfeld and Keesing 2000; Randolph and Dobson 2012), both at the level of *Borrelia* and of the *Borrelia* genospecies. These new insights introduce an extra dimension to the dilution effect hypothesis for Lyme borreliosis in Europe and pose important implications for the role of biodiversity in disease ecology. This study therefore strongly emphasizes the need to consider the different *Borrelia* genospecies as distinct pathogens and to study the species assemblages of the host community in high risk areas to assess human disease risk.

Appendix 2.1.

Primers and probes used in the PCR analyses to detect *Borrelia burgdorferi* s.l. and *Borrelia* genospecies infections.

Target gene/primer & probe	Amplicon length	Sequence
OspA (Outer membrane Protein A, *B. burgdorferi* s.l.)	± 139bp	
B-OspA_modF		5'-AAT ATT TAT TGG GAA TAG GTC TAA-3'
B-OspA_borAS		5'-CTT TGT CTT TTT CTT TRC TTA CA-3'
B-OspAmodPatto		5'-Atto520-AAG CAA AAT GTT AGC AGC CTT GA-BHQ1-3'
FlaB (Flagelin B, *B. burgdorferi* sl.)	± 89bp	
B-FlaB-F		5'-CAG AIA GAG GTT CTA TAC AIA TTG AIA TAG A-3'
B-FlaB-Rc		5'-GTG CAT TTG GTT AIA TTG CGC-3'
B-FlaB-Rt		5'-GTG CAT TTG GTT AIA TTG TGC-3'
B-FlaB-Patto		5'-Atto425-CAA CTI ACA GAI GAA AXT AAI AGA ATT GCT GAI CA-Pho-3'

X = BHQ-1-dT

BHQ = Black Hole Quencher

Appendix 2.2

Proportions of *Borrelia* genospecies detected in the genospecies community in nymphs from the Kempen region and the diversity (exponent of Shannon index) of the genospecies communities, per forest type (mean ± SE). n: number of stands used per forest type. Mean is given in percentage.

Borrelia genospecies	pine				oak			
	without shrub (n=20)		with shrub (n=32)		without shrub (n=19)		with shrub (n=22)	
B. afzelii	82.2	(±5.2)	85	(±3.3)	61.9	(±7)	62.9	(±6.7)
B. garinii	6.2	(±2.9)	7.1	(±2.3)	12.5	(±4.6)	12.5	(±3.3)
B. burgdorferi s.s.	5.5	(±3.3)	4.5	(±1.8)	15.7	(±5.9)	16.3	(±3.9)
B. valaisiana	1.3	(±0.9)	0.8	(±0.6)	5.3	(±2.3)	6.1	(±2)
B. spielmanii	3.8	(±3.75)	2.6	(±1.5)	4.6	(±2.8)	1.5	(±1.5)
B. bavariensis	1	(±1)	0	(±0)	0	(±0)	0.6	(±0.6)
diversity of *Borrelia* genospecies community	1.4	(±0.5)	1.5	(±0.5)	2	(±0.8)	2.2	(±1)


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3 Year-to-year variation in the density of *Ixodes ricinus* ticks and the prevalence of the rodent-associated human pathogens *Borrelia afzelii* and *B. miyamotoi* in different forest types.

After: Ruyts S. C., Tack W., Ampoorter E., Coipan E. C., Matthysen E., Heylen D., Sprong H., Verheyen K. Year-to-year variation in the density of *Ixodes ricinus* ticks and the prevalence of the rodent-associated human pathogens *Borrelia afzelii* and *B. miyamotoi* in different forest types. Ticks and Tick-borne diseases, accepted (IF 3.23)

3.1 Abstract

The human pathogens *Borrelia afzelii*, which causes Lyme borreliosis and *B. miyamotoi*, which causes relapsing fever, both circulate between *Ixodes ricinus* ticks and rodents. The spatiotemporal dynamics in the prevalence of these pathogens have not yet been fully elucidated, but probably depend on the spatiotemporal population dynamics of small rodents. We aimed to evaluate the effect of different forest types on the density of infected nymphs in different years and to obtain more knowledge about the spatial and temporal patterns of ticks and tick-borne pathogens. We analysed unfed nymphal ticks from 22 stands of four different forest types in Belgium in 2009, 2010, 2013 and 2014 and found that the density of nymphs in general and the density of nymphs infected with *B. afzelii* and *B. miyamotoi* varied yearly, but without temporal variation in the infection prevalence. The yearly variation in density of infected nymphs in our study thus seems to be caused most by the variation in the density of nymphs, which makes it a good predictor of disease risk. The risk for rodent-associated tick-borne diseases also varied between forest types. We stress the need to elucidate the contribution of the host community composition to tick-borne disease risk.

3.2 Introduction

Both *Borrelia afzelii*, which causes Lyme borreliosis, and *B. miyamotoi*, which causes relapsing fever, circulate in the same tick species and the same vertebrate hosts (Hanincová et al. 2003a; Cosson et al. 2014). In Europe, *Ixodes ricinus* is the main vector for *B. afzelii* transmission to humans (Piesman and Gern 2004) and especially the host-seeking nymphs contribute most to the Lyme borreliosis risk (Barbour and Fish, 1993). In addition, this tick species is an important carrier for *B. miyamotoi*, and as with other tick-borne pathogens, *B. afzelii* regularly co-occurs with *B. miyamotoi* in the same tick individuals (Gern et al. 2010; Cosson et al. 2014; Kjelland et al. 2015).

The different genospecies of *B. burgdorferi* s.l. (‘*Borrelia’*) and *B. miyamotoi* each appear to be associated with a particular host species, or a range of hosts. *Borrelia afzelii* is commonly transmitted to ticks by small rodents, such as the wood mouse (*Apodemus sylvaticus* Linnaeus, 1758) and the bank vole (*Myodes glareolus* Schreber, 1780) (Hanincová et al., 2003; Humair et al., 1995). Also Eurasian red squirrels (*Sciurus vulgaris* Linnaeus, 1758) and European hedgehogs (*Erinaceus europaeus* Linnaeus, 1758) have been suggested to transmit *B. afzelii* to ticks (Skuballa et al. 2012; Pisanu et al. 2014). Like *B. afzelii*, *B. miyamotoi* appears to be associated with rodents (Barbour et al. 2009; Taylor et al. 2013; Cosson et al. 2014).

A recent European meta-analysis including 44 hosts, however, showed that only a few host species (small rodents, thrushes and roe deer) feed the majority of *I. ricinus* individuals (Hofmeester et al. 2016). Roe deer are generally the most important feeding host for female ticks in Europe and are important in the maintenance and reproduction of *I. ricinus* populations (Gray, 1998; Hofmeester et al., 2016; Ruiz-Fons and Gilbert, 2010). In most regions, larvae mainly feed on small rodents, and rodents are generally responsible for the majority of *Borrelia* infections in *I. ricinus* larvae (Hofmeester et al. 2016). The densities of small rodents such as wood mouse and bank vole in our study region, but also of other important host species such as roe deer, are positively correlated with the presence of a shrub layer and are higher in broadleaved forests than in coniferous forests (Tack et al. 2012a; Tack 2013). Furthermore, infection prevalence of nymphs with *B. afzelii* tends to be higher in pine than in oak forests (see Chapter 2), which suggests that small rodents feed more larvae in pine than in oak forests, relative to other host species. The densities of nymphs are also highest in structure rich broadleaved forests, as shown in Chapter 2 and

other studies (Gray et al. 1998; Tack et al. 2012b). Besides the type of forest, also the availability of seeds influences the occurrence and population dynamics of rodents. A negative effect of rodent abundance on human Hantavirus disease incidence in Belgium has already been observed in a study by (Tersago et al. 2009), which showed that abundant seed production of oak and beech precedes disease epidemics, due to an increase in bank vole abundance. Increased rodent abundance due to increased seed supply is shown to affect the density of nymphs (Ostfeld et al. 2001; Ostfeld et al. 2006; Tack 2013; van Duijvendijk 2016). Therefore, it is expected that the spatial and temporal differences in population dynamics of small mammals are important in explaining the density of infected nymphs, which is a commonly used tick-borne disease risk measure (Ostfeld et al. 2006).

The temporal dynamics in the prevalence of many important tick-borne pathogens, such as the Lyme borreliosis bacteria, remain largely unclear. In the light of the reported rise in incidence of tick-borne diseases in recent years, the study of the ecology and the spatial and temporal patterns of ticks, hosts and tick-borne pathogens is becoming increasingly important (Gray et al. 2009; Randolph 2010; Estrada-Peña et al. 2011). With our temporal survey, we provide data on the annual variability of the impact of forest characteristics on the density of ticks and the infection prevalence of the rodent-associated pathogens *B. afzelii* and *B. miyamotoi*.

3.3 Material and methods

3.3.1 Forest stand selection

This study was performed in the two study sites Postel (‘site P’) and Averbode-Hertberg (‘site AH’). The 22 forest stands we used in this study were selected in the framework of the study of Tack et al. (2012b) and were also studied in Chapter 2. The studied stands included five pine stands without a shrub layer, six pine stands with a shrub layer, six oak stands without a shrub layer and five oak stands with a shrub layer. In our study region, the years 2006, 2007 and 2011 were mast years of pedunculate oak and 2011 was a mast year of beech (Nussbaumer et al. 2016). Corsican pine experienced a high seed crop in 2012 and 2013 and Scots pine in 2013 (Verstraeten A., personal communication). No data for these pine species are available for our region before 2009.

3.3.2 Data collection

Questing nymphs were sampled three to four times per year in a fixed representative part of each forest stand between June and October in 2009, 2010, 2013 and 2014. For the exact procedure of tick sampling, we refer to Chapter 2. The differences in structure and composition of the herbaceous community between the different stands were negligible so that the sampling could be performed in a standardized way (Tack et al. 2012a). Nymphs were removed from the blanket after sampling each transect and transferred to vials containing 70% ethanol and afterwards stored at -22 °C. The ticks from 2009 and 2010 were collected in the framework of another study (Tack 2013) and were not collected by the same person as the ticks from 2013 and 2014. Differences in the sampling techniques of these persons and daily and seasonal variation in abiotic factors (temperature, humidity) may have affected the number of ticks caught. However, all stands were sampled with comparable intensity and in the same period each year. The stands were sampled in a random order each time, to account for daily fluctuations in temperature and humidity during the sampling sessions. To account for seasonal differences in tick abundance, we pooled nymphs per forest stand for all sampling occasions in the same year. We counted and pooled nymphs from all sampling occasions from each year per forest stand. From each pool, 35 individual nymphs were randomly selected to examine for infection with *Borrelia* genospecies and *B. miyamotoi*. For the procedure of DNA extraction of the individual nymphs and the simultaneous detection of *Borrelia* and *B. miyamotoi* by multiplex qPCR, and for the identification of *Borrelia* genospecies, we refer to the methods described in Hansford et al. (2014). The molecular tools we used are not suited to detect infection intensities of the pathogens. As the conventional *Borrelia*-PCR followed by Sanger sequencing is less sensitive than our duplex *Borrelia*-qPCR, we could not assign a genospecies to all ticks that were *Borrelia*-positive by qPCR. A more sensitive test, that would assign all *Borrelia*-positive samples to a genospecies, is not available yet, as far as we know. To correct for this shortcoming, we approximated the infection prevalence of nymphs with each *Borrelia* genospecies for each plot by following the approach described by Hofmeester et al. (2017). We proportionally assigned the unidentifiable sequences per stand to the different *Borrelia* genospecies using the proportion of each genospecies detected in the nymphs from that stand as a weighting factor. We assumed that the probability to successfully identify a genospecies is equal for all *Borrelia* genospecies, but

we acknowledge that it is possible that the qPCR will be more sensitive to the most prevalent genospecies.

3.3.3 Statistical analysis

All analyses were conducted in R version 3.3.1 (R Core Team 2017). DON is the average yearly density of nymphs per plot. The nymphal infection prevalence (NIP) is the proportion of infected nymphs per year, averaged over all sampling occasions per year per plot, and the density of infected nymphs (DIN) is the product of DON and NIP. We calculated NIP and DIN for the *Borrelia* complex (subscript 'sl'), for each *Borrelia* genospecies and for *B. miyamotoi*. Due to low numbers for *Borrelia* genospecies other than *B. afzelii*, only NIP_{sl}, DIN_{sl}, $NIP_{afzelii}$, $DIN_{afzelii}$, $NIP_{miyamotoi}$, and $DIN_{miyamotoi}$ were included in the statistical analyses.

We used linear mixed-effect models (*lme*) from the package *nlme* (Pinheiro et al. 2015) to explore the effect of sampling year and forest characteristics on the response variables DON, NIP_{sl}, DIN_{sl}, $NIP_{afzelii}$, $DIN_{afzelii}$, $NIP_{miyamotoi}$ and $DIN_{miyamotoi}$. As fixed effects, we used sampling year (levels '2009', '2010', '2013', '2014'), the dominant tree species ('pine' or 'oak'), the presence of a shrub layer ('yes' or 'no') and all two-way interactions. We added forest stand as a random effect to take into account the repeated measures in each stand. Significance of the predictor variables in all model fits were assessed using analysis of variance (ANOVA) with Chi-square (χ^2) test and we checked for heterogeneity of the residuals following the approach described in Zuur *et al.* (2009). Finally, to estimate if changes in DON correlate to changes in NIP, we performed a Spearman Rank Correlation using the package *Hmisc* (Harrell et al., 2016) on DON and NIP_{sl}, $NIP_{afzelii}$ and $NIP_{miyamotoi}$.

We did not statistically test the effect of weather variables such as precipitation and temperature on the tick-borne disease risk, since our sample size of four years and 22 stands was too low.

3.4 Results

In the 22 forest stands, a total of 21,376 questing *I. ricinus* nymphs were collected. We used 3,080 nymphs for further analysis. Overall, 17.63% of the analysed nymphs was infected with at least one pathogen. We identified six different *Borrelia* genospecies in 341 of the 471 (72.4%) infected nymphs, namely *B. afzelii*, *B. garinii*, *B. burgdorferi* s.s., *B.*

valaisiana, *B. spielmanii* and *B. bavariensis* (Appendix 3.1), but we were unable to identify the genospecies in 130 *B. burgdorferi* s.l.-positive nymphs. Thirteen nymphs were co-infected with *Borrelia* and *B. miyamotoi*. For eight of these co-infected nymphs, *B. miyamotoi* occurred together with *B. afzelii*. The *Borrelia* genospecies in the remaining five cases of co-infection could not be identified.

Figure 3.1 visualizes DON, $NIP_{afzelii}$ and $DIN_{afzelii}$ in each year. We found a significant effect of year ($p < 0.01$), dominant tree species ($p < 0.01$), presence of a shrub layer ($p = 0.01$) and the interaction between year and the presence of a shrub layer ($p < 0.01$) on DON. Highest values of DON were observed in 2010 and lowest in 2014 (Table 3.1 and Fig. 3.1). DON was consistently higher in oak forests than in pine forests (Fig. 3.1, Table 3.1). DON was significantly higher in stands with a shrub layer than in stands without a shrub layer in 2009 and 2010, but no difference was found in 2013 and 2014.

The variables NIP_{sl}, $NIP_{afzelii}$ and $NIP_{miyamotoi}$ did not show significant temporal variation (Table 3.1). $NIP_{afzelii}$ was significantly higher in pine forests ($p < 0.01$), consistently throughout the years (Table 3.1 and Fig. 3.1). We found no correlation between DON and NIP_{sl} ($p = 0.17$, $\rho = -0.15$), between DON and $NIP_{afzelii}$ ($p = 0.24$, $\rho = -0.13$) or between DON and $NIP_{miyamotoi}$ ($p = 0.32$, $\rho = -0.11$).

Table 3.1 The effect of sampling year, dominant tree species and presence of a shrub layer and their two-way interactions on density of nymphs (DON), nymphal infection prevalence of *Borrelia burgdorferi* s.l. (NIP_{sl}), *B. afzelii* ($NIP_{afzelii}$) and *B. miyamotoi* ($NIP_{miyamotoi}$), and density of nymphs infected with *B. burgdorferi* s.l. (DIN_{sl}), *B. afzelii* ($DIN_{afzelii}$) and *B. miyamotoi* ($DIN_{miyamotoi}$). Values represent F-values obtained by ANOVA (* $p < 0.05$). Higher F-values indicate higher variation in the response variable.

	year	tree	shrub	tree:shrub	tree:year	shrub:year
DON	13.27*	20.29*	8.32*	0.2	2.02	5.90*
NIP_{sl}	0.16	4.17	<0.01	0.38	0.03	0.65
DIN_{sl}	3.82*	6.54*	6.36*	1.04	0.29	2.49
$NIP_{afzelii}$	0.63	9.43*	0.9	0.5	1.42	0.18
$DIN_{afzelii}$	1.65	1.59	1.49	1.12	0.19	0.7
$NIP_{miyamotoi}$	0.27	0.06	<0.01	0.71	0.6	1
$DIN_{miyamotoi}$	2.38	6.85*	0.2	0.5	0.36	1.56

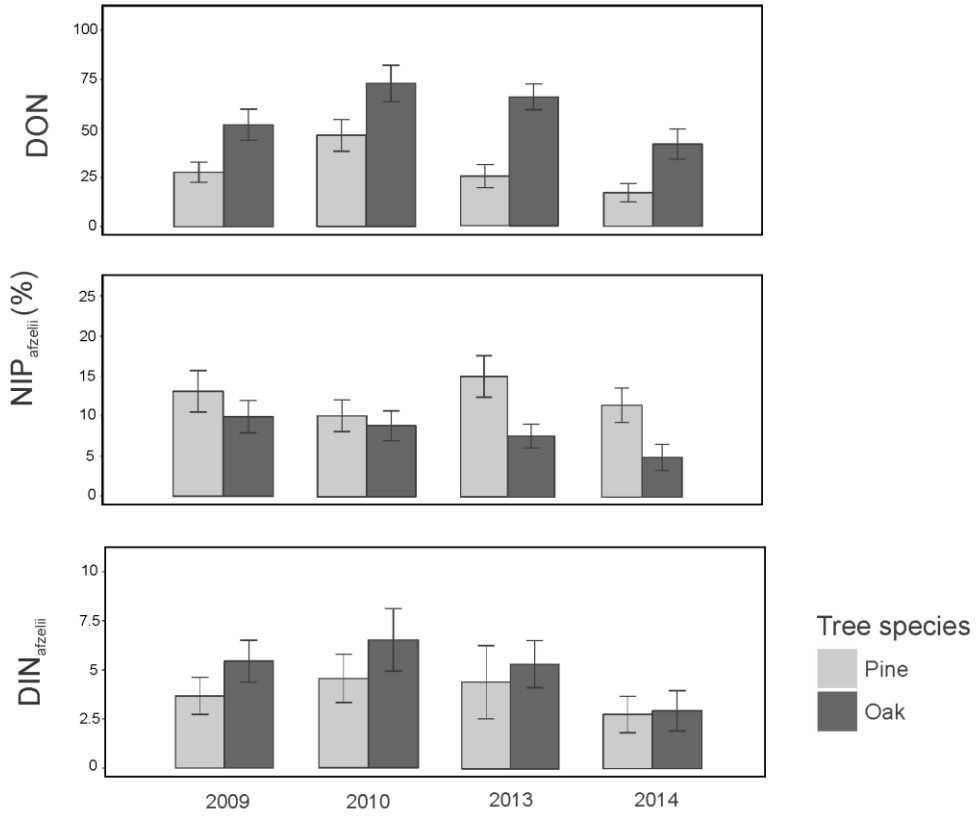

Fig. 3.1 The density of nymphs (DON), nymphal infection prevalence of the rodent associated pathogen *Borrela afzelii* ($NIP_{afzelii}$) and density of nymphs infected with *B. afzelii* ($DIN_{afzelii}$) in the different sampling years averaged over pine and oak stands (mean ± SE).

Like DON, DIN_{sl} significantly differed among years (p = 0.02), with highest values in 2010 and lowest in 2014 (Table 3.1). $DIN_{miyamotoi}$ and $DIN_{afzelii}$ did not show significant temporal variation (Table 3.1). Like DON, DIN_{sl} (p = 0.02) and $DIN_{miyamotoi}$ (p = 0.01) were higher in oak forests than in pine forests, consistently throughout the years (Table 3.1).

3.5 Discussion

In this temporal survey, we looked at the inter-annual dynamics in tick densities and the infection prevalence of tick-borne bacteria, with special attention to the rodent-associated human pathogens *B. afzelii* and *B. miyamotoi*, in relation to forest types in Belgium. Our results indicate that the risk of rodent-associated tick-borne disease varies both between different types of forest and between years. This spatiotemporal variation can be related to

the response of both ticks and hosts to the biotic and abiotic conditions influenced by the dominant tree species, and can be predicted by the density of nymphs.

In our study, the rodent-associated pathogens *B. afzelii* and *B. miyamotoi* were the most common bacteria in the investigated nymphs. The bird-associated *Borrelia* genospecies *B. garinii* and *B. valaisiana* occurred at low infection prevalence in our study sites. Together, this suggests that rodents are most likely the most important feeding hosts for larvae in our study area, as stated by Hofmeester et al. (2016). In our study, *B. miyamotoi* displayed co-infection with *B. afzelii*, which supports the assumption that they share the same hosts (Barbour et al. 2009; Taylor et al. 2013; Cosson et al. 2014).

Our results show that DON, but not NIP, displays inter-annual fluctuations. Some European studies have reported that an increased supply of acorns can increase the population density of wood mouse and bank vole the next year (Tack 2013; van Duijvendijk 2016). Moreover, they show that this increased rodent density leads to more feeding opportunities for larvae and a high DON one year later, while NIP remains stable. Also densities of other host species, such as roe deer, red squirrel and wild boar, may increase after a high seed crop of oak, beech or pine (Wauters and Lens 1995; Tixier and Duncan 1996; Wauters et al. 2004; Cutini et al. 2013). Oak experienced a high seed crop in 2006, 2007 and 2011, beech in 2011 and pine in 2012 and 2013. Based on this, we would expect DON to be highest in the years 2009, 2013 and 2014. However, DON is highest in 2010 and 2013. Yearly variation in weather conditions such as temperature and the amount of precipitation can also influence DON. Since ticks are sensitive to desiccation (Needham and Teel 1991), they will be more prone to death in dry conditions, or will seek shelter in the litter layer or lower vegetation which makes it more difficult to collect them with the standard sampling methods and thereby biasing the results. In our study, it is not possible to conclude if mast years or weather conditions affect DON, as these and other possible influencing factors are not accounted for.

Lyme borreliosis incidence has increased significantly the last decades in many European countries (Hofhuis et al. 2006; Ducoffre 2010; Sprong et al. 2012). We found no clear pattern in DON, NIP_{sl} or DIN_{sl} but rather DON and DIN_{sl} varied from one year to the other. Similar to our results, Estrada-Peña et al. (2011) detected no specific temporal trend at the European level in the prevalence of *Borrelia* genospecies and relate the prevalence of genospecies across Europe to temperature and vegetation stress, which are important

drivers of both tick and host populations. Like in other studies (Jouda et al. 2004; James et al. 2013; Vourc'h et al. 2016), but contrary to Tälleklint and Jaenson (1996), we found no correlation between DON and NIP. As NIP in our study did not vary from year to year, the temporal variation in DIN resembles the temporal variation in DON. This confirms that DON can be a good predictor of disease risk, as already suggested by e.g. Jaenson et al. (2009). The relationship between DON and NIP, however, can depend on the specific host community composition (van Buskirk and Ostfeld 1995; Tälleklint and Jaenson 1996b; Kurtenbach et al. 2006).

In accordance with other studies (Tack et al. 2012b) and as shown in Chapter 2, we found a higher DON in oak forests and a higher $NIP_{afzelii}$ in pine forests. The higher DON in oak stands can be explained by the more favourable biotic and abiotic conditions for ticks in oak forests than in pine forests, such as a better microclimate or a higher abundance of hosts (Gray et al. 1998). Previous research has shown that oak forests in our study region harbour higher densities of small rodents and roe deer compared to pine forests, and thus more feeding opportunities for ticks (Tack et al. 2012a; Tack 2013). Although the densities of small rodents are higher in oak than in pine forests, it is possible that, perhaps due to their wide ecological tolerance (Douglass et al. 1992), wood mouse and bank vole contribute more to the host community in pine than in oak forests, relative to other host species. This way they feed relatively more larvae in pine forests. Squirrels are also generally more abundant in pine than in oak forests (Wauters and Lens 1995). Since squirrels are, like mice and voles, believed to be associated with *B. afzelii* (Humair et al. 1995; Hanincová et al. 2003a; Pisanu et al. 2014), this might explain the higher $NIP_{afzelii}$ in pine than in oak stands.

3.6 Conclusions

From our results, we may conclude that the density of nymphs can be used to predict yearly variation in tick-borne disease risk. We found that the effect of the dominant tree species on the density of nymphs, which reflects changes in biotic and abiotic conditions, is consistent through time. In this study, we did not directly examine the host community of the ticks. Further research should therefore try to determine the exact contribution of the different host species and of the whole host community to the enzootic cycle of human pathogens, and to test the effect of weather conditions and different host community compositions to the tick-borne disease risk.

Appendix 3.1

The infection prevalence (%) of *Ixodes ricinus* nymphs with *Borrelia miyamotoi* or a distinct *Borrelia* genospecies in 2009, 2010, 2013 and 2014 in each studied forest type, averaged over all forest stands from that forest type. We approximated the nymphal infection prevalence of the *Borrelia* genospecies to correct for the samples that were positive in RT-PCR but could not be identified to genospecies level, as written in the text.

Bacteria	**Year**	**Pine**		**Oak**	
		without shrub	**with shrub**	**without shrub**	**with shrub**
B. afzelii	**2009**	17.9	9.1	9.0	11.1
	2010	10.6	10.0	9.1	8.8
	2013	16.0	16.0	9.6	6.3
	2014	15.3	13.5	6.3	6.0
B. garinii	**2009**	0	1.3	4.8	2.0
	2010	0	3.5	0.5	0.6
	2013	0	1.0	1.5	2.9
	2014	0	1.8	0.6	7.4
***B. burgdorferi* s.s.**	**2009**	3.3	3.6	0	1.7
	2010	5.4	1.8	1.2	4.3
	2013	0	0.6	4.0	0.6
	2014	1.2	0.9	0	0
B. valaisiana	**2009**	0	0.8	0.6	0
	2010	0	1.4	0.6	0
	2013	0	0	0.6	0.6
	2014	0	1.0	1.7	0
B. spielmanii	**2009**	0	0	0	0
	2010	0	0	0	0
	2013	0	0	0.5	1.1
	2014	0.7	0.0	1.3	1.5
B. bavariensis	**2009**	0	0	0	0
	2010	0	0	0	0.6
	2013	0	0	0	0
	2014	0	0	0	0
B. miyamotoi	**2009**	0.6	3.8	3.8	2.3
	2010	2.9	1.9	3.3	3.4
	2013	3.4	2.9	3.8	1.7
	2014	1.7	3.3	1.9	2.3


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4 Melting pot of tick-borne zoonoses: the European hedgehog contributes to the maintenance of various tick-borne diseases in natural cycles urban and suburban areas

After: Jahfari S. and Ruyts S.C., Frazer-Mendelewska E., Jaarsma R., Verheyen K., Sprong H. (2017). Melting pot of tick-borne zoonoses: the European hedgehog contributes to the maintenance of various tick-borne diseases in natural cycles urban and suburban areas. Parasites & Vectors, 10(1), 134 (IF 3.080)

4.1 Abstract

European hedgehogs (*Erinaceus europaeus*) are urban dwellers and host both *Ixodes ricinus* and *I. hexagonus*. These ticks transmit several zoonotic pathogens like *Borrelia burgdorferi* s.l. (‘*Borrelia’*), *Anaplasma phagocytophilum*, *Rickettsia helvetica*, *Borrelia miyamotoi* and “*Candidatus* Neoehrlichia mikurensis”. It is unclear to what extent hedgehogs in (sub)urban areas contribute to the presence of infected ticks in these areas, which subsequently pose a risk for acquiring a tick-borne disease. Engorged ticks from hedgehogs were collected from (sub)urban areas via rescue centres in Belgium. Ticks were screened individually for presence of *Borrelia* genospecies, *B. miyamotoi*, *A. phagocytophilum*, *R. helvetica* and “*Ca.* N. mikurensis”. Infection rates of the different pathogens in hedgehog ticks were calculated and compared to infection rates in questing ticks. Both *I. hexagonus* and *I. ricinus* of all life stages were found on the 54 investigated hedgehogs. Only a few hedgehogs carried most of the ticks, with six of the 54 hedgehogs carrying more than half of all ticks. *Anaplasma phagocytophilum*, *R. helvetica*, *B. afzelii*, *B. bavariensis* and *B. spielmanii* were found significantly more in ticks from hedgehogs in comparison to questing *I. ricinus*. European hedgehogs seem to contribute to the spread and transmission of tick-borne pathogens in urban areas. The relatively high prevalence of *B. bavariensis*, *B. spielmanii*, *B. afzelii*, *A. phagocytophilum* and *R. helvetica* in engorged ticks suggests that hedgehogs contribute to their enzootic cycles in (sub)urban areas. The extent to which hedgehogs can independently maintain these agents in natural cycles and the role of other hosts (rodents and birds) remain to be investigated.

4.2 Introduction

The *Borrelia* genospecies are most commonly transmitted to humans by *Ixodes ricinus*; a tick species that quests in the vegetation to find a host for its blood meal. Other ixodid tick species, such as *I. hexagonus*, have been shown to transmit *Borrelia* genospecies to its host species too (Skuballa et al. 2012). Contrary to *I. ricinus*, *I. hexagonus* does not quest but in its off-host phase, mainly stays in the nests of its host species, which is primarily the West European hedgehog (*Erinaceus europaeus* Linnaeus, 1758). The European hedgehog is a nocturnal insectivorous mammal commonly found throughout Western Europe (Reeve 1994). They seem to have adjusted to a wide variety of habitats and occur in rural, suburban, and urban areas but generally prefer grassland with sufficient edge habitats. Hedgehogs can reach up to nine times higher densities in urban areas with parks and garden, than in rural areas, with lowest densities in forests and open grassland fields and agricultural land without cover such as shrubs or dead wood (Huijser 1999; Young et al. 2006; Hubert et al. 2011). Since they are one of the most successful urban adapters, hedgehogs and *I. hexagonus* could contribute to the spread and persistence of pathogens in a (sub)urban habitat via secondary enzootic cycles, even when the contact between *I. hexagonus* and humans is low (Pfäffle et al. 2011; Skuballa et al. 2012).

Only a few studies have been performed on the reservoir competence of the European hedgehog. These studies have shown that these mammals can be infected with different *Borrelia* genospecies (Gern et al. 1997; Skuballa et al. 2007; Skuballa et al. 2012) as well as other tick-borne pathogens, such as *Anaplasma phagocytophilum* (Skuballa et al. 2010; Silaghi et al. 2012), tick-borne encephalitis virus (TBEV) (Labuda and Randolph 1999) and *Rickettsia helvetica* (Speck et al. 2013). The role of the European hedgehog and both ixodid tick species feeding on it in the transmission cycle of many tick-borne pathogens like *Borrelia miyamotoi* and "*Candidatus* Neoehrlichia mikurensis" is not completely illuminated, yet (Krawczyk et al. 2015).

In this study, we aim to investigate the prevalence of *Borrelia* genospecies, *B. miyamotoi*, *A. phagocytophilum*, "*Ca.* N. mikurensis" and *R. helvetica* in the different stages of the *I. hexagonus* and *I. ricinus* tick species sampled from European hedgehogs from Belgium. Furthermore, we aim to investigate the role of these tick species and that of the hedgehog in the enzootic cycle of the different disease pathogens. By using epidemiological analysis and comparing the infection prevalences of the different pathogens from engorged ticks

collected from hedgehogs with questing nymphs from the vegetation, we aim to i) determine the reservoir status of the European hedgehog for *Borrelia* genospecies, *B. miyamotoi*, *A. phagocytophilum*, "*Ca.* N. mikurensis" and *R. helvetica*, and ii) find indications for the vector competence of *I. hexagonus* for tick-borne pathogens.

4.3 Methods

4.3.1 Hedgehog and tick sampling

Since European hedgehogs are legally protected in Belgium, the current investigation was carried out on ticks sampled from hedgehogs that were brought to the rescue centres of Herenthout and Heusden-Zolder in the Kempen, Belgium. In general, these hedgehogs were captured in gardens and urban areas by civilians. To grant the hedgehogs an easy and full recovery, removal of all ectoparasites upon arrival at the rescue centre is a standard procedure. Because it would be detrimental for their recovery, it was not possible to analyse blood samples of the hedgehogs. For this study, attached ticks of all life stages were collected by the centres' volunteers in 2014 (both centres) and 2015 (only Herenthout) between the end of April and the end of October. Tick specimens were stored in 70% ethanol at room temperature until further investigation. Ticks were identified to species and life stage (Arthur 1963). The number of attached ticks (tick burden) was recorded for each hedgehog. Since only hedgehogs that harboured ticks were used in this study, there is no data on the percentage of hedgehogs that were infested by ticks. Age (adult or juvenile) was determined based on size (Skuballa et al. 2012) for all hedgehogs, except two. The questing *I. ricinus* ticks that we used in this study were caught by drag-sampling the vegetation in a suburban forest in the same region as where the hedgehogs were collected, are part of a previously published study (Heylen et al. 2016).

4.3.2 Sample preparation and molecular detection of tick-borne pathogens

All ticks were processed individually. The questing *I. ricinus* ticks that are part of a previously published study (Heylen et al. 2016) were processed and analysed by using the same protocols. Nucleic acids were extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The extracted DNA was stored at -20 degrees Celsius until further use. Ticks were tested individually for presence of *Borrelia*, *B. miyamotoi*, *A. phagocytophilum*, "*Ca.* N. mikurensis" and *R.*

helvetica DNA using two separate multiplex real-time PCR assays as described before (Stenos et al. 2005; Jahfari et al. 2012; Heylen et al. 2013a; Heylen et al. 2016), followed by sequencing for species identification. For the identification of *Borrelia* genospecies a conventional PCR assay targeting the 5S-23S intergenic region (IGS) was performed. *Borrelia* genospecies identification was determined by comparison of sequences to isolate in-house molecular databases (Coipan et al. 2013a). As genospecies identification was successful for only 43.4% of the *Borrelia*-positive sequences, we proportionally assigned these unidentifiable sequences in each life stage per tick species to the different *Borrelia* genospecies present in each stage, using the proportion of each genospecies detected in that stage as a weighting factor, as described in Chapter 3. For confirmation of *B. miyamotoi* conventional PCR targeting *glpQ* gene was done (Hovius et al. 2014). The *groEL* gene of *A. phagocytophilum* was amplified and sequenced (Jahfari et al. 2014). For all conventional PCR's, both strands of PCR products were sequenced by BaseClear (Leiden, the Netherlands). The molecular tools we used are not suited to detect infection intensities of the pathogens.

4.3.3 Statistical tests

All statistical tests were performed using R version 3.2.0 (R Core Team 2017) and all graphs were made with the package *ggplot2* (Wickham 2009). To test the differences in distribution of tick species, tick burden and infection prevalence of the different pathogens in ticks on hedgehogs from different age classes, Kruskal-Wallis tests were employed. The number of mixed tick species infestations (both tick species on the same hedgehog) was compared to the number of single species infestations (only *I. ricinus* or *I. hexagonus*) with Pearson's Chi-squared test. With the *prop.test* function, we tested if the pathogens in the ticks occurred more frequently alone or co-existing with a different pathogen in the same tick. Afterwards we compared the infection prevalence of the pathogens in *I. hexagonus* with the prevalence in *I. ricinus.* Finally, for a species to be a reservoir host of a pathogen, it has to be able to transmit the pathogen to ticks feeding on it. This can be evaluated by allowing an uninfected tick feeding on the host and afterwards analysing the tick for infection ('xenodiagnosis'). To assess the transmission capabilities of the hedgehog for each pathogen, we compared the infection prevalence in the engorged ticks collected from hedgehogs with the infection prevalence in questing *I. ricinus* from the same region, and used this as a proxy to evaluate the reservoir status of the hedgehog. In order to evaluate

the importance of a host species to transmit a pathogen, it is best to compare engorged *I. ricinus* larvae with questing *I. ricinus* larvae and nymphs.

As the amount of engorged *I. ricinus* larvae is too low to perform these analyses (n = 7), we decided to compare the infection prevalence of each pathogen in engorged *I. ricinus* larvae and nymphs collected from hedgehogs with the infection prevalence in host-seeking *I. ricinus* nymphs and adults. This way, we compare ticks that fed once (engorged larvae and questing nymphs) or twice (engorged nymphs and questing adults), and omit ticks that had the chance to feed three times (engorged adults). Engorged adults have a higher chance to be infected than a questing tick anyway, because a questing tick has never fed more than twice. The difference between the pathogen communities in engorged and questing ticks is thus that the engorged ticks will have certainly fed, at least once, on hedgehogs, while the chance that the questing ticks will have fed on hedgehogs is rather low. Differences between the infection prevalence of the pathogens in engorged and questing ticks may then reflect the importance of the hedgehog in the transmission of pathogens. A higher infection prevalence of a certain pathogen in engorged larvae and nymphs will then suggest that the hedgehog has an important role in the transmission of that pathogen.

4.4 Results

Of the 54 hedgehogs investigated, 24 were adults and 28 were juveniles. For two hedgehogs, age was not determined. Both *I. hexagonus* and *I. ricinus* ticks of all life stages were found on the hedgehogs. The number of ticks per hedgehog ranged from one to 167. Most hedgehogs in our study carried only few ticks, while only few individuals harboured the majority of the ticks. Six of the 54 hedgehogs carried more than half of all ticks (624/1205) and only 15 hedgehogs carried 25 or more ticks. Tick burden did not significantly differ between hedgehog age classes ($p = 0.97$). In total, we collected 1205 ticks and found significantly more *I. hexagonus* ($n = 1132$) than *I. ricinus* ($n = 73$, $p < 0.05$). The most common life stage of *I. hexagonus* retrieved from the hedgehogs were nymphs ($n = 586$, $p = 0.03$). Of *I. ricinus*, all life stages were equally common ($p = 0.07$, Fig. 4.1). Some hedgehogs were found to harbour both species of ticks ($n = 10$), but infestations with only one tick species were more common ($n = 44$, $p < 0.05$).

Of the 1205 collected ticks, two (one *I. ricinus* and one *I. hexagonus*) got lost during sample preparation; hence the molecular analyses were performed on 1203 ticks. A total of 859

(71.4%) ticks was infected with at least one of the tested pathogens. Of these infected ticks, 524 (61%) had a single infection, and 335 ticks (39%) were infected with more than one pathogen of another genus. *Anaplasma phagocytophilum* and *R. helvetica* were the two most common pathogens and occurred in 466 ticks (38.7% of all analysed ticks or 54% of all infected ticks) coming from 34 hedgehogs and in 481 ticks (40% of all analysed ticks or 56% of all infected ticks) coming from 37 hedgehogs, respectively (Table 4.1, Appendix 4.1). An infection with *Borrelia* occurred in 297 ticks (24.7% of all analysed ticks or 34.6% of all infected ticks) from 28 hedgehogs. We were able to identify the *Borrelia* genospecies in 129 (43.4%) of these infected ticks, of which *B. afzelii* (n = 80), *B. spielmanii* (n = 28) and *B. bavariensis* (n = 17) were the most common. *Borrelia turdi* occurred once in both tick species and *Borrelia garinii* and *B. valaisiana* each in one *I. ricinus* tick. An infection with *B. miyamotoi* occurred in 20 ticks from five hedgehogs. Only three *Ixodes ricinus* ticks from two hedgehogs were infected with *"Ca.* N. mikurensis" (Appendix 4.1, Fig. 4.2). The pathogen prevalence per tick species is depicted in Figure 4.2. *Ixodes ricinus* seems to be more likely infected with at least one pathogen (59/72, 81.9%) than *I. hexagonus* (800/1131, 70.7%) but the difference between the two tick species was only marginally significant ($p = 0.06$). More specifically, the infection prevalence of *A. phagocytophilum*, "*Ca.* N. mikurensis", *B. afzelii*, *B. garinii*, *B. valaisiana* and *B. turdi* was highest in *I. ricinus* while infection with *R. helvetica* was highest in *I. hexagonus* ($p < 0.05$, Fig. 4.2). For the infection prevalence of *B. miyamotoi*, *B. spielmanii* and *B. bavariensis*, no difference between the tick species could be observed. There was no difference in infection prevalence between adult and juvenile hedgehogs for any of the detected pathogens ($p > 0.05$).

Co-infections of other pathogens with *Borrelia* were investigated. For *I. ricinus*, 37 of the 59 infected ticks (62.7%) carried two (n=31) or three (n=6) pathogens. The most common co-infection in *I. ricinus* (24/37) was with *Borrelia* and *A. phagocytophilum*. Of the 800 infected *I. hexagonus* ticks, 298 (37.3%) had a co-infection composed of two (n=232) or three (n=65) pathogens. Co-infections of *A. phagocytophilum* and *R. helvetica* (102/298), *A. phagocytophilum* and *Borrelia* (86/298) and *A. phagocytophilum*, *R. helvetica* and *Borrelia* (64/298) occurred most often. One *I. hexagonus* tick was infected with four pathogens: *A. phagocytophilum*, *R. helvetica*, *Borrelia* and *B. miyamotoi*. All pathogens were found more often co-existing with another pathogen in a tick, than as the single pathogen infecting the tick ($p < 0.05$).

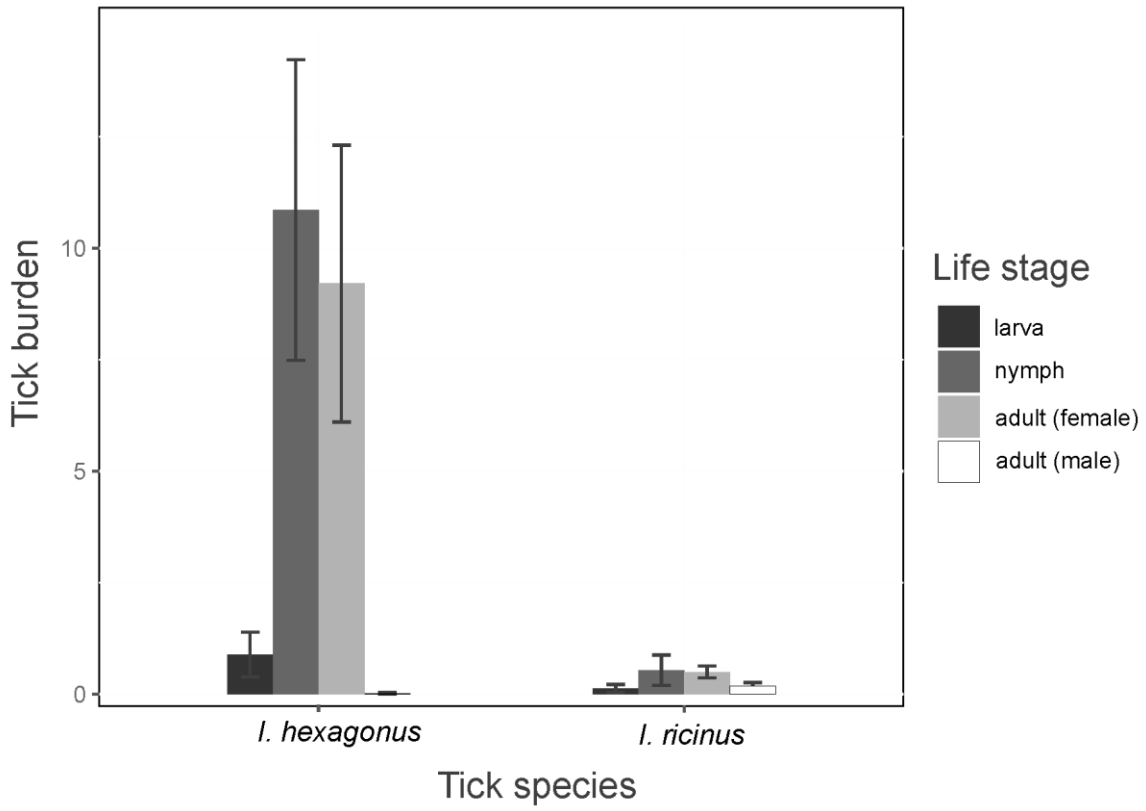

Fig. 4.1 The distribution of the different life stages of *Ixodes ricinus* and *Ixodes hexagonus* collected from 54 hedgehogs in the Kempen, Belgium (mean ± SE).

Ixodes ricinus larvae and nymphs from hedgehogs were infected more often (28/35) than questing *I. ricinus* nymphs and adults (367/1874, $p < 0.05$). We could not detect any difference in prevalence of *R. helvetica*, *B. miyamotoi*, *B. garinii* and *B. valaisiana*. For all other pathogens, infection prevalence was significantly higher in the engorged ticks from the hedgehogs ($p < 0.05$).

Table 4.1 The number (#) of *Ixodes ricinus* and *Ixodes hexagonus* ticks infected with a certain pathogen, for all life stages together or for larvae (L), nymphs (N) or adults (A) separately, and the percentage (%) of infected ticks of the two species on all analyzed ticks from that species per life stage.

		# ticks analyzed		*B. burgdorferi* s.l.	*B. miyamotoi*	*R. helvetica*	*A. phagocytophilum*	*"Ca.* N. mikurensis"
I. hexagonus	**L**	**48**	**#**	3	2	10	2	0
			%	6.3	4.2	20.8	4.2	0
	N	**585**	**#**	166	8	192	279	1
			%	28.4	1.4	32.8	47.7	0.2
	A	**498**	**#**	91	7	267	137	0
			%	18.3	1.4	53.6	27.5	0
	all	**1131**	**#**	260	17	469	418	1
			%	23	1.5	41.5	37	0.09
I. ricinus	**L**	**7**	**#**	1	0	0	3	0
			%	14.3	0	0	42.9	0
	N	**28**	**#**	20	0	3	23	2
			%	71.4	0	10.7	82.1	7.1
	A	**37**	**#**	16	3	9	22	0
			%	43.2	8.1	24.3	59.5	0
	all	**72**	**#**	37	3	12	48	2
			%	51.4	4.2	16.7	66.7	2.8

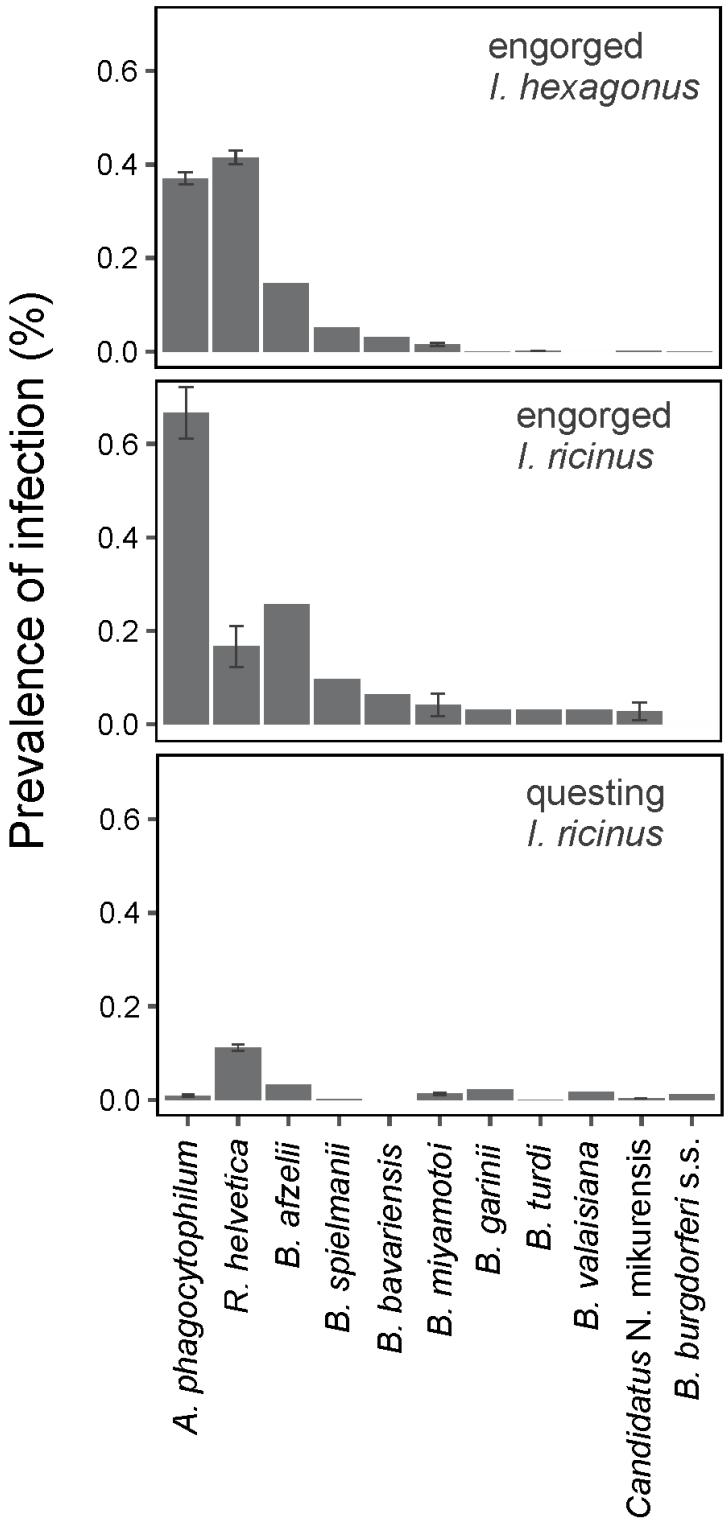

Fig. 4.2 The prevalence of the distinct pathogens in engorged *Ixodes ricinus* and *I. hexagonus* ticks collected from hedgehogs and in questing *I. ricinus* collected from the vegetation (mean ± SE). No SE was calculated for the *Borrelia* genospecies, since we used the prevalence of each genospecies corrected for the unidentifiable sequences per life stage per tick species, as written in the text.

Afterwards we repeated these analyses for *I. hexagonus* collected from hedgehogs and compared the larvae and nymphs of this tick species with the questing *I. ricinus* nymphs and adults collected from the vegetation. This enables us to interpret more comprehensively the reservoir role of the hedgehog for the different pathogens, and the vector competence of *I. hexagonus*. We observed that *B. garinii* and *B. valaisiana* were more prevalent in the questing *I. ricinus* ticks. No significant difference in infection prevalence between questing or engorged ticks could be detected for *B. turdi*, *B. miyamotoi* and "*Ca.* N. mikurensis". The prevalence of all other pathogens, including *R. helvetica*, is higher in the engorged than the questing ticks. Furthermore, when comparing just the ticks collected from hedgehogs that carried 25 or more ticks, we obtain the same outcome.

For *A. phagocytophilum*, *R. helvetica*, *B. bavariensis* and *B. miyamotoi*, the distribution of the infections was clustered in some hedgehogs, with most hedgehogs harbouring no, or only few infected ticks, while only few hedgehogs were responsible for the majority of the infected ticks (Fig. 4.3). Twelve of the 17 ticks infected with *B. bavariensis* and 16 of the 20 ticks infected with *B. miyamotoi* came from one individual hedgehog (hedgehog #18). Hedgehog #33 harboured 125 ticks of which 118 were infected with *A. phagocytophilum* (25.3% of all *A. phagocytophilum* infections). Still, there are hedgehogs that harbour many ticks, while no or few or these ticks are infected with one of these pathogens (Fig. 4.3).

Of the *A. phagocytophilum* positive ticks, 43 were sequenced of which 33 *I. hexagonus* and 10 *I. ricinus* from 18 different individual hedgehogs. All the *groEL* sequences of the *A. phagocytophilum* isolates clustered with the zoonotic ecotype, ecotype I (Jahfari et al. 2014).

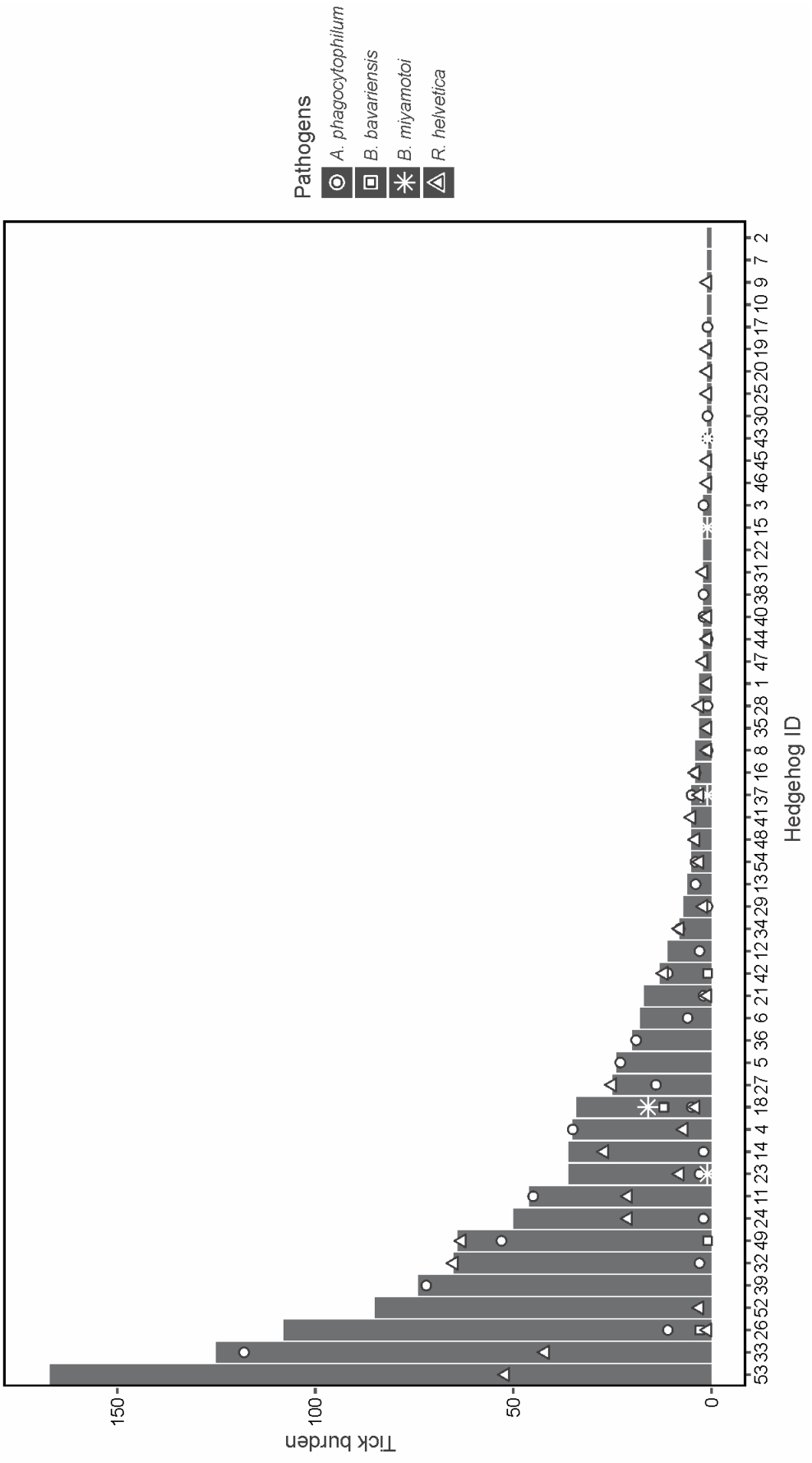

Fig. 4.3 The tick burden per hedgehog with the number of ticks per hedgehog harbouring infection with *Anaplasma phagocytophilum*, *Rickettsia helvetica*, *Borrelia bavariensis* and *Borrelia miyamotoi*.

4.5 Discussion

Our results confirm that hedgehogs are a host of all three stages of *I. hexagonus* and *I. ricinus*. Still, more *I. hexagonus* were found feeding on hedgehogs than *I. ricinus* ticks. The aggregation of ticks on hedgehogs varied vastly between the individual hedgehogs, as only a few hedgehogs were recorded to carry most of the ticks. Such aggregated distribution, in which most individuals carry only few parasites and only a few individuals carry the majority of parasites, is a well-known principle in parasitology (Shaw et al. 1998; Hughes and Randolph 2001). This means that just a few hedgehogs contribute to tick maintenance, similar to what is seen on rodents (van Duijvendijk 2016). This seems to be especially the case for *I. hexagonus*, and to a lesser extend for *I. ricinus*, since the burdens of *I. ricinus* on hedgehogs appears to be relatively low. Moreover, it is less likely that hedgehogs can maintain the *I. ricinus* life cycle as the sole host species because, even though it can feed all life stages of this generalist tick species. Hedgehog densities in forested areas, the preferred habitat of *I. ricinus*, are probably too low to feed enough ticks, especially compared to the densities of wood mouse and bank vole, species that are generally responsible for the feeding of the majority of *I. ricinus* larvae (Huijser 1999; Verkem et al. 2003; Hubert et al. 2011). Namely, if all *I. ricinus* stages should rely only on the hedgehog to feed on, many ticks would starve and perish since the amount of encounters with this host would be low. We think, rather, that a host community without large mammals but composed only of small or medium-sized mammals such as rodents, birds and hedgehogs (like in (sub)urban area's and parks), can already be sufficient to complete the life cycle of *I. ricinus*. This because, as we show, large mammals are not the only hosts adult *I. ricinus* ticks feed on. More research is needed, however, to elucidate the role of hedgehogs in the life cycle of this generalist tick species. Other animals that live close to human settlements, such as foxes, squirrels and martens could possibly also serve as a host for adult ticks (Sréter et al. 2005; Millins et al. 2015). Moreover, domestic animals, such as dogs and cats, are known to carry adult females (Ogden et al. 2000). The role of wildlife and domesticated animals in tick-borne disease risk, however, still needs to be addressed. Furthermore, the ticks we investigated were collected from animals that were sick or needed (medical) assistance in some way. This might have influenced the results from this study, and future research should investigate ticks from hedgehog populations in nature.

Since 71.4% of the ticks retrieved from hedgehogs were infected by at least one pathogen, hedgehogs can be considered as epidemiologically important wildlife species. Moreover,

39% of all infected ticks carried more than one pathogen of another genus. High prevalence of tick-borne pathogens *B. bavariensis*, *B. spielmanii*, *B. afzelii*, *A. phagocytophilum* and *R. helvetica* in engorged *I. hexagonus* and *I. ricinus* ticks obtained from *E. europaeus*, indicates that hedgehogs contribute to pathogen maintenance in natural cycles in urban and suburban areas. For *B. bavariensis*, *B. spielmanii*, *B. afzelii*, *A. phagocytophilum* and *R. helvetica*, the infection prevalence was higher in the engorged ticks of both species, in comparison to the infection rates in questing ticks from the same region. This indicates that the hedgehog is a possible reservoir host of these pathogens and contributes to their enzootic cycle. On the other hand, "*Ca.* N. mikurensis" infection rate was not significantly higher in questing *I. ricinus* ticks than in engorged hedgehog ticks, indicating that hedgehogs do not play a main role in the maintenance of the enzootic cycle of this pathogen.

Engorged *I. ricinus* ticks tend to be more infected with any pathogen in comparison to engorged *I. hexagonus*, except for *R. helvetica*, which was significantly more prevalent in *I. hexagonus* ticks. Perhaps this observation can be subscribed to transmission pathway of *R. helvetica*, which occurs transovarially as well as transstadially. Therefore, ticks in nature are usually thought to be the main reservoir and vectors of *R. helvetica* (Socolovschi et al. 2009). However, since transovarial transmission rates are less than 100%, vertebrate hosts like the hedgehog can act as an amplifier of this pathogen, playing a vital role in transmission cycles. The pathogens that are present in engorged *I. ricinus* ticks can originate from a previous blood meal from another host species, while the pathogens *I. hexagonus* carries are most probably coming from the hedgehog, since hedgehogs are their preferred host species. This way infection prevalence in engorged *I. ricinus* can be higher than engorged *I. hexagonus*, when they fed in a previous stage on a host species that functions as an efficient reservoir species for some of the investigated pathogens, such as small rodents or birds.

Remarkably, the infection of some pathogens such as *B. bavariensis*, *B. miyamotoi*, *R. helvetica* and *A. phagocytophilum* seems to be clustered per individual hedgehog, meaning that only a few hedgehogs contribute to the gross of the infected ticks. *Borrelia miyamotoi* is known to give short-term systemic infection in rodents, therefore making rodents excellent but transitory hosts of this bacterium (Burri et al. 2014). Vertebrates other than rodents may also become infected: *B. miyamotoi* DNA was also found in the tissue of an European greenfinch and a great tit (Wagemakers et al. 2017). The clustering of infected

fed ticks on only one hedgehog in this study indicates that *B. miyamotoi* might cause a short-term systemic infection in hedgehogs as well. The role of these animals in the transmission cycle is not clear; they could be transitory hosts. Another possible explanation for the fact that many ticks were infected with the same pathogen on the same hedgehog could be co-feeding transmission (Randolph 2011; Voordouw 2015). With this route of transmission, the co-feeding ticks act as reservoirs and vectors. The host is only a transient bridge, bringing together infected and uninfected ticks in both space and time, thereby facilitating pathogen exchange. The host does not necessarily have to be infected himself (Randolph 2011; Voordouw 2015). The bird associated *Borrelia* genospecies, *B. garinii* and *B. valaisiana*, were each detected in one *I. ricinus* adult tick, and *B. turdi* occurred in one *I. hexagonus* female and one *I. ricinus* nymph. We can thus confirm the indication that hedgehogs are no reservoir hosts for the bird associated, only for the rodent-associated, *Borrelia* genospecies (Skuballa et al. 2012).

Hedgehogs and their host-specific parasite *I. hexagonus* seem to play a role in maintaining some pathogens, like *B. bavariensis*, *B. spielmanii*, and *A. phagocytophilum* in cryptic cycles. The generalist feeding behaviour of *I. ricinus* and the low prevalence of these pathogens in questing *I. ricinus* suggest that they do not play a main role in the maintenance of the enzootic cycle of these pathogens. However, when feeding on hedgehogs *I. ricinus* may still be infected by *I. hexagonus*-associated pathogens and transmit them to humans. *Borrelia bavariensis* can cause neurological disease in humans (Coipan et al. 2016), and *B. spielmanii* has been linked to erythrema migrans in humans. Both pathogens have already been linked to hedgehogs (Skuballa et al. 2007; Skuballa et al. 2012). Moreover, co-infection of *R. helvetica* and *Borrelia* has been shown in neuroborreliosis patients (Koetsveld et al. 2015). Also, co-infections are thought to affect the severity of disease and influence clinical outcomes in some cases (Swanson et al. 2006). Since hedgehogs seem to contribute to co-infection rates in ticks, this poses an increased health risk. Moreover, it has been shown that co-infections can reduce or increase the virulence of one or multiple pathogens, depending on the mechanistic details of the pathogens' exploitation of the host or tick (Brown et al. 2002). The effect of the co-infections in the ticks on the virulence of the pathogens in the ticks has not been investigated but should be addressed in further research. The variant of *A. phagocytophilum* detected in these samples were all linked to human cases of anaplasmosis (ecotype I) (Jahfari et al. 2014).

4.6 Conclusions

From these findings we conclude that hedgehogs are important components in the enzootic cycle of a diverse set of human pathogens, thereby contributing to the maintenance of various tick-borne diseases in (sub)urban areas. Humans are likely to come into contact with ticks infected with one or several of these pathogens while gardening or recreating in parks (Mulder et al. 2013). This poses a potential human health risk. Most hedgehogs, however, carry only few ticks and hedgehog densities are relatively low, thus hedgehogs will probably infect only few ticks with a certain pathogen. Further research is necessary to elucidate the interaction between hedgehog densities, tick burden and tick infection prevalence and to assess the precise impact of hedgehogs on the enzootic cycle of the various tick borne human pathogens, and the associated human health risk.

Appendix 4.1

The number of *Ixodes ricinus* and *I. hexagonus* ticks infected with a certain pathogen, for all life stages together or for larvae (L), nymphs (N) or adults (A) separately.

tick species	stage	collected	analysed	*Borrelia burgdorferi* s.l.							
				positive ticks	identified genospecies	*B. afzelii*	*B. spielmanii*	*B. bavariensis*	*B. turdi*	*B. valaisiana*	*B. garinii*
I. ricinus	L	7	7	1	0	0	0	0	0	0	0
	N	29	28	20	7	4	1	1	1	0	0
	M	10	10	4	1	0	0	0	0	0	1
	F	27	27	12	8	4	2	1	0	1	0
	all	**73**	**72**	**37**	**16**	**8**	**3**	**2**	**1**	**1**	**1**
I. hexagonus	L	48	48	3	1	0	0	1	0	0	0
	N	586	585	166	85	54	22	9	0	0	0
	M	1	1	1	0	0	0	0	0	0	0
	F	497	497	90	27	18	3	5	1	0	0
	all	**1132**	**1131**	**260**	**113**	**72**	**25**	**15**	**1**	**0**	**0**
all	**all**	**1205**	**1203**	**297**	**129**	**80**	**28**	**17**	**2**	**1**	**1**

tick species	stage	collected	analysed	*B. miyamotoi*	*R. helvetica*	*A. phagocytophilum*	*"Ca.* N. mikurensis"
I. ricinus	L	7	7	0	0	3	0
	N	29	28	0	3	23	2
	M	10	10	1	4	5	0
	F	27	27	2	5	17	0
	all	**73**	**72**	**3**	**12**	**48**	**2**
I. hexagonus	L	48	48	2	10	2	0
	N	586	585	8	192	279	1
	M	1	1	0	0	0	0
	F	497	497	7	267	137	0
	all	**1132**	**1131**	**17**	**469**	**418**	**1**
all	**all**	**1205**	**1203**	**20**	**481**	**466**	**3**


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5 Molecular detection of tick-borne pathogens *Borrelia afzelii, Borrelia miyamotoi* and *Anaplasma phagocytophilum* in Eurasian red squirrels (*Sciurus vulgaris*)

After: Ruyts S. C., Frazer-Mendelewska E., Van Den Berge K., Verheyen K., Sprong H. (2017). Molecular detection of tick-borne pathogens *Borrelia afzelii*, *Borrelia miyamotoi* and *Anaplasma phagocytophilum* in Eurasian red squirrels (*Sciurus vulgaris*). European Journal of Wildlife Research, 63(3), 43 (IF 1.264)

5.1 Abstract

Eurasian red squirrels (*Sciurus vulgaris*) are common hosts of ixodid ticks and could thus carry tick-borne disease agents. The relative contribution of the red squirrel, a medium-sized rodent species, to the transmission dynamics of tick-borne pathogens in Europe yet remains unclear. We analysed spleen and liver samples from 45 dead squirrels collected in Flanders, Belgium, and detected the presence of *Borrelia burgdorferi* s.l. in the spleen of two squirrels (4.4%). One of the sequences could be identified as *B. afzelii*. *Borrelia miyamotoi* was detected in the spleen of three squirrels (6.7%) and *Anaplasma phagocytophilum* in four spleen samples (8.9%). Both *A. phagocytophilum* ecotype I and II were found. We could not detect the presence of "*Candidatus* Neoehrlichia mikurensis" or tick-borne encephalitis virus in any of the squirrels. Our results suggest that Eurasian red squirrels can host *B. afzelii*, as already proposed by previous studies, but we could not confirm the previous established association between squirrels and *B. burgdorferi* sensu stricto. Our results demonstrate the epidemiological importance of the red squirrel, particularly in (sub)urban areas, since they can harbour a similar community of tick-borne pathogens as do mice and voles and can act as hosts for *A. phagocytophilum* ecotype I, which has important implications for human health risk.

5.2 Introduction

For some common host species, such as the medium-sized rodent Eurasian red squirrel (*Sciurus vulgaris* Linnaeus, 1758), the exact contribution in the ecology of Lyme borreliosis has not yet been determined. Some studies, however, suggest that red squirrels act as reservoir hosts for transmitting the *Borrelia burgdorferi* s.l. (‘*Borrelia’*) genospecies *B. afzelii* and *B. burgdorferi* sensu stricto (s.s.) to ticks (Humair and Gern 1998; Pisanu et al. 2014). Furthermore, rodents can carry the emerging pathogens *Borrelia miyamotoi*, “*Candidatus* Neoehrlichia mikurensis”, *Anaplasma phagocytophilum* and tick-borne encephalitis virus (TBEV) (Mansfield et al. 2009; Burri et al. 2014; Obiegala et al. 2014). The relative contribution of the red squirrel to the transmission dynamics of the above-mentioned tick-borne pathogens in Europe thus remains to be elucidated. This is of high epidemiological importance, since red squirrels can reach higher densities in (sub)urban areas than in forested areas (see Rézouki et al. (2014) and references therein). This way they can potentially pose an important human health risk by maintaining and spreading tick-borne pathogens in ixodid ticks in areas close to human habitation. In this study, we tried to detect the presence of DNA of the tick-borne pathogens *Borrelia*, *B. miyamotoi*, “*Ca.* N. mikurensis”, *A. phagocytophilum* and TBEV in tissue samples of spleen and liver of dead squirrels. The presence of DNA in these organs points to systemic disseminated infection in the host animal, which indicates that the pathogen has dispersed throughout the body Furthermore, we aimed to confirm the suspected association of previous studies between the red squirrel and the *Borrelia* genospecies *B. afzelii* and *B. burgdorferi* s.s.

5.3 Material and methods

As a part of a surveillance study, the Flemish Research Institute for Forest and Nature collected dead red squirrels, which were victims of road traffic, throughout Flanders, Belgium, from 2010 to 2014. The bodies were frozen at -20°C until dissection. For this study, we selected the 52 animals in which the internal organs were not visibly affected by decay or scavengers. Most of the squirrels we used in this study were collected at roads in the provinces Antwerpen (19/52) and Limburg (18/52), areas with a high incidence of tick bites and Lyme borreliosis compared to other Flemish provinces (Linard et al. 2007; tekennet.wiv-isp.be). Other animals came from the province Oost-Vlaanderen (12/52), West-Vlaanderen (3/52) or Vlaams-Brabant (4/52), the latter province having a high incidence of tick bites and Lyme borreliosis as well (Linard et al. 2007; tekennet.wiv-

isp.be). No information regarding the surrounding environment was recorded. Tissue samples were taken from liver and spleen from 52 animals. Seven animals, of which two were females and five were males, were collected between December and February and 45 animals, of which 25 were females and 20 were males, were collected between March and November. This is the activity period of *Ixodes ricinus*, an important ixodid tick species that acts as a vector of the pathogens under study between vertebrate hosts and humans in Europe (Dantas-Torres et al. 2012; Coipan et al. 2013b). Since the infection status of a reservoir host (and the number of ticks feeding on it) is an important factor in interpreting the reservoir role of the host, the infection status in the period that ticks are most active is crucial (Hofmeester et al. 2016). Moreover, the presence of a pathogen in the environment can be maintained by persistent infection in (long-living) host animals. It is not yet clear if squirrels are capable of maintaining persistent infections. Therefore, we decided to report the results on the animals collected during tick activity season and during winter as separate groups, to better interpret the reservoir role of the squirrel.

Spleen and liver were collected and frozen at -80°C. For the detection of DNA of *Borrelia, B. miyamotoi, A. phagocytophilum* and "*Ca.* N. mikurensis", and the identification of the *Borrelia* genospecies, we refer to the methods described in the previous Chapter and in Stenos et al. (2005), Jahfari et al. (2012), Heylen et al. (2013), Coipan et al. (2013a) and Heylen et al. (2016). We used primers that can detect all genospecies of *Borrelia* (Heylen et al. 2013b), and all ecotypes of *Anaplasma phagocytophilum* (Jahfari et al. 2014). For the detection of TBEV, real-time qPCR reactions were done in a final volume of 20 µl with TaqMan® Fast Virus 1-Step Master Mix (Thermo Fisher scientific, USA), 5 µl of sample and 0.4 µM for all primers and 0.4 µM probe. The primers for TBEV we used are described in Klaus et al. (2010). An internal control was added to all samples, with 20 min reverse transcription step at 50°C, denaturation at 95°C for 30 s and 55 cycles of 95°C for 10 s and 60°C for 30 s. The amplification was performed on a Roche LightCycler 480 instrument. The DNA of bacteria (such as *Borrelia*) in a wide range of organisms and tissues is fairly stable (*personal observation*), and degradation does not happen easily. Therefore, the number of days that the squirrels were dead before collection, which is limited, will most likely not influence the prevalence of the pathogens or the ability of the real-time qPCR to detect the pathogens in this study.

We used the prop.test function in R version 3.3.2 (R Core Team 2017) to test whether infection occurred more in male or female squirrels.

5.4 Results

Based on the PCR analysis of the tissue samples of the 45 animals collected in the tick activity period, we could detect DNA of at least one of the selected pathogens in seven animals (15.6%), of which six were males and one was female. Males were found to be infected more often than females ($p = 0.048$). All positive samples were spleens. *Borrelia* DNA was detected in the spleen of two squirrels (4.4%), DNA of *B. miyamotoi* was found in the spleen of three squirrels (6.7%) and *A. phagocytophilum* DNA in four spleen samples (8.9%). One *Borrelia* sequence was identified as *B. afzelii*, by conventional PCR followed by sequence identification. We were unable to determine the identity of the other isolate. Two sequences of *A. phagocytophilum* were identified as ecotype II and one sequence as ecotype I. The third isolate could not be further typed. Co-infection occurred in the spleen of two animals. One animal harboured DNA of *B. miyamotoi* and *A. phagocytophilum* ecotype I and one animal harboured DNA of *B. afzelii* and *B. miyamotoi*. Of the eight squirrels collected in winter, all animals except one tested negative for all tick-borne pathogens investigated. For this male squirrel collected in December, both spleen and liver tested positive for *Borrelia* infection. DNA of "*Ca.* N. mikurensis" or TBEV could not be detected in any of the samples. All *Borrelia*-positive squirrels originated from a province with high incidence of tick bites and Lyme borreliosis (Limburg and Vlaams-Brabant).

5.5 Discussion

The prevalence of *Borrelia* in the tissue samples of red squirrels in our study is low (4.4%), in contrast to the infection prevalence in mice such as *Apodemus* spp., which is on average 17% (Hofmeester et al. 2016). Our results suggest that squirrels can host *B. afzelii*, as already proposed by previous studies (Humair and Gern 1998; Pisanu et al. 2014). These studies report a higher *Borrelia* prevalence in tissue samples from dead red squirrels and state that *B. burgdorferi* s.s. was the most common genospecies, which we cannot confirm. These studies, however, used skin tissue to detect presence of pathogen DNA. It is possible that *B. burgdorferi* s.s., or other *Borrelia* genospecies, were present in the skin of the animals we investigated, but absent from the liver and spleen, because it did not (yet) disperse throughout the body. The differences in infection prevalence of *Borrelia* in

squirrels and the detection of certain genospecies between Switzerland, France and Flanders can also be due to differences in eco-epidemiological aspects, such as e.g. host composition, tick-host contact rates or tick population dynamics. Because we were able to detect DNA of *B. afzelii* in the spleen and liver of one individual collected in December, it seems that *B. afzelii* can cause a persistent infection in squirrels. The methods we used in this study, however, do not allow us to make statements about the absence of pathogens, only about the presence. We emphasize the need to study the presence of pathogens in skin tissue of squirrels, as well as in liver and spleen and in ticks that fed on squirrels, to assess the role of red squirrels in the transmission of tick-borne pathogens. Moreover, for a species to be a reservoir host of a pathogen, it has to be able to transmit the pathogen to ticks feeding on it. This can be evaluated by allowing an uninfected tick feeding on the host and afterwards analysing the tick for infection ('xenodiagnosis'). Further research should investigate the infection prevalence of tick-borne pathogens in ticks that fed on squirrels.

Male squirrels were infected with tick-borne pathogens more often than females. Earlier studies have shown that at least for some small rodent species, such as bank vole and wood mouse, males tend to have higher infestation levels with ticks than females. The higher infestation of males has been linked to their larger home ranges (Tälleklint and Jaenson 1997; Brunner and Ostfeld 2008) and to high testosterone levels, which has been shown to weaken the immune response (Hughes and Randolph 2001). This may cause males to be infected with tick-borne pathogens more often than females. Red squirrel males have larger home ranges than females (Wauters and Dhondt 1992; Wauters et al. 2004).

Humair & Gern (1998) found a higher prevalence of *B. burgdorferi* s.s. in ticks collected from red squirrels than in questing ticks, and concluded that the red squirrel is probably an important reservoir host for this genospecies. The conclusions from that study, however, are based on a low sample size (only six squirrels). One study (Pisanu et al. 2014) investigated tissue samples of a large amount of squirrels (273 individuals) but the animals were collected throughout the year in a large region, without specifying infection prevalence per season or region. Hofmeester and colleagues (Hofmeester et al. 2016) therefore stress that the results from these two studies are not conclusive.

Previous research showed that rodents can act as reservoir hosts for *B. miyamotoi* and "*Ca.* N. mikurensis", but are probably only accidental hosts of *A. phagocytophilum*, only rarely transmitting the pathogen to attached ticks (Burri et al. 2014; Obiegala et al. 2014). Jahfari

et al. (2014) detected *A. phagocytophilum* ecotype III in the spleen of a wood mouse and in two ixodid ticks that had fed on a wood mouse. Ecotype III was not detected in any other host species except rodents, and only at very low infection prevalence, and rodents did not carry other ecotypes than ecotype III (Jahfari et al. 2014). Contrary to this, we did not find ecotype III in the tissue samples of red squirrel in our study. This may be due to the fact that ecotype III is probably mainly maintained in an enzootic cycle involving small woodland rodents such as mice and voles and *I. trianguliceps*, an ixodid tick species that is not likely to feed on squirrels (Bown et al. 2003). All human cases of anaplasmosis are linked only to *A. phagocytophilum* ecotype I, no other ecotype has been detected in humans (Jahfari et al. 2014). We show here that red squirrels can carry *A. phagocytophilum* ecotype I, which has the highest zoonotic potential, and ecotype II, which has been associated to wild ungulates (Jahfari et al. 2014).

5.6 Conclusions

In our study, we find that the Eurasian red squirrel, which is a medium-sized rodent species, can harbour a comparable community of tick-borne pathogens as do small rodents such as mice and voles. Our results demonstrate the epidemiological importance of the red squirrel since they can carry *A. phagocytophilum* ecotype I. The exact role of the red squirrel in the human health risk remains unclear. Future research should assess the transmission potential of different pathogens to ticks and the reservoir competence of this ubiquitous European host of ixodid ticks.


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6 Low probability of a dilution effect for Lyme borreliosis in Belgian forests

After: Ruyts S.C., Landuyt D., Ampoorter E., Heylen D., Ehrmann S., Matthysen E., Sprong H., Verheyen K. Low probability of a dilution effect for the rodent associated Lyme borreliosis pathogen *Borrelia afzelii* in different forest types in Belgium, Europe. Ticks and Tick-borne diseases, submitted (IF 3.23)

6.1 Abstract

An increasing number of studies have investigated the consequences of biodiversity loss for vector-borne disease risk. As host species differ in their ability to transmit the Lyme borreliosis bacteria *Borrelia burgdorferi* s.l. (‘*Borrelia’*) to ticks, increased host diversity can decrease disease prevalence by increasing the proportion of dilution hosts, which transmit pathogens less efficiently. Previous research shows that Lyme borreliosis risk differs between forest types and suggests that a higher diversity of host species might dilute the contribution of small rodents to infect ticks with *B. afzelii*, a common *Borrelia* genospecies. However, empirical evidence for a dilution effect in Europe is largely lacking. We tested the dilution effect hypothesis in 19 Belgian forest stands of different forest types. We used both empirical data and a Bayesian belief network to investigate the impact of the proportion of dilution hosts on the density of ticks infected with *B. afzelii*, and identified the key drivers of the density of infected ticks, a measure of human infection risk. Densities of ticks and *B. afzelii* infection prevalence differed between forest types, but the model indicated that the density of infected ticks is hardly affected by a change in dilution. The most important variables in explaining variability in disease risk were related to the density of ticks. Combining empirical data with a model-based approach supported decision making to reduce tick-borne disease risk. We found a low probability of a dilution effect for Lyme borreliosis in a Northwestern European context and emphasize that under these circumstances Lyme borreliosis prevention should rather aim at reducing tick-human contact rate instead of attempting to increase the proportion of dilution hosts.

6.2 Introduction

In Chapter 2, we have indirectly tested the dilution effect hypothesis for the different *Borrelia* genospecies in Europe and found indications that Lyme borreliosis risk can differ between different forest types. *Ixodes ricinus* nymphs were more likely infected with *B. afzelii* in pine forests than in oak forests and infections with other genospecies tended to occur more often in oak forests. These findings suggest that a higher diversity of host species might diminish the influence of small rodents to infect ticks with *B. afzelii*, the most common *Borrelia* genospecies. We thus expect to see a dilution effect for *B. afzelii* in oak forests, caused by a lower proportion of rodents in the host community, and a higher host diversity, in oak forests compared to pine forests. However, the relations between forest types, host community composition and disease risk have not been adequately studied so far. This research gap makes it impossible to verify the validity of the dilution effect hypothesis for Lyme borreliosis in Europe.

This study aims to unravel the relationship between host community composition and Lyme borreliosis risk and tests the dilution effect hypothesis for Lyme borreliosis in a European context, using the density of infected nymphs, a widely applied disease risk measure (Ogden and Tsao 2009). We focus on *Borrelia afzelii*, the most common *Borrelia* genospecies in our study region, as shown in Chapters 2 and 3, whose enzootic cycle is mainly driven by rodents. We use empirical models to study relationships in the field and a literature-based Bayesian belief network model to (1) gain more insights into the mechanisms that drive these field observations in different forest types, and to (2) position our study within the full range of conditions that can be observed in the field.

6.3 Material and methods

6.3.1 Study site

For this study, we used the *I. ricinus* ticks that were collected from forest stands in site AH and site P in the framework of the studies described in Chapters 2 and 3. In site AH, we investigated 10 stands: two pine stands without a shrub layer, three pine stands with a shrub layer, three oak stands without a shrub layer, and two oak stands with a shrub layer. In site P, we sampled nine stands: three pine stands without a shrub layer, three pine stands with a shrub layer, and three oak stands with a shrub layer. Oak stands with a shrub layer are supposed to contain the highest host diversity, and pine stands without a shrub layer

supposedly the lowest (Carnus et al. 2006; Du Bus de Warnaffe and Deconchat 2008). In each stand, we chose a sampling plot of 0.2 ha in the center of each stand, representative for that forest type, for the collection of the data.

6.3.2 Data collection

6.3.2.1 Ticks

In the studies described in Chapters 2 and 3, we only examined the density of (infected) questing *I. ricinus* nymphs. The nymphal life stage most often transmits *Borrelia* infection to humans, so this is the most interesting life stage to examine in the light of human health. The density of infected nymphs depends on the density of infected larvae, which is the product of the density of larvae and the proportion of larvae that fed on a competent host. Here, we aim to gain more insight in the mechanism of the dilution effect by evaluating the impact of the proportion of larvae that fed on competent host species on the density of nymphs and by interpreting how variation in certain variables will affect the density of infected nymphs. Therefore, in this study, we examine both the density of questing larvae and the density of questing nymphs.

Questing *Ixodes ricinus* ticks were collected by dragging a 1 m² flag through the vegetation along six transects of 25 m in the sampling plot in each stand in June, July and September in 2013 and in 2014. Larval *I. ricinus* are less than 1 mm large and have only six legs, and are distinguished in the field from nymphs which are larger (1.2 to 1.5 mm) and have eight legs (Hillyard 1996). We visually estimated the total amount of larvae attached to the flag at the end of the six transects in 2013. In 2014, we collected all nymphs attached to the flag at the end of each transect in plastic vials containing 70% ethanol, following the procedure described in Chapter 2. Nymphs from the different sampling occasions were pooled per forest stand. The density of larvae (DOL) and nymphs (DON) for each stand was calculated as the number of larvae and nymphs, respectively, per 100 m², averaged over the three sample occasions.

6.3.2.2 Borrelia *genospecies*

We randomly selected 35 nymphs per pool for further analysis. For the molecular detection of *Borrelia* infection in the nymphs and identification of the *Borrelia* genospecies, we followed the same approach as described in Chapter 3. For each genospecies, we calculated

the nymphal infection prevalence (NIP) as the percentage of nymphs infected with a certain genospecies. In this study, we focused on the percentage of nymphs infected with *B. afzelii*. The density of nymphs infected with *B. afzelii* (DIN) is the product of NIP and DON.

6.3.2.3 Host community

To estimate the density of the different host species for ticks, we used a combination of different sampling methods in the sampling plots in 2014 and 2015. For a detailed description of the sampling procedures, we refer to the supplementary material (Appendix 6.1). In 2014, we used live traps to estimate densities of small mammals (mice, voles and shrews) and used roe deer resting site as our approximate roe deer density per sampling plot. Bird species were identified by sound and densities were estimated by point counts. We only focused on thrushes (*Turdus* sp.), Eurasian wren (*Troglodytes troglodytes* Linnaeus, 1758) and European robin (*Erithacus rubecula* Linnaeus, 1758) since they are the forest bird species who contribute the most to the tick population (Marsot et al. 2012). In 2015, footprint tunnels and bite marks on hazelnuts and pine cones were used to assess presence of hedgehogs and squirrels, respectively. We used camera traps and bait to detect a diverse set of vertebrates such as wild boar (*Sus scrofa* Linnaeus, 1758), red fox (*Vulpes vulpes* Linnaeus, 1758) and stone marten (*Martes foina* Erxleben 1777). Since red squirrel, European hedgehog, red fox and stone marten occur in relatively low densities in European forests and have large home ranges (Heydon et al. 2000; Verkem et al. 2003; Wauters et al. 2004; Young et al. 2006), it is not feasible to quantify densities in small forest stands. Therefore, we used data on presence or (observed) absence in the sampling plots to approximate of the density of these species in our small stands. In the stands where we recorded wild boar, we set their abundance at 10 individuals, as this is the average group size in our study region (Verkem et al. 2003). As a result of differences in life history traits, the spatiotemporal dynamics of the populations of the medium sized and large host species recorded in 2015 are expected to vary less than those of the smaller host species sampled in 2014 (Begon et al. 2006). Therefore, we assumed that our estimation of the population density of the host species recorded in 2015 is representative for their densities in 2014. In our study, we considered mice, voles, squirrels and hedgehogs as competent hosts for *B. afzelii* transmission based on our studies described in Chapters 4 and 5 and other studies (Humair and Gern 1998; Skuballa et al. 2007; Pisanu et al. 2014; van Duijvendijk et al. 2015). All other recorded host species were considered to be dilution hosts.

We multiplied the estimated density for each host species with its average larval burden in Europe, as reported by Hofmeester et al. (2016), to approximate the importance of a particular host species for the feeding of larvae. This way, the proportion of dilution hosts in the total host community, taken into account their average larval burden, thus represents the proportion of larvae in a particular stand that could potentially feed on dilution hosts. This index was called the 'potential dilution', as opposed to the 'realized dilution', that represents the actual number of larvae that feeds on a dilution host. The potential dilution in our study is an estimation of the realized dilution. We further used the estimated host densities and their potential larval burden to calculate an exponential Shannon Wiener index (eH). Species richness (SR) was calculated as the total number of recorded species. For oak stands with a shrub layer from site P, we eliminated one stand in the calculation of eH and potential dilution, because of lack of data on mice, voles and shrews (Appendix 6.1).

6.3.3 Data analysis

All statistical analyses were performed in R version 3.4.0 (R Core Team 2017). The risk for human exposure to Lyme borreliosis depends on the density of host-seeking infected ticks (acarological risk) and on the human-tick contact rate (Jaenson et al. 2009). Here, we studied acarological risk and used DOL from 2013, DON from 2014, NIP from 2014 and DIN from 2014.

First, we examined the effect of forest type on DOL, DON, NIP and DIN and on the host community indices SR, eH and potential dilution. We used univariate linear mixed-effect models (*lmer*) with a Gaussian error distribution from the *lme4* R-package (Bates et al. 2014). Forest site was used as a random effect to account for the hierarchical design of our study, forest type ('pine without shrub', 'pine with shrub', 'oak without shrub', 'oak with shrub') was used as fixed effect. For each response variable, we compared the null model containing only the random effect with the model containing the fixed effect. Then, to evaluate the dilution effect hypothesis, we assessed the effect of the host community (SR, eH and potential dilution) on DOL, DON, NIP and DIN with univariate *lmer*, using the same procedure as described above. For each analysis, we checked for heterogeneity of the residuals following the approach described in Zuur et al. (2009). We used the Akaike Information Criterion adjusted for sample size (AIC_C) (Hurvich and Tsai 1989) to select the model with the highest probability of observing the data (smallest AIC_C). Finally, we

calculated the marginal R^2 (R^2m), i.e. the fraction of the variation explained by the fixed structure, and the conditional R^2 (R^2c), i.e. the fraction of the variation explained by the fixed and random structure.

6.3.4 Bayesian belief network modelling

Bayesian belief networks (BBNs) are probabilistic graphical models that model a system by accounting for all causal relations among the system's variables and by quantifying those relations through conditional probability distributions (Jensen and Nielsen 2007). The ability to explicitly account for uncertainties makes this technique highly suitable for modelling systems when uncertainty levels are high (Landuyt et al. 2013), like in the case of the enzootic cycle of *Borrelia* in Europe. Through probabilistic inference, BBNs can propagate these uncertainties through the model while making predictions for one of the model's variables given some data on the others. For more details on the modelling technique, see Jensen and Nielsen (2007).

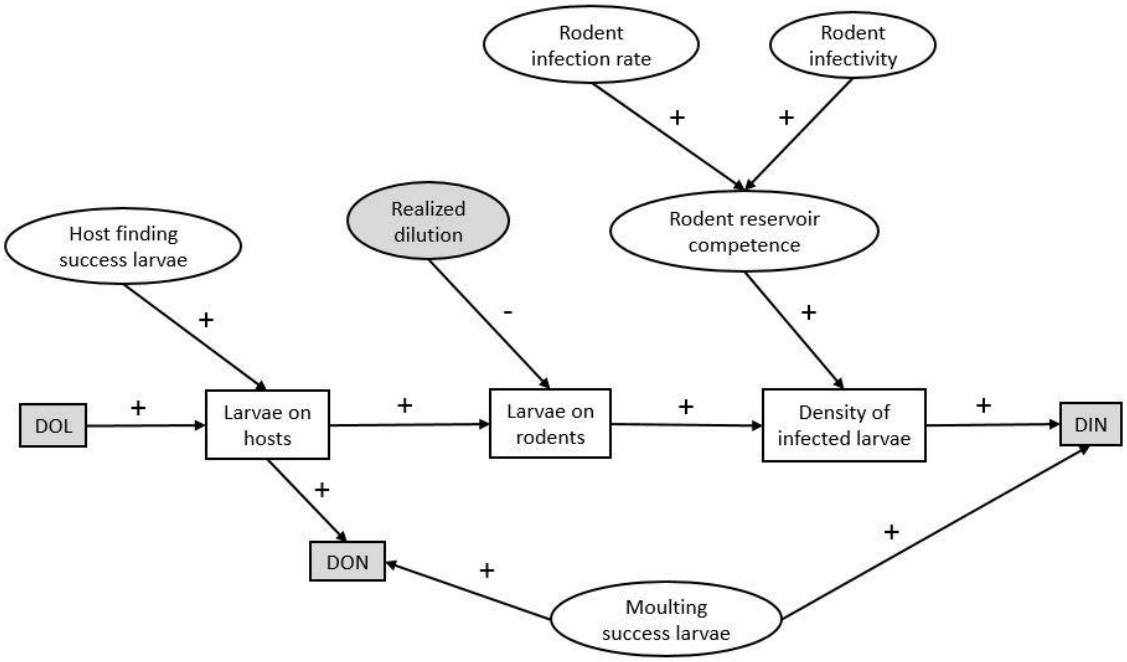
BBNs have a history in medical diagnosis problems, deriving the type of disease from observed symptoms or test results. However, after their introduction, they have been used in other domains as well, especially for cases where uncertainties prevail and where systems can be represented by a relatively simple linear chain of causes and consequences. The major advantages of BBNs include their transparency (they can increase system understanding), their explicit accounting for uncertainties and the possibility to include all kinds of data (expert knowledge, literature data, field data). The major disadvantages include their simplicity (they might not be suited for modelling complex systems with feedback loops) and information loss through discretization of all variables (transferring continuous variables and equations into discrete counterparts) (Jensen and Nielsen 2007).

We constructed a BBN model to evaluate the impact of dilution of pathogens by hosts and to predict acarological risk under variable conditions. We based our model on published theories and data on Lyme borreliosis ecology in Europe, focusing on *B. afzelii* as single pathogen. We used the simple but realistic scenario in which rodents, squirrels and hedgehogs are the only source of *B. afzelii* infection of nymphs in a certain habitat, and no infected nymphs are transported from nearby habitat patches. The structure of the network was defined based on relationships described in the literature (e.g. see Piesman and Gern 2004) and is visualized in Figure 6.1. The network represents the turnover of larvae in one

year to nymphs infected with *B. afzelii* the next year in a certain location. In this model, the 'realized dilution', the actual number of larvae that feeds on a dilution host, is estimated by the potential dilution, the proportion of dilution hosts in the total host community. The model's output node is the risk measure DIN which directly depends on the density of larvae the previous year, on the probability that they are infected by their host, and on the probability that they will moult into nymphs. We used BBN to explore the conceptual model of the dilution effect, contrary to hypothesis testing. Therefore, the structure of BBN is the structure of the conceptual model. We directly translated the conceptual model of the dilution effect into an operational BBN and explored how the operational model corresponds to the conceptual model.

As all relations among the system's variables could be described through mathematical equations, conditional probability distributions were populated through sampling, using the "Equation To Table" tool available in Netica (Norsys Software Corporation 1998). Figure 6.1 shows the equations that were used to construct the model. The input nodes of the model (DOL, 'host finding success larvae', 'realized dilution', 'rodent infection rate', 'rodent infectivity' and 'moulting success larvae') require prior probability distributions or, if available, a fixed state (e.g. DOL = 150). The probability distribution of the other nodes depends on the state or probability distribution of the other nodes. As a result of missing prior knowledge on some of the model's input nodes, including DOL, 'host finding success larvae', 'moulting success larvae' and 'realized dilution', uniform prior probability distributions were used for these variables. As empirical data will be inserted into the variables DOL and 'realized dilution' for model simulations, these prior probabilities did not affect model predictions reported below. Probability distributions for the network's input nodes 'rodent infection rate' and 'rodent infectivity' were defined based on values found in the literature (Humair et al. 1999; Huegli et al. 2002; Buffet et al. 2012; Gassner et al. 2013; van Duijvendijk 2016; Vourc'h et al. 2016). The number of infected nymphs that feed on rodents and transmit the pathogen to the rodents, influences the rodent infection rate (van Duijvendijk et al. 2015). Our model, however, only considers the turnover from the density of uninfected larvae to the density of infected nymphs, which is influenced by the rodent infection rate at that particular time step. Therefore, in our BBN we do not consider the impact of NIP or DIN on rodent infection rate. In this study, we collected empirical data for the nodes DOL, DON, DIN and 'realized dilution'. We entered the

empirical data for these nodes depending on the specific aspect we wanted to simulate with the model.



Variable	Equation
Larvae on hosts	DOL * Host finding success larvae
Larvae on rodents	Larvae on hosts * (1 – Realized dilution)
Rodent reservoir competence	Rodent infection rate * Rodent infectivity
Density of infected larvae	Larvae on rodents * Rodent reservoir competence
DON	Larvae on hosts * Moulting success larvae
DIN	Density of infected larvae * Moulting success larvae

Fig. 6.1 The graphical structure of the Bayesian belief network model and the equations underlying the relationships between the variables. All square nodes in the network represent densities of ticks, the oval shaped nodes are parameters that affect the density nodes when moving to the next stage, and reflect survival and infection probability of ticks. The input variables density of larvae in 2013 (DOL), density of nymphs in 2014 (DON), density of infected nymphs in 2014 (DIN) and realized dilution, variables for which we have empirical data, are depicted in grey. Realized dilution: proportion of larvae that feeds on dilution hosts. An increase in realized dilution will decrease the amount of larvae on rodents and subsequently DIN and Lyme borreliosis risk. The (+) and (-) signs above each arrow represent the nature (positive or negative, respectively) of the relation between the variables.

To compare model predictions with empirical data, several scenarios were evaluated using the model, with each scenario representing one of the studied forest types in one of the forest sites. For each scenario, Netica was used to calculate the probability distribution of realized dilution given the empirical DOL, DON and DIN as input variables, averaged over all stands for each scenario. Afterwards, to interpret the relationship between DIN and realized dilution, we only inserted information on DOL and DON into the model, and assessed potential changes of DIN as a result of varying realized dilution from 0% to 90-100%. In pine forests without a shrub layer in site P, the average empirical density of nymphs in 2014 was higher than the average empirical density of larvae in 2013. Probably we underestimated the density of larvae in 2013 because of imperfect sampling due to unverified accidental conditions such as human error or weather. Since it is not a realistic situation, we did not include this forest type as a scenario in the BBN. To investigate the sensitivity of DIN to changes in any other node, we performed a sensitivity analysis using Netica.

6.4 Results

A total of 2924 nymphs were collected in the 19 forest stands in 2014 and we counted 9020 larvae in the forest stands in 2013. We examined 665 nymphs for *Borrelia* spirochetes, of which 104 (15.64%) were found to be infected. In each infected nymph, only one genospecies could be identified. We identified five different *Borrelia* genospecies in 72 (69.23%) infected nymphs, namely *B. afzelii*, *B. garinii*, *B. burgdorferi* s.s., *B. valaisiana* and *B. spielmanii*. For the other 32 infected nymphs, genospecies could not be defined. The genospecies *B. afzelii* was most common, occurring in 66.36% of all infected nymphs, or in 10.08% of all nymphs. This corresponds to a mean (± SE) NIP of 10.1% (± 1.8) over all stands. DIN ranged from 0 to 10.6, with a mean DIN of 2.1 (± 0.6). The second most common genospecies in the examined nymphs was *B. garinii*, which occurred in 2.7% (± 1) of all nymphs. The mean prevalence *B. spielmanii* and *B. valaisiana* was 1% (± 0.6) and 0.9% (± 0.5), respectively, *B. burgdorferi* s.s. occurred in 0.6% (± 0.3) nymphs.

We found that the model that included forest type as an explanatory variable explained the variation in DOL and NIP better than the null model (Fig. 6.2a, c; Appendix 6.2). More larvae were caught in oak than in pine stands. DON also shows a trend towards higher densities in oak stands compared to pine stands (Fig. 6.2b). NIP was higher in pine than in oak stands. Forest type had no effect on DIN (Fig. 6.2d, Appendix 6.2).

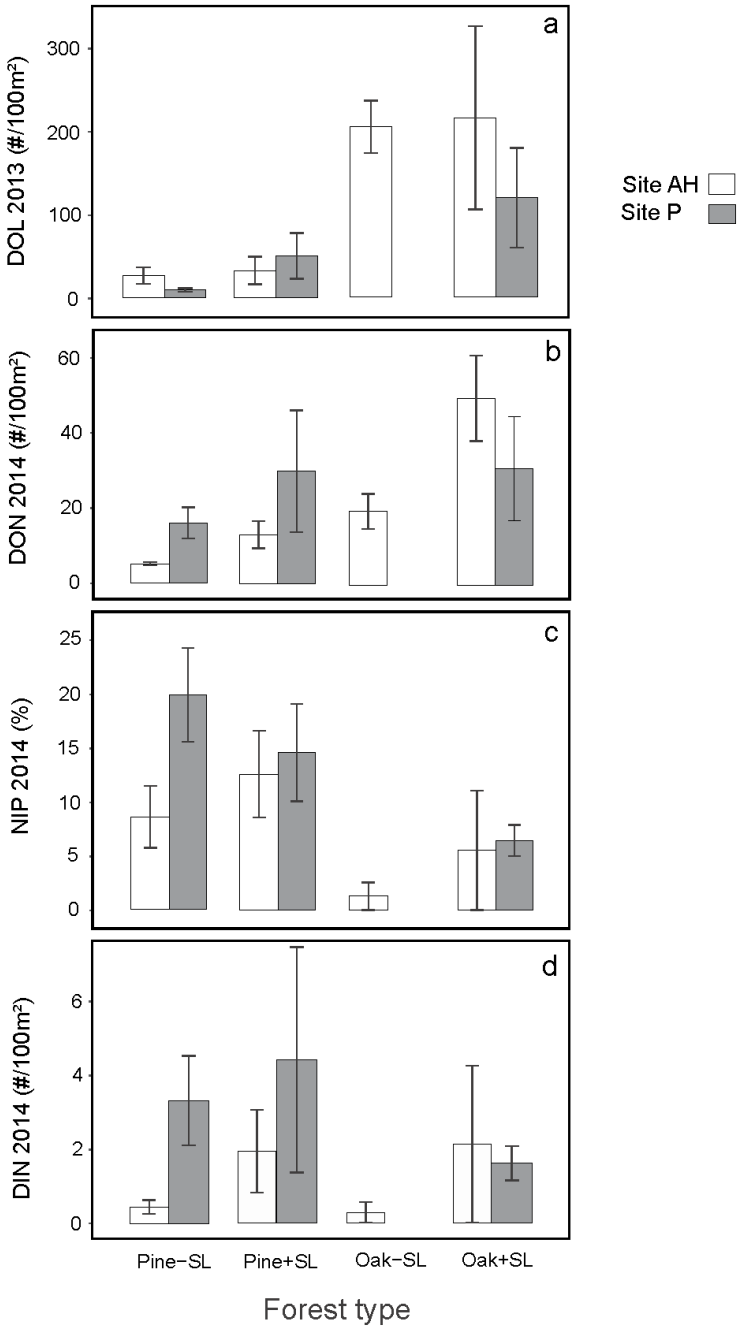


Fig. 6.2 The mean densities of larvae (DOL, a), densities of nymphs (DON, b), nymphal infection prevalence (NIP, c) and densities of infected nymphs (DIN, d) (± SE) for *Borrelia afzelii* in pine and oak stands, with a shrub layer (+SL) or without a shrub layer (-SL) in site AH and site P, averaged over all forest stands per forest type.

The host community that we observed in the stands during our sampling campaigns consisted of bank vole, wood mouse, common/crowned shrew (*Sorex araneus/coronatus*), pygmy shrew (*S. minutus* Linnaeus, 1758), roe deer, wild boar, squirrel, hedgehog, fox, stone marten (*Martes foina* Erxleben 1777), wren, robin, common blackbird (*Turdus merula* Linnaeus, 1758) and song thrush (*T. philomelos* Brehm 1831) (Appendix 6.3). We grouped these last two bird species as 'thrushes' (*Turdus* spp.). For the species groups 'rodents', 'shrews', 'birds' and 'predators' we summed the observed densities of the two rodent species, the two shrew species, the three groups of birds and the two predators, respectively. We could not detect a difference in host community measures between the different forest types (Appendix 6.3). However, the density of small rodents appears to be lowest in pine stands without a shrub layer in both sites (Fig. 6.3). We could not detect a difference in potential dilution between the different forest types, but the potential dilution appeared to be higher in pine than in oak stands (Fig. 6.4). The models containing SR, eH or potential dilution as explanatory variable did not explain the variation in DOL, DON, NIP or DIN better than the null models (Appendix 6.4).

In the BBN, we see that for some scenarios, the predicted probability of realized dilution does not correspond to the potential dilution (Appendix 6.5). For the oak stands in site AH, realized dilution is higher than the potential dilution, while for the pine stands in site P, potential dilution is much higher than the realized dilution. In three scenarios (Fig. 6.5a-c), DIN changes only little with varying realized dilution while in the other three scenarios (Fig. 6.5d-f), DIN decreases with increasing realized dilution. The five most-influencing variables all strongly relate to the density of ticks (Fig. 6.6). The input variables related to the infection prevalence of ticks, such as reservoir competence of rodents and realized dilution, caused a much smaller reduction in relative variance.

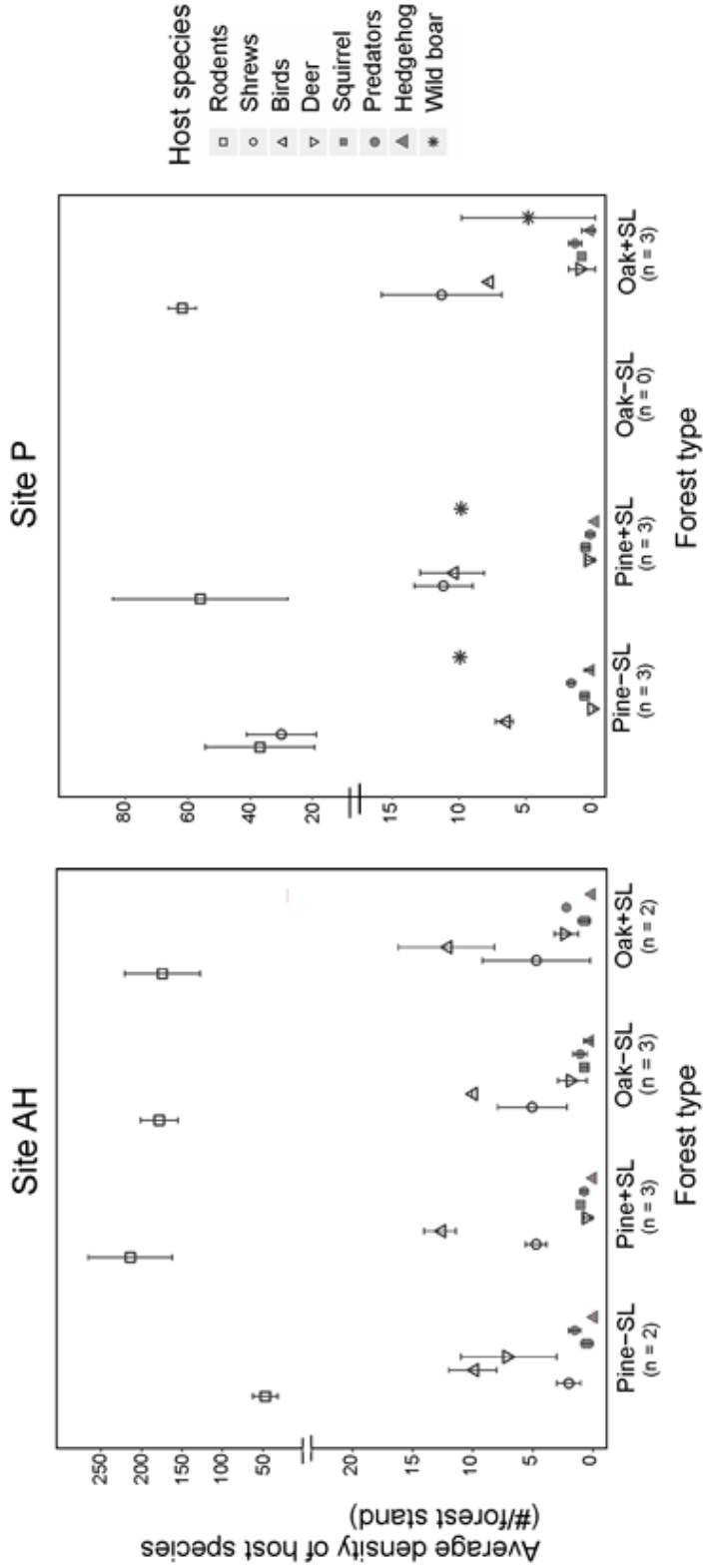


Fig. 6.3 The composition of the host community in site AH and site P in pine and oak stands, with (+SL) or without (-SL) a shrub layer (n = number of forest stands per forest type). Bars represent mean density of individuals (±SE), averaged over all forest stands of that particular forest type. Note the different scaling on the Y-axes of the two plots.

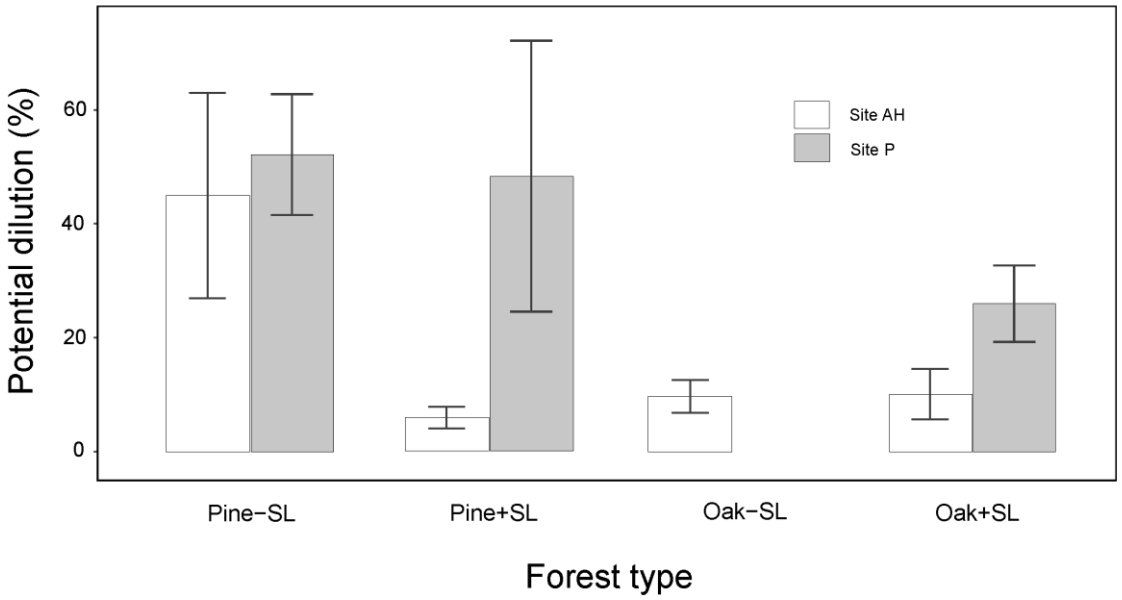


Fig. 6.4 The mean percentage (± SE) of the host community that consists of dilution hosts for *Borrelia afzelii* (potential dilution) in pine and oak stands, with a shrub layer (+SL) or without a shrub layer (-SL) in site AH and site P. Percentages are averaged over all forest stands per forest type.

6.5 Discussion

This study investigated the composition of the host community of ticks in different forest types. We examined the presence of a dilution effect for the rodent associated Lyme borreliosis genospecies *Borrelia afzelii* using both empirical data as well as a model, and identified the most important variables that influence acarological risk. Based on a Bayesian belief network, we found that the most important variables in explaining variation in DIN in our study are related to the density of ticks, instead of to the proportion of dilution hosts. We found no effect of SR, eH or potential dilution on the risk measures DOL, DON, NIP or DIN and thus cannot confirm the dilution effect hypothesis.

The most common *Borrelia* genospecies in the nymphs from our study sites was the rodent-associated *B. afzelii*, which confirms our assumption that Lyme borreliosis in our region is to a large degree rodent-driven. We found *B. afzelii* in 10.1 % of the collected nymphs.

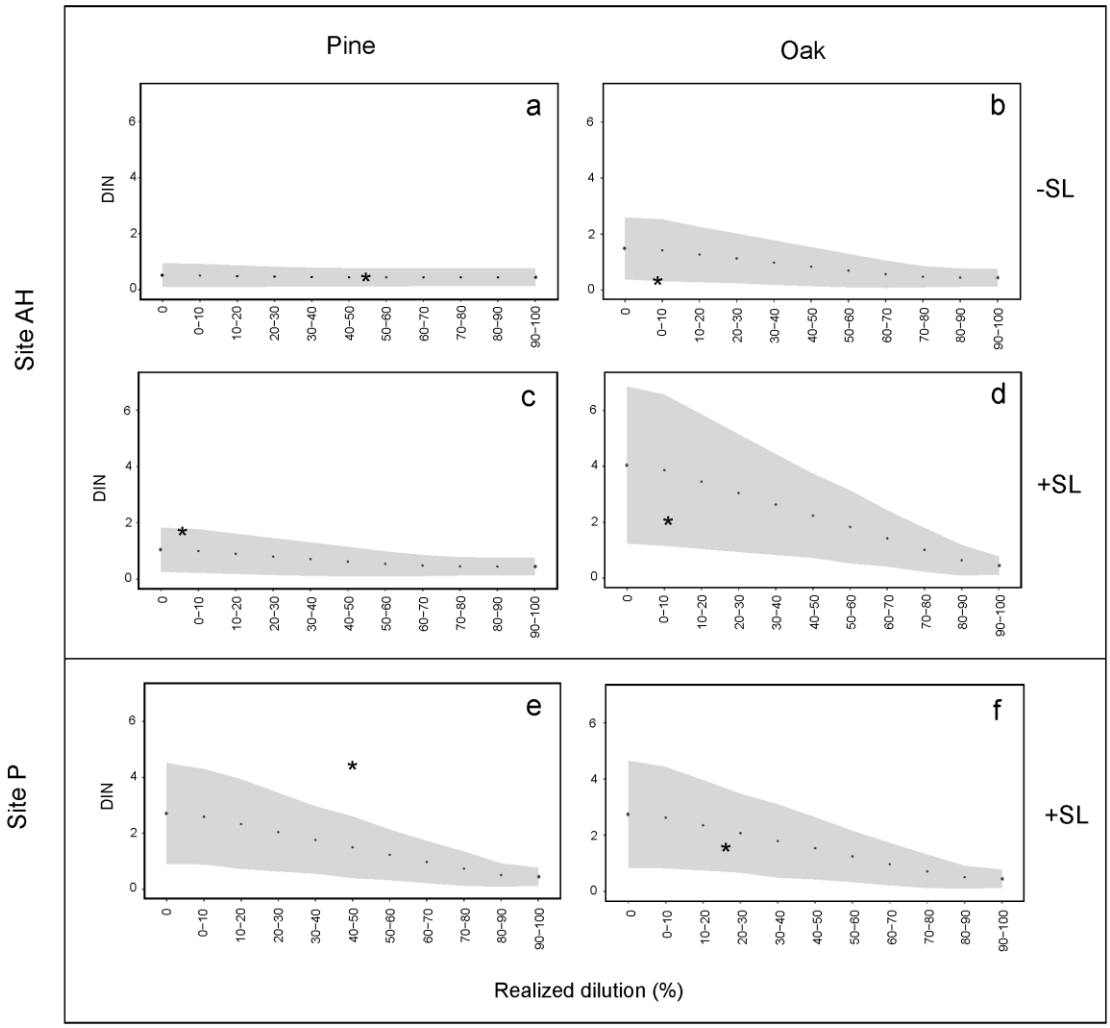


Fig. 6.5 Relation between density of infected nymphs (DIN) and realized dilution (%) as predicted by the model for six scenarios representing different forest types in different forest sites. Scenarios were set by inserting average measured densities of larvae (DOL) and nymphs (DON) into the model for scenario. Asterisks depict measured potential dilution and DIN values for each scenario. The grey area represents the standard deviation. The different scenarios include pine stands without a shrub layer (a) and with a shrub layer (c) and oak stands without a shrub layer (b) and with a shrub layer (d) in site AH and pine stands with a shrub layer (e) and oak stands with a shrub layer (f) in site P.

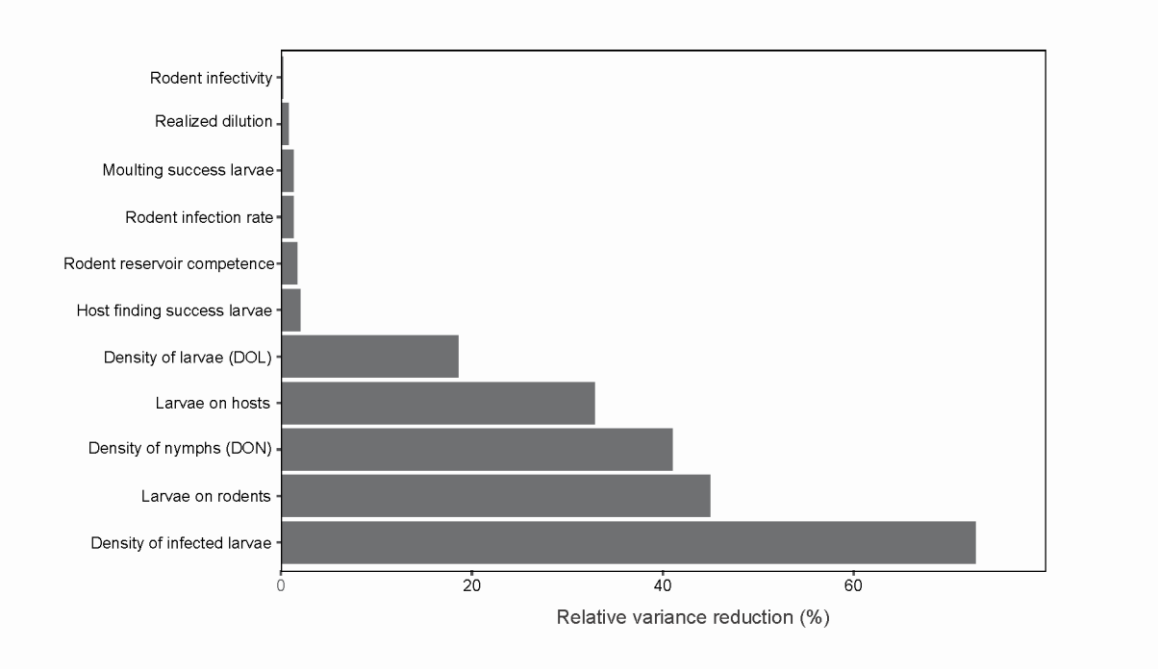


Fig. 6.6 Relative reduction in variance of the model predictions (density of infected nymphs (DIN)) by inserting evidence in one of the network's variables, calculated for each variable through a sensitivity analysis of the model.

We found a clear trend towards higher tick densities in oak stands compared to pine stands. Tick abundance is mainly determined by humidity and the availability of hosts (Gray et al. 1998; Tagliapietra et al. 2011). The density of rodents appears to be lowest in pine stands without a shrub layer. Since small rodents are important feeding hosts for larval ticks (Hofmeester et al. 2016), this could explain the apparent lower density of nymphs in pine stands, compared to oak stands. Moreover, we found that NIP was higher in pine than in oak stands, which seems to indicate that the contribution of small rodents to the feeding of ticks is higher in pine stands, and thus that dilution is highest in oak stands, as suggested in Chapters 2 and 3. The density and composition of the host communities we surveyed, however, do not seem to support the assumption that broadleaved forest stands contain a higher host diversity and hence a higher probability for dilution than coniferous stands (Carnus et al. 2006; Du Bus de Warnaffe and Deconchat 2008). Contrastingly, the potential dilution appears to be higher in pine stands than in oak stands. It appears that dilution in our study is mainly driven by the varying density of rodents, since the densities of other host species, multiplied by their possible average larval burden, is comparable between the different forest types. Although there are more dilution hosts in pine stands than in oak stands for ticks to feed on, larvae seem to feed relatively more on rodents than on dilution

hosts in pine stands compared to oak stands. Our results indicate that in our study region, the difference in the density of dilution hosts between habitats is probably less important in establishing a dilution effect. Instead, the mechanism of the dilution effect is probably driven by densities of transmission competent hosts, an aspect that is often neglected in literature (Ostfeld and Keesing 2000; LoGiudice et al. 2003). Yet, although our empirical data showed a trend towards higher potential dilution in pine compared to oak stands, the BBN model predicted the opposite. Realized dilution in oak stands is predicted to be higher than the potential dilution. The potential dilution in the oak stands is thus probably an underestimation of the realized dilution.

Although we estimated the host community composition adequately, there are some uncertainties in our calculations as we used relatively small sampling plots that do not reflect the home range of larger host species and the average larval burden per host species from literature. Moreover, the relation between the host community composition and realized dilution might be more complex than initially assumed. The potential dilution is only an estimation of the host use by ticks, taking into account the densities of the host species and their average larval burden. The average larval burden for each host species did not incorporate the impact of different habitat types on host use by larvae, and is thus only an approximation of the actual larval burden for our study. The realized host use does not only depend on the availability and suitability of host species, but also on the tick-host contact rate (Randolph and Craine 1995; Levi et al. 2016). In habitats with comparable host communities but with different composition and structure of the vegetation, and different microclimate, contact between ticks and hosts could differ because of host behavior or tick questing behavior (Craine et al. 1995; Randolph and Craine 1995; Randolph and Storey 1999). This differential host use by ticks in different habitats might explain the higher realized dilution in oak stands, despite the higher rodent densities. Furthermore, host species other than wood mouse, bank vole, squirrel and hedgehog could be able to transmit *B. afzelii* to feeding larvae. More research is needed to establish the importance of different vertebrate species in Europe in the feeding of *I. ricinus* and the transmission of different *Borrelia* genospecies. Further research on the dilution effect in Europe should take into account not only the host community composition, but also the effect of vegetation features and microclimate on larval burden on hosts. By comparing our empirical results with the model predictions, we were able to explore the complexity of the relationship between the host community composition, the habitat and the dilution effect.

An increase in the realized dilution, and thus a decrease in the density of larvae that feed on competent host species, could theoretically decrease DIN and the subsequent disease risk. However, we found that DIN was low in many scenarios and insensitive to a change in realized dilution. This is in accordance with the sensitivity analysis of the BBN model, which indicates that realized dilution has only limited influence on DIN. The most important variables in explaining variation in DIN are related to the density of ticks, which makes DON a good predictor of acarological risk, as found in Chapter 3 and suggested by others (Jaenson *et al.* 2009; Coipan *et al.* 2013). Since the variability in DON is generally much larger than the variability in NIP, which in our study varies between 0 and 0.30, DON has a higher weight in the calculation of DIN than NIP or realized dilution.

6.6 Conclusions

From our results we conclude that it may not be feasible to focus on the dilution effect to reduce Lyme borreliosis risk in our study region, and possibly in other European regions with similar forest communities. Instead, Lyme borreliosis prevention should aim to reduce tick densities and the contact rate between ticks and humans, which appear to be the most important variables in explaining acarological risk (Jaenson et al. 2009). An increase in host diversity did not decrease DIN in our study sites. Moreover, adding different species to the host community can even increase Lyme borreliosis risk, because different host species are associated with different *Borrelia* genospecies, which lead to different manifestations of Lyme borreliosis. An increase in host diversity could thus increase Lyme borreliosis risk by increasing the prevalence of *Borrelia* genospecies that give rise to more severe clinical manifestations than *B. afzelii*, which is associated with skin manifestations. The bird-related *B. garinii*, for example, can cause neuroborreliosis (Balmelli and Piffaretti 1995). Future research should focus on establishing the importance of less studied host species in the enzootic *Borrelia* cycle and the behavior of ticks and hosts in different forest types and vegetation features. We emphasize the need to reduce tick-human contact rate, for example by guiding visitor flows through forests or frequently mowing the vegetation along forest trails. These are actions that are easier and more effective for forest managers to take than increasing the dilution probability (Verheyen and Ruyts 2016).

Appendix 6.1: The host sampling protocol.

The sampling campaigns to estimate presence or density of hosts occurred in 2014 for small mammals, birds and European roe deer (*Capreolus capreolus* Linnaeus, 1758). In 2015 we used additional techniques to estimate densities of wild boar (*Sus scrofa* Linnaeus, 1758), red fox (*Vulpes vulpes* Linnaeus, 1758), stone marten (*Martes foina* Erxleben 1777), European hedgehog (*Erinaceus europaeus* Linnaeus, 1758) and Eurasian red squirrel (*Sciurus vulgaris* Linnaeus, 1758). The sampling was performed in a 0.2 ha sampling plot in the center of each forest stand, and densities of hosts in each sampling plot were representative for that particular forest stand.

Live traps

To estimate the relative population densities of small mammals (wood mouse (*Apodemus sylvaticus* Linnaeus, 1758), bank vole (*Myodes glareolus* Schreber 1780), pygmy shrew (*Sorex minutus* Linnaeus, 1758) and common/crowned shrew (*S. araneus/coronatus*)), trapping sessions were performed in each forest stand at the end of August and the beginning of September 2014. Each session consisted of four consecutive nights, in which traps (Trip-Trap live traps, Procter Brothers Ltd, Pantglas Industrial Estate, UK) were checked two to three times per night, starting from 21h, with an interval of two to three hours. Prior to the trapping session, traps were prebaited for two days. In the sampling plots, 49 traps were placed in a 7 x 7 grid, with 7 m distance in between the traps. We replaced the original plastic nest box of each trap with a larger wooden box (6 cm × 7 cm × 18 cm) and a wire-meshed opening at the bottom to reduce stress in the captured animals. A mixture of oat flakes, raisins and peanut butter was used as bait. Shrews are particularly vulnerable to accidental mortality during live-trapping, so each trap was supplied with meal worms to increase survival (Do et al. 2013). Captured rodents were identified to the species level, and released at the spot after being marked by clipping their fur. Shrews were immediately released without marking, to prevent mortality due to stress. We estimated relative rodent abundance in each stand using the mark-recapture data of the individuals following the approach described by Schnabel (1938) (e.g. Ryan 2011; Hager and Stewart 2013; Tack 2013). When the densities of captured rodents were too low to perform these calculations, we used the number of captured individuals in each sampling plot over all trapping sessions as our measure of relative rodent population density. As we did not mark the shrews, shrew population density was estimated using the number of captured

individuals. In one oak stand with a shrub layer in site P, the densities of small mammals are underestimated, since the traps were often accidently closed by foraging wild boar. Therefore, we eliminated this forest stand when investigating the relationships between host community composition and forest type or disease risk.

Resting sites

During sampling of ticks in 2014, we counted the number of resting sites of European roe deer in each sampling plot. Resting sites of roe deer could be detected as oval shaped patched of slightly scraped soil, often accompanied by other marks such as hoof prints, or depressions in the vegetation. We used the maximum amount of resting sites of the three sampling occasions for each forest stand as a proxy for the density of roe deer as performed by Bíró et al. (2006) and Tack et al. (2012).

Point counts

We estimated the density of common blackbird (*Turdus merula* Linnaeus, 1758), song thrush (*T. philomelos* Brehm 1831), Eurasian wren (*Troglodytes troglodytes* Linnaeus, 1758) and European robin (*Erithacus rubecula* Linnaeus, 1758) for each forest stand using point counts (Bibby et al. 1998). Each stand was visited three times in 2014 (beginning of April, end of April, middle of May), between sunrise and midday on dry days, and numbers of individual were counted for each detected bird species. We positioned ourselves in the middle of the sampling plot, waited for one minute to allow the birds to settle down after our arrival, and counted the number of individuals per species we heard within the limits of the sampling plot during eight minutes. Individuals were identified to species level based on their song. We distinguished between different individuals of the same species based on the location of the song. When we saw a bird flying off and moving to another position to sing, we took this into account to reduce the bias of overestimating the number of individuals. However, this was challenging in dense vegetation. We multiplied the number of individuals per forest stand per sampling occasion by two, to account for the females, since songs usually originate from males (Williams 2004). We then used the maximum amount of individuals in a sampling plot, over all sampling occasions, as a proxy of the density of a bird species per forest stand.

Feeding marks

The average density of Eurasian red squirrels in our study region varies from 0.1 squirrel per ha for small forest fragments, to 2.2 per ha in large forests, and their home range is approximately 2 to 5 ha large in pine forests and more than 10 ha in broadleaved forest (Verbeylen et al. 2003; Wauters et al. 2004). Since red squirrels occur in relatively low densities in Belgian forests and have large home ranges, it is not feasible to attempt to quantify densities in small forest stands. Therefore, we used data on presence or absence of squirrels as an approximation of squirrel density in our forest stands.

We recorded the presence/absence of red squirrels using feeding marks on hazelnuts and pine cones (Gurnell et al. 2001). We hung a small wooden tray in five trees in each forest stand and provided each tray with five hazelnuts in May 2015, and visited the forest stands again in October. Feeding marks on hazelnuts from squirrels could be distinguished from those of birds or other rodents such as mice, as squirrels tend to split hazelnuts in half, while on the top of one of the halves, marks from their lower incisors are often visible (Olsen 2013). Additional to the hazelnut feeding planks, remains of pine cones on the forest floor were investigated for feeding signs typical of squirrels (Olsen 2013). Feeding signs of squirrels on hazelnuts or pine cones in a forest stand indicated that at least one squirrel was present in that stand.

Footprint tunnels

Presence/absence of European hedgehogs was surveyed using footprint tunnels (Yarnell et al. 2014). In each tunnel (Mammal Society Hedgehog Tube Kit, Envisage Wildcare Ltd, UK) we put cat food in the middle, as bait, and placed ink pads on either side of the bait. Near the opening of the tunnel, at both ends, we placed a piece of white paper. Hedgehogs were attracted to the bait and, when visiting the tunnel, left footprints on the paper after returning from the middle of the tunnel to the opening. In May 2015, two tunnels were placed, approximately 50 m apart, in each forest stand, and were checked on five consecutive mornings. Bait was replaced if necessary and papers were replaced if they were damaged or if footprints of any animal were recorded. As the densities of hedgehogs in forests are generally low and rarely exceed 1 per ha (Huijser 1999; Young et al. 2006; Hubert et al. 2011), it is not feasible to attempt to quantify densities in small forest stands. We used presence-absence data rather than an approximation of relative hedgehog density.

Camera traps

Using camera traps with passive infrared (PIR) sensors, we assessed the presence of stone marten, fox and wild boar in the different forest stands. The PIR sensor responds to changes in infrared energy (heat) emitted by background temperature and a moving object, and triggers the camera if the difference exceeds a pre-set threshold (Rovero et al. 2013). Within each forest stand, one camera trap (Browning Recon Force, series BTC-2, Promotheus group LLC, USA) was deployed for 23 weeks, and moved to a new, random, location within the same sampling plot twice, to increase detection of host species. The camera was hung facing north and parallel to the ground on a tree at a height of 40 cm above ground level. At a distance of about two meters, we placed a stick of one meter length and embedded the top of the stick with a lure, consisting of a mixture of peanut butter and fish oil. If necessary, we pruned the vegetation in front of the camera (as far as 5 m, and 1 m broad) to optimize the detection of species and reduce triggering of the sensor by moving vegetation (Meek et al. 2014). When triggered, the camera took three 'rapid fire shots', and stayed inactive for 10 seconds before it could take a next set of pictures. Since martens and foxes occur in relatively low densities in Belgian forests and have large home ranges (Verkem et al. 2003), it is not feasible to attempt to quantify densities in small forest stands. Therefore, we used data on presence or absence of stone marten and red fox as an approximation of their density in our forest stands. In the stands where we recorded wild boar, we set the density of wild boar at 10 individuals, as this is the average group size as seen on the camera images during our sampling campaign.

Appendix 6.2

Comparison of alternate univariate generalized linear mixed effect models (*lmer*) for the effect of forest type on acarological risk measures (A) and host community measures (B). The models contain the random effect, i.e., forest site, and the fixed effect, i.e. forest type. Densities of animals used for the calculation of exponential Shannon Wiener diversity (eH) and potential dilution are estimated densities multiplied by the average larval burden for that particular species, or species group, in Europe. The potential dilution is calculated as the percentage of dilution hosts in the total host community, based on the densities of the hosts. ΔAICc represents the difference between the Akaike Information Criterion corrected for small sample sizes (AICc) for each model and the model with the lowest AICc and is 0 for the model that best explains the variation in the data. R^2m refers to the marginal R^2, i.e. the fraction of the variation explained by the fixed structure; R^2c refers to the conditional R^2, i.e. the fraction of the variation explained by the fixed and random structure. DOL: the density of larvae in 2013; DON: the density of nymphs in 2014; NIP: the nymphal infection prevalence in 2014; DIN: the density of infected nymphs in 2014.

A. Acarological risk

	DOL			DON		
	ΔAICc	**R^2m**	**R^2c**	**ΔAICc**	**R^2m**	**R^2c**
null model	5.84	0.00	0.00	0.00	0.00	0.00
forest type	0.00	0.61	0.61	4.41	0.32	0.32

	NIP			DIN		
	ΔAICc	**R^2m**	**R^2c**	**ΔAICc**	**R^2m**	**R^2c**
null model	0.25	0.00	0.09	0.00	0.00	0.05
forest type	0.00	0.48	0.49	8.60	0.15	0.15

B. Host community measures

	SR			eH			potential dilution		
	ΔAICc	**R^2m**	**R^2c**	**ΔAICc**	**R^2m**	**R^2c**	**ΔAICc**	**R^2m**	**R^2c**
null model	0.00	0.00	0.00	0.00	0.00	0.54	0.00	0.00	0.23
forest type	9.00	0.12	0.12	5.84	0.14	0.68	6.11	0.25	0.40

Appendix 6.3

The empirical densities of host species observed per forest stand. The densities are estimated using various techniques (see main text for more detail) and represent abundance per sampling plot per forest stand.

Site	Forest stand	Tree species	Shrub layer	Bank vole *(Myodes glareolus)*	Wood mouse *(Apodemus sylvaticus)*	Common/crowned shrew *(Sorex araneus/coronatus)*	Pygmy shrew *(Sorex minutus)*	Roe deer *(Capreolus capreolus)*	Wild boar *(Sus scrofa)*	Squirrel *(Sciurus vulgaris)*	Hedgehog *(Erinaceus europaeus)*	Fox *(Vulpes vulpes)*	Stone marten *(Martes foina)*	Wren *(Troglodytes troglodytes*	Robin *(Erithacus rubecula)*	Thrushes *(Turdus spp.)*
AH	**AH3**	**oak**	**no**	66.9	81.5	1	9	0	0	1	1	0	1	4	4	2
AH	**AH42**	**oak**	**yes**	152.6	65.2	5	4	3	0	0	0	1	1	6	4	6
AH	**AH10**	**pine**	**yes**	292.5	23.4	2	1	0	0	1	0	1	0	4	4	2
AH	**AH11**	**pine**	**yes**	104.0	71.5	0	6	0	0	1	0	1	0	4	4	6
AH	**AH17**	**pine**	**no**	42.4	20.0	3	0	3	0	1	0	1	1	4	4	0
AH	**AH18**	**pine**	**no**	2.0	29.6	0	1	11	0	0	0	0	1	4	4	4
AH	**AH19**	**pine**	**yes**	69.3	78.1	1	4	1	0	1	0	0	0	4	6	4
AH	**AH25**	**oak**	**no**	119.3	103.1	0	5	1	0	0	0	0	0	6	4	0
AH	**AH26**	**oak**	**no**	137.2	23.7	0	0	4	0	1	0	1	1	4	4	2
AH	**AH40**	**oak**	**yes**	79.0	45.7	0	0	1	0	1	0	1	1	4	2	2
P	**P4**	**oak**	**yes**	NA	NA	NA	NA	3	10	0	0	1	0	0	4	0
P	**P5**	**oak**	**yes**	37.3	29.9	11	5	0	10	1	0	1	0	2	4	2
P	**P6**	**oak**	**yes**	23.3	35.0	3	4	2	0	1	1	1	1	4	2	2
P	**P7**	**pine**	**no**	53.7	17.1	1	14	0	10	1	0	1	0	4	4	0
P	**P8**	**pine**	**no**	10.0	2.0	6	19	0	10	0	1	1	1	2	4	0
P	**P9**	**pine**	**no**	13.0	16.6	11	41	0	10	1	0	1	1	2	4	0
P	**P10**	**pine**	**yes**	1.0	0.0	2	11	0	10	0	0	0	0	4	2	0
P	**P11**	**pine**	**yes**	16.0	67.0	6	8	0	10	1	0	0	0	6	4	4
P	**P12**	**pine**	**yes**	51.3	35.2	5	2	1	10	1	0	0	1	2	4	6

Appendix 6.4

Comparison of alternate univariate generalized linear mixed effect models (*lmer*) for the effect of host community measures on acarological risk measures. The models contain the random effect, i.e., forest site, and the fixed effect, i.e. host community measure. Densities of animals used for the calculation of exponential Shannon Wiener diversity (eH) and potential dilution are estimated densities multiplied by the average larval burden for that particular species, or species group, in Europe. The potential dilution is calculated as the percentage of dilution hosts in the total host community, based on the densities of the hosts. ΔAICc represents the difference between the Akaike Information Criterion corrected for small sample sizes (AICc) for each model and the model with the lowest AICc and is 0 for the model that best explains the variation in the data. R^2m refers to the marginal R^2, i.e. the fraction of the variation explained by the fixed structure; R^2c refers to the conditional R^2, i.e. the fraction of the variation explained by the fixed and random structure. DOL: the density of larvae in 2013; DON: the density of nymphs in 2014; NIP: the nymphal infection prevalence in 2014; DIN: the density of infected nymphs in 2014.

	DOL			**DON**		
	ΔAICc	**R^2m**	**R^2c**	**ΔAICc**	**R^2m**	**R^2c**
null model	0.00	0.00	0.00	0.00	0.00	0.00
SR	3.16	0.01	0.01	1.40	0.10	0.10

	NIP			**DIN**		
	ΔAICc	**R^2m**	**R^2c**	**ΔAICc**	**R^2m**	**R^2c**
null model	0.00	0.00	0.09	0.00	0.00	0.05
SR	2.91	0.02	0.09	1.34	0.10	0.12

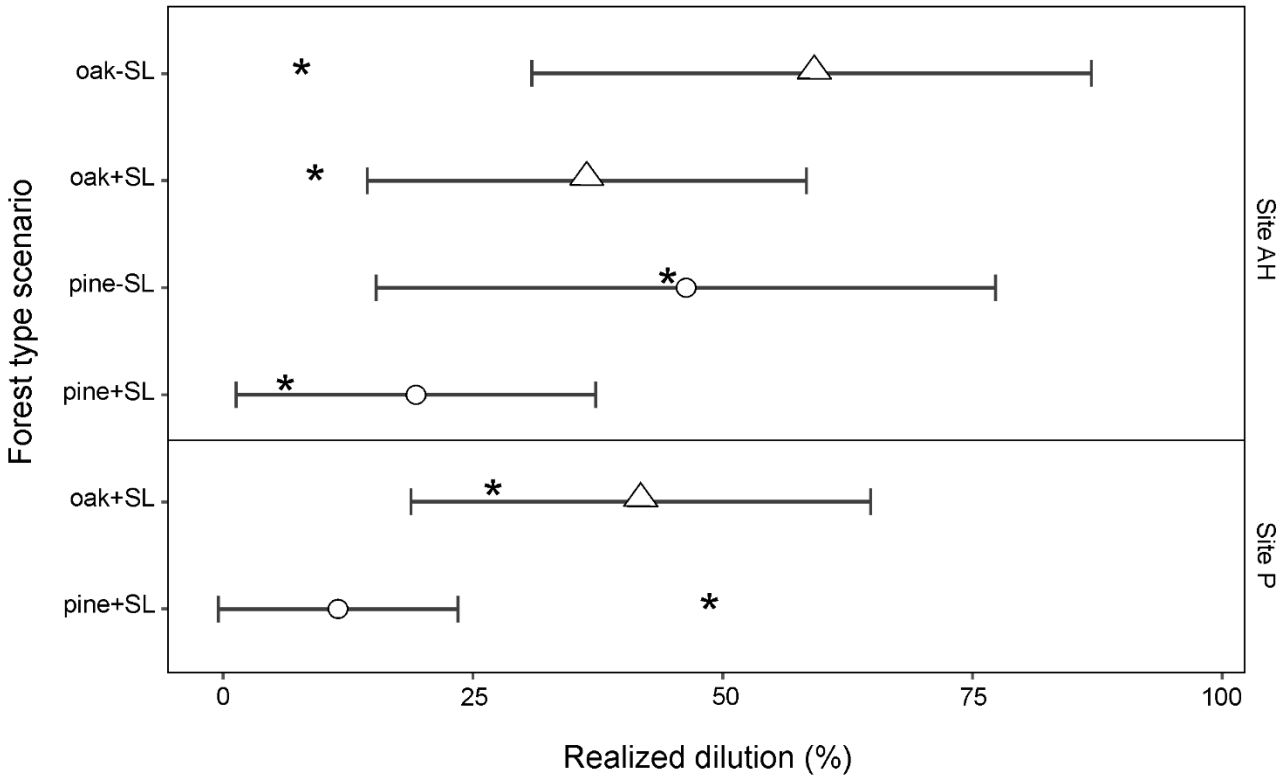
	DOL			**DON**		
	ΔAICc	**R^2m**	**R^2c**	**ΔAICc**	**R^2m**	**R^2c**
null model	0.00	0.00	0.00	0.00	0.00	0.00
eH	2.88	0.03	0.03	2.55	0.05	0.05

	NIP			**DIN**		
	ΔAICc	**R^2m**	**R^2c**	**ΔAICc**	**R^2m**	**R^2c**
null model	0.00	0.00	0.14	0.00	0.00	0.07
eH	2.43	0.10	0.10	0.54	0.16	0.16

Appendix 6.4 (continued)

	DOL			DON		
	ΔAICc	**R^2m**	**R^2c**	**ΔAICc**	**R^2m**	**R^2c**
null model	0.00	0.00	0.00	0.00	0.00	0.00
potential dilution	1.38	0.11	0.11	3.24	0.01	0.01

	NIP			DIN		
	ΔAICc	**R^2m**	**R^2c**	**ΔAICc**	**R^2m**	**R^2c**
null model	0.00	0.00	0.14	0.00	0.00	0.07
potential dilution	2.36	0.10	0.10	3.27	0.01	0.03



Appendix 6.5

The mean (± SD) realized dilution (%) for the six scenarios of forest types, given the average density of larvae (DOL), nymphs (DON) and infected nymphs (DIN), averaged over all forest stands from that scenario. The different scenarios include pine and oak stands, with a shrub layer (+SL) or without a shrub layer (-SL) in site AH and in site P. The triangles represent the mean realized dilution of oak stands; the circles represent the mean realized dilution of pine stands. The potential dilution in each scenario is depicted with an asterisk.


8℃ 04/18/2015 10:57AM AH40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7 General discussion and conclusions

A large body of evidence demonstrates the positive effects on human well-being of getting in contact with nature (MEA 2005; Luck et al. 2011; Cardinale et al. 2012; Carrus et al. 2015). People have a need to recreate in natural areas such as forests and (sub)urban green spaces (Hansmann et al. 2007; Keniger et al. 2013; Wolch et al. 2014). Although interacting with nature in green environments delivers a range of measurable benefits for human health and well-being (Keniger et al. 2013), it brings people in close contact with ticks and tick-borne pathogens.

Tick-borne diseases are a growing public health concern globally as their incidence is rising (Dantas-Torres et al. 2012). However, the spatiotemporal dynamics of the hazard, namely questing ticks infected with zoonotic pathogens such as the Lyme borreliosis bacteria, remain largely unclear. Studying the ecology of Lyme borreliosis and the spatial and temporal patterns of tick density, host community composition and the prevalence of tick-borne pathogens is therefore becoming increasingly important (Gray et al. 2009; Randolph 2010; Estrada-Peña et al. 2011). We focused on pathogens causing Lyme borreliosis, but we also investigated the transmission dynamics of other common tick-borne pathogens. Several tick-borne pathogens cycle between the same vector and vertebrate host species. *Ixodes ricinus*, the most common tick species that readily bites humans in Europe, acts as a vector for many human pathogens such as the Lyme borreliosis genospecies (e.g. *Borrelia afzelii*), as well as *Borrelia miyamotoi*, *Anaplasma phagocytophilum*, "*Candidatus* Neoehrlichia mikurensis", *Rickettsia helvetica* and the tick-borne encephalitis virus (Randolph 2001; Coipan et al. 2013b). These pathogens can cause similar non-characteristic viral-like symptoms in human patients, which makes proper diagnosis challenging (Jahfari et al. 2016). Furthermore, many of these pathogens, alone or together, can be transmitted to ticks by the same host species, such as e.g. small rodents (Coipan 2016). With this PhD thesis, we aimed to fill gaps in the knowledge concerning Lyme borreliosis ecology and tested the dilution effect hypothesis, which claims that an increase in host diversity decreases Lyme borreliosis risk, in a European context.

More specifically, we first investigated the spatiotemporal variation in the density of *I. ricinus* infected with *Borrelia burgdorferi* s.l. (‘*Borrelia*’) and the prevalence of the different *Borrelia* genospecies in different forest types (Chapters 2 and 3). In the second part of this thesis, we assessed the role of two poorly studied host species in the transmission cycle of Lyme borreliosis and other tick-borne diseases (Chapters 4 and 5). Afterwards, we combined the acquired knowledge from the previous two parts with data on the host community composition in the different forest types to test the dilution effect hypothesis (Chapter 6).

7.1 Host community composition and Lyme borreliosis risk

7.1.1 Spatiotemporal dynamics in Lyme borreliosis risk in different forest types in the Kempen

In the first part of the thesis (Chapters 2 and 3), we studied forest stands that represent the different stages in the process of forest conversion in the Kempen (northern Belgium) - from pine (*Pinus* spp.) stands without a substantial shrub layer to oak (*Quercus* spp.) stands with a shrub layer. We showed that Lyme borreliosis risk was significantly affected by forest type. The density of nymphs was higher in the oak than in the pine stands. The higher tick densities in oak stands can be related to the response of ticks and their hosts to the specific biotic and abiotic conditions in the different forest stands, which are influenced by the dominant tree species and assumed to be more favourable for ticks and their hosts under oak (Gray et al. 1998). We found that the nymphal infection prevalence of *B. afzelii*, the most common Lyme borreliosis genospecies in patients in Western Europe (Jahfari et al. 2017b), was higher in the pine stands while the diversity of *Borrelia* genospecies was higher in the oak stands. Infected nymphs tended to harbour *B. afzelii* more often in pine stands, while *B. garinii* and *B. burgdorferi* ss. infection appeared to be more prevalent in oak stands. Because of the specific associations between hosts and *Borrelia* genospecies (Kurtenbach et al. 2002), the higher prevalence of *B. afzelii* in pine stands seems to reflect a difference in host use by ticks in the different forest types. In pine stands, larvae seem to feed more often on small rodents, which transmit *B. afzelii*. In oak stands, the larvae seem to feed more on other types of hosts, such as birds, which transmit *B. garinii* and *B. valaisiana* (Humair et al. 1999; Hanincová et al. 2003a; Hanincová et al. 2003b; Heylen et al. 2014).

The density of nymphs fluctuated from year to year but did not increase from 2009 to 2014. As the prevalence of *B. afzelii* in the studied nymphs did not show any temporal variation, the density of infected nymphs in a certain year, and thus the Lyme borreliosis risk, will resemble the density of nymphs in that year. The density of nymphs, therefore, can be used to predict the disease risk. The observed absence of an increase in nymph densities is in accordance with the reported stable incidence of tick bites and Lyme borreliosis in Belgium (Vanthomme et al. 2012; Bleyenheuft et al. 2015).

7.1.2 The role of hedgehogs and squirrels in the transmission of tick-borne pathogens

In the second part of the thesis, we investigated the role of two poorly studied tick hosts in the enzootic cycle of Lyme borreliosis and other tick-borne diseases: the European hedgehog (*Erinaceus europaeus* Linnaeus, 1758; Chapter 4) and the Eurasian red squirrel (*Sciurus vulgaris* Linnaeus, 1758; Chapter 5). We collected ticks from hedgehogs from (sub)urban areas and found that most hedgehogs carried only few ticks, while a few hedgehogs harboured many. Hedgehogs carried all three life stages of *I. hexagonus* and *I. ricinus*, but *I. hexagonus* was the most common tick species feeding on hedgehogs. Even though hedgehogs can feed all life stages of *I. ricinus* and can thus theoretically maintain the tick's life cycle as the sole host species, hedgehog densities in forested areas, the preferred habitat of this generalist tick species, are probably too low to actually allow ticks to complete their entire life cycle on hedgehogs. We analysed 1203 ticks (*I. ricinus* as well as *I. hexagonus*) from 54 hedgehogs and detected *Borrelia miyamotoi*, *B. afzelii*, *B. bavariensis*, *B. spielmanii, Anaplasma phagocytophilum* and *Rickettsia helvetica* in both tick species. *Borrelia afzelii*, *B. bavariensis*, *B. spielmanii, A. phagocytophilum* and *R. helvetica* were found significantly more in ticks collected from hedgehogs than in questing *I. ricinus* from a suburban forest in the same region (Heylen et al. 2016), which suggests that hedgehogs contribute to the enzootic cycles of these pathogens.

In the tissue samples (spleen and liver) of 45 dead Eurasian red squirrels, we found *B. afzelii*, as already proposed by previous studies, as well as *B. miyamotoi* and *Anaplasma phagocytophilum*. We could not confirm the previously observed association between squirrels and *B. burgdorferi* s.s. (Pisanu et al. 2014). Our results demonstrate the epidemiological importance of red squirrel in (sub)urban areas, seeing that the squirrels can harbour similar tick-borne pathogens as mice and voles.

In forests, squirrels and hedgehogs probably do not play a major role in the transmission cycles of tick-borne pathogens because of their low density. In (sub)urban areas, however, they may play a major role in the disease risk due to their higher abundances (see e.g. Hubert et al. 2011; Rézouki et al. 2014). Humans are thus likely to encounter ticks infected with one or several pathogens while gardening or recreating in parks (Mulder et al. 2013).

7.1.3 A dilution effect in European forests?

The Bayesian belief network model that we employed in Chapter 6 to elucidate the relationship between the host community and Lyme borreliosis risk showed that a decrease in the number of larvae that feed on competent host species, i.e. the realized dilution, can - in theory - decrease the density of infected nymphs and the subsequent disease risk. According to the dilution effect hypothesis, such an increase in realized dilution and a decrease in the density of infected nymphs are caused by an increase in host diversity. In our study sites, however, we did not find a relationship between the host diversity and the density of infected nymphs. Therefore, we could not confirm the dilution effect hypothesis.

There was no difference in host diversity between the studied forest types, but the density of small transmission-competent rodents was lowest in the pine stands without shrub layer. As the densities of the other host species in those pine stands without shrub layer were similar to the other forest types, larvae were more likely to feed on dilution hosts in the pine forests without shrub layer, which leads to a high potential dilution in these stands. Yet, the prevalence of the rodent-associated *B. afzelii* was higher in nymphs from pine stands than in nymphs from oak stands (cf. 7.1.1 and Chapters 2 and 3), and the model demonstrated that larvae more often fed on small rodents than on dilution hosts in pine stands. Seeing the higher rodent densities in the oak forests and similar densities of dilution hosts, the realized dilution was higher in oak stands than in pine stands.

The realized host use by larval ticks does not only depend on the availability and suitability of host species, but also on the tick-host contact rate (Randolph and Craine 1995; Levi et al. 2016). In habitats with comparable host communities but with a different composition and structure of the vegetation and a different microclimate, contact between ticks and hosts can differ because of differences in the behaviour of hosts or questing ticks (Craine et al. 1995; Randolph and Craine 1995; Randolph and Storey 1999). Furthermore, host species

other than the ones included in our model (i.e. wood mouse, bank vole, squirrel and hedgehog) could be able to transmit *B. afzelii* to feeding larvae.

The dilution effect hypothesis for *Borrelia*, as described by Ostfeld and Keesing (2000), did not apply to our study forests, and possibly neither to other similar forest communities in Europe. Because of the multiple *Borrelia* genospecies in Europe and the host-genospecies associations, the interactions between *Borrelia*, ticks and hosts appear to be much more complex in Europe than in North America. An increase in host diversity can increase or decrease the prevalence of individual genospecies, depending on the response of the associated host species. If a dilution effect of increased host diversity is to occur in Europe, it will most likely occur at the level of specific *Borrelia* genospecies, rather than on the overall prevalence of *Borrelia*. Hence, we strongly emphasize to the need to consider the different *Borrelia* genospecies as distinct pathogens in studies on the impact of host community composition on Lyme borreliosis risk.

7.2 Recommendations for (forest) management

In our study region, the effects of forest conversion and a higher host diversity on the density of infected nymphs are probably small. However, forest management can play a role in reducing Lyme borreliosis risk.

Since there is a low probability of a dilution effect in our forest sites, increasing the host diversity (e.g. through changing the composition and structure of forests) in order to lower the density of infected ticks is not an efficient management strategy. Moreover, increasing host diversity can even increase tick-borne disease risk, because different host species are associated with different tick-borne pathogens. An increase in host diversity could thus increase Lyme borreliosis risk by increasing the prevalence of *Borrelia* genospecies that give rise to more severe clinical manifestations than *B. afzelii*, which is associated with skin manifestations. The bird-related *B. garinii*, for example, can cause neuroborreliosis (Balmelli and Piffaretti 1995). The most important variables in explaining variation in the density of infected nymphs were related to the density of larvae and nymphs, rather than to the infection prevalence of *Borrelia* or *B. afzelii*. This confirms that the density of nymphs is a good predictor of disease risk, as suggested before (Jaenson et al. 2009; Coipan et al. 2013b). The homogeneous pine forests harboured lower densities of ticks than the more natural, mixed forests dominated by oak trees. However, it is certainly not advisable to

reverse the ongoing forest conversion towards more species-rich and structurally diverse forests as a disease prevention measure. Mixed forests are better able to deliver a whole suite of ecosystem services such as biodiversity conservation, prevention of soil degradation, watershed protection and recreation (van der Plas et al. 2016). Smart management of (converted) forests involves planning and conducting forest management in a fully integrated manner through which a maximum number of synergies between multiple ecosystem services, such as economic and commercial purposes, human health, recreation and regulating functions, are strived for (Führer 2000). Such integrated smart management may thus provide the most effective measure to optimize multiple ecosystem services, including human health and well-being. The results from this thesis could be extrapolated to other forest types, if taken into account the biotic (hosts) and abiotic conditions (temperature, humidity) that determine tick density. However, the interaction between the vegetation and the host community composition appears to determine the prevalence of *Borrelia* genospecies in the ticks, which influences the severity of Lyme borreliosis in humans. More research is needed to elucidate the exact impact of vegetation on the host use by ticks.

Verheyen and Ruyts (2016) explained how smart management of forests can effectively decrease Lyme borreliosis risk. The risk for human exposure to Lyme borreliosis not only depends on the density of infected ticks (acarological risk) but also on the human-tick contact rate (Jaenson et al. 2009). Therefore, Verheyen and Ruyts (2016) defined Lyme borreliosis risk as the probability of a person making contact with at least one host-seeking tick infected with *Borrelia* along a 100 m forest trail. At low densities of infected ticks (smaller than two per 100 m^2), a reduction in the density of infected ticks can lower the disease risk (Fig. 7.1). For higher densities of infected ticks (above two per 100 m^2), the Lyme borreliosis risk can only be substantially decreased by decreasing the human-tick contact rate (Fig. 7.1). The mean density of infected nymphs in the forest stands studied in this thesis (cf. Chapter 2) was 5.9 per 100 m^2. Therefore, for our study region, decreasing the human-tick contact rate is a more effective, and probably also less expensive, management strategy than decreasing the density of infected ticks.

The human-tick contact rate in a forest depends on the number of visitors and the probability of making contact with questing ticks (Verheyen and Ruyts 2016). As recreation and education are important ecosystem services of forests, forest management should not

aspire to decrease the amount of people visiting a forest site. Guiding the visitor flow, on the other hand, can be a useful management action. Effective measures to direct the visitors are, for example, points of attraction such as parking lots and bird observation towers, marked out routes that visitors have to follow and the presence of well-maintained trails versus trails of much poorer quality (van Marwijk et al. 2010).

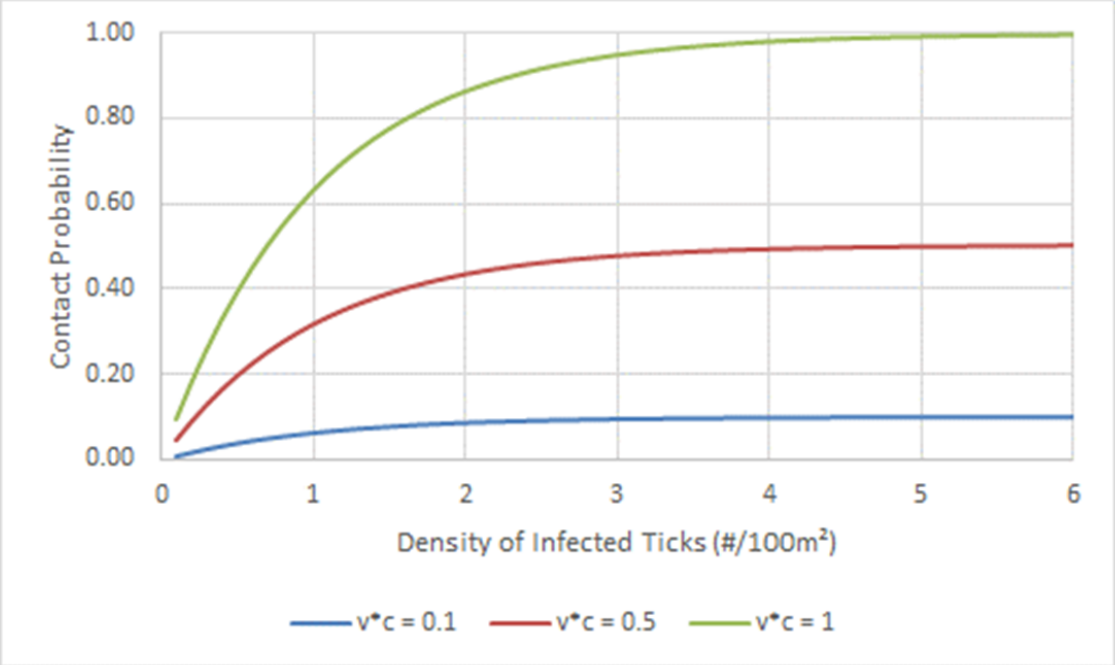

Fig. 7.1 The contact probability with at least one infected tick along a 100 m forest trail section (Y-axis) as a function of different densities of infected ticks (X-axis) and for different combined visitor passage and contact probabilities (coloured lines). The probability of a person making contact with at least one *Borrelia*-infected tick along a 100 m forest trail is calculated as (v*c)*[1 – exp(-p*DT)]. v: the probability of at least one visitor passage per hour; c: the contact probability with questing ticks; p: the prevalence of *Borrelia*; DT: the density of *Ixodes ricinus* nymphs and adults along a 1m wide and 100 m long forest trail section. Figure taken from Verheyen and Ruyts (2016).

The greatest numbers of questing nymphal and adult ticks can be found at 50 to 70 cm above ground level (Mejlon and Jaenson 1997). The probability of contact with questing (infected) ticks when walking on a forest trail is thus strongly related to the height of the vegetation along the trail. When vegetation is kept low, or when paths are wide, the risk of a tick bite is expected to be limited (Verheyen and Ruyts 2016). Providing information and education for the public can be an effective tool to reduce the human-tick contact rate and the probability of Lyme borreliosis after a tick bite. The knowledge on how to recognize ticks and efficiently remove them from the body, for example, can be provided on information panels, on a website or via a mobile app (Beaujean and Sprong 2016; Eisen and Gray 2016). There is seasonal variation in the density of (infected) nymphs, with a peak in late spring – early summer (Tälleklint and Jaenson 1996a; Perret et al. 2000; Randolph et al. 2002), which coincides with a peak in the number of tick bites in Belgium (TekenNet: tekennet.wiv-isp.be). In this period, people often take advantage of the good weather to recreate in forests. Therefore, Lyme borreliosis prevention campaigns could be aimed predominantly at this period. The seasonal pattern of tick activity and *Borrelia* prevalence in Belgium, however, has not yet been thoroughly investigated.

In a Dutch study, most people (43%) were bitten by ticks in the forest, but an unexpected large number of people (31%) reported tick bites from their gardens (Mulder et al. 2013). Since humans can encounter (infected) ticks not only in forests, but also in (sub)urban green areas, management action should be taken in these environments as well. The above-mentioned management actions for forests (mowing paths, giding visitors) can be applied to city parks as well, but are often not possible in gardens and smaller green areas. Providing information will especially be an important tool, because the public is often not aware of the possibility tick bites in (sub)urban areas.

7.2.1 A one Health approach

The results presented in this thesis are relevant for human health issues in that we show that the risk for Lyme borreliosis can be predicted by the density of nymphs. The density of infected nymphs is a well-established disease measure, and it is reasonable that this variable will predict the risk adequately. However, molecular tests to identify infection in ticks are very resource demanding: they are expensive, labour intensive and time consuming. Collecting ticks, on the contrary, is less expensive and does not require a lot of time. Therefore, monitoring the density of ticks (nymphs) is a valuable method to predict Lyme

borreliosis risk. Since we show in Chapter 3 that the density of nymphs fluctuates from year to year, such monitoring campaigns should best be done each year, to predict the risk more accurately. We did not examine seasonal variation, which is also an important aspect to investigate (Randolph et al. 2002). Moreover, in this thesis, we emphasize the relationship between vegetation features and tick densities, as in previous studies. Since we suggest that the contact rate between humans and ticks is the most important variable determining human health risk, the results we provide can be used to reduce risk, or human-tick contact, in high-risk areas (such as mixed oak forests with a substantial shrub layer) as discussed above.

Tick-borne diseases may be difficult to control due to their complex epidemiology. An integrated, interdisciplinary approach for the management of Lyme borreliosis (a one Health approach, see Dantas-Torres et al. (2012)) requires an increased communication between different fields such as ecology, zoology, epidemiology, molecular biology, medical and veterinary sciences, climatological and social studies and forestry.

The results from this thesis can be integrated in a one Health framework, for example by improving communication between ecologists and physicians. It is crucial to not only inform the public about Lyme borreliosis ecology through prevention campaigns but also to provide physicians with the latest research results. Diagnosis of Lyme borreliosis, or other tick-borne infectious diseases, if often challenging (Stanek et al. 2011). However, proper diagnosis can be increased, for example, by asking the patient not only if they visited forests (and if so, which type of forests) but also if they visited city parks or often work in their garden, or if they have a pet. The role of domestic animals and some wildlife species in the Lyme borreliosis enzootic cycle has yet to be determined. Certainly the role of the different host species in (sub)urban areas is an aspect that requires further attention. For example, dogs or cats could carry infected ticks closer to humans, or even act as reservoir hosts for *Borrelia* genospecies and infect uninfected ticks. This aspect requires the combined knowledge of ecologists, veterinarians and physicians: a veterinarian that finds an (infected) tick on a dog may alert the pet's owner to seek medical assistance when he feels ill.

Mapping Lyme borreliosis risk is an important tool in disease prevention and management. The model that we present in Chapter 6 is not suitable to directly estimate health risk and tick-borne disease incidence, because of the variables that we used to build the model.

Instead, you can perfectly integrate a BBN based on the model and the results we present in this thesis in a one Health framework. For example, variables that can predict human-tick contact rate in a forest such as population density, recreation opportunities in a forest and number of visitors can be added to the model. With such a model and GIS tools, a map can be build that can assess Lyme borreliosis risk in any forest type in Flanders/Belgium. Predictions would still be uncertain to some extent, but BBN can take this into account.

7.3 Suggestions for further research

The findings in this thesis have contributed to the current knowledge concerning Lyme borreliosis ecology in a European setting. Nevertheless, several issues remain to be addressed in further research.

We demonstrated that forest composition and structure influence the density of nymphs and the nymphal infection prevalence of *Borrelia* genospecies, and that the effect of tree species on the density of nymphs is consistent through time. However, the time series used in this thesis only consists of four years, interrupted by two years. A longer, uninterrupted investigation of tick densities and the prevalence of tick-borne pathogens can more thoroughly elucidate the spatiotemporal patterns in tick-borne disease risk. Moreover, research investigating the within-year variation in the density of (infected) ticks in Belgium is lacking and is an important aspect in predicting Lyme borreliosis risk. In addition, as the densities of different host species can affect the densities of ticks and the prevalence of the different *Borrelia* genospecies, it is essential to quantify the exact host community composition. The prevailing weather conditions and the seed crop of tree species in the studied years can influence host densities and should also be taken into account.

We found that hedgehogs and squirrels can contribute to the transmission cycle of some common tick-borne pathogens, including the Lyme borreliosis pathogen *B. afzelii*. The exact role of these hosts in the human health risk remains unclear. Further research is necessary to elucidate the interaction between host density, tick burden and tick infection prevalence and to assess the precise role of hedgehogs and squirrels in the enzootic cycle of the various tick-borne human pathogens and the associated human health risk in forests as well as in (sub)urban areas. The role of some other widespread host species in the maintenance of ticks and the transmission of pathogens, in forests and (sub)urban areas, is also unclear. For example, data on the tick burden and the reservoir competence for many

common tick-borne pathogens of the common/crowned shrew complex (*Sorex araneus/coronatus*), pygmy shrew (*S. minutus* Linnaeus, 1758), stone marten (*Martes foina* Erxleben, 1777), red fox (*Vulpes vulpes* Linnaeus, 1758) and wild boar (*Sus scrofa* Linnaeus, 1758) are scarcely available (Hofmeester et al. 2016). We also showed that, besides the pathogens causing Lyme borreliosis, other pathogens such as *B. miyamotoi*, *A. phagocytophilum* and *R. helvetica*, which cause similar disease symptoms and may be transmitted together (Jahfari et al. 2016), share the same enzootic cycles including the same tick and host species. Our data did not allow us to fully investigate the spatiotemporal dynamics of all the observed tick-borne pathogens or of all *Borrelia* genospecies. We therefore stress the need to identify the different components of the enzootic cycle of the different common tick-borne diseases and shed more light on the mechanisms of their transmission cycles.

There are some limitations attached to the molecular methods we used in this thesis to detect the presence of *Borrelia* genospecies and other pathogens. First, in this thesis, we did not the variation within *Borrelia* genospecies, but only compared different genospecies. In a broader genospecies community, the different *Borrelia* genospecies will possibly display higher within-genospecies variation (Begon et al. 2006). At this moment, we have insufficient knowledge in how far the genetic diversity within a *Borrelia* genospecies correlates with the population size of the genospecies. Second, because of the difference in sensitivity between the qPCR and the conventional PCR that we used to detect presence of *Borrelia* and identify *Borrelia* genospecies, respectively, not all *Borrelia*-positive samples could be assigned to a genospecies. Further research could design and use a more sensitive test that would assign all *Borrelia*-positive samples to a genospecies. Third, we did not determine infection intensity of ticks. The amount of propagules of a pathogen per tick (or host) could affect the infectivity and virulence of the tick (see e.g. Råberg (2012)) which may influence transmission dynamics of the pathogen and human health risk. Moreover, co-infection of pathogens in a tick could influence the virulence of the tick or host (Brown et al. 2002). These aspects of the transmission pathogens between ticks and hosts have not been addressed in this thesis, but should be investigated in further research.

A limitation of our study on the effect of the host community on Lyme borreliosis risk is the relatively small size of the investigated forest stands. Forest stands of approximately 1 ha are adequate to study spatiotemporal patterns in the populations dynamics of mice and

voles, which are important feeding hosts for larval ticks (Hofmeester et al. 2016). Larger host species, such as foxes or martens, have a larger home range and may cross different habitats while ranging. To study spatiotemporal dynamics of such hosts and to assess their effect on the spatiotemporal dynamics of Lyme borreliosis risk, larger study sites should be considered. In addition, we showed that the habitat type can also influence Lyme borreliosis risk by influencing the host use by ticks. The effect of different habitat types on tick behaviour, host behaviour and the occurrence of the dilution effect has not received much attention so far, but deserves to be more thoroughly investigated. Moreover, we did not investigate how different herb layer compositions influence tick abundance and the prevalence of *Borrelia* genospecies in tick. Further research on the dilution effect in Europe should therefore take into account not only the host community composition, but also the effect of vegetation features, herb layer composition and microclimate on the larval burden on hosts, in order to elucidate the relationships between potential and realized dilution. Besides looking at the density of (infected) ticks and *Borrelia* genospecies prevalence between forest types and different vegetation features, the surveillance of Lyme borreliosis risk, or the risk for other tick-borne diseases, should compare sites at a larger scale such as regions, provinces and countries. This has not been addressed in this thesis but could be integrated by using tools such as TekenNet, which collects data of tick bites and subsequent medical observation from Belgium (tekennet.wiv-isp.be).

The greater part of Lyme borreliosis research has focused on the Lyme borreliosis risk in forests. However, we showed that also (sub)urban green spaces can pose a human health risk. While most tick bites reported in a Dutch survey occurred in forests (43 %), almost one third of the tick bites were reported from gardens (Mulder et al. 2013). Moreover, hedgehogs and squirrels, two host species that carry multiple human pathogens, are well adapted to urbanization and often reach higher densities in cities than in forests (e.g. see Hubert et al. 2011; Rézouki et al. 2014). Therefore, we emphasize the need to study Lyme borreliosis risk in (sub)urban green areas and investigate the contribution of urban-dwelling hosts to the transmission cycle of tick-borne pathogens and the maintenance of a tick's life cycle.


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Summary

Tick-borne diseases are a growing public health concern globally as their incidence is rising. However, the spatiotemporal dynamics of questing ticks infected with human pathogens such as the Lyme borreliosis bacteria, remain largely unclear. We investigated the transmission dynamics of common tick-borne pathogens, focusing mainly on *Borrelia burgdorferi* sensu lato (‘*Borrelia’*) and the impact of ecological interactions between ticks, hosts and forest types on Lyme borreliosis risk. Forest composition and structure have been shown to affect the density of ticks, but their impact on the prevalence of *Borrelia* in the ticks has not been investigated so far. The study of the impact of forest type on Lyme borreliosis risk is particularly important in the context of the ongoing conversion of homogeneous coniferous forests to more natural, mixed forests dominated by indigenous broadleaved trees in many regions in Europe.

According to the dilution effect hypothesis, postulated in North America, a forest with a high diversity of hosts for ticks contains more hosts that are poorly capable of transmitting *Borrelia* to ticks compared to a forest with a species-poor host community, consisting mostly of transmission-competent host species. This hypothesis has not yet been tested in Europe, where different types of hosts transmit different *Borrelia* genospecies.

In the Kempen, northern Belgium, we studied forest stands of four forest types: oak or pine stands, with or without a substantial shrub layer. These forest types are representative for the different stages in the process of forest conversion. In this part of the study, we focused on *Borrelia afzelii*, the most common *Borrelia* genospecies in Lyme borreliosis patients in Western Europe and transmitted to ticks by small rodents. We found that the density of *Ixodes ricinus* ticks or the infection prevalence of *Borrelia* in ticks from our study sites did not increase from 2009 to 2014, similar to the reported stable incidence of Lyme borreliosis and tick bites in Belgium. The density of ticks, rather than the infection prevalence of *B. afzelii*, was more important in explaining variation in the density of infected ticks and can thus be used as a predictor of disease risk. The density of ticks was higher in oak stands than in pine stands, but the prevalence of *B. afzelii* was highest in pine stands. We could not confirm the dilution effect hypothesis; the density of infected ticks, a commonly used

risk measure in the literature, was not correlated with host diversity, and the host diversity did not differ between the forest types. Our results indicate differential host use by ticks in different habitats, with larvae feeding more often on small rodents in pine stands and more on other types of hosts, such as birds, which transmit genospecies other than *B. afzelii*, in oak stands.

Besides in forests, the favourable habitat of *I. ricinus*, humans are also exposed to ticks and human pathogens in (sub)urban green spaces. We found many common human pathogens, such as *Borrelia* genospecies, *Borrelia miyamotoi* and *Anaplasma phagocytophilum*, in squirrels and ticks from hedgehogs collected in urban settings. Hence, humans are likely to encounter ticks infected with one or several pathogens while gardening or recreating in parks.

We conclude that focusing on the dilution effect to reduce Lyme borreliosis risk is not an effective management option in our study region, and possibly in other European regions with similar forest communities. Instead of decreasing Lyme borreliosis risk, adding host species to the host community can even increase disease risk, by increasing the prevalence of *Borrelia* genospecies that give rise to clinical manifestations of Lyme borreliosis such as neuroborreliosis that are more severe compared to skin manifestations (mainly caused by *B. afzelii*). Lyme borreliosis prevention should therefore aim to reduce tick densities and the contact rate between ticks and humans. The risk for human exposure to Lyme borreliosis not only depends on the density of infected ticks but importantly also on the human-tick contact rate. Our results suggest that decreasing the density of (infected) ticks will rarely lead to a substantially lower Lyme borreliosis risk. Forest management can decrease the human-tick contact rate and the subsequent Lyme borreliosis risk by directing visitor flows, e.g. along points of attraction or marked-out routes, and by mowing the vegetation along trails.

Samenvatting

De toenemende incidentie van ziekten overgedragen door teken is - wereldwijd - een bron van zorgen en er is nog maar weinig kennis over en inzicht in de ruimtelijke en temporele dynamieken in de densiteit van geïnfecteerde teken. Daarom onderzochten wij de transmissiedynamieken van veel voorkomende pathogenen overgedragen door teken, met speciale aandacht voor *Borrelia burgdorferi* sensu lato (‘*Borrelia’*), en de impact van de ecologische interacties tussen teken, hun gastheren en bostypes op het risico op de ziekte van Lyme. We weten dat de samenstelling en structuur van bossen een invloed hebben op de densiteit van teken, maar de impact van verschillende bostypes op de infectiegraad van teken met *Borrelia* was tot dusver niet onderzocht. In verschillende regio’s in Europa worden homogene naaldbossen omgevormd naar meer natuurlijke, gemengde bossen gedomineerd door inheemse loofboomsoorten. In het kader van deze bosomvorming is onderzoek naar de impact van bostype op het risico op de ziekte van Lyme erg belangrijk.

De ‘dilutiehypothese’ werd beschreven in Noord-Amerika en stelt dat een bos met een hoge diversiteit aan tekengastheren meer gastheren bevat die inefficiënt zijn in het overdragen van *Borrelia* naar teken (‘dilutiegastheren’) in vergelijking met een soortenarme gastheergemeenschap, die voornamelijk bestaat uit transmissie-competente gastheren. Deze hypothese werd nog niet eerder getest in Europa, waar de verschillende soorten gastheren geassocieerd zijn met verschillende genotypes van *Borrelia*.

We bestudeerden vier bostypes die verschillende stadia in het proces van bosomvorming vertegenwoordigen: eiken- of dennenbestanden met of zonder goedontwikkelde struiklaag, in de Kempen (Noord-België). We focusten op *Borrelia afzelii*, het meest voorkomende *Borrelia*-genotype in patiënten met de ziekte van Lyme in West-Europa en overgedragen door kleine knaagdieren zoals muizen. We zagen geen verandering in de densiteit van teken en de infectiegraad met *Borrelia* tussen 2009 en 2014, wat overeenkomt met de stabiele incidentie van tekenbeten en de ziekte van Lyme in België in de laatste decennia. De variatie in densiteit van geïnfecteerde teken in de bestudeerde bossen werd voornamelijk beïnvloed door de variatie in tekendensiteit, niet door de variatie in infectiegraad. We

kunnen het risico op de ziekte van Lyme hier dus voorspellen aan de hand van de tekendensiteit. De tekendensiteit was hoger in eikenbestanden, maar de infectiegraad van *B. afzelii* was het hoogst in dennenbestanden. De dilutiehypothese was niet geldig in de bestudeerde bossen; de densiteit van geïnfecteerde teken was niet gecorreleerd met de diversiteit aan gastheren. En hoewel de infectiegraad verschilde tussen de bostypes was de gastheerdiversiteit gelijk in de verschillende bostypes. Onze resultaten tonen dat teken de gastheren op een andere manier gebruiken in de verschillende bostypes. Larven voeden zich vaker op kleine knaagdieren in dennenbestanden terwijl ze zich in eikenbestanden vaker voeden op andere gastheren (bv. vogels), die andere genotypes dan *B. afzelii* overdragen.

Bossen zijn de geprefereerde habitat van *I. ricinus*, maar mensen kunnen ook blootgesteld worden aan teken en hun pathogenen in (sub)urbane groene ruimtes. In eekhoorns en in teken vanop egels die werden ingezameld in stedelijke gebieden vonden we verschillende veelvoorkomende pathogenen (*Borrelia*-genotypes, *Borrelia miyamotoi*, *Anaplasma phagocytophilum*). Het is dus mogelijk om in aanraking te komen met een teek die geïnfecteerd is met één of meerdere pathogenen in een stadspark of tuin.

Vertrouwen op het ‘dilutie-effect’ om het risico op de ziekte van Lyme te verlagen in ons studiegebied (of vergelijkbare regio’s in Europa) blijkt dus geen effectieve beheermaatregel. Een hogere diversiteit van soorten in de gastheergemeenschap kan zelfs leiden tot een hoger ziekterisico, door de hogere infectiegraad van *Borrelia*-genotypes die leiden tot ernstigere klinische manifestaties van de ziekte van Lyme (bv. neurologische stoornissen) dan *B. afzelii*, die voornamelijk huidaandoeningen veroorzaakt. Preventie van de ziekte van Lyme wordt dus beter gericht op het verlagen van de tekendensiteit en het contact tussen teken en mensen. Het risico op de ziekte van Lyme hangt immers niet enkel af van de densiteit van geïnfecteerde teken, maar wordt in belangrijke mate ook beïnvloed door de waarschijnlijkheid van het oplopen van een tekenbeet: het contact tussen mensen en teken. Het verlagen van de densiteit aan (geïnfecteerde) teken leidt slechts zelden tot een verlaagd ziekterisico. Bosbeheerders kunnen de mate van contact tussen mens en teek, en dus het ziekterisico, verlagen door de vegetatie langs de paden kort te houden (frequent maaien) en bosbezoekers gericht te sturen (gemarkeerde routes en aantrekkelijke centrale voorzieningen).

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Websites

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Curriculum vitae

Education

2009–2011	MSc in Biology, Ecology & Evolution, K.U. Leuven, Faculty of Sciences
2006–2009	BSc in Biology, K.U. Leuven, Faculty of Sciences
2001-2006	Secondary School (Mathematics-Sciences), Sancta-Maria, Leuven

Professional experience

January 2013–present	PhD research at Ghent University, Faculty of Bioscience Engineering, Department of Forest and Water Management, Forest & Nature Lab

Scientific publications

Publications in international journals with peer review cited in the Science Citation Index (IF = impact factor for 2016)

Ruyts S.C., Landuyt D., Ampoorter E., Heylen D., Ehrmann S., Matthysen E., Sprong H., Verheyen K. Low probability of a dilution effect for the rodent associated Lyme borreliosis pathogen *Borrelia afzelii* in different forest types in Belgium, Europe. Tick-borne diseases, submitted (IF 3.23)

Ruyts S. C., Tack W., Ampoorter E., Coipan E. C., Matthysen E., Heylen D., Sprong H., Verheyen K. Year-to-year variation in the density of *Ixodes ricinus* ticks and the prevalence of the rodent-associated human pathogens *Borrelia afzelii* and *B. miyamotoi* in different forest types. Ticks and Tick-borne diseases, accepted (IF 3.23)

Ehrmann S., Prinz M., Ruyts S.C., Brunet J., Cousins S.A.O., Deconchat M., Decocq G., De Frenne P., De Smedt P., Diekmann M., Gallet-Moron E., Gärtner S., Hansen K., Kolb A., Lenoir J., Lindgren J., Naaf T., Paal T., Panning M., Scherer-Lorenzen M., Valdès A., Verheyen K., Liira J. Habitat properties are key drivers of *Borrelia burgdorferi* s.l. prevalence in *Ixodes ricinus* populations of deciduous forest fragments. Parasites & Vectors, accepted (IF 3.080)

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Ruyts S.C., Ampoorter E., Coipan E.C., Baeten L., Heylen D., Sprong H., Matthysen E., Verheyen K. (2016). Over teken, gastheren en het 'verdunningseffect': hoe bosomvorming het risico op de ziekte van Lyme kan veranderen. ANTenne 10 (1), 6-11.

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Chapters in books

Verheyen K., Ruyts S.C. (2016) How can forest managers help to reduce the risk for Lyme borreliosis? In Braks M. A., van Wieren S. E., Takken W., Sprong H. (eds) Ecology and prevention of Lyme borreliosis, Wageningen Academic Publishers, Wageningen, The Netherlands, pp 239-247.

Scientific activities

Participation in congresses, symposia or workshops

Participation with oral presentation

13 December 2016. Tips voor (bos)beheerders om tekenbeten te voorkomen. TickTactics III. Wageningen, the Netherlands.

3 October 2016. Longitudinal survey of the effect of forest types on the abundance of *Ixodes ricinus* ticks. Onehealth-EcoHealth Workshop

22 September 2016. De relatie tussen teken, *Borrelia burgdorferi* s.l. genotypes, biodiversiteit en bostypes. Teken en tekenziekten in Vlaanderen' Gent, Belgium.

13 February 2016. Effect van bosomvorming op de ziekte van Lyme (survey in de Antwerpse Kempen). ANKONA-ontmoetingsdag. Antwerp, Belgium.

10-11 February 2015. *Ixodes ricinus* and *Borrelia burgdorferi* s.l. populations are maintained by a few common host species. Netherlands Annual Ecology Meeting. Lunteren, the Netherlands

28 March 2014. Het effect van bosomvorming op het voorkomen van de ziekte van Lyme. Starters in het natuur- en bosonderzoek. Brussels, Belgium

Participation with poster presentation

12-13 December 2014. Monitoring the host community of ticks in different forest types to investigate risk of Lyme disease. ZOOLOGY 2014 Congress. Liège, Belgium.

Participation without presentation

20 November 2014. Current Themes in Ecology 2014. Wageningen, the Netherlands.

1 November 2013. Workshop Lyme preventie – Integreer in Natuurbeheer. Bilthoven, Netherlands.

22-29 September 2013. International Summer School on functional significance of forest biodiversity. Białowieża, Poland.

Supervision of MSc thesis students

2014-2015	Florian Martens (UGent). Quantifying the host community of the *Ixodes ricinus* ticks in different forest types in the Campine. Supervisors: Prof. dr. Dries Bonte, Prof. dr. Ir. Kris Verheyen.
	Marie Cours (UGent). The link between the small mammal community and the prevalence of *Borrelia burgdorferi* s.l. genospecies in *Ixodes ricinus* ticks in different forest types. Supervisors: Prof. dr. Dries Bonte, Prof. dr. Ir. Kris Verheyen.