Tick tactics

Interactions between habitat characteristics, hosts and microorganisms in relation to the biology of the sheep tick *Ixodes ricinus*

Fedor Gassner

Propositions

1. Allowing cattle to graze in woodlands for the purpose of landscape management can have unforeseen benefits by reducing the risk of Lyme borreliosis.

   (this thesis)

2. Ticks infected with *Borrelia* bacteria are more active than *Borrelia*-uninfected ticks.

   (this thesis)

3. "A stitch in time saves nine" is especially true considering the four-fold increase of Lyme borreliosis in The Netherlands over the past 1.5 decades[†]; intensified research on the ecology of Lyme borreliosis is the first step towards understanding the factors that have contributed to this increase, which are unknown to date.

   † Hofhuis, A., M.G. Harms, J.W.B. van der Giessen, H. Sprong, D.W. Notermans, and W. van Pelt. 2010. De ziekte van Lyme in Nederland 1994 – 2009: Aantal huisartsconsulten blijft toenemen. Is voorlichting en curatief beleid genoeg? Infectieziekten Bulletin 21(3): 84-87.

4. The strong dependence of *Ixodes ricinus* on microclimate and day length[‡] may cause a variation in *Borrelia* transmission risk through the intensity of contact between the tick and its host, which varies diurnally and seasonally.

   ‡ Perret, J. L., P. M. Guerin, P. A. Diehl, M. Vlimant, and L. Gern. 2003. Darkness induces mobility, and saturation deficit limits questing duration, in the tick *Ixodes ricinus*. The Journal of Experimental Biology 206: 1809-15.

5. There are no examples in Nature of providing loans, hence humans should not make lending part of their daily life.

6. With a near-infinite amount of readily-accessible information on the internet, a true shortcoming is a medium that aids us to formulate the right query.

Propositions belonging to the thesis, entitled "Tick tactics".
Fedor Gassner
Wageningen, 2010-11-29

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Interactions between habitat characteristics, hosts and microorganisms in relation to the biology of the sheep tick *Ixodes ricinus*

Fedor Gassner

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Interactions between habitat characteristics, hosts and microorganisms in relation to the biology of the sheep tick *Ixodes ricinus*

Fedor Gassner

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Abstract

The sheep tick *Ixodes ricinus* (L.) is known to transmit a large number of pathogens of medical and veterinary importance, including the bacterium *Borrelia burgdorferi* sensu lato, the causative agent of Lyme borreliosis. *Ixodes ricinus* is found predominantly in woodlands, which provide suitable hosts and microclimates for tick survival. Three blood meals from a wide array of possible hosts are needed to complete the tick life cycle within 2 to 6 years. Ticks can acquire *B. burgdorferi* s.l. during a blood meal on an infected host and can infect a new host during the next blood meal. In this thesis, interactions between hosts, habitat characteristics, and microorganisms in relation to the biology of *I. ricinus* were investigated. A longitudinal study that was initiated at 24 sites across The Netherlands revealed that ticks infested with *B. burgdorferi* s.l. were found at all sites, but with strong spatial and temporal variation. This variation could be partially attributed to habitat characteristics. Substantial variations in the bacterial diversity within *I. ricinus* nymphs were also observed between habitats. Molecular genetic analyses indicated the presence of several potentially pathogenic and non-pathogenic bacteria. The interactions of these bacteria with hosts, ticks and other micro-organisms that reside in the tick need to be established in the future. It is known that rodent populations can mediate *B. burgdorferi* s.l. circulation in nature. Therefore, we studied the potential effects of rodents on the spatial variation of *Borrelia* infections in host-seeking ticks. Wood mice (*Apodemus sylvaticus*) and bank voles (*Myodes glareolus*) were abundant at most sites, but had varying tick burdens and *Borrelia*-infection prevalence between sites. It is argued that the infection prevalence in host-seeking *I. ricinus* ticks in the vegetation depends on the degree of contact between ticks and mice, which is, in turn regulated by habitat characteristics.

Larger hosts, such as ruminants, are important for tick reproduction. Because domestic cattle is frequently used in the management of forested areas in The Netherlands, the effect of such management on tick populations was addressed. Results from this thesis show that the presence of cattle in a woodland resulted in a locally reduced tick density, whereas *Borrelia* infection prevalence remained the same compared to ungrazed woodlands. The tick-reducing effect of cattle was attributed to the negative effect of cattle on rodent populations. Effects of other mechanisms such as habitat alteration by the cattle are arguable and require further study. A central issue in the ecology of Lyme borreliosis is the mechanism by which *Borrelia* species circulate between the life stages of ticks and their hosts. This circulation is strongly affected by the chance that the tick will find a host. It was found that that *B. burgdorferi* s.l.-infected *I. ricinus* nymphs displayed increased walking activity and had a greater resistance to desiccation than uninfected ticks. These *Borrelia*-mediated effects can contribute to increased chances of host finding by the tick, and thereby to an increased transmission of *Borrelia* species. In conclusion, *Borrelia*-infected ticks were present at all locations studied, but with a dynamic heterogeneity, which is partly influenced by habitat characteristics and rodent hosts. The introduction of large herbivores could become an advantageous management strategy to help reduce the incidence of Lyme borreliosis, and further experiments confirming the mechanisms underlying this relationship would be valuable. The established *B. burgdorferi* s.l.-mediated behavioural

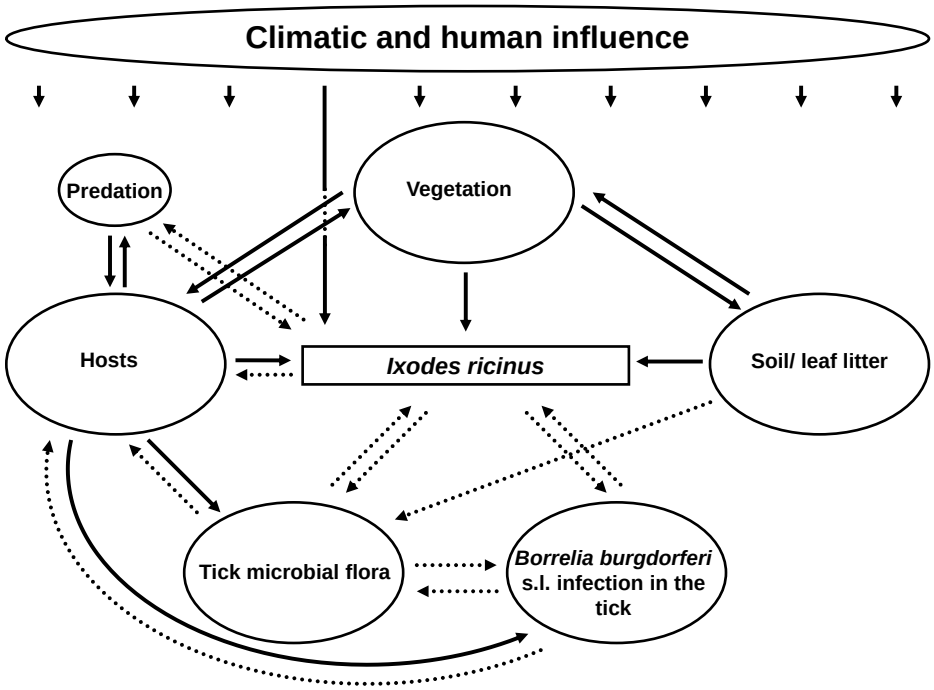
changes in *I. ricinus* contribute to the understanding of the ecology of Lyme *borreliosis*, and create opportunities for future studies on tick-parasite interactions.

Contents

Chapter 1

General introduction

Fedor Gassner



Introduction

The agents of vector-borne diseases are transmitted to various host species via pathogen-carrying organisms acting as vectors. In human and animal vector-borne diseases these vectors are usually arthropods such as mosquitoes, fleas, flies, midges, true bugs or ticks. If these vectors transmit pathogens from animals to humans, the disease is called a vector-borne zoonosis. Pathogens of vector-borne zoonoses can circulate among wildlife, unnoticed by the human population. Usually, clinical human cases are only the tip of the "zoonotic iceberg", whereas the bulk of the vector borne pathogens circulates in nature (Randolph and Šumilo 2007). An example of a typical vector-borne zoonosis of which the bulk of causative agents circulates in nature is Lyme borreliosis (or Lyme disease), which is widespread throughout the northern hemisphere (Steere 1994, Stanek and Strle 2003, Smith and Takkinen 2006, Stanek and Strle 2009).

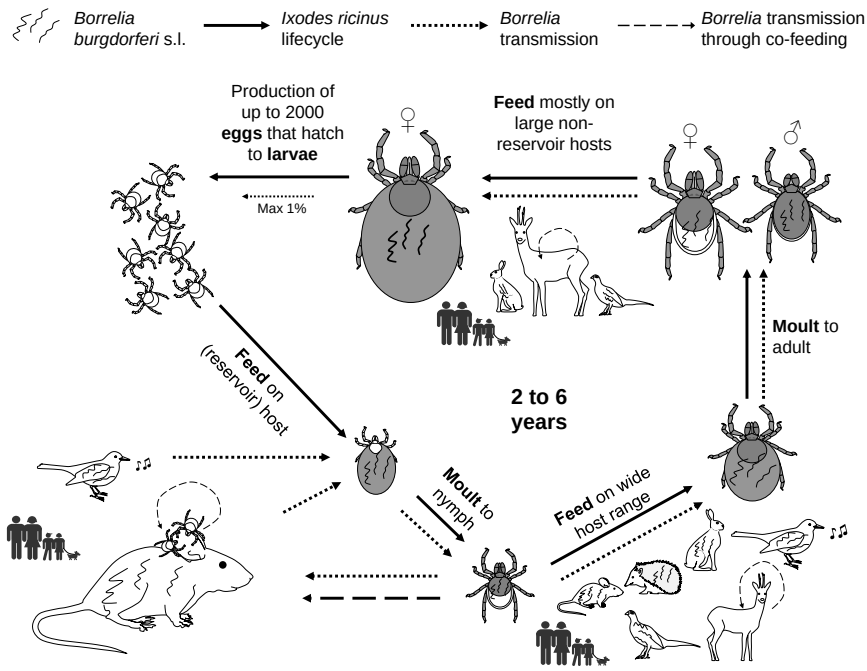


Figure 1.1. Life cycle of *Ixodes ricinus* in relation to the natural circulation of *Borrelia burgdorferi* s.l. Note that all tick life stages feed on humans apart from their natural hosts. The hosts that are drawn with *Borrelia* represent reservoir hosts that can re-infect ticks through a systemic infection. The depicted hosts represent the diversity of hosts, the true host range may be larger in nature.

Lyme borreliosis is caused by the bacterium *Borrelia burgdorferi* sensu lato, which is transmitted by the bite of ticks of the *Ixodes ricinus* species complex. In The Netherlands, the incidence of Lyme borreliosis has increased four-fold since 1994, reaching 22,000 cases in 2009 (Hofhuis et al. 2006, Smith

and Takkinen 2006, Hofhuis et al. 2010). The vector in western Europe is the sheep tick *Ixodes ricinus* (L.), which is primarily found in woodlands. These woodlands provide hosts, a suitable moist microclimate and a litter layer for optimal survival of ticks. Three blood meals from a wide array of possible hosts are needed to complete the 2-6-year tick life cycle, which comprises eggs, larvae, nymphs and adults (Sonenshine 1991, Randolph et al. 2002). Tick larvae can acquire *B. burgdorferi* s.l. during a blood meal on an infected host, notably small rodents and birds, and can infect a new host during the subsequent nymphal and adult blood meal (Fig. 1.1). To find a blood meal, all life stages of *I. ricinus* climb up the herbal vegetation, where they wait in ambush for a passing host: which includes rodent species, ungulates, birds, but also humans (Gern et al. 1998, Gray 1998). This process is referred to as questing, where larvae, nymphs and adults each prefer a specific height depending on their desiccation resistance and host preference, up to ~1.5 m for adults (Mejlon and Jaenson 1997).

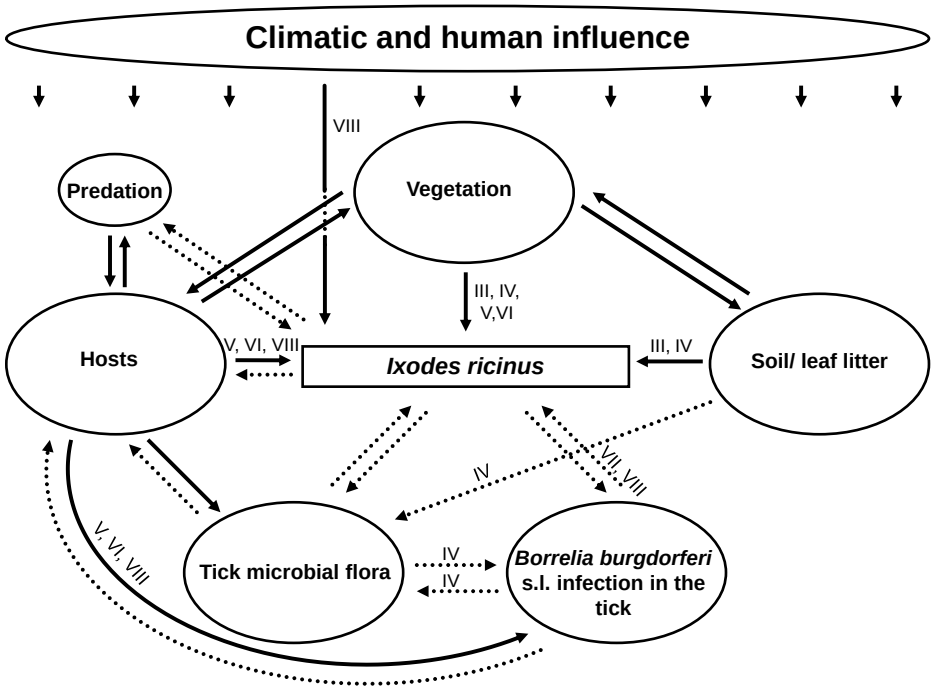


Figure 1.2. Components of the ecology of *Ixodes ricinus* ticks. Solid lines indicate pathways that have been extensively studied, dotted lines indicate interactions that are unstudied or scarcely studied. Roman numbers at the arrows refer to which chapter in this thesis covers the specific interaction. Modified with permission from Gassner and van Overbeek (2007).

The risk for humans of contracting Lyme borreliosis will ultimately depend on the density of infected ticks that can transmit the disease to humans, and on human behaviour in tick infested areas. The density of *I. ricinus* is determined by the fitness of individuals in the population as well as host availability, whereas the activity of the ticks affects their chances of contacting a

host. Both fitness and activity of *I. ricinus* depend on a network of interrelated factors (Fig. 1.2). These include human influences such as land use, wildlife management and the use of pesticides. Climatic factors can affect tick survival, developmental time and activity, but may also indirectly affect ticks through effects on hosts, vegetation and soil traits. Most of the well-established interactions indicated by solid arrows in Figure 1.1 are extensively studied. Although the global mechanisms behind these interactions are well understood, more research is needed for specific mechanisms on effects of these interactions as caused by temporal changes in tick densities and on the abundance and distribution of *Borrelia* pathogens in tick vectors. Other connections in the network are less well established (dotted arrows, Fig. 1.2). For example, only few examples of interactions between *B. burgdorferi* s.l. and other microorganisms in the tick have been described (Ginsberg 2008) (Fig. 1.2, line IV). Moreover, very little is known about interactions between *I. ricinus* and *B. burgdorferi* s.l (Fig. 1.2, lines VII, VIII). Therefore, it is important to study how spatial and temporal distribution of *I. ricinus* and the influence of host and pathogen interactions influence the ecology of Lyme disease, with a special focus on natural habitats in The Netherlands.

Problem definition and research questions
In this thesis, I address the spatial and temporal distribution of *I. ricinus* and their bacterial infections. Moreover, I studied the interactions between rodents, cattle and *B. burgdorferi* s.l.- infected ticks on the one hand, and interactions between *I. ricinus* and *B. burgdorferi* s.l. on the other.

To address these issues, I have formulated following research questions:

1. What is the spatial and temporal distribution of ticks and their associated microbial communities?

2. What is the impact of environmental factors (e.g., large herbivores and rodents) on the spatial distribution of *I. ricinus* and their *Borrelia burgdorferi* sensu lato infections?

3. Does infection with *Borrelia burgdorferi* sensu lato affect tick behaviour and development?

Thesis outline and hypotheses

In **Chapter 2,** the manifestation of the Lyme borreliosis in Europe is reviewed, and the potential effects of climate change and tick control on *I. ricinus* populations are discussed.

Chapter 3 presents a study that was initiated to address the hypothesis that tick populations and their *Borrelia* species infections are not homogeneous in time and space. During monthly sampling efforts that were undertaken by volunteers, ticks were collected in two transects at 24 locations across The Netherlands for a period of 1.5 years, and *Borrelia* species infections in these ticks were determined. Vegetation variables were recorded to explain possible variation in tick densities between the 24 locations.

Chapter 4 focuses on the microbial diversity in ticks, with the hypothesis that microbial diversity in ticks differs between habitats and correlates with *B. burgdorferi* s.l. infections. A broad-range molecular screening essay was applied to make an inventory of the bacterial community composition in ticks. Moreover, dominant bacterial clones were selected for sequence analyses to assess the identity of bacteria that can be found inside ticks by sequence analyses.

Chapter 5 addresses the effects of large herbivores on tick populations; It was hypothesized that cattle introduced into woodlands can amplify tick populations and *Borrelia* infection rates in ticks. To investigate whether differences occured between grazed and ungrazed habitats, ticks and rodents were sampled in identical woodland sites, where cattle were either present or absent. The presence or absence of *B. burgdorferi* s.l. was determined in collected host-searching ticks. Additionally, the rodent population in the grazed and ungrazed habitats was assessed and tick burdens on captured rodents were established.

In **Chapter 6**, the interactions between rodents and ticks are addressed. Rodents are assumed to play a key role in the circulation of *B. burgdorferi* s.l. in nature (Gern et al. 1998). Therefore, I hypothesized that *B. burgdorferi* s.l. infections in host-searching *I. ricinus* nymphs in the vegetation are proportional to infections in rodents. In this study, ticks and rodents were collected in six habitats in The Netherlands. All host-searching ticks, rodents and ticks feeding on rodents were counted and analysed for *B. burgdorferi* s.l. infections.

Chapter 7 focuses on the behaviour of nymphal *I. ricinus* in relation to *B. burgdorferi* s.l. infections. The success of infected ticks in finding a host is an important factor in the ecology of Lyme borreliosis. Therefore, it can be expected that *B. burgdorferi* s.l. can mediate *I. ricinus* to enhance the chances of to contact a host. Especially because many parasites throughout the animal kingdom are able to manipulate their host to promote their own dispersal (Dobson 1988, Hurd 2003, Schaub 2006, Lefèvre and Thomas 2008, Libersat et al. 2009). Here, I hypothesized that *B. burgdorferi* s.l. infections increase the activity of *I. ricinus* nymphs. The behaviour of field-collected as well as laboratory-reared *Borrelia*-infected and uninfected *I. ricinus* nymphs was digitally monitored in a laboratory based arena to compare the behaviour of infected and uninfected ticks.

Chapter 8 addresses the survival and development of *I. ricinus* in relation to *B. burgdorferi* s.l. infections. Results obtained from Chapter 7 and from several field studies indicate that *Borrelia*–infected nymphs show behaviour that exposes them to desiccating conditions to a greater extent than uninfected nymphs. Therefore, it is hypothesised that *I. ricinus* nymphs that are infected with *B. burgdorferi* s.l. are more resistant to desiccation. To test this hypothesis, field-collected *I. ricinus* nymphs were subjected to desiccating conditions and subsequently tested for infections with *B. burgdorferi* s.l. The findings of Chapter 7 on activity and energy storage of *I. ricinus* also lead to the hypothesis that infection with *B. burgdorferi* s.l. has a positive effect on the feeding and development of *I. ricinus*. To test this hypothesis, *B. burgdorferi* s.l

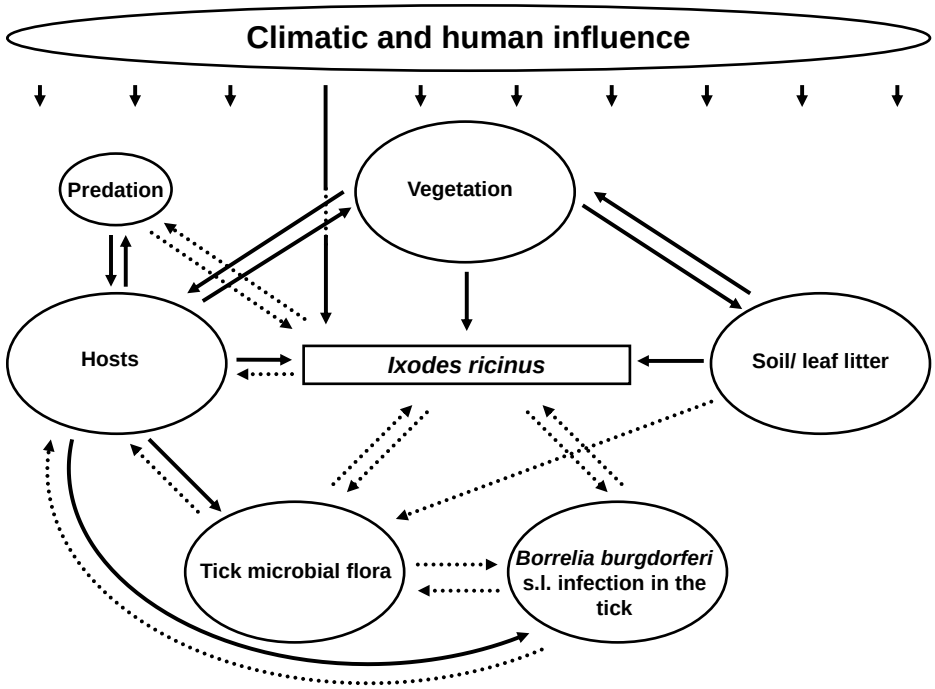
infected and uninfected field-collected wood mice (*Apodemus sylvaticus*) were housed in the laboratory. Naturally-feeding *I. ricinus* larvae were allowed to bloodfeed and moult into nymphs. After all naturally-attached larvae had fed to repletion, laboratory-reared ticks were allowed to bloodfeed on the same rodents. Feeding and development of both groups of larvae were studied in relation to *B. burgdorferi* s.l. infections in the ticks.

In **Chapter 9**, it is discussed how the findings of this thesis contribute to a better understanding of the ecology of Lyme borreliosis, with special emphasis on The Netherlands. Furthermore, I discuss how the findings of this thesis have consequences on existing knowledge of *B. burgdorferi* s.l. transmission to humans and how they can contribute to the development for the future prevention of Lyme borreliosis. Finally it is proposed that, based on he findings of this thesis, future research should be aimed at resolving the mechanistic background and consequences of parasite-mediated changes in *I. ricinus* on the circulation of *B. burgdorferi*.

Chapter 2

Lyme disease in Europe: Facts and no fiction

Fedor Gassner and Leo S. van Overbeek



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Abstract
Lyme disease is an emerging disease in The Netherlands and elsewhere in Europe. Infections of *Ixodes ricinus* ticks with the Lyme disease-causative agents have been found almost throughout Europe. Remarkable are the large differences in infection rates between different regions. It is supposed that observed heterogeneity in infected tick numbers is characteristic for the way *Borrelia* species are acquired by, and transmitted via *I. ricinus* ticks. A factor directly affecting microbial communities in the tick gut, including populations of *Borrelia* species, is blood from hosts. Especially smaller animals like birds and rodents, often called 'reservoir hosts', play an important role in transmission of *Borrelia* species between vector and hosts. Local environmental factors will largely impact these groups of smaller animals and modulate their populations. Factors like soil, vegetation, predation and human interferences will have strong influences on local and temporal fluctuations of reservoir hosts and supposedly also on *Borrelia*-species-infected tick numbers. Local differences in these factors cause differences in infection rates in ticks and it was therefore proposed that large fluctuations in infected tick numbers may occur at small-scale level; so-called 'hot spots' of infected ticks. The concept of hot spots needs further attention as it is important for control of infected ticks in natural areas via locally applied and integrative approaches.

Introduction.

As mentioned in one of the most fundamental works on ticks "The biology of ticks" (Sonenshine 1991), the first records of ticks as nuisance to humans date back hundreds of years B.C. and were described by ancient Greek and Roman authors. The first record of ticks as species were done by Linnaeus in the Systema Naturae from 1758. Ticks they have been found encapsulated in fossil amber from at least 50 million years ago. It was only since the early 1900's that ticks recognized to transmit diseases to cattle and humans. Although symptoms of Lyme disease, also called Lyme borreliosis, in Europe were first described in 1883 (Matuschka et al. 1995), no link with ticks as vector was made until late 20th century. It revealed that the major vectors of Lyme borreliosis were species belonging to the hard tick genus *Ixodes*. The causative agent *Borrelia burgdorferi* was originally isolated in the United States in 1982 and a link was made between this pathogen and the Lyme disease symptoms Erythema migrans in the town of Old Lyme in 1975 (Burgdorfer et al. 1982). In Europe, *Borrelia* species were detected in various *Ixodes* tick species which were obtained from museum specimens dating back to 1896 (Hubbard et al. 1998). Although historical evidence provides information on infected ticks up to about a century ago; genetic variation within *Borrelia* species isolates and *Ixodes scapularis* populations indicate that this relationship must exist over much longer periods; at least since the last ice-age. Similar relationship is also expected between European *Ixodes* tick vectors, which are closely related to their nearctic counterparts, and *Borrelia* species occurring in ticks, although it must be taken into account that European *Borrelia* species diversity is higher than found in the United States (Kurtenbach et al. 2006).

By the late 20th century Lyme borreliosis has developed into the most prevalent tick-borne zoonosis in the northern hemisphere. The primary vectors of Lyme borreliosis are hard ticks belonging to the *I. ricinus* species complex, which is widely distributed over the palearctic as well as the nearctic regions of the northern hemisphere. In North America, the westerly distributed Black Legged Tick (*I. pacificus*) and the deer tick (*I. scapularis*) are the primary vectors of Lyme borreliosis (Piesman et al. 1999). In Eurasia, mainly the sheep tick *I. ricinus* and the more eastern distributed taiga tick *I. persulcatus* are considered as primary vectors of Lyme borreliosis, although other tick species may also play a role in the enzootic cycle of the Lyme borreliosis agent, but have been rarely linked to clinical cases of Lyme disease (Gray 1998). *I. ricinus* also acts as vector of other tick borne diseases such as babesiosis, human granulocytic ehrlichiosis and tick borne encephalitis (Gray 2002), which are not as generally prevalent as Lyme borreliosis and limited to certain geographic regions (Piesman et al. 1999).

Lyme borreliosis is a disease with a wide array of symptoms, possibly causing skin, hart, joint and neurological disorders and can be acquired by the bite of an infected tick (Stanek and Strle 2003). An early symptom of infection is erythema migrans, a red migrating skin rash which occurs in most, but not all infections after the bite of an infected tick. Different *Borrelia* species cause different symptoms in humans and not all *Borrelia* species have been proved to be pathogenic for humans. Occasionally, clinical symptoms have been observed in various domestic animals.

In this chapter, the emerging character of Lyme borreliosis will be discussed from an ecological perspective of *I. ricinus*. The aetiology of the Lyme-causative agents belonging to the *Borrelia* species complex will be discussed here, with emphasis on heterogeneous distribution of infected ticks in natural areas and possible remedies to reduce incidences of Lyme borreliosis. Occasionally other *Ixodes* species will be mentioned because parallels in *Borellia* species infections exist between paleartic and nearctic *Ixodes* species.

Ecology of *Ixodes ricinus*

The primary vector of Lyme borreliosis in Europe, *I. ricinus* (Fig. 2.1) has a 3-stage lifecycle, larva, nymph and adult, which is typical for all species of the hard tick family, *Ixodidae*. *I. ricinus* ticks strictly depend on a blood meal for moulting and entrance into the next developmental stage.

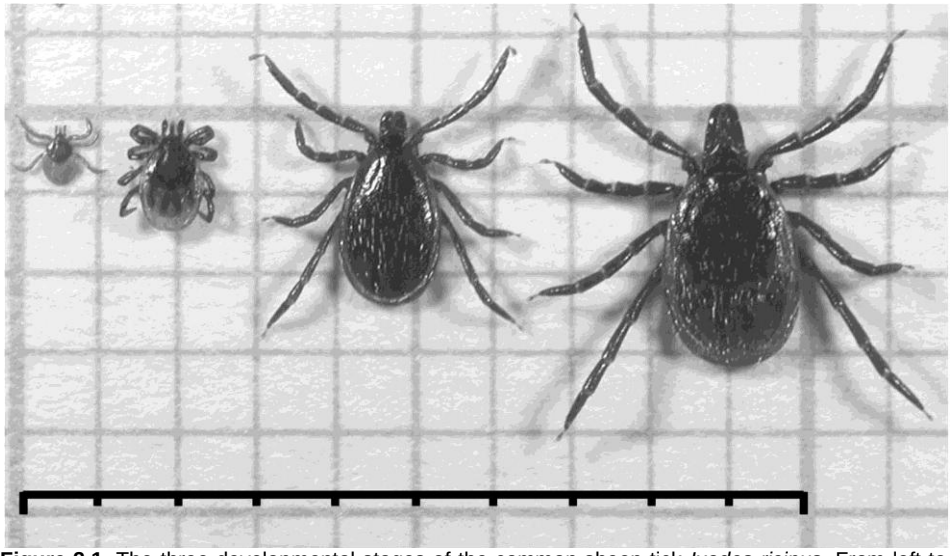

Figure 2.1. The three developmental stages of the common sheep tick *Ixodes ricinus*. From left to right: larva; nymph; adult male; adult female. The scale bar indicates 1 cm.

The host is found by specific foraging behaviour usually called questing, where the tick detects various cues from the host, like CO_2, skin volatiles, movement and heat (Sonenshine 1991). In contrast to many other blood feeding arthropods, *I. ricinus* ticks have a limited action radius and spatial dispersion is mainly driven by transportation via the host. Before questing, *I. ricinus* ticks climb into the vegetation waiting for suitable hosts. When a host passes, the tick grasps the fur and crawls to preferred feeding sites such as ears, neck and skin between the legs. *I. ricinus* ticks attaches to the skin and uses specialized mouthparts (Fig. 2.2) and salivary excretions to anchor itself into the skin where it remains undetected until full engorgement (Sonenshine 1991). Engorgement usually lasts between 3 to 7 days. Insemination usually takes place within the vegetation (Sonenshine 2004) during questing or in some cases on the host. Females normally oviposit between 3 to 27 days after feeding and will lay between 500 to 3000 eggs. Eggs are preferably deposited in the leaf litter or in

the top bottom layer of the forest floor, and can remain unhatched for months. Development of all tick life stages and eggs is optimal at temperatures between 8 - 11 ºC.

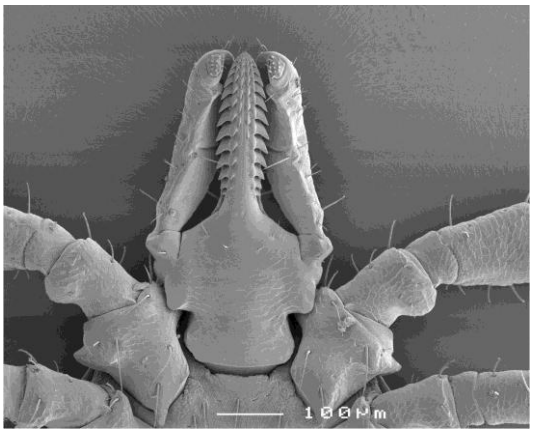

Figure 2.2. Scanning electron micrograph of nymphal *Ixodes ricinus* hypostome (picture by A. van Aelst).

Once hatched, larvae prefer small rodents and birds for feeding, but they occasionally also feed on larger hosts (Gray 1998) including humans (Gray 2002). Occurrence of larvae is usually restricted to lower vegetation because of their host preference and susceptibility to desiccation, this in contrast to nymphs and adults which reside higher in vegetation (Mejlon and Jaenson 1997). The occurrence of larvae lower in the vegetation makes it difficult to collect them for research. Most commonly applied method is to collect ticks from the vegetation by dragging with a cotton blanket (Fig. 2.3). It must therefore be accounted for that collected larvae numbers will, in general, be underestimated in comparison with those of nymphs and adults. For example in our studies at several locations within The Netherlands, no larvae were captured in a pine forest over a 5 month period, whereas many were found feeding on rodents in the same forest simultaneously. After their first blood meal, which takes generally between 3 - 5 days, larvae will retreat into the forest floor and will develop into which takes between 24 days to sometimes several months, depending on seasonal and climatic conditions.

The newly developed nymphs have a much wider host range than larvae, ranging from small rodents and birds to reptiles and various ungulates. Wider host range in combination with relatively less susceptibility to desiccation, allows questing higher in the vegetation than larvae (Mejlon and Jaenson 1997). Once attached to a host, nymphs will feed between 4 to 7 days, followed by a next moulting period in the forest floor, which can last up to one year before they appear as sexually mature adults. Adult ticks are generally found higher in the vegetation in comparison to both other stages and can even be found up to 1.5 meters above the forest floor, which is about the height of a larger-sized animal (Mejlon and Jaenson 1997). Adult ticks have a different host range than larvae and nymphs, especially larger animals like hedgehogs, hares and ungulates (Gray 1998), whereas they rarely found on smaller rodents. These larger hosts are important to complete the tick lifecycle and therefore these large animals are called 'reproduction' hosts. For all stages, questing periods

are usually in spring, early summer and autumn depending on photoperiod, temperature and humidity. The duration of the life cycle is flexible because of temperature-dependent differences in development and the possibility to enter into an inactive stage, such as developmental diapause and quiescence, required to overcome unfavourable climatic conditions or absence of hosts.

The entire life cycle from egg to adult is usually between 2 and 4 years but may take up to 7 years, mainly depending on local climate and daylength at different latitudes and altitudes. *I. ricinus* ticks thrive in climates with cooler summers and milder winters which explains why this species mainly occur in the northern hemisphere (Gray 1998). A study on tick phenology in Western Europe revealed that all tick cohorts (i.e., individuals of the same developmental stage and age) always recruited to the next developmental stage during autumn (Randolph et al. 2002). Furthermore, this study revealed that all tick stages showed clear peaks in questing activity that were assigned to hatching or transition from inactive to active stages. In general, larvae were found questing from early May until end of August showing highest peak in summer time. Nymphs were generally found questing from mid February until end of November showing highest peak between March and July and adult females were found questing throughout the year, without any clear peak in questing activity and occurrence at lower abundance than individuals of both other stages. These observations correspond with those found in The Netherlands where larvae were most active from May until late November, nymphs from March until November and adults throughout the year with lowest activity in winter time (Smit et al. 2003). This reveals that *I. ricinus* tick activity is not constant over the year but shows clear peaks.

Factors important for *I. ricinus* tick activity are temperature and relative air humidity. In general ticks become active above 80% of relative air humidity and at temperatures of 4 ºC or higher. Commonly, saturation deficit is used as parameter for tick activity. Saturation deficit combines air temperature with humidity and represents the 'drying power' exposed on ticks. Ticks are usually found to be active at saturation deficits of 4 mmHg and higher (Perret et al. 2000). However, susceptibility of ticks to desiccation not only depends on the saturation deficit alone but also on stored energy present in tick bodies. It was found that ticks were more active at lower saturation deficits shortly after a blood meal (Randolph and Storey 1999). This can be explained by the fact that ticks can actively accumulate water from the air and for this they need sufficient energy coming from nutrients in blood.

It is expected that *I. ricinus* ticks are more active during night because the saturation deficit is optimal and many nocturnal host animals will be active. It is plausible to believe that ticks are at least as active at night compared to during the day (Mejlon and Jaenson 1997). However, most studies were done at day time and one may wonder whether obtained tick numbers are representative for the populations present in the studied areas. Daytime collection will lead to serious underestimation of tick abundance (Randolph and Storey, 1999). This was also confirmed by our observation made in a forest near the village Renkum, in The Netherlands, where higher tick numbers of all stages were found at night than during the day. However, most studies were done with the purpose to study transmission of Lyme borreliosis -causative

agents to humans and therefore daytime collection of ticks is more appropriate because most people visit natural areas at day and not at night.

For the lifecycle of *I. ricinus* ticks climatic conditions, coverage by vegetation, composition of the forest floor (soil and leaf litter layer), and presence of suitable hosts are the most important parameters. It is clear that these parameters will fluctuate in time and space and thus it can not be expected that tick activity and abundance are the same for all places at all times. This has important consequences for the transmission of tick borne pathogens because tick numbers will fluctuate in time and space.

Figure 2.3. Blanket dragging method used for collection of questing ticks in vegetation (picture by W. Takken).

Borrelia species and transmission to hosts

Lyme borreliosis -causative agents are the most important pathogens transmitted via *Ixodes* species ticks to humans. Responsible for Lyme disease is a group of closely related species belonging to the bacterial genus *Borrelia*. This complex consists of three main clusters: Lyme borreliosis *Borrelia* species (consisting of appx 12 species); New world Tick Borne Relapsing Fever *Borrelia* (TBRF) and Old world TBRF *Borrelia*. The TBRF agents are widely distributed over the northern hemisphere and are mainly transmitted via body lice and ticks (Rebaudet and Parola 2006), and will not be further discussed in this chapter.

Worldwide 33 species are known within the *Borrelia* species complex (http://www.bacterio.cict.fr/b/borrelia.html). At least seven of these are known to occur in Europe: *B. afzelii*, *B. burgdorferi sensu stricto, B. garinii*, *B. valaisiana*, *B. lusitaniae, B. spielmanii* and *B. bisettii. B. spielmanii* and *B. bisettii* were recently described (Derdáková and Lencáková 2005). It can not be excluded that new *Borrelia* species will even be found in the near future, for example species that have remained undetected to date, or more virulent strains of already known species which are newly formed through horizontal gene transfer (Kurtenbach et al. 2006). Within the *Borrelia* species complex, interspecies genetic variation is large, with special emphasis on *B. garinii*, which can be subdivided in several genospecies based on differential expression of the outer surface protein A (Osp-A). *B. afzelii*, *B. burgdorferi sensu stricto* and *B. garinii* are in particular responsible for symptoms in humans (Stanek and Strle 2003),

whereas the role of the other species in development of Lyme borreliosis symptoms is more disputable. When dealing with disease transmission via *I. ricinus* ticks, it is often referred to the Lyme borreliosis -causative species of the *Borrelia* complex, whereas the others are often ignored. Because the role of other *Borrelia* species in the establishment of the Lyme borreliosis -causative species in vectors as well as their transmission to hosts is largely unknown, they still may be important. Therefore, '*Borrelia* species' is preferred above '*B. burgdorferi* sensu lato', because it covers all species within the *Borrelia* species complex, including the ones that are supposed not to be pathogenic and those from which pathogenic traits has not been elucidated yet.

Once inside *I. ricinus* ticks, *Borrelia* species cells will establish in the midgut and can thus be transferred to the next developmental stage. In principle tick infection with *Borrelia* species cells can take place after each blood meal, which explains why highest incidences in *Borrelia* species infections are generally found in adults followed by nymphs and lowest in larvae. Cells of *Borrelia* species present in the blood meal can colonize the tick midgut and from there cells can migrate via the haemolymph to the salivary glands. *Borrelia* outer surface proteins (Osp) present in cells of *Borrelia* species and TROSPA molecules expressed by the tick play an important role in the settlement and migration from the midgut to the salivary glands. Especially OspC was recently found to be indispensable in this process (Goettner et al. 2006, Fingerle et al. 2007). Once present in the salivary glands, other molecular mechanisms including factors expressed by the tick can affect the transmission of the *Borrelia* cells as well as attachment of the tick to the host (Hovius et al. 2007). Time of migration of *Borrelia* species cells in tick vector differs per species; in *I. scapularis* it was found to take place within 36 - 48 hours (De Silva and Fikrig 1995). However, for *B. afzelii* in *I. ricinus*, dissemination from the midgut to the salivary glands is much faster and can result in *Borrelia* transmission in 24 hours (Crippa et al. 2002). Therefore, it is generally advised to remove attached ticks within 24 hours after attachment.

From many different studies it has become clear that there is a relationship between certain hosts and *Borrelia* species. For example *B. afzelii, B. burgdorferi* s.s. and the *B garinii* Osp-A 4 genotype are mainly associated with rodents and *B. valaisiana* and other Osp-A types of *B. garinii* are more associated with different bird species (Kurtenbach et al. 2002). Preference of *Borrelia* species for particular hosts may be explained by differences in host immune responses for different *Borrelia* species. In Europe, at least 9 small and, 7 medium sized mammals were able to transmit *Borrelia* species to *I. ricinus* ticks and are therefore considered reservoir hosts (Gern et al. 1998). Furthermore, manly rodents (Humair et al. 1999) and birds (Hanincová et al. 2003) account for the natural circulation of the spirochetes in nature. Many reproduction hosts, including common host such as ungulates (Telford III et al. 1988, Tälleklint and Jaenson 1994) and rabbits (Matuschka et al. 2000) are generally incompetent as reservoir. However, some reproduction hosts can also serve as reservoir, e.g., pheasants (Kurtenbach et al. 1998). Feeding all stages of *I. ricinus*, presence of such hosts could maintain tick populations by feeding adult females, enabling them to lay their eggs. In addition, such infected reproduction hosts can infect all feeding tick stages and thereby maintain a tick

population with a circulation of *Borrelia* species in absence of other reservoirs and reproduction hosts.

Another infection route of ticks can happen through co-feeding. Here hosts that are poor reservoirs can receive *Borrelia* species cells in the local bloodstream around a feeding infected tick. Uninfected ticks feeding nearby can thereby pick up the bacteria without the need of an systemic infection in the host (Ogden 1997). Such effects can be expected to contribute significantly to the infection prevalence in tick populations, especially if co feeding occurs in a mix of infected nymphs and uninfected larvae, which can sometimes feed in aggregations of over 50 larvae in an aggregated feeding area, such as the neck, the nose or the ears, in combination with one or more nymphs (Gassner, unpublished data).

Finally, a last process may contribute *Borrelia burgdorferi* s.l. in an area is transoverial transmission, whereas it has not been established that vertically transmitted spirochetes in larvae are infective to new hosts. This is certainly a phenomenon that needs further study, since 1,9% of larvae are infected Europe wide (Rauter and Hartung 2005).

The role of different animal groups has major implications on transmission of Lyme borreliosis -causative species causative agents in nature. The group of smaller animals, like birds and rodents, needs therefore particular attention because of their proposed role in transmission of Lyme borreliosis -causative species ultimately leading to infections in humans.

The detection of Lyme disease-causing agents in ticks and animal tissues

To investigate the behaviour of different *Borrelia* species in ticks and vertebrates it is important to discriminate Lyme-disease-causing-*Borrelia* species from others. Nowadays a myriad of detection techniques are available to screen for the presence of different bacterial species in environmental samples. However, *Borrelia* species need special attention for the following reasons: 1) *Borrelia* species cells can not always be recovered from environmental samples by preceding culturing steps, in spite the fact that these cells are still often viable, 2) cells from different *Borrelia* species closely resemble each other and it is hard to differentiate between different species, especially those that are involved in Lyme borreliosis. Therefore dark field microscopy and cultivation-based detection methods are not always suitable for specific detection of *Borrelia* species in environmental samples. There, culture-independent detection techniques like molecular (PCR –based) and serological techniques are more appropriate. A commonly applied technique used for detection of *Borrelia* species in tick samples is the so called reverse line blot method (Rijpkema et al. 1995, Schouls et al. 1999). The reverse line blot detection is based on extraction of total DNA from ticks followed by PCR with *Borrelia* genus-specific primers which incorporate biotin to the PCR product. The PCR products are hybridized to a membrane with *Borrelia* species-specific probes. This method appeared to be applicable in many different studies involving *Borrelia* species infection in ticks, but it has some drawbacks. First, only a limited number of *Borrelia* species can be determined in one single sample and second, this method is qualitative and not quantitative. Because many different pathogens are supposed to be present in ticks, besides *Borrelia* species, it is often important to measure the relative abundance of all pathogens

in the same sample. Therefore, detection in quantitative multiplex settings may be more appropriate. Although these techniques have not been applied in tick research yet, it is expected that they will soon come available in our laboratories and will provide deeper insight in interactions within microbial communities in ticks.

Microbial communities in *I. ricinus*.

Microbial communities in tick guts may influence establishment and reproduction of invading pathogens, like those belonging to the *Borrelia* species complex, in ticks. The microbial composition of the *I. ricinus* midgut will be influenced by different environmental factors (Fig. 2.3). The most important factor influencing microbial communities in ticks will be blood from hosts. Blood composition and micro-organisms present in blood will determine the microbial composition of tick gut communities. Because ticks are rather immobile it is expected that local variation in animal hosts will strongly determine the microbial composition in ticks. Other environmental factors like vegetation, soil type, predation and human activities are also important because these factors can locally influence the composition of small animal populations. The effect of local parameters on microbial composition of tick guts and their possible effect on pathogen invasion and establishment are so far rarely explored and may explain more in detail why large fluctuations in *Borrelia* species infection rates occur in different regions over the world.

An illustrative example of the indirect effect of vegetation on the occurrence of *Borrelia* species in ticks is shown in a study done on acorn mast. Acorn mast is a periodical phenomenon where oak populations simultaneously produce larger than average amounts of acorns. A modelling study done in North America and based on 15 years of field observations indicated that a mast season can clearly affect reservoir hosts populations resulting in higher than normal tick numbers and *Borrelia* species infection rates (Ostfeld et al. 2006a). Since acorn mast is a common phenomenon also in Europe as well, it is expected that it will have the same effect here. Not only acorn mast drives host populations, but also presence of leaves, fruits and other seeds may cause large temporal fluctuations in tick densities through alteration of host densities. Differences in *Borrelia* species infection rates in ticks from areas with different plant communities was found (Kampen 2004). Therefore, vegetation type must be considered as an important factor affecting *I. ricinus* tick populations and *Borrelia* species inside these ticks.

Soil and leaf litter composition of forest floors may also be important factors affecting *Borrelia* species infection rates in ticks. Soil type will have a direct effect on plant composition and thus indirectly affecting local animal communities. However, the leaf litter layer is an important environment for ticks as well because ticks lay their eggs in the leaf litter layer and also they spend a considerable period of their life span in this layer, e.g., for transstadial development. Size, pH and humidity of the leaf litter layer are therefore important factors directly affecting success of tick reproduction and thus *Borrelia* species infection rates in ticks.

As indicated in Figure 2.4, the role of many factors affecting *I. ricinus* - *Borrelia* species interactions are not fully understood yet. The most prominent one is direct effect of different micro-organisms present in ticks on tick survival.

Although speculative, a limited number of studies has focused on this interaction and it must be taken into account that presence of certain micro-organisms might even be responsible for changes in reproduction and behaviour (Lefcort and Durden 1996). This has already been shown for several other pathogens in numerous arthropod vectors (Hurd 2003) and requires further attention in tick research.

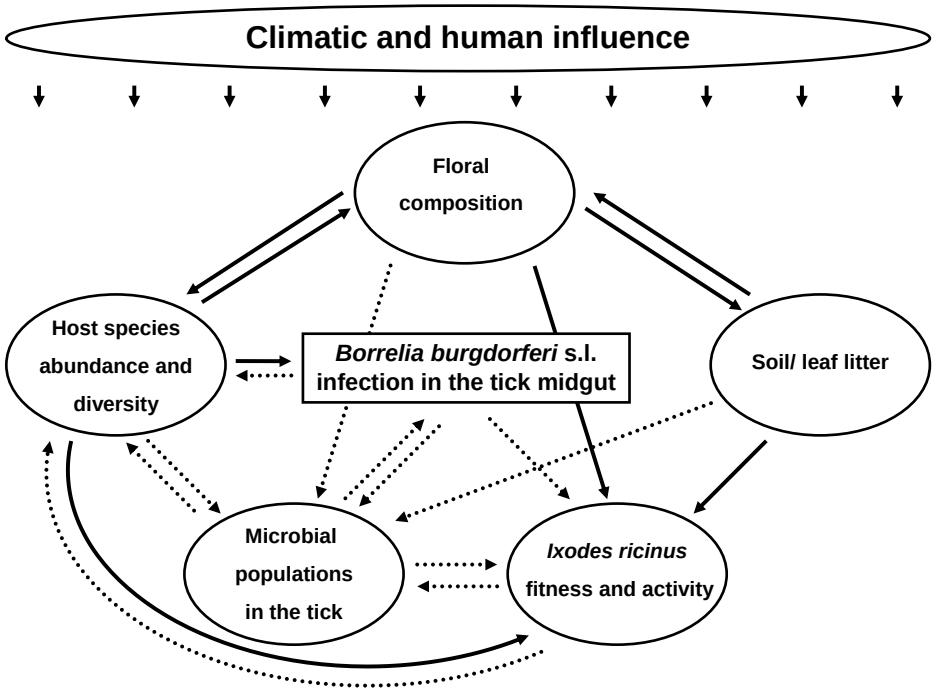

Figure 2.4. Natural factors affecting microbial composition and *Borrelia* species establishment in *Ixodes ricinus* ticks. Solid lines indicate direct effects on microbial communities and dotted lines presumptive indirect effects.

Spatial and temporal variations in *Borrelia* species infection rates in ticks

Local environmental factors will have a strong influence on survival and reproduction of ticks. However, increase in tick numbers does not necessarily have to lead to higher infection rates. To understand how *Borrelia* species are able to accumulate in reservoir hosts and ticks, it is first important to understand at which spatial and temporal scale level infection rates varies in ticks.

Large spatial variations in *Borrelia* species infection rates were found in different coutries in Europe (Smit et al. 2003, Jouda 2004, Kampen 2004, Ferquel et al. 2006, Wielinga et al. 2006) and locally in North America (van Buskirk and Ostfeld 1998). In a Europe-wide meta analysis study the existence of large differences in infection rates was demonstrated (Rauter and Hartung 2005). Especially the difference between western and eastern European countries was striking; 14 % tick infection rates in eastern Europe and 24 % tick infection rates in western Europe. Infection rates in ticks ranged from low (1.5% in Slovenia) to very high (49% in Slovakia and Switzerland). Furthermore, only 2

out of 110 studies included in this meta analysis showed uninfected ticks, which emphasizes that *Borrelia* species infections in ticks are found almost all over Europe. Of all *Borrelia* species found, *B. afzelii* and *B. garinii* were the most common ones. However, not in all studies these were the most dominant species found. For example in a study done in Germany it revealed that *B. valaisiana* was more abundant than *B. garinii* and *B. afzelii* (Kampen 2004). It is questionable whether this meta study will fully reflect the actual incidences of *Borrelia* species infection rates over Europe. One should realize that most, if not all studies were performed in regions with a history of Lyme borreliosis. Still, it is clear that differences in *Borrelia* species infections can fluctuate over larger areas and time scales, as is the same for the occurrence of different *Borrelia* species.

Within The Netherlands, differences in *Borrelia* species infection rates in ticks were found at smaller spatial scale, infection rates varied per region, and were between 5% and 30% (De Boer et al. 1993, Rijpkema et al. 1994, Rijpkema and Bruinink 1996, Rijpkema et al. 1996, Smit et al. 2003, Wielinga et al. 2006). This indicates that infected tick rates may vary locally, e.g., per forest. What is the minimal scale-level where differences in *Borrelia* species infections occur? One study revealed that tick densities and *Borrelia* species infection rates fluctuated within a single woodland (Zeman 1999). Is it possible that incidences of high infection rates occur very locally, e.g., at a scale of a few square meters? These so called 'hot spots' may explain why such variation exists between and within woodlands. Furthermore, it is important to understand how humans acquire Lyme borreliosis in natural areas and also to understand how the causative agents spread through natural areas and may disappear again. The question is why variations in tick infection rates at smaller scale levels are usually not published in detail.

The existence of variation of infected ticks is a fascinating subject and requires further attention. From an ecological perspective, it may explain the role of reservoir hosts and the effect of ecological interaction on such populations on the spread of Lyme borreliosis through nature and transmission to humans. For practical applications 'hot spots' may be important for application of control measures aimed to reduce spread and accumulation of pathogens belonging to the *Borrelia* species complex and possibly also of other pathogens present in *I. ricinus* ticks.

An extreme scenario: The Netherlands.

Recently, a third country was added to the list of high incidence countries. A survey of erythema migrans and tick bite cases in The Netherlands showed a simultaneous dramatic increase over a period of 10 years (Hofhuis et al. 2006). This study was performed on a local scale and shows remarkable regional differences in incidence (Fig. 2.5). On average, the incidence of erythema migrans nearly tripled from 39 cases per 100.000 inhabitants in 1994 to 103 cases per 100.000 inhabitants in 2005. At the same time, tick bites consultations at hospitals and General practitioners also increased, from 191 per 100.000 inhabitants in 1994 to 446 per 100.000 inhabitants 2005. The latter observation is most likely an underestimation since many people do not attend medical help for a tick bite.

To date, there is no clear scientific explanation for this dramatic increase. There is a clear lack of nationwide tick population and *Borrelia burgdorferi* s.l. monitoring, which is common for most European countries. The available data from infected tick populations in The Netherlands do not show extreme differences with other European countries and vary between 5% and 30% with temporal as well as spatial differences (De Boer et al. 1993, Rijpkema et al. 1994, Rijpkema and Bruinink 1996, Rijpkema et al. 1996, Smit et al. 2003, Wielinga et al. 2006). To date, no long term study is available, where ticks have been monitored for example during a 10 year period. Though it is expected that tick populations are becoming more infected (Takken, unpublished data), with locally high prevalence. Apart from an increase in populations of infected ticks, also increase in recreation, creation of ecological connections between nature areas, changes in host abundance and recent milder winters might have caused the increase in Lyme borreliosis. Perhaps, infection prevalence is locally boosted by occurrence of transmission hotspots, as discussed before in this chapter.

The European distribution of *Borrelia burgdorferi* s.l. and *Ixodes ricinus* overlaps residential areas.

A possible explanation of the observed increase in several European countries can be explained by the fact that infected *Ixodes ricinus* ticks are situated in populated areas. For example, an internet survey in The Netherlands in 2006 and 2007 revealed that many people acquired a tick bite in their own gardens (W. Takken, personal communication). Europe wide, infected *Ixodes ricinus* are found in recreational areas near or in cities (Table 2.1). This phenomenon brings people more into contact with infected ticks, the question should be raised on how this process has developed. For example, did we build our cities in areas where infected ticks were already present, or did they advance into these areas after the expansion of the city's boundaries? Apparently, for city parks and private gardens, it can be assumed that these have been newly constructed so that present ticks have been brought in by possible vectors. These urban vectors can be for example rats, mice, squirrels and various birds such as thrushes and blackbirds (Matuschka et al. 1997, Humair and Gern 1998, Humair et al. 1998, Kurtenbach et al. 1998).

Table 2.1. Lyme borreliosis vector *Ixodes ricinus* in European urban areas.

Country	Urban area type	Reference
Finland	Urban recreational areas	(Junttila 1999)
Germany	City outskirts, recreational sites and private gardens	(Maetzel et al. 2005)
Poland	Recreational sites near city	(Michalik et al. 2003)
The Netherlands	Urban park	(Wielinga et al. 2006)
United Kingdom	Urban park	(Guy and Farquhar 1991)
	Recreational site	(Robertson 2000)

Off course, the question remains whether we expand our urban and recreation areas into tick habitats, or whether the ticks and their pathogens are advancing into human territory. A combination of both may also be true, but it shows that monitoring of tick and *Borrelia burgdorferi* s.l. populations in urban areas is of great importance for monitoring and predicting Lyme borreliosis.

Lyme borreliosis and climate change.

Over the past few decades, with emphasis on the past few years, climate change has become a very popular topic in the media, in science and for the general public. Evidently, the climate is changing with various effects worldwide, changing risks for human health through direct effects of thermal stress and extreme weather events. Many other indirect effects can occur through via agriculture, air pollution and by various infectious (vector borne) diseases (McMichael et al. 2006). In Europe, climate change has been predicted to cause increased drought in the southern parts and warmer winters with wetter summers in more northern countries. To date, climate change effects are most dominantly seen in increased winter temperatures and increased growing season length (IPCC, 2001) in:(in:Lindgren and Jaenson 2006).

As described earlier in this chapter, local climatic conditions can be of great influence on survival and behaviour of *Ixodes ricinus* in a direct as well as indirect way (Fig. 2.4). Consequently, effects of climate change can be expected to have amplified effects on populations of infected ticks since climate can affect several the same time. It should be mentioned that these effects can be positive as well as negative, for example considering habitat suitability for the vector. A recent modelling study that correlated habitat suitability showed that climatic factors such as temperature and summer rainfall can have different effects depending on the region, where some areas were predicted to face stable or increasing *Ixodes ricinus* densities and other areas showed opposite trends (Estrada-Peña and Venzal 2006).

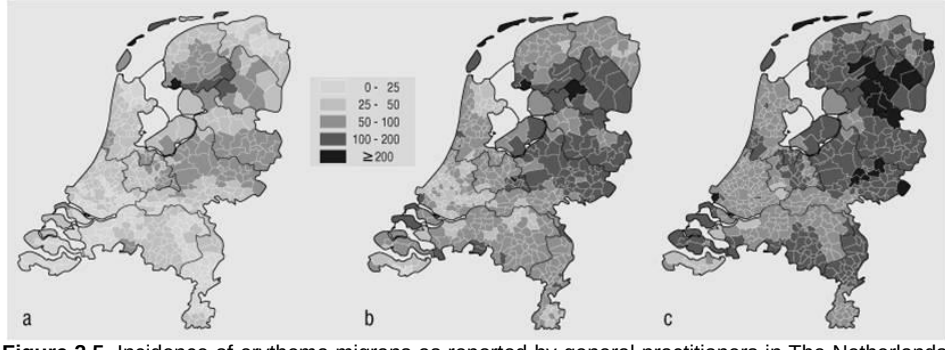

Figure 2.5. Incidence of erythema migrans as reported by general practitioners in The Netherlands in 1994 (A), 2001 (B) and 2005 (C) per 100.000 inhabitants. (Reproduced with permission from Hofhuis et al., 2006).

Climatical conditions depend on the latitude and altitude of an area and thereby determine the boundaries of Lyme borreliosis endemic regions. With a changing climate, these boundaries are expected to shift to other altitudes and latitudes. For *Ixodes ricinus,* an increase in distribution and abundance has

been linked to climatic change in various studies (Lindgren et al. 2000, Daniel et al. 2003, Hubálek 2005).

Since an increase in ticks is positively correlated to Lyme borreliosis incidence (Stafford et al. 1998, Hubálek et al. 2003), climate change that increases tick distribution and density is expected to increase incidence of Lyme borreliosis (Bennet et al. 2006). Additionally, behaviour of the general public should also be taken into account since warmer summers generally increases the exposure of recreationists to ticks, where increase of ticks can come together with an increase of recreation activity (Mavin et al. 2005, Joss et al. 2007). Recently, a report by WHO has also stressed the role of climatic change in Europe (Lindgren and Jaenson 2006), but concluded that emergence of Lyme borreliosis cannot be exclusively be attributed to climatic factors. Based on Climate changes alone, the distribution of *Ixodes scapularis* and thereby the incidence of Lyme borreliosis in North America was predicted to increase substantially in the coming decades (Brownstein 2005). Further research is needed in Europe to make reliable predictions.

Control of ticks in nature.

Regarding the emerging character of Lyme borreliosis and other tick borne diseases such as Tick Borne Encephalitis (Randolph and Šumilo 2007), tick control is still a relatively neglected subject in Europe. Surprisingly little scientific attention is paid to direct control of ticks in Europe compared to the United States and Africa, where ticks transmit livestock diseases which can cause severe economical damage regionally (Jongejan and Uilenberg 2004).

For Europe little information is available of what economical damage tick borne diseases cost annually, taking in account e.g., damage to livestock, health care costs for humans as well as pets and economical loss to a decrease of recreation incomes in known endemic areas. An accurate assessment together with the alarming signals described earlier in this chapter, could lower the threshold for the European governments to take action.

Tick control does not necessarily have to be aimed at large scale applications of chemical pesticides like dichlorodiphenyltrichloroethane (DDT) or various pyrethroid pesticides. Nowadays, Integrated Pest Management (IPM) is widely applied in agriculture and may be adopted for application in nature as well. Several strategies can be combined to control ticks; e.g., biological agents in combination with forestry management practices. Biological control of ticks can be done with entomopathogenous fungi which were shown to be effective against *I. scapularis*. (Kaaya and Hassan 2000, Benjamin et al. 2002). Application of these fungi has not been tested before on a larger scale, but may offer opportunities in an IPM-like approach.

Other tick control strategies are use of impregnating materials that enable contact with hosts such as nesting materials, four posters[1] and bait boxes with a chemical or biological control agent. These methods are already applied and offer opportunities to control ticks in different developmental stages

[1] Four posters are bait stations with four pesticide impregnated bristles which rub the ears, antlers and neck of deer that are attracted to the offered food. Four posters are commercially available in the United states.

(Lane 2001, Ostfeld et al. 2006b). Control of potential reservoirs for *Borrelia* species and vegetation management are alternative measures to control ticks, although these measures are more invasive (Wilson et al. 1988, Buskirk 1995). Other methods such as trapping ticks with pheromones or other chemical attractants, will be most effective on the host, for example by preventing ticks to mate on a reproduction host (Sonenshine 2004). The effect of other type of traps will be limited due to the relatively short range motility of ticks from the place where they moulted compared to for example free flying mosquitoes. An IPM-like approach may be the best option to control ticks in nature. However, most methods are in an experimental stage and not yet ready for larger-scale applications in Europe.

Apart from control focussing at ticks and tick hosts control of Lyme disease risk can also be focussed on education and information of the general public, including professional groups such as forest workers. Indeed, the general public has heard off Lyme disease, but very few know exactly what the risks are. For example, two common misconceptions are that ticks can jump like a flea and will drop from a tree. Knowing that ticks will only cling on to a person if one touches it's questing position and that ticks very seldom drop from trees and generally do not climb up higher than approximately 1,5 meter (Mejlon and Jaenson 1997) can alter one's behaviour and perception, especially during recreation.

One effective way to inform the general public is through the media, where moments of high risk can be given together with methods for personal protection and basic information on Lyme borreliosis (Lindgren and Jaenson 2006). For example, tick removal needs clear instruction since it's a precarious action due to the strongly barbed tick hypostome (Fig. 2.2) since inadequate removal of the tick may cause faster transmission of the disease agent.

A potentially interesting measure could be a short tick warning item which can be embedded in national weather forecasts, in newspapers, on radio, television and the internet during high risk periods. Information on personal protection could also be focussed on risk groups such as forest workers and (youth) groups. Possibly, once local hotspots are identified, local warnings in newspapers and at the entrances of nature areas could also be effective.

Concluding remarks

Lyme disease is emerging and hard to control in natural areas. The causative agents and the primary vectors of Lyme borreliosis are commonly observed in many countries in Europe. Occasional high incidences of *Borrelia* species infections in ticks reveal the heterogeneous nature of these infections in *I. ricinus* ticks. It is important to understand the local environmental factors affecting accumulation of *Borrelia* species in ticks and their natural hosts for development of measures to control ticks in nature. There is a clear need for long term studies that describe year round fluctuations in ticks and their pathogens. Additionally, spatial heterogeneity needs to be further assessed in order to assess Lyme borreliosis risk. Microbial communities associated with ticks may play an important role in modulation of *Borrelia* species populations in the vector. The role of different microbial species in the establishment and reproduction of *Borrelia* species in *I. ricinus* ticks is so far unexplored and may reveal new opportunities to combat Lyme borreliosis.

Chapter 3

Geographic and temporal variation in population dynamics of *Ixodes ricinus* and associated *Borrelia* infections in The Netherlands

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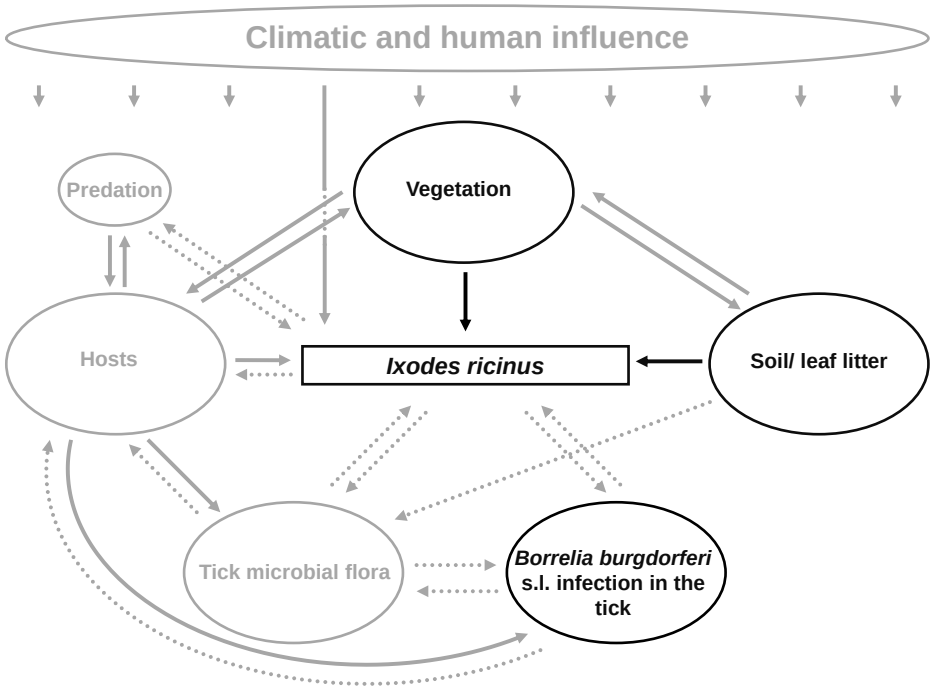

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Abstract
In a country-wide investigation of the ecological factors that contribute to Lyme borreliosis risk, a longitudinal study on population dynamics of the sheep tick *Ixodes ricinus* and their infections with *Borrelia burgdorferi* sensu lato was undertaken at 24 sites in The Netherlands from July 2006 to December 2007. Study sites were mature forests, dune vegetations or new forests on land reclaimed from the sea. Ticks were sampled monthly and nymphal ticks were investigated for the presence of *Borrelia*. *Ixodes ricinus* was the only tick species found. Ticks were found in all sites, but with significant spatial and temporal variations in density between sites. Peak densities were found in July and August, with lowest tick numbers collected in December and January. In some sites, questing activities of *I. ricinus* nymphs and adults were observed in the winter months. Mean monthly *Borrelia* infections in nymphs varied from 0 to 29.0% (range: 0-60%), and several sites had significantly higher mean nymphal *Borrelia* infections than others. Four genospecies of *Borrelia burgdorferi* s.l. were found, with *B. afzelii* being dominant at most sites. *Borrelia* infection rates in nymphal ticks collected in July, September and November 2006 were significantly higher (23.7%, $P<0.01$) than those in the corresponding months of 2007 (9.9%). The diversity in *Borrelia* genospecies between sites was significantly different ($P<0.001$). Habitat structure (tree cover) was an effective discriminant parameter in the determination of *Borrelia* infection risk, as measured by the proportion of nymphal ticks infected with *B. burgdorferi* s.l.. Thickness of the litter layer and moss cover were positively related to nymphal and adult tick densities. The study shows that *Borrelia*-infected ticks are present in many forest and dune areas in The Netherlands and suggests that in such biotopes, which are used for a wide variety of recreational activities, the infection risk is high.

Introduction

Lyme borreliosis has become a vector-borne disease of great significance in the northern hemisphere since its discovery in 1976. The disease, caused by spirochaetes of the *Borrelia burgdorferi* complex, is transmitted by hard ticks of the *Ixodes ricinus* complex. In Europe, *I. ricinus* L. is the dominant vector and its ecology has been reviewed by Gray (1998). The species is found predominantly in forested habitats, where microclimate and host composition are suitable for survival of the four life stages, egg, larva, nymph and adult, the last three requiring a single blood meal from a vertebrate host (mamalian or avian). Should the host be a reservoir of *Borrelia* bacteria, it can infect the ticks, which remain infective throughout successive lifestages. *Borrelia burgdorferi* s.l. is a species complex with at least 15 genospecies, of which seven are present in western Europe (Hubalek and Halouzka 1997, Vennestrom et al. 2008). The mean infection rate of *I. ricinus* nymphs with *B. burgdorferi* s.l. in Europe is approximately 10%, but varies from zero to over 30% (Rauter and Hartung 2005). *Borrelia* infection rates in ticks can exhibit great temporal and spatial fluctuations (Rauter and Hartung 2005), which are ascribed to variations in environmental conditions such as animal host density and climate (Gray 1998). In addition to *Borrelia*, *I. ricinus* may also be infected with several other human pathogens such as *Rickettsia* spp., *Anaplasma* (*Ehrlichia*) spp., *Babesia* spp. and *Bartonella* spp. as well as, in some regions, tick-borne encephalitis (TBE) virus. In particular, co-infections of *Borrelia* and *Rickettsia* spp. are common (Christova et al. 2003, Schouls et al. 1999, Skarphedinsson et al. 2007).

Although the ecology of Lyme borreliosis is reasonably well understood, the factors that determine the observed large variations in tick density, *Borrelia* infections and other determinants for Lyme borreliosis are not (Killilea et al. 2008, Maetzel et al. 2005). For example, a strong correlation between Lyme borreliosis risk and forested areas has been reported (De Mik et al. 1997, Estrada Pena 2001, Jaenson et al. 2009), but risk level varies considerably between forests (Killilea et al. 2008). The abundance of deer is thought to contribute to risk variation, but factors such as human behavior, litter layer, abundance of small animals and micro- as well as macroclimate may also affect risk level. In The Netherlands, Lyme borreliosis has developed into an important disease in the last decade, with an estimated 17,000 recorded cases in 2005 (Hofhuis et al. 2006). The relationship between tick activity and seasonal and regional variation in *Borrelia* infection rates of ticks is poorly understood. The present study was undertaken to examine the spatial and temporal dynamics of *I. ricinus* across The Netherlands, and to establish their relationship to *Borrelia* infection rates of ticks collected at selected study sites. The effects of habitat characteristics on the spatial variation in tick densities and *Borrelia* infections in ticks are discussed.

Materials and methods

Description of study sites

Twenty-four sites, distributed across The Netherlands were selected for tick collections (Fig. 3.1). Criteria for selection were a) likelihood of finding *I. ricinus* ticks and b) the availability of a team of volunteers to make monthly collections. Within each location, two marked transects of 100m^2 were monitored consistently. Geographic coordinates of each transect were established with

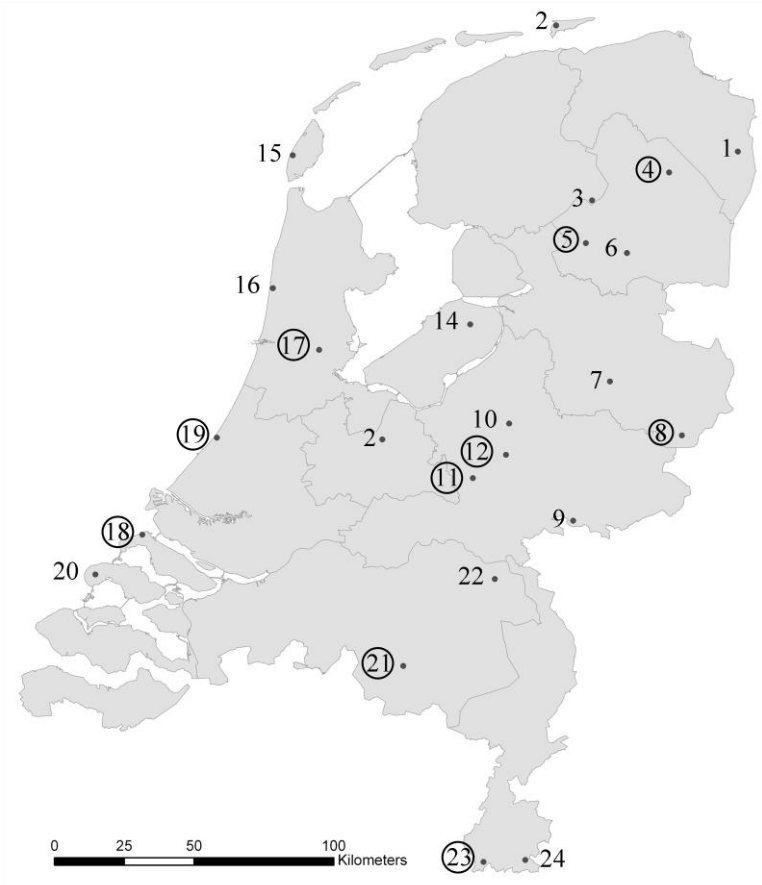


Figure 3.1. Map of The Netherlands, showing the location of the 24 study sites where *Ixodes ricinus* was collected. Encircled sites represent the sentinel sites referred to in the study.

GPS, and a description of the habitat was made and classified according to Braun-Blanquet (1964) and Weeda et al. (2005) (Tables 3.1 and 3.2). Ten of the 24 sites, evenly distributed across The Netherlands and representing the different habitat classes present among the 24 sites, were chosen for detailed analysis of *Borrelia* infections of nymphal ticks.

Tick collections

Ticks were collected once per month between July 2006 and December 2007. Teams of experienced volunteers, recruited through the Association for Environmental Education (IVN) and familiar with ecological investigations, were given a training session by the researchers at the start of the first sampling period. They were visited by one of the authors after six months to verify the quality of the sampling procedure. At each of the 24 sites (Table 3.1), tick collections were made by dragging a white cotton cloth (1m^2) over the two marked transects (Daniels et al. 2000). The cloth was inspected for the presence of ticks at intervals of 25 m (Tälleklint and Jaenson 1996). Larvae,

Table 3.1. Plant communities (associations) of all 48 transects in the 24 sites, after Braun -Blanquet (1964) and Weeda et al. (2005). Nr. corresponds to the sites in Fig. 3.1. Coordinates are given in degrees latitude and longitude. The types are described *Ticks from 10 selected sentinel sites were insepected for *B. burgdorferi* s.l. infections bimonthly during the study period.

Nr	Location	Coordinates transect 1	Vegetation type transect 1	Coordinates transect 2	Vegetation type transect 2
1	Bellingwedde	53°04'26N, 7°07'36E	Betulo Quercetum roboris	53°04'26N, 7°07'37E	Betulo Quercetum roboris
2	Schiermonnikoog	53°29'34N, 6°09'46E	Fago Quercetum	53°29'44N, 6°09'55E	Cladonio Pinetum sylvestris
3	Appelscha	52°55'33N, 6°20'34E	Eurhynchiumpraelongum Pseudosderopodium-purum-[Vaccinio-Piceetea]	52°55'35N, 6°20'45E	Prunus serotina [Dicrano-Pinion]
4*	Gieten	53°00'54N, 6°45'18E	Betulo Quercetum roboris deschampsietosum	53°00'56N, 6°45'12E	Fago Quercetum holcetosum
5*	Ruinen	52°47'07N, 6°18'22E	Leucobryo-Pinetum deschampsietosum	52°47'07N, 6°18'22E	Betulo - Quercetum roboris vaccinietosum
6	Hoogeveen	52°47'08N, 6°18'28E	Deschampsio Fagetum typicum	52°45'05N, 6°31'26E	Betulo Quercutum roboris vaccinietosum
7	Nijverdal	52°20'18N, 6°25'27E	Leucobryo Pinetum vaccinietosum	52°20'18N, 6°25'27E	Cladonio Pinetum sylvestris dicranetosum polyseti
8*	Haaksbergen	52°09'50N, 6°47'54E	Betulo Quercetum roboris molinietosum	52°09'50N, 6°47'54E	Betulo Quercetum roboris cladonietosum
9	Montferland	51°53'29N, 6°13'10E	Leucobryo-pinetum vaccinietosum	51°55'42N, 6°13'23E	Quercion roboris
10	Apeldoorn	52°12'27N, 5°53'31E	Leucobryo Pinetum vaccinietosum	52°12'54N, 5°54'26E	Betulo Quercetum roboris vaccinietosum
11*	Ede*	52°01'41N, 5°41'49E	Betulo-Quercetum roboris cladonietosum	52°01'41N, 5°41'49E	Betulo-Quercetum roboris cladonietosum
12*	Hoog Baarlo	52°06'20N, 5°52'29E	Betulo Quercetum roboris vaccinietosum	52°06'20N, 5°52'29E	Betulo Quercetum roboris vaccinietosum

Table 3.1, continued from previous page

Nr	Location	Coordinates transect 1	Vegetation type transect 1	Coordinates transect 2	Vegetation type transect 2
13	Bilthoven	52°09'22N, 5°13'40E	Leucobryo-Pinetum deschampsietosum	52°09'27N, 5°13'47E	Betulo - Quercetum roboris deschampsietosum
14	Dronten	52°31'38N, 5°41'06E	Fraxino-Ulmetum typicum	52°31'31N, 5°41'09E	Fraxino-Ulmetum typicum
15	Texel	53°04'11N, 4°44'26E	Fago Quercetum holcetosum	53°04'11N, 4°44'26E	Betulo Quercetum roboris dryopteridetosum
16	Bergen aan zee	52°38'40N, 4°38'05E	Crataego Betuletum pubescentis	52°38'48N, 4°39'15E	Carex arenaria Calamagrostis epigejos-[Dicrano-Pinion]
17*	Twiske	52°26'52N, 4°53'35E	Artemisio- salicetum albae agrostietosum stoloniferae	52°26'52N, 4°53'35E	Lychnido-Hypericetum Tetrapteri orchietosum morionis
18*	Kwade Hoek	51°50'25N, 3°58'58E	Rhamno Crataegetum	51°50'25N, 3°58'58E	Rhamno Crataegetum
19*	Wassenaar	52°09'32N, 4°21'41E	Carex arenaria - Calamagrostis epigejos-[Dicrano-Pinion]	52°09'23N, 4°21'38E	Hippophao Ligustratum typicum
20	Schouwen	51°42'37N, 3°44'22E	Fago- Quercetum convallarietosum	51°42'34N, 3°44'32E	Fago Quercetum holcetosum
21*	Veldhoven	51°25'19N, 5°20'02E	Betulo-Quercetum roboris molinietosum	51°25'19N, 5°20'02E	Betulo-Quercetum roboris deschamsitosum
22	Cuijck	51°42'14N, 5°48'51E	Fago-Quercetum molinietosum	51°42'15N, 5°48'57E	Carici-Elongatae Alnetum rubetosum idaei
23*	Eijsden	50°47'23N, 5°44'14E	Stellaria Carpinetum polystichetosum	50°48'54N, 5°44'45E	Stellario - Carpinetum allietosum
24	Vaals	50°47'38N, 5°57'19E	Stellario - Carpinetum allietosum	50°45'48N, 5°58'57E	Luzulo Luzuloides Fagetum

nymphs and adult ticks were collected using forceps, placed in 70% ethanol and dispatched by mail to the research team in Wageningen, where all ticks were recounted and identified to species and life stage.

Determination of Borrelia *infections in ticks*

At two-month intervals, starting in July 2006, nymphal ticks from 10 of the 24 sites (see *Description of study sites*) were examined for the presence of *Borrelia* infections. Detection was done using PCR followed by the reverse line blot (RLB) procedure, modified after Schouls et al. (1999) for identification of the *Borrelia* species (Van Overbeek et al. 2008). If fewer than 30 nymphs were collected, all ticks were examined; if more than 30 nymphs were found, a random sample of 30 ticks was selected for *Borrelia* analysis. On two occasions, in May and September 2007, nymphal ticks collected from all (n=24) sites were examined for *Borrelia* infections. DNA extraction was performed as described by Schouls et al. (1999) and 60µl of each extract was stored at -80 °C until further analyses.

Table 3.2. Habitat characteristics of all 48 transects in the 24 sites. Nr. corresponds to the sites in Figure 3.1. Litter is thickness of litter layer in cm; the other variables are coverage on a 1 to 10 scale of the moss layer, herbal layer, brush layer and tree layer respectively (Braun-Blanquet, 1964, Weeda et al. 2005).

Nr	Location	Transect 1					Transect 2				
		Litter	Moss	Herb	Brush	Tree	Litter	Moss	Herb	Brush	Tree
1	Bellingwedde	1	8	6	8	1.0	1	6	1	8	0.5
2	Schiermonnikoog	1	8	1	1	1.0	1	3	1	8	5.0
3	Appelscha	8	7	3	8	8.0	8	4	8	7	10.0
4	Gieten	1	8	3	8	3.0	1	3	6	8	5.0
5	Ruinen	3	2	2	6	7.5	6	7	5	7	17.5
6	Hoogeveen	1	1	1	8	20.0	8	5	6	4	10.0
7	Nijverdal	6	7	1	7	5.0	5	8	5	1	0.0
8	Haaksbergen	4	8	5	8	4.0	7	8	3	6	1.0
9	Montferland	8	8	6	7	5.0	1	8	5	7	2.0
10	Apeldoorn	3	8	4	7	15.0	3	8	4	7	15.0
11	Ede	8	8	4	4	14.0	8	6	4	4	18.7
12	Hoog Baarlo	2	8	3	8	3.6	7	8	3	5	2.8
13	Bilthoven	3	5	3	5	6.0	5	3	4	6	6.5
14	Dronten	1	8	4	8	0.0	1	6	4	8	0.0
15	Texel	1	3	6	6	2.0	1	6	5	7	3.0
16	Bergen aan zee	1	8	8	7	1.0	1	8	1	8	2.0
17	Twiske	1	8	1	1	0.0	1	1	1	1	0.0
18	Kwade Hoek	1	7	4	1	0.0	1	4	8	1	0.0
19	Wassenaar	2	6	7	8	1.0	6	7	5	1	1.0
20	Schouwen	2	7	4	8	5.0	2	4	5	8	8.0
21	Veldhoven	4	8	4	7	9.0	7	7	4	3	6.0
22	Cuijck	3	7	7	7	12.5	6	7	8	4	10.0
23	Eijsden	6	8	3	8	0.0	2	8	4	8	0.0
24	Vaals	2	8	6	8	0.0	1	8	4	8	10.0

Data analysis

Statistical analyses were performed using Genstat software (release 12.1).

Generalized linear models (GLM) were used to analyse temporal and spatial variation in tick densities. Because tick densities are counts, a Poisson

distribution with a logarithm link function was chosen. Tick densities were used as response variates, temporal units (e.g., year and month) and location were used as explanatory variables in the regression models. Analyses of spatial and temporal variation in nymphal infection with *B. burgdorferi* s.l. were performed with a GLM with a binomial distribution and a logit link function, and infections were linked to the total number of nymphs analysed in each observation. Post hoc t-probabilities were calculated to test pair-wise differences between sites, months and years. Relationships amongst habitat characteristics, tick densities and *B. burgdorferi* s.l. infection prevalence were calculated using GLM as described above, but here using the sum of a given variable over the 18-month study period as response variables and the vegetation characteristics given in Table 3.2 as explanatory variables. In all GLM analyses, overdispersion was taken into account where appropriate. Differences in composition of the dominant species *B. afzelii, B. garinii, B. burgdorferi* s.s. and *B. valaisiana* between sites were analysed using loglinear regression on a contingency table that was derived from Table 3.3, including ticks with co-infections. The presence of a significant interaction between location and species indicates that species composition differs between sites. Stepwise removal and pairwise comparison of sites was used to reveal which sites differed significantly. In all analyses, effects were considered to be significant at a level of $P \leq 0.05$.

Results

Population dynamics

Ixodes ricinus was found in all sites studied. In Dronten (site #14), the species was found on only six of 18 sampling dates and in very low numbers. In Ede (site #11) and Hoog Baarlo (site #12), ticks were found on all sampling occasions. There was a strong seasonal variation in tick density, with a single peak in the summer, and reduced activity of larvae and adults in the winter (Fig. 3.2).

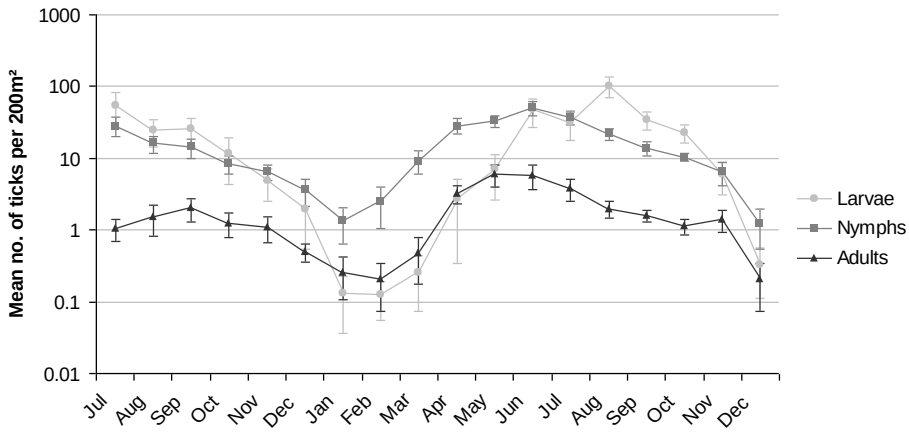


Figure 3.2. Mean monthly number of *Ixodes ricinus* collected per 200 m^2 of habitat in 24 sites in The Netherlands from July 2007 to December 2007. L – larvae; N – nymphs; A – adults (males + females). Error bars represent standard errors of the mean.

At this time of the year, however, activity of nymphs was still observed, with a mean density of 1.4 (January) and 2.5 (February) per 200m^2 respectively. Comparing the months of July-December 2006 and 2007, significantly more larvae and adults were collected in 2007 than in 2006 (Larvae: P=0.001; Adults: P=0.002). Comparing the same months, there was a significantly higher nymphal density in 2007 than in 2006 (P=0.01). Considering month-to-month variations, larval densities were significantly different in all months (P=0.01) except for November 2006 and 2007, September 2006 and October 2007, and July 2007 and September 2007. Adult densities were significantly higher in September 2006, July 2007 and August 2007 than in all other months (P= 0.013; P<0.001 and P<0.001, respectively).

During the 18 months of the study, and considering the 24 study sites, there was a significant effect of the thickness of the litter layer and moss cover on numbers of nymphs and adults per location (Nymphs: P=0.002 and P=0.01and Adults: P=0.027 and P=0.026, respectively). Tree, shrub and herbal coverage, separately, did not have an effect on the number of ticks at each site.

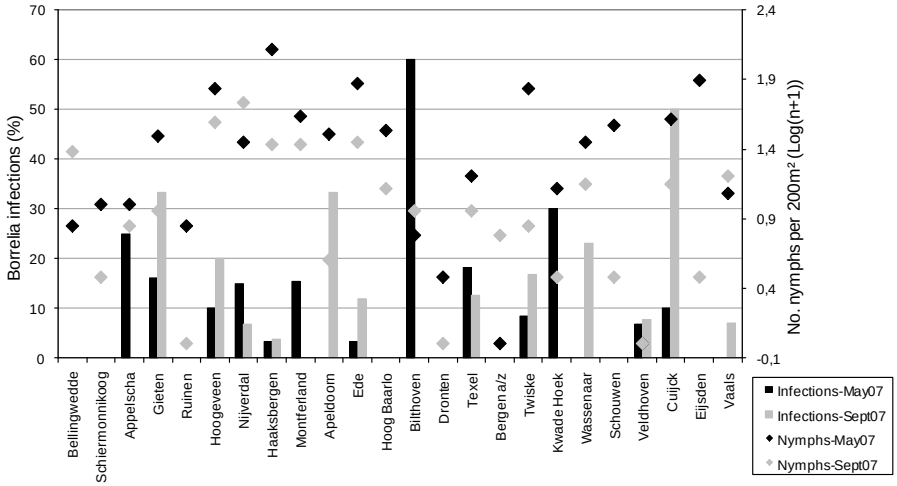


Figure 3.3. *Borrelia* infections of nymphal *Ixodes ricinus* collected in 24 study sites in May and September 2007 Black bars represent infection percentage in May 2007, grey bars represent infection percentage in September 2007. Nymphal densities are represented by black and grey diamonds for May and September 2007 respectively.

Borrelia burgdorferi *sensu lato infection rates*

The average infection rate with *Borrelia burgdorferi* s.l. of all nymphal ticks combined in May 2007 was 9.1% (n=421) and in September 2007 10.3% (n=275). The range of *Borrelia* infections in May was 3.3 - 60.0% and in September 3.9 - 50.0%. In eight of the 24 sites, no infected ticks were detected (Fig. 3.3). In seven sites, nymphs contained *Borrelia* only in one of these two months, while nymphs from nine sites had *Borrelia* infections in both months. Nymphs from four sites had *Borrelia*-positive ticks in May, but none in September. Three sites had only *Borrelia*-positive nymphs in September. The

mean *Borrelia* infection rate of all sites was similar in May and September 2007 (P=0.885). Infection rates in these months differed between sites, although these were at the border of significance (P=0.051) (Fig. 3.3). Furthermore, there was no relationship between *Borrelia* infection rate and tick density (P=0.264) (Fig. 3.4).

Considering the two months in which *Borrelia* infections were examined in nymphal ticks from all 24 study sites, there was a highly significant negative effect of tree cover on *Borrelia* infection rate of nymphal *I. ricinus* (P<0.001); the more open the forest, the more ticks were infected. A similar effect was observed on the pooled data of the 10 sentinel sites over the 18-month study period.

Table 3.3. *Borrelia* species distribution in *I. ricinus* collected in 10 sentinel locations from July 2006 to December 2007. Different letters in the last column indicate significant differences in *Borrelia* species diversity (loglinear regression: p<0.001).

Location	Number of ticks examined	Average % of ticks infected	*B. afzelii*	*B. garinii*	Sensu lato only	*B. burgdorfgeri sensu stricto*	*B. valaisiana*	*B. ruski* (*B. afzelii* like)	no. with co-infections	Co-infection (no.)	Significance of *Borrelia* diversity
Ede	329	15.5	43	3	3	1	4	0	3	Bg+Bs(1) Bg+Bv(2)	a
Eijsden	105	10.5	8	0	0	1	2	0	0	-	a
Gieten	135	19.3	14	6	3	2	1	1	2	Bg+Ba(1) Bg+Br(1)	b
Haaksbergen	174	7.5	4	1	2	5	1	0	0	-	b
Hoog Baarlo*	193	6.7	4	3	1	1	4	0	0	-	b
Kwade Hoek	119	38.7	41	1	1	1	7	0	5	Bv+Ba(4) Bg+Ba(1)	a
Ruinen	102	9.8	8	1	0	1	0	0	0	-	a
Twiske	167	13.2	14	0	1	3	3	0	0	-	a
Veldhoven	273	8.8	23	0	1	0	0	0	0	-	a
Wassenaar	47	4.3	2	0	0	0	0	0	0	-	a
Total examined and infected	1,644		161	15	12	15	22	1	10		
% infected of all sites combined		13.1									
Frequency (%) of *Borrelia* genospecies			71.2	6.6	5.3	6.6	9.7	0.4	4.4		

* 13 infections in 2006, no infections in 2007

Considering the 10 sentinel sites only, where nymphal ticks had been examined for *Borrelia* infections at 8-week intervals from July 2006 to December 2007, the mean *Borrelia* infection rate over 18 months was 13.7%

(n=1,644) and differed significantly between sites (P<0.001) (Table 3.3). There was a significant difference between infection rate for corresponding months in 2006 and 2007 (Fig. 3.5; P<0.001). No *Borrelia* infections could be detected in the nymphal ticks collected in January 2007 (n=22). In March and May 2007, *Borrelia* infections rates were 1.3% and 6.1%, respectively. Remarkably, the *Borrelia* infection rate of nymphal ticks from Hoog Baarlo in 2006 was 16.0% (n=58), while in 2007 it was 0% (n=112).

In the 10 sentinel sites, four *Borrelia* genospecies were identified (Table 3.3). *B. afzelii* was the dominant species in the complex (71.2%, P<0.001), and *B. ruski* [a *B. afzelii*-like isolate (Alekseev et al. 2001, Wielinga et al. 2006)] the most uncommon, being found on one occasion only. Twelve *Borrelia* infections (5.3%) could not be identified at species level. The distribution of *Borrelia* genospecies over the study sites was heterogeneous, but differed significantly between sites (P<0.001) (Table 3.3). For example, *Borrelia burgdorferi* s.s. was the main genospecies in Haaksbergen (Site #8) whereas *B. valaisiana* was more abundant in Kwade Hoek (site #18) than in other sites and *B. garinii* prevailed in Gieten and Hoog Baarlo. Infections with two *Borrelia* genospecies were found in 4.4% of all ticks examined (Table 3.3).

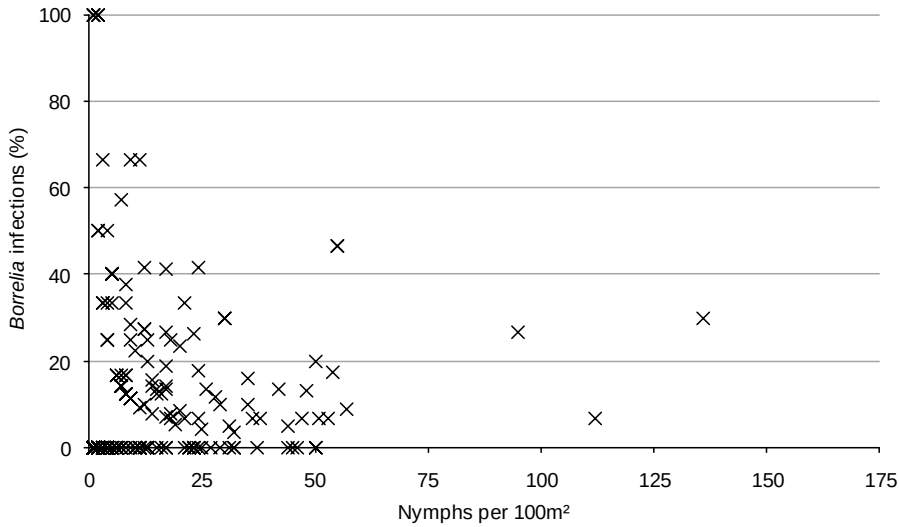


Figure 3.4. Relationship between *Borrelia* infection rates of nymphal ticks and tick density. Data points represent monthly nymphal densities on 100 m² transects, where at least one nymph was collected and analysed (n=215 data points).

Discussion

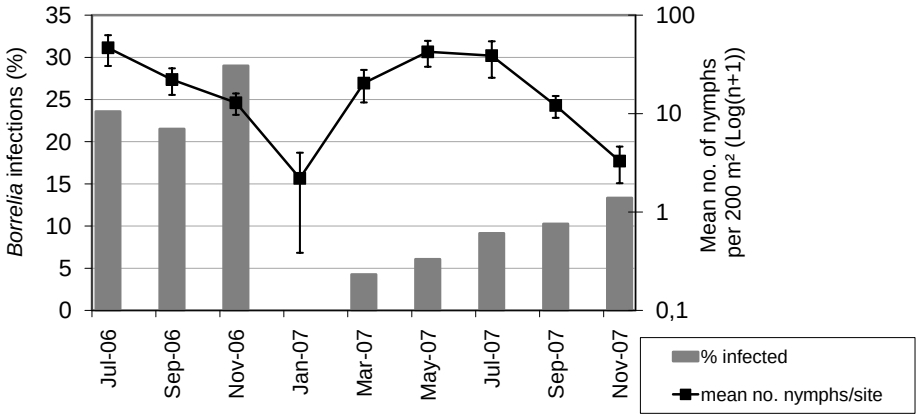
The present study provides, for the first time in The Netherlands, a country-wide and seasonal overview of *I. ricinus* populations and their corresponding *B. burgdorferi* s.l. infections. *Ixodes ricinus* was found at all 24 study sites, but with a large spatial and temporal variation. This is not surprising, because *I. ricinus* depends on several factors that determine habitat suitability. Vegetation structure, micro as well as macro climate and host composition and

abundance can affect not only tick density, but also the proportion of ticks infected with *Borrelia* (Eisen et al. 2006, Gray et al. 2009, Gray et al. 1998). European studies having extensive geographical coverage are scarce, but have taken place in the UK (Pietzsch et al. 2005, Scharlemann et al. 2008), Switzerland (Jouda et al. 2004) and Sweden (Jaenson et al. 2009). The latter authors concluded that the distribution of *I. ricinus* in Sweden corresponds to the distribution of deciduous trees other than birch or aspen, and that proliferation of ticks is aided by availability of deciduous vegetation that is favourable to mammals and birds, which serve as hosts for the ticks. From these and our own study it became evident that *I. ricinus* has a wide distribution within the northern temperate climate zone, and that most forest ecosystems below 1600 m in altitude are suitable for *I. ricinus* (Estrada-Pena et al. 2006).

There was a strong seasonal variation in questing tick abundance, with a single peak in early June, and lowest abundance in January. This is not surprising, as summer climate is favourable for *I. ricinus* and historic winter temperatures in western Europe were generally too low for tick activity. However, the relatively high numbers of ticks caught between December 2006 and February 2007 (Fig. 3.2) challenge this paradigm. The winter of 2007 was relatively warm (http://www.knmi.nl/, accessed 22 January 2010) so that a portion of the tick population remained active. Dautel et al. (2008) similarly reported unusually high tick activity in Germany throughout January/February and ascribed this to the extremely mild winter of 2006/2007. It has been suggested that climate change has contributed to this shift in seasonal dynamics (Gray et al. 2009, Lindgren and Gustafson 2001).

There is some evidence that the abundance of ticks has increased in the UK (Scharlemann et al. 2008) and in The Netherlands (Hofhuis et al. 2006) in recent years, but because reliable historical data on tick abundance and distribution are missing, we can only suggest that, based on the growing trend in tick bites on humans in The Netherlands and on red grouse in the UK, tick populations may be escalating. Possible reasons for this are expansion of nature reserves, increased abundance of wildlife, the continuing reduction in the use of pesticides in agriculture and forestry (Horne and Fielding 2002) and climate change (Gray et al. 2009).

In the selection of our study sites, we gave preference to areas characterized as coniferous/decidious woodland and/or nature reserve, as these were considered to be prime biotopes for *I. ricinus* in similar ecozones of Europe (Estrada-Pena et al. 2006, Jaenson et al. 2009). In our study, large variation in tick abundance between the 24 sites was found, with consistent associations between tick density and sites. Diuk-Wasser et al. (2006) report similar phenomena from the USA, where abundance of the black-legged tick *I. scapularis* appeared associated with specific sites, having different host abundances. In Europe, similar associations between sites and tick density were also reported (e.g., Jouda et al. 2004). In our study, habitat characteristics varied considerably between the 24 sites. These habitat differences can facilitate different microclimate conditions and variable host abundance and diversity. Thickness of litter layer and cover of the moss layer were the only



	Jul-06	Sep-06	Nov-06	Jan-07	Mar-07	May-07	Jul-07	Sep-07	Nov-07
No. nymphs analyzed	466	178	129	22	204	425	388	121	33
% infected	23.6	21.5	29.0	0.0	4.3	6.1	9.1	10.3	13.3
Range of infections (%)	0-46.6	0-40	0-100	0.00	0-8.3	0-30	0-25	0-33.3	0-20

Figure 3.5. Bimonthly mean number. of nymphs collected per 200 m^2 of habitat and the corresponding *Borrelia* infection rate (%) from 10 sentinel sites in The Netherlands between July 2006 and December 2007. Error bars represent standard errors of the mean.

measured parameters that were positively correlated with tick density. This confirms that these are essential for tick survival since they serve as refuge for ticks during periods of unsuitable climatic conditions (Randolph and Storey 1999) and shelter tick eggs and blood fed moulting ticks during the largest part of their life. In addition, macroclimatic conditions in The Netherlands could explain differences in tick phenology between inland and coastal areas (Smit et al. 2003). However, these differences are considered marginal in explaining differences in tick population size (Gray, 1991) and were not included in our study.

Apart from habitat characteristics, host composition and abundance may also affect tick populations. In western Europe, *I. ricinus* has been found to feed on a wide variety of mammals and birds (Humair et al. 2007, Matuschka et al. 1991). In particular, small rodents (mice and voles) and birds are important hosts for larval stages. There are no published data on small mammals for our study sites, although several recent studies in The Netherlands showed that mice and voles are abundant in many locations (Gassner et al. 2008, Jagers op Akkerhuis et al. 2003, Smit et al. 2003). Similarly, roe deer are present at most study sites (S. van Wieren, pers. comm.), and are considered to be a primary host for nymphal and adult *I. ricinus* (Pichon et al. 2006, Tälleklint and Jaenson 1997, Vor et al. 2010). We conclude that overall, none of the study sites were unsuitable for *I. ricinus*, and that the large variations in abundance between the sites was most likely due to variation in the composition of the vegetation and abundance and species composition of host animals.

The observed differences in *Borrelia* infections, with variations from 3 to 60% on some monthly sampling occasions, fall within data reported from

elsewhere in western Europe (Rauter and Hartung 2005). Although there was no significant correlation between *Borrelia* infection rate and study site for the 24 sites in May and September 2007, the 10 sentinel sites, where ticks were analysed more frequently, can be grouped into classes with medium to high *Borrelia* infections (e.g., Kwade Hoek, Gieten, Ede) and those with low to medium *Borrelia* infections (e.g., Hoog Baarlo, Haaksbergen, Wassenaar). A high proportion of the rodent hosts in Kwade Hoek is infected with *Borrelia* spp. (F.G., unpublished data), which may explain why on some occasions more than 50% of nymphal ticks collected at this site were infected. On several sampling occasions *B. burgdorferi* s.l. was not found in ticks. This could have been due to the fact that the number of ticks sampled may have been too low to find infections. Also, annual fluctuations in *Borrelia* infection rates of ticks are common (Mejlon, 1993, Eisen et al. 2004, Wielinga et al. 2006) and may explain the absence of *B. burgdorferi* s.l. from these sites.

Four genospecies of *B. burgdorferi* s.l. were present, with *B. afzelii* being the most abundant. This suggests that *I. ricinus* in The Netherlands frequents small rodents, these being the most important reservoir host of *B. afzelii* (Hanincova et al. 2003, Piesman and Gern 2004, Richter et al. 2004). In Kwade Hoek, a relatively high frequency of *B. valaisiana* was found, which suggests that in this area birds may be an important host for *I. ricinus* next to the available rodents (Hanincova et al. 2003, Taragel'ova et al. 2008). At several other sites, the *Borrelia* species diversity was different from the national trend (Table 3.3). The reasons for these differences in species diversity may be related with the fact that several different mammalian and avian host species are involved in the *Borrelia* life cycle (Gern, 2008, Kurtenbach et al. 1998). The 12 *Borrelia* isolates that could not be further identified to genospecies, may possibly comprise European *Borrelia* species that could not be detected by the set of probes used in our RLB, such as *B. spielmanii* and *B. bavariensis*. Co-infections of *Borrelia* spp. in *I. ricinus* were infrequent and associated with study sites that had the highest *Borrelia* infection rates.

The present study supports the reported high incidence of early symptoms of Lyme borreliosis in The Netherlands (Hofhuis et al. 2006) by the fact that relatively high abundances of *Borrelia*-infected ticks were found in many study sites. The 24 sites investigated in this study are representative of many natural biotopes in the country and are all accessible to the general public. With *Borrelia* infection rates in ticks varying from 3–60%, ticks that attach to the body are potential sources of *Borrelia* infections.We could not identify a singular factor that serves as prediction high risk areas for *Borrelia* transmission, although tick abundance was in many cases determined by habitat type, especially thickness of the leaf litter and moss cover, and infection rates were associated with tree cover. The results from this study suggest that more accurate predictions of tick abundance and the associated risk of Lyme borreliosis, including the level of human exposure to infected ticks, are needed to improve the quality of public health risk monitoring programmes. In particular the factors that cause strong annual variation in *Borrelia* infection rate of *I. ricinus* deserve specific attention.

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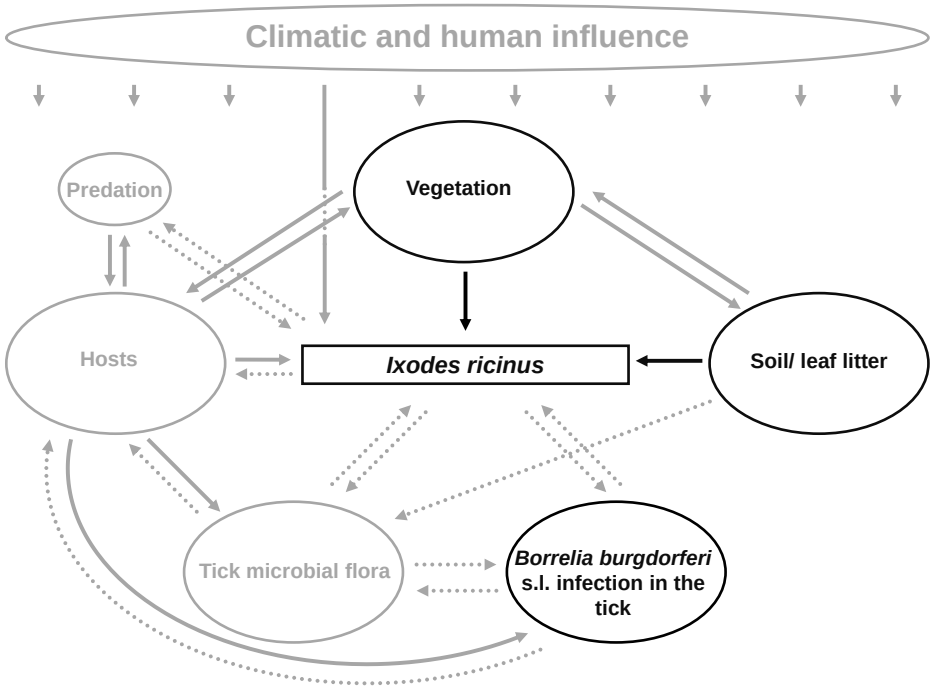
Chapter 4

Diversity of *Ixodes ricinus* tick-associated bacterial communities from different forests.

Leo S. van Overbeek, **Fedor Gassner**, Carin Lombaers van der Plas, Pieter Kastelein, Ulisses Nunes-da Rocha and Willem Takken



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Abstract
Nymphal *Ixodes ricinus* ticks (n=180) were collected from three different areas in The Netherlands to investigate the effect of forest composition on tick-associated microbial communities. Sampled habitats differed in thickness of leaf litter and humus layers and vegetation associations and were located near Amsterdam (Beech-Oak), Ede (Birch-Oak) and Veldhoven (Birch-Oak). Analysis of nine 16S rRNA gene clone libraries made from individual ticks showed nearest matches with presumed pathogens *Candidatus* NeoEhrlichia mikurensis and *Rickettsia australis* and arthropod endosymbionts *Wolbachia pipientis* and *Candidatus* Midichloria mitochondrii. Total bacterial species diversity (Shannon index) and *Borrelia* species infections were determined in *I. ricinus* by, respectively, PCR-denaturing gradient gel-electrophoresis and PCR-reverse line blot with probes specific for *B. burgdorferi sensu stricto*, *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. lusitaniae* and *B. ruski*. Bacterial diversity differed significantly per area and was lowest in Ede. In contrast, *Borrelia* species-infected ticks were more abundant in Ede, *Candidatus* NeoEhrlichia mikurensis-infected ticks in Ede and Veldhoven, and *R. australis* infected ticks in Amsterdam. *B. afzelii* was the most common *Borrelia* species found in all three areas. Bacterial tick diversity was influenced by local differences in forest structure, which is proposed to modulate animal populations that are commonly parasitized by *I. ricinus*.

Introduction

Lyme borreliosis is a widespread phenomenon occurring in many different countries, especially in the Northern Hemisphere. The causative agents of Lyme borreliosis belong to a group of related *Borrelia* species within the class of Spirochaetes that are transmitted mainly via ticks of the genus *Ixodes*. Major agents causing disease in humans are *Borrelia burgdorferi sensu stricto* (ss), *B. afzelii* and *B. garinii*. Other *Borrelia* species closely related to this group are *B. valaisiana*, *B. lusitaniae*, *B. spielmanii* and *B. bisettii*. Currently, this group consists of at least 11 different species. Some of these species are also suspected to be human pathogens. The principal vector of *Borrelia* species in Europe is *Ixodes ricinus* (Rauter & Hartung, 2005). The prevalence of *Borrelia* species infections as well as the species composition in *I. ricinus* ticks are highly variable in time and space. *Borrelia afzelii*, for example, is often the most prevalent species found in *I. ricinus* (Rijpkema et al. 1995, Schouls et al. 1999, Jenkins et al. 2001, Fraenkel et al. 2002, Kipp et al. 2006, Wielinga et al. 2006). However, in some studies prevalence is lower or this species is even absent (Nohlmans et al. 1995, Kirstein et al. 1997, Kurtenbach et al. 1998, De Michelis et al. 2000, Lenčová et al. 2006, Fingerle et al. 2007).

The location and presence of vertebrate animals that are parasitized by *I. ricinus* determine which *Borrelia* species prevails (Kurtenbach et al. 2006). *Borrelia garinii* and *B. valaisiana* were more common in *I. ricinus* ticks feeding on birds (Kurtenbach et al. 1998, Comstedt et al. 2006, Kipp et al. 2006) and B. *lusitaniae* in ticks feeding on lizards (Richter et al. 2006, Amore et al. 2007). Small rodents play an important role in the transmission of B. *afzelii*, and birds and rodents in the transmission of B. burgdorferi ss from and to *I. ricinus* (Gray 1998, Kurtenbach et al. 2002, Ginsberg et al. 2005, Rauter and Hartung 2005). The geographic distribution of different *Borrelia* species in *I. ricinus* largely depends on local environmental factors. In particular, variations in populations of vertebrate animals that are parasitized by *I. ricinus* play an important role (Kurtenbach et al. 2006, Gassner and van Overbeek 2007).

Other bacterial species are also commonly found in *I. ricinus*, some of which are pathogenic to humans as well. *Ehrlichia* species were found in *I. ricinus* ticks collected in Germany (Hildebrandt et al. 2002), Norway (Jenkins et al. 2001, Stuen et al. 2006), Poland (Stańczak et al. 2004), The Netherlands (Schouls et al. 1999, Wielinga et al. 2006), Tunisia and Morocco (Sarih et al. 2005) and Switzerland (Leutenegger et al. 1999). The taxonomy of *Ehrlichia* and *Anaplasma* species has been changed (Dumler et al. 2001) and many species previously classified as *Ehrlichia* nowadays belong to *Anaplasma*. *Anaplasma* (*Ehrlichia*) *phagocytophilum* is the most commonly found *Anaplasma* species in *I. ricinus*. An *Ehrlichia*-like species (Schotti variant; Pan et al. 2003) was found in *I. ricinus* in The Netherlands (Schouls et al. 1999) and in rats and *I. ovatus* ticks in Japan and was renamed as *Candidatus* NeoEhrlichia mikurensis (Kawahara et al. 2004). Other species detected in *Ixodes* ticks are *Bartonella* species (Schouls et al. 1999), *Coxiella burnetii* (Movila et al. 2006), *Francisella tularensis* (Wicki *et al*. 2000), *Pasteurella* species (Stojek and Dutkiewicz 2004), *Rickettsia* species (Benson et al. 2004), *Wolbachia pipientis* (O'Neill et al. 1992, Benson et al. 2004) (Sarih et al. 2005) and *Candidatus* Midichloria mitochondrii (Beninati et al. 2004, Sassera et al. 2006). Bacteria belonging to the group of Gammaproteobacteria were isolated

on semi-selective media from *I. ricinus* ticks collected in Poland (Stojek and Dutkiewicz 2004). These culturable bacteria taxonomically differ from the species commonly observed with molecular techniques. It is likely that different taxonomical groups in ticks can be accessed by application of different techniques.

Many bacteria present in *I. ricinus* are difficult to culture or are even unculturable. This makes characterization of bacteria present in ticks a daunting task. Molecular detection techniques are most suitable to determine which species are present in ticks. PCR-reverse line blot (RLB) hybridization of *Borrelia* species-specific oligonucleotides with *Borrelia* genus-specific PCR amplicons made from total tick extracts is a commonly applied method to screen for the presence of different *Borrelia* species in ticks (Rijpkema et al. 1995). Later, probes specific for other species were included in the PCR-RLB set-up to allow screening for other pathogens as well (Gubbels et al. 1999, Schouls et al. 1999, Georges et al. 2001, Christova et al. 2003, Wielinga et al. 2006). PCR-RLB is most suitable for the screening of different bacterial groups expected to be present in ticks. DNA sequencing of random clone libraries made from PCR amplicons of tick DNA extracts is an option to screen for dominant bacterial groups present in *Ixodes* species (Benson et al. 2004). The advantage of DNA sequencing of clone libraries over PCR-RLB is that it allows detection of the most dominant species present, including those that had not been associated with *I. ricinus* previously. Molecular fingerprinting techniques in combination with 16S rRNA gene sequencing were applied to determine species diversity in ticks and to elucidate the most dominant groups present in ticks (Schabereiter-Gurtner et al. 2003, Halos et al. 2006, Moreno et al. 2006). Molecular finger-printing techniques can be used to investigate shifts in microbial communities in *I. ricinus*, e.g., from individuals collected at different locations.

The prevalence of many other pathogenic and nonpathogenic bacteria present in *I. ricinus* varies depending on geographic location, as for *Borrelia* species. Local environmental factors affecting the survival and establishment of different groups of animals that can act as hosts for *I. ricinus* will influence the microbial community composition in *I. ricinus*. The aim of this study was to investigate to what extent geographically separated and structurally different habitats can influence bacterial composition in *I. ricinus*. From other studies, it is already known that different habitats can influence the distribution of different pathogens in *I. ricinus* (Kirstein *et al*. 1997, Rauter and Hartung 2005, Wielinga *et al*. 2006). It is, however, unknown whether this is the case for other bacterial groups present in *I. ricinus*. Information about environmental factors affecting bacterial communities in *I. ricinus* is important to elucidate factors that contribute to the transmission of pathogens from vertebrate animals to *I. ricinus*. The establishment and survival of pathogens in *I. ricinus* is expected to be mediated by other microorganisms residing in ticks. The present study focuses on potential differences in bacterial communities in ticks collected from different habitats in The Netherlands using PCR-denaturing gradient gel-electrophoresis (DGGE). Concomitantly, these ticks were also screened for the presence of *Borrelia* species by PCR-RLB. From a selection of these ticks, the most dominant bacterial groups were identified by 16S rRNA gene PCR amplification, sequencing and BLAST -assisted database searches.

Materials and methods

Location and characterization of tick sampling areas

Three forest habitats from different places in The Netherlands were selected on the basis of frequent reports on the presence of *I. ricinus* ticks. These areas were Amsterdam water supply dunes (A), Ede, deSysselt (E) and Veldhoven, Hoogeind (V). Within each area, two locations covering an area of 20 m x 70 m were chosen for tick collections and the GPS coordinates were: W 9° 98.63', N 48° 39.64' for A1; W 10° 01.18', N 48° 46.67' for A2; W 17° 56.71', N 44° 88.93' for E1; W 17° 58.45', N 44° 88.02' for E2; W 15° 13.45', N 38° 16.36' for V1 and W 15° 02.19', N 38° 16.48' for V2. For each location, local vegetation was described by estimating the coverage of different layers of plant species. Three vegetation layers were considered as most relevant: a soil covering layer containing mosses, grasses and herbs, an intermediate layer containing all shrubs and a tree layer. Vegetation type was classified in accordance with a modified system of Braun-Blanquet (1928), described in Stortelder et al. (1999) which is a common system to classify different vegetation types into associations.

Table 4.1. Forest floor characteristics of three different natural areas in The Netherlands used for *Ixodes ricinus* tick collections.

		Humus layer**				Leaf litter layer			
Location*	Soil type	Thickness (cm)	Water content (%)	Organic matter content (%)	pH	Thickness (cm)	Water content (%)	Organic matter content (%)	pH
A1	sand	25[b]	1.0	5.8[b]	4.2[b]	7	6.1	58	4.7
A2		25[b]	0.6	4.2[b]	4.2[b]	7	6.3	60	4.7
E1	loamy sand	10[a]	1.0	9.8[c]	3.7[a]	4	6.7	72	4.3
E2		12[a]	1.0	8.1[c]	3.7[a]	7	7.6	92	4.3
V1	sand	26[b]	1.8	1.8[a]	4.1[b]	6	7.6	78	4.7
V2		26[b]	2.0	2.2[a]	4.4[b]	9	6.3	73	4.4

*A, Amsterdam water supply dunes; E, Ede, deSysselt; V, Veldhoven, Hoogeind; 1 location 1; 2 location 2.

*Statistically different; a < b < c; P < 0.01.

Forest floor composition at all six locations was determined by measuring leaf litter and humus layers at four different places per location. Composite samples of 100 g per location were drawn, both from the leaf litter and from the humus layers. From all samples pH was measured in suspensions made of 20 g subsamples in 20 ml demineralized water. Dry weights and organic matter contents were determined from 10 g subsamples that were heated overnight at 110 and 550 1C, respectively. Differences in weight before and after heat treatments were used for calculations of percentages of dry weight and organic matter content.

Collection of ticks

Ticks were collected on three successive days during daytime (between 8 am and 1 pm) in October 2006. The period before sampling was relatively warm and dry for over one month with daily oscillating temperatures between 18 and 22 °C during the day, and 8 and 15 °C at night. In each location four tracts of 50

m were chosen, with a maximum distance of 2 m between each tract. Ticks were collected by dragging a 1 m^2 white cotton blanket over the vegetation layer, and in each location, a total of 200 m^2 was sampled. Every 25 m, the blanket was visually inspected for the presence of ticks. The ticks were removed from the blanket and transferred into 70% ethanol. The developmental stage of each tick was determined and all ticks were stored in vials containing 70% ethanol at 4 °C for no longer than 1 month before DNA extraction and molecular analyses.

DNA extraction from ticks

Total DNA was extracted from 30 individual nymphal ticks per location, resulting in a total of 180 ticks from all six locations. Total DNA was extracted according to the procedure described in Schouls et al. (1999). Briefly, ticks were removed from the vials and dried for 20 min on tissue paper. Individual ticks were then transferred to Eppendorf tubes containing 100 µl of 0.7 M NH_4 OH, boiled for 20 min and immediately chilled on ice. Tubes were centrifuged at 10.000 g for 20 s and heated at 90 °C with open caps for 20 min to allow ammonia to evaporate. Supernatants containing tick DNA were immediately stored at 80 °C until further analyses.

DNA amplification and molecular analysis

Total tick DNA was amplified with Phi 29 (Amersham Biosciences, NJ, USA) prior to further analyses. Therefore, 0.5 µl (approximately 5 ng of DNA) of tick DNA extract was included in a volume of 4.5 µl reaction mixture with Phi 29 and amplified, all according to the protocol provided by the manufacturer.

Construction of tick clone libraries and database comparisons.

Phi 29-amplified tick DNA samples from nine ticks (three from each area) were PCR amplified using primers 27F and 1492R (Rochelle et al. 1992) in 50 µl PCR mixtures containing 200 nM of each primer, 100 µM of each dNTP, 1x Super Taq PCR buffer (HT Biotechnology, Cambridge, UK) and 2 units of Super Taq DNA polymerase (HT Biotechnology). PCR conditions were: one cycle at 94 °C, 4 min (initial denaturation step); 35 cycles at 94 °C, 1 min (denaturation); 55 °C, 1 min (annealing); 72 °C, 2 min (primer extension) and one cycle at 72 °C, 5 min (final extension step). PCR products were purified and checked for their expected length of about 1,500 bp in agarose gels stained with ethidium bromide. Purified PCR products were then cloned into the pGEM-T vector (Promega, Leiden, NL) and introduced into *Escherichia coli* JM109 by transformation, according to the protocol provided by the manufacturer. Clones with the expected of 1,500-bp inserts were subjected to sequencing with primer 1492R using the service of Greenomics, Plant Research International B.V., The Netherlands. The resulting sequences (approximately 450 bp DNA) were checked for chimeras using the Check Chimera tool and non-chimeric sequences were assessed for similarities to database sequences available at the NCBI web site (http://www.ncbi.nlm.nih.gov) by BLAST searching.

Table 4.2. Vegetation in three different natural areas in The Netherlands used for *I. ricinus* tick collections

		Percentage coverage and association*					
Plant species		A1	A2	E1	E2	V1	V2
Trees							
Scots Pine	*Pinus sylvestris*			60			5
Down Birch	*Betula pubescens*			20	20		
Common Beech	*Fagus sylvatica*			<5	20		
Pedunculate Oak	*Quercus robur*	50	50	20	20	40	40
Sycamore	*Acer pseudoplatanus*		10				
Shrubs							
Norway Spruce	*Picea abies*			<5			
European Larch	*Larix decidua*			0	<5		
Rowan	*Sorbus aucuparia*		< 5	20	<5	<5	
Black Cherry	*Prunus serotina*	10	10	20	20	5	5
Blackberry	*Rubus fruticosus*					5	
Honeysuckle	*Lonicera periclymenum*					5	
Herbs							
Lady Fern	*Athyrium filix-femina*			<5	<5		
Common Bracken	*Pteridium aquilinum*	20	20				
Sheep Fescue	*Festuca ovina*			40	40		90
Purple Moore Grass	*Molinea caerulea*					90	10
Blueberry	*Vaccinium myrtillus*			40	40		
Mosses							
Schreber's Moss	*Pleurozium schreberi*	< 5	10	60	5	15	15
Cap Moss	*Polytrichum commune*						<5
Vegetation association, subassociation							
Fago-Quercetum roboris pteridietosum		X	X				
Betulo-Quercetum roboris vaccinietosum				X			
Betulo-Quercetum roboris cladonietosum					X		
Betulo-Quercetum roboris molinietosum						X	
Betulo-Quercetum roboris cladonietosum							X

*A, Amsterdam water supply dunes; E, Ede, deSysselt; V, Veldhoven, Hoogeind; 1, location 1; 2, location 2.

PCR-DGGE analysis.

PCR amplifications were performed on 100 ng of Phi 29-amplified tick DNA samples in standard 50µl reaction mixtures according to Van Elsas & Wolters (1995), using primers 968F, with GC clamp (Muyzer et al.,1993), and 1401R, both directed towards the 16S ribosomal RNA gene region V6 (Heuer et al.,

1997; Heuer & Smalla, 1999). PCR amplification was performed in a PTC-100 (MJ Research Inc., Ma, USA) thermocycler. PCR products were checked for the expected length of 450 bp in 0.8 % agarose gels and bands were visualized under UV after staining with ethidium bromide. For DGGE, polyacrylamide gels (6%) were prepared with denaturing gradients of 45 – 65 % (100 % denaturant consists of 7 M Urea and 40 % formamide). Gels were loaded with 15 µl volumes prepared by mixing 10 µl PCR product (approximately 200 ng) with 5 µl loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol FF, 30 % glycerol). All gels were run in a PhorU2 apparatus (Ingeny, Goes, NL) at 60 °C, 100 V for 15 h. After running, gels were stained with SYBR gold (Molecular Probes, Leiden, NL) and fingerprints from individual lanes were analysed using the Molecular Analyst software (version 1.61, BioRad, Veenendaal, NL). Normalization of the gels was achieved using a marker, consisting of 16S rRNA gene amplicons from *Enterobacter cloaceae* BE1, *Listeria innocua* ALM 105, *Arthrobacter* sp. AR1 and *Burkholderia cepacia* P2, which was loaded at three different positions in the gel. Migration distance and intensity of individual bands in the fingerprints were chosen as the parameters used for calculation of Shannon diversity index (H'). The Shannon diversity index combines diversity and evenness of bacterial species and increases with the number of species (individual bands in fingerprints) and the more even distribution of biomass (total band size) over the different bacterial species in each sample.

PCR-DGGE fingerprints from all 180 ticks were inspected for the presence of specific bands comigrating with PCR-amplified products of clone inserts that gave nearest matches with *Candidatus* Neoehrlichia mikurensis and *R. australis* (see later) and with PCR products of 16S rRNA genes of *B. burgdorferi ss*, *B. afzelii*, *B. garinii* and B*. valaisiana*. Ticks containing one or more of these bands in their fingerprints were scored positive for the presence of these species.

Reverse line blot

For detection of different *Borrelia* species in ticks, the PCR-RLB procedure described in Rijpkema et al. (1995) and adapted according to Wielinga et al. (2006) was applied with two modifications. These modifications were: (1) use of primers B-5Sbor modified (biotin-5'-ATGGTACTGCGAGTTCGCGG-3') and 23Sbor modified (5'-TTTCGCTCGCCACTACTAAGG-3') to generate *Borrelia* genus-specific PCR products from Phi 29-amplified tick DNA samples, and (2) chemiluminescence detection and signal quantification from hybridized filters with a chemidoc XRS camera and accompanying software (Bio-Rad Laboratories, Veenendaal, The Netherlands). These modifications were applied to reduce nonspecific annealing of primers and to improve signal sensitivity from hybridized filters.

Statistical analyses

Statistical analyses were performed on (1) thickness, water content, organic matter content and pH of humus and leaf litter layers, with two replicates per area; (2) *Borrelia* species-, *Candidatus* NeoEhrlichia mikurensis- and *R. australis*-infected ticks, with two replicates per area; and (3) bacterial diversity and evenness (Shannon index, *H'*) in individual ticks deter mined by PCR-DGGE, containing 30 replicates per location. Comparisons in thickness, water

content, organic matter content, pH, infected tick numbers and diversity (H') values between different locations and areas were made by ANOVA. (GenStat 9.1, Rothamsted Experimental Station, UK), and least significant difference (LSD) values were calculated.

Results

Forest floor and vegetation characteristics of the study areas

Amsterdam water supply dunes. The soil in A was characterized as sand (dune sand). The thickness of humus and leaf litter layers were, respectively, 25 cm and 7 cm at both locations. In the humus layer, water content was higher in A1 (1.4 %) than in A2 (0.6 %), also was organic matter content (5.8 % in A1 and 4.2 % in A2), whereas pH was the same at both locations, 4.2. In the leaf litter layer water content in A1 was lower (6.1 %) than in A2 (6.3 %), also was organic matter content (58% in A1 was and 60 % in A2), whereas pH was the same at both locations, 4.7 (Table 4.1). The vegetation layer covering the forest floors at both locations in A consisted of Schreber's Moss (*Pleurozium schreberi*; < 5% in A1 and 10% in A2) and Common Bracken (*Pteridium aquilinum*; 20% at both locations). Rowan (*Sorbus aucuparia*) only occurred in the shrub layer of A2 (<5 %) and was absent in that of A1, whereas Black Cherry (*Prunus serotina*) was present at both locations (10 %). Sycamore (*Acer pseudoplatanus*) was only present in the tree layer in A2 (10 %) and absent in A1, whereas Pedunculate Oak (*Quercus robur*) was present at both locations (50 %). Vegetation association at both locations was *Fago-Quercetum roboris pteridietosum* (Beech-Oak association, subassociated with Common Bracken) (Table 4.2).

Ede, de Sysselt. The soil in E was characterized as loamy sand. The thickness of humus layers were different at both locations (10 cm in E1 and 12 cm in E2), also were the leaf litter layers (4 cm in E1 and 7 cm in E2). In the humus layers at both locations water content was the same (1.0 %), organic matter content was higher in E1 (9.8 %) than in E2 (8.1 %) and pH was the same at both locations, 3.7. In the leaf litter layer, water content in E1 was lower (6.7 %) than in E2 (7.6 %), organic matter content in E1 was lower (72 %) than in E2 (92 %) and pH was the same at both locations, 4.3 (Table 4.1). The vegetation layer covering forest floors at both locations consisted of Schrebers's Moss (60 % in E1 and 5 % in E2), Blueberry (*Vaccinium myrtillus*; 40 % at both locations), Sheep Fescue (*Festuca ovina*; 40 % at both locations) and Lady Fern (*Athyrium filix-femina*; < 5 % at both locations). Black Cherry (20 % at both locations), Rowan (20 % in E1 and < 5 % in E2), European Larch (*Larix decidua*; < 5 % in E2), and Norway Spruce (*Picea abies*; < 5 % in E 1) were the plants present in the shrub layers. Pedunculate Oak (20 % at both locations), Common Beech (*Fagus Sylvatica*; < 5 % in E1 and 20 % in E2), Down Birch (*Betula pubescens*; 20 % at both locations) and Scots Pine (*Pinus Sylvestris*; 60 % in E1) were present in the tree layers. Vegetation associations at both locations were *Betulo-Quercetum roboris vaccinietosum* (Birch-Oak association, subassociated with blueberry; E1) and *Betulo-Quercetum roboris cladonietosum* (Birch-Oak association, subassociated with mosses; E2) (Table 4.2).

Veldhoven, Hoogeind. The soil in V was characterized as sand ('Enkheerd' soil). The thickness of humus layers were the same at both locations (26 cm) whereas that of the leaf litter layers differed (6 cm in V1 and in

Area[1]	Library[2]	Number of clones	Nearest match	Accession	Identity (%)	Nearest match type strain[3]	Accession	identity (%)
A	Ai	3	*Rickettsia* sp. IP1	AF394904	98	*Rickettsia australis* (T)	L36101	99
	Aii	3	*Rickettsia* sp. IP1	AF394904	99	*Rickettsia australis* (T)	L36101	99
	Aiii	2	*Wolbachia* sp. Avit16SWol	AY007549	98	*Wolbachia pipientis* (T)	X61768	94
		1	*Wolbachia* sp.	U83098	98	*Anaplasma phagocytophilum* (T)	AF172167	87
E	Ei	2	*Candidatus* Neoehrlichia mikurensis	AB213021	100	*Candidatus* Neoehrlichia mikurensis (T)	AB084582	100
		5	*Ehrlichia* sp. Belluno	AY098730	99	*Candidatus* Neoehrlichia mikurensis (T)	AB084582	99
	Eii	8	*Candidatus* Neoehrlichia mikurensis	AB213021	100	*Candidatus* Neoehrlichia mikurensis (T)	AB084582	100
	Eiii	1	*Candidatus* Neoehrlichia mikurensis	AB213021	100	*Candidatus* Neoehrlichia mikurensis (T)	AB084582	100
		1	*Ehrlichia* sp. Belluno	AY098730	100	*Candidatus* Neoehrlichia mikurensis (T)	AB084582	99
		1	*Candidatus* Midichloria mitochondrii	AJ566640	99	*Rhizobium vitis* (T)	X67225	89
		1	*Pelomonas soli*	EF660749	100	*Pelomonas saccharophila* (T)	AB021407	100
		1	Rickettsiales bacterium It86	AF525482	99	*Ensifer meliloti* (T)	X67222	88
V	Vi	4	*Candidatus* Neoehrlichia mikurensis	AB213021	100	*Candidatus* Neoehrlichia mikurensis (T)	AB084582	100
		5	*Ehrlichia* sp. Belluno	AY098730	99	*Candidatus* Neoehrlichia mikurensis (T)	AB084582	99
		1	*Acinetobacter calcoaceticus*	AY211134	99	*Acinetobacter calcoaceticus* (T)	ATCC 23055T	95
	Vii	7	*Candidatus* Neoehrlichia mikurensis	AB213021	100	*Candidatus* Neoehrlichia mikurensis (T)	AB084582	100
		11	*Ehrlichia* sp. Belluno	AY098730	99	*Candidatus* Neoehrlichia mikurensis (T)	AB084582	99
	Viii	7	*Candidatus* Neoehrlichia mikurensis	AB213021	100	*Candidatus* Neoehrlichia mikurensis (T)	AB084582	100
		13	*Ehrlichia* sp. Belluno	AY098730	99	*Candidatus* Neoehrlichia mikurensis (T)	AB084582	99

Table 4.3. Taxonomic identity of cloned 16S rRNA gene amplicons from Ixodes ricinus tick DNA extracts.

[1] A, Amsterdam water supply dunes; E, Ede, de Sysselt; V, Veldhoven, Hoogeind.

[2] i-iii, libraries made from individual ticks.

[3] Nearest matches with sequences present in public databases (http://www.ncbi.nlm.nih.gov), accessed by Blast searches.

V2 9 cm). In the humus layer water content in V1 was lower (1.8 %) than in V2 (2.0 %), organic matter content was lower in V1 (1.8 %) than in V2 (2.2 %) and pH was also lower in V1 (4.1) than in V2 (4.4). In the leaf litter layer water content in V1 was higher (7.6 %) than in V2 (6.3 %), organic matter content in V1 was higher (78 %) than in V2 (73 %) and pH was also higher in V1 (4.7) than in V2 (4.4) (Table 4.1). The vegetation layers covering the forest floors at both locations consisted of Schreber's Moss (15 % at both locations), Hair Cap Moss (*Polytrichum commune*; < 5 % in V2), Purple Moore Grass (*Molinea caerulea*; 90 % in V1 and 10 % in V2) and Sheep Fescue (90 % in V2). Black Cherry (20 % at both locations), Blackberry (*Rubus fruticosus*; 5 % in V1), Honeysuckle (*Lonicera periclymenum*; 5 % in V1) and Rowan (< 5 % in V1) were the shrubs, and Pendunculate Oak (40 % at both locations) and Scots pine (5 % in V2) the trees present in V. Vegetation associations at both locations were *Betulo-Quercetum roboris molinietosum* (Birch-Oak association, subassociated with Purple Moore Grass; V1) and *Betulo-Quercetum roboris cladonietosum* (Birch-Oak association, subassociated with mosses; V2) (Table 4.2).

Most striking differences between the three areas were: thickness of the leaf litter layer in E that was less than half of the size present in A and V and the organic matter content in the humus layer in E that was about the double the amount present A and V, whereas pH was lower in E than in A and V (Table 4.1). Diversity in vegetation was higher in E (13 plant species) than in V (10 plant species) and A (6 plant species) and vegetation association in A was different from those in E and V (Table 4.2).

Collection of I. ricinus *ticks*

In total 391 *I. ricinus* ticks were collected from the three areas in the following developmental stages: adult (40; 21 male and 19 female), nymph (292) and larvae (59). Highest tick numbers were collected from V (162), then from A (116) and finally from E (113). Large differences were found per location: V1 (106), E1 (71), A1 (60), A2 (56), V2 (56) and E2 (42). Number of nymphs collected from the different locations were: V1 (82), E1 (48), V2 (49), A2 (45), A1 (38) and E2 (30). Nymphs were most abundant among all collected ticks and only these were used for further analysis. From each location 30 nymphs were selected and subjected to PCR-DGGE and PCR-RLB and from each area three nymphs were selected for cloning of 16S rRNA gene amplicons.

Molecular identification of bacteria present in ticks

Clone libraries made from PCR amplified 16S rRNA genes of nine randomly selected ticks, three from each area, were used for identification of the most dominant species. Sequences of 77 clone inserts were compared with sequences present in databases for nearest matches with strains and type strains (Table 4.3). Among inserts from all nine clone libraries, nearest matches with nine different strains and eight different type strains were found. In 29 inserts from six clone libraries (Ei, Eii, Eiii, Vi, Vii and Viii) nearest matches (100 % similarity) with *Candidatus* NeoEhrlichia mikurensis were found and in 35 inserts from five libraries (Ei, Eiii, Vi, Vii, Viii) nearest matches (99 % similarity) with *Ehrlichia* sp. Belluno were found. Inserts that gave nearest matches with *Ehrlichia* sp. Belluno and with *Candidatus* NeoEhrlichia mikurensis all gave nearest matches with *Candidatus* NeoEhrlichia mikurensis type strain indicating

that both strains are closely related. Nearest match with *Rickettsia* species IP1 (99 % similarity with type strain *Rickettsia australis*) was found in six inserts from clone libraries Ai and Aii. In clone library Aiii, two inserts gave nearest matches with *Wolbachia* species Avit (94 % similarity with type strain *Wolbachia pipiensis*) and one with *Wolbachia* species (87 % similarity with type strain *Anaplasma phagocytophilum*). In clone library Eiii, one insert gave nearest match with *Rickettsiales* bacterium It86 (88 % similarity with type strain *Ensifer meliloti*), one with *Candidatus* midichloria mitochondrii (89 % similarity with type strain *Rhizobium vitis*), one with *Pelomonas soli* (100 % similarity with type strain *Pelomonas saccharophila*) and one with *Acinetobacter calcoaceticus* (95 % similarity with type strain *A. calcoaceticus*).

Bacterial diversity in ticks

DNA extracts from ticks were first amplified by Phi 29, prior to PCR-DGGE and PCR-RLB analyses and construction of 16S rRNA gene clone libraries. Amplification with Phi 29 appeared to be an essential step before PCR-DGGE analysis. Preliminary tests with PCR-DGGE done on non-amplified tick DNA extracts resulted in fingerprints with no, or occasionally a few bands. Inclusion of a Phi 29 amplification step before PCR-DGGE resulted in much higher band numbers, sometimes even up to 13 bands per fingerprint (Fig. 4.1). Performance

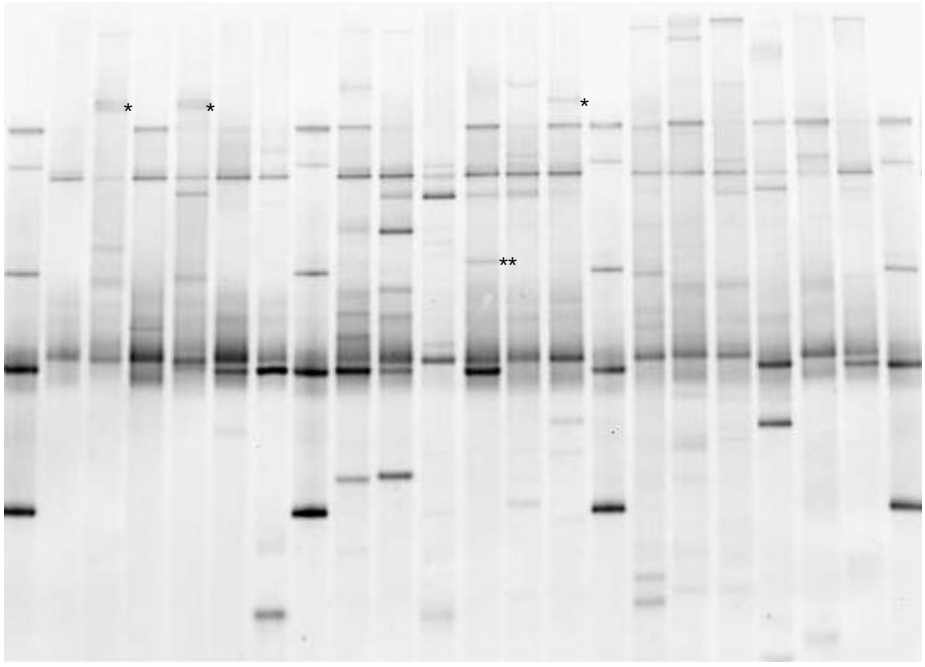

Figure 4.1. Bacterial PCR-DGGE on 18 individual ticks from three different habitats in The Netherlands. M, marker; E, Ede deSysselt; V, Veldhoven Hoogeind; A, Amsterdam water supply dunes. A specific band co-migrating with *Candidatus* Neoehrlichia mikurensis is marked with * and a band co-migrating with *Rickettsia australis* with **

of PCR-DGGE was strongly improved with a preceding Phi 29 amplification step. As a consequence, however, even slight contaminations might be co-amplified. Therefore, Phi 29-amplifications were periodically made from the same extracts to check for differences in PCR-DGGE fingerprints and no differences were observed at any moment. Also, clone libraries (see later), constructed from Phi 29-amplified tick extracts did not show any hits in the NCBI database with species commonly used in our laboratories like *E. coli*.

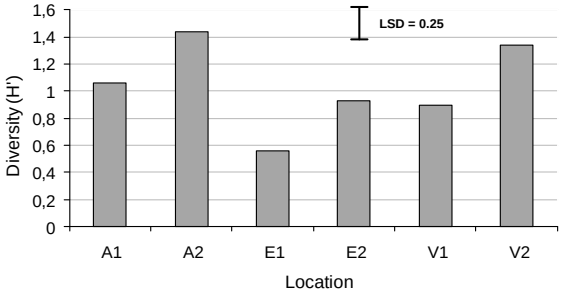

Figure 4.2. Shannon diversity (*H'*) of bacterial communities present in ticks from three different natural areas in The Netherlands. A, Amsterdam water supply dunes; E, Ede, de Sysselt; V, Veldhoven, Hoogeind; 1, 2 different locations within each area. LSD = 0.25; P < 0.001.

PCR-DGGE profiles from 18 different ticks, six from each area, differed per area (Fig. 4.1). PCR-DGGE profiles of all 180 ticks differed per location and in total 55 bands were present at different positions in the gels. Intensities of the bands were different, also for bands at the same position in different lanes, indicating that bacterial population densities differed per tick. The number of bands in each lane, representative of bacterial communities in one tick, varied between 1 and 13 bands. Band numbers differed between locations; between 2 and 10 for A1, E2, V1 and V2; between 1 and 13 for A2 and between 2 and 7 for E1. The Shannon diversity index (H'), calculated from the number of bands (species) and intensity (biomass) of all 180 ticks was between 0 and 2.15 (Fig. 4.2). Significantly lower diversity per area was observed in E (0.743), followed by V (1.114) and finally in A (1.251). Bacterial diversity in ticks also differed per location and significantly lower diversity in ticks was found in E1 (0.556), followed by E2 (0.930), V1 (0.892), A1 (1.061) and finally by V2 (1.335) and A2 (1.441). This indicates that microbial diversity differed per area and even within.

Table 4.4. Presence of *Candidatus* Neoehrlichia mikurensis and *Rickettsia australis* in *Ixodes ricinus* ticks collected from three different habitats in The Netherlands

	Number of positive ticks and percentage (between brackets) for each location**	
Location*	*Candidatus* Neoehrlichia mikurensis	*Rickettsia australis*
A1	1 (3.3)	10 (33)
A2	2 (6.7)	6 (20)
E1	5 (16.7)	2 (6.7)
E2	4 (13.3)	3 (10)
V1	3 (10)	2 (6.7)
V2	6 (20)	5 (16.7)

*A, Amsterdam water supply dunes; E, Ede deSysselt; V, Veldhoven Hoogeind; 1,2 different locations within each area.

** Total number of ticks analyzed: 180, 30 per location.

PCR amplified products of inserts from four different clones, two showing 99% match with *Ehrlichia* sp. Belluno and two showing 100% match with *Candidatus* Neoehrlichia mikurensis, all gave a band with identical mobility in DGGE gels. In 21 tick PCR-DGGE fingerprints, a band comigrating with the *Candidatus* Neoehrlichia mikurensis specific band was found, indicating that these ticks were positive for *Candidatus* Neoehrlichia mikurensis. The highest number of ticks positive for *Candidatus* Neoehrlichia mikurensis was found in V2, followed by, respectively, E1, E2, V1, A2 and A1 (Table 4.4). Between locations, no significant differences in the number of *Candidatus* Neoehrlichia mikurensis-infected ticks were found. PCR-amplified products of four clone inserts from two different ticks showing nearest matches with *R. australis* gave bands with the same mobility in DGGE gel. In a total of 28 tick fingerprints, a band was found that comigrated with this *R. australis*-specific band, indicating that these ticks were all positive for *R. australis*. The highest number of *R. australis*-positive ticks was found in A1, followed by A2, V2, E2 and finally the same in E1 and V1 (Table 4.4). Between locations, no significant differences were found between numbers of R. australis-infected ticks. In one fingerprint of a tick from E1, *Candidatus* Neoehrlichia mikurensis- and *R. australis*- specific bands were found, indicating that a double infection was present in this tick. PCR-amplified 16S rRNA genes of *B. burgdorferi*, *B. afzelii*, *B. garinii* and *B. valaisiana* gave bands with identical mobility in DGGE gel. In none of the 180 tick fingerprints a band comigrating with this *Borrelia* species-specific band was found, indicating that *Borrelia* species were absent or cell numbers were below the threshold level for detection by PCR-DGGE (see later). *Candidatus* Neoehrlichia mikurensis and *R. australis* were the most prevalent species found in ticks.

Borrelia species present in ticks

PCR**-**RLB analyses on all 180 Phi 29-amplifiedtick tick DNA extracts yielded 41 positive signals with all three *Borrelia* genus-specific probes (Table 4.5). The number of *Borelia* genus-positive ticks differed per area; the highest were found in E (18), then in A (13) and finally in V (10). Also, this number differed per location and was highest in E2 (12), followed by A1 (7), E1 (6), A2 (6), V1 (6) and V2 (4) and these differences were not significant (Table 4.5). In total, 36 of these positive ticks were also positive for one, and at four occasions for two probes specific for *B. burgdorferi* ss, *B. garinii*, *B. afzelii* and/ or *B. valaisiana*. No positive reactions were observed with the probes specific for *B. ruski* and *B. lusitaniae*. Ticks positive for *B. afzelii* (34) were highest in number, followed by those positive for *B. valaisiana* (1) and *B. garinii* (1). Of the four tick DNA extracts that gave positive reactions with two probes: two were positive for *B. afzelii* and *B. valaisiana*, one for *B. garinii* and *B. valaisiana* and one for *B. garinii* and *B. burgdorferi* ss. At one occasion only positive signals were observed with the three *Borrelia* genus-specific probes and not with the six species specific probes, indicating that this tick harboured a different species of *Borrelia*.

Table 4.5. *Borrelia* species-infected *Ixodes ricinus* ticks collected from three different natural areas in The Netherlands.

Location[*]	Number positive for *Borrelia* genus[**]	*Borrelia* species present in ticks[***]						
		Ba	*Bg*	*Bv*	*Ba* + *Bv*	*Bg* + *Bv*	*Bg* + *Bb*	Unknown
A1	7	5	1			1		
A2	6	3		1	2			
E1	6	5					1	
E2	12	12						
V1	6	5						1
V2	4	4						

[*] A, Amsterdam water supply dunes; E, Ede, de Sysselt; V, Veldhoven, Hoogeind; 1 and 2, different locations within each area.

[**] Number of ticks that were positive with *Borrelia* genus-specific primers; total number of ticks analyzed: 180, 30 per location.

[***] Number of ticks that were positive in PCR-RLB for *Borrelia* species-specific probes; *Ba, Borrelia afzelii; Bg, B. garinii; Bv, B.valaisiana; Bb, B. burgdorferi sensu stricto*, Unknown, no response with any of the *Borrelia* species-specific probes tested. including *B. lusitaniae* and *B. ruskii* (*Borrelia afzelii*-like).

Discussion

Ixodes ricinus ticks collected from three different natural habitats in The Netherlands differed in bacterial diversity. Ticks collected from location E1 showed lowest bacterial diversity, indicating that these ticks harboured a few dominating species. Highest numbers of *Borrelia* species-, *Candidatus* NeoEhrlichia mikurensis- and *R. australis*-infected ticks also differed per habitat; *Borrelia* species-infected ticks were most abundant in E, *Candidatus* NeoEhrlichia mikurensis-infected ticks in E and V and *R. australis*-infected ticks in A. That these species did not prevail all together in the same habitat would indicate that different factors are responsible for the transmission of these (presumed) pathogens to ticks. Most likely, reservoir hosts for ticks present in these habitats carry different bacterial communities, and this may explain why there is so much variation in PCR-DGGE fingerprints between habitats and even within. It is an intriguing question as to whether certain environmental factors in these habitats could prevent successful colonization and establishment of particular bacterial species in ticks. From this study there is no unequivocal evidence for the existence of these factors, although it is remarkable that the area lowest in the number of *Candidatus* NeoEhrlichia mikurensis-infected ticks (A) was highest in *R. australis*-infected tick numbers.

In order to assess whether environmental factors drive bacterial diversity in ticks, differences in forest floor composition and vegetation between habitats were compared. Major differences were found between E and the two other study areas with respect to thickness of the leaf litter layer and organic matter content in the humus layer. The forest floor structure is important for tick survival. Female adults lay eggs therein and ticks will retreat in the leaf litter layer for transstadial development after each blood meal and when conditions are unfavourable for questing (Randolph and Storey 1999). Vegetation associations also differed per study area and differences were largest between

A and both the other areas. Differences in vegetation type determine the occurrence of tick populations because some are more favourable for tick survival and activity than others (Randolph and Storey 1999). Vegetation is also important as a food and protection source of local tick hosts. Ticks mostly acquire bacteria from vertebrate animals because blood is their only food source (Kurtenbach et al. 2006, Gassner and van Overbeek 2007). Variation in animal host species composition is an important factor in determining tick-associated bacterial communities. *Borrelia afzelii* is a rodent-associated species (Gray 1998, Kurtenbach et al. 2002, Kurtenbach et al. 2006) and was most prevalent in ticks in this study, which would indicate that rodents played an important role in transmission of *Borrelia* and other species to and from ticks. If and how different *Borrelia* species would interact with each other in ticks and reservoir hosts remains so far unresolved. From observations made with coinfected ticks it seems likely that interactions occur. Namely, it was reported that incidences of coinfections were slightly higher than calculated from single occurrences (Rar et al. 2005, Wielinga et al. 2006). Apparently, there is an interaction between different *Borrelia* species present in the same tick. This, in combination with the fact that *Borrelia* species have different transmission rates in different reservoir hosts (Kurtenbach et al. 1998), indicates that transmission of *Borrelia* species between ticks and reservoir hosts in nature is highly complex.

Geographic differences in tick bacterial composition have been observed before (Kirstein et al. 1997, Wielinga et al. 2006). In the study by Wielinga et al., ticks were collected from four different habitats in The Netherlands during two to four successive years. Because ticks were collected over a longer period in time, it must be assumed that differences found in *Borrelia*-, *Anaplasma*- and *Ehrlichia*-species infection rates included both spatial and temporal variations. In a study on *Ixodes scapularis* populations in different natural habitats in New York State, bacterial diversity in individual ticks at different developmental stages and nutritional status (unfed, partially or fully engorged) were investigated by temporal temperature-gradient gel electrophoresis (Moreno et al. 2006). It was found that both developmental stage and nutritional status influenced the bacterial composition of the ticks. In our study, we examined *I. ricinus* ticks that were in the same developmental stage and nutritional status and that had been collected within a short time frame, thus excluding factors other than geography and vegetation type. Surprisingly, differences in tick bacterial diversity were also found between separate locations within the three habitats. Distances between locations in different habitats never exceeded 300 m, which indicates that differences in tick bacterial composition occurred at a smaller scale. (Wielinga et al. 2006) also reported on small-scale level variations in tick infection rates of different pathogens. Small-scale level variations in bacterial diversity in ticks is an important finding as it indicates that *Borrelia* species and possibly also other pathogens are heterogeneously distributed over habitats.

A majority of the clones from tick clone libraries gave nearest matches in the database with *Candidatus* NeoEhrlichia mikurensis. This species was proposed to be a new member in the family of Anaplasmataceae (Kawahara et al. 2004) and has been found before in ticks from The Netherlands (Schouls et al. 1999), in rats and shrews in China (Pan et al. 2003), in rats and different

mice species in Japan (Kawahara et al. 2004, Naitou et al. 2006), and in rodents and roe deer in Italy (Beninati et al. 2006). Members of this species are likely to occur in ticks and possibly rodents act as reservoir hosts. In two clone libraries, nearest matches were found with *R. australis* in the database. Recently, this bacterium was isolated from a patient from north-eastern Australia suffering from Queensland tick typhus (Unsworth et al. 2007), and to our knowledge this species has not been detected in European ticks before. *Candidatus* NeoEhrlichia mikurensis and *R. australis* are species dominating in cell number in ticks. Analysis of the clone libraries reveals that the majority of ticks in our study carries pathogens other than Lyme borreliosis agents and highlights the ability of these ticks to transmit various pathogens to humans. Although likely, it is still not proven that all observed species represent actual pathogens. So far, detection of these pathogens is based on a relatively small part of the genome and additional tests for the presence of virulence genes on isolates or tick DNA extracts should provide more information on the true nature of these species.

Nearest matches with typical endosymbionts were also found in the clone libraries. In one clone library, hits were found with *W. pipientis* and in another one with *Candidatus* Midichloria mitochondrii. *Wolbachia pipientis* is a commonly found member of bacterial communities residing in arthropods (O'Neill et al. 1992, Lo et al. 2006), 1992; *Candidatus* Midichloria mitochondrii was recently proposed as a new member in the group of *Rickettsia*les (Sassera et al. 2006). This species has been found in *I. ricinus* ticks before (Beninati et al. 2004, Lo et al. 2006) and it must be regarded as a common member of tick-associated bacterial communities. It is the first described species that invades and persists in mitochondria present in eukaryotic cells (Sassera et al. 2006). It is interesting that no hits related to *Borrelia* species were found in the clone library. Possibly, *Borrelia* species are not dominating members of the bacterial com munities present in unfed and questing ticks. However, cell numbers of *Borrelia* species may be higher in *I. ricinus* when ticks are fully engorged, which was the case in *I. scapularis* (Moreno et al. 2006). Hits with two other bacterial species, *P. soli* and *A. calcoaceticus* belonging to, respectively, the groups of Beta- and Gammaproteobacteria, were also found in the clone libraries. All other species found in the clone libraries from ticks belonged to the Alphaproteobacteria and this group must be regarded as the most dominant bacterial group associated with *I. ricinus* in this study. Dominance of the group of Alphaproteobacteria in *Ixodes* species is possibly not a general phenomenon as was shown in *I. scapularis* from Massachusetts, where a much higher diversity of associated bacterial species was found (Benson et al. 2004).

In conclusion, geographic differences were found in tick-associated bacterial communities. It is likely that *Borrelia* species and also other pathogens like *Candidatus* NeoEhrlichia mikurensis and *R. australis* in ticks will differ per habitat. The observation of the endosymbiotic species *W. pipiensis* and *Candidatus* Midichloria mitochondrii in ticks makes the group of Alphaproteobacteria living in association with *I. ricinus* important. Differences in habitat structure, in particular the leaf litter composition and vegetation, may affect the presence and survival of ticks, and hence affect the risk of disease transmission. The observed differences in bacterial species composition and

infection rates in the ticks are likely to be influenced by habitat structure as well as by the resident reservoir vertebrate hosts of *Borrelia* species.

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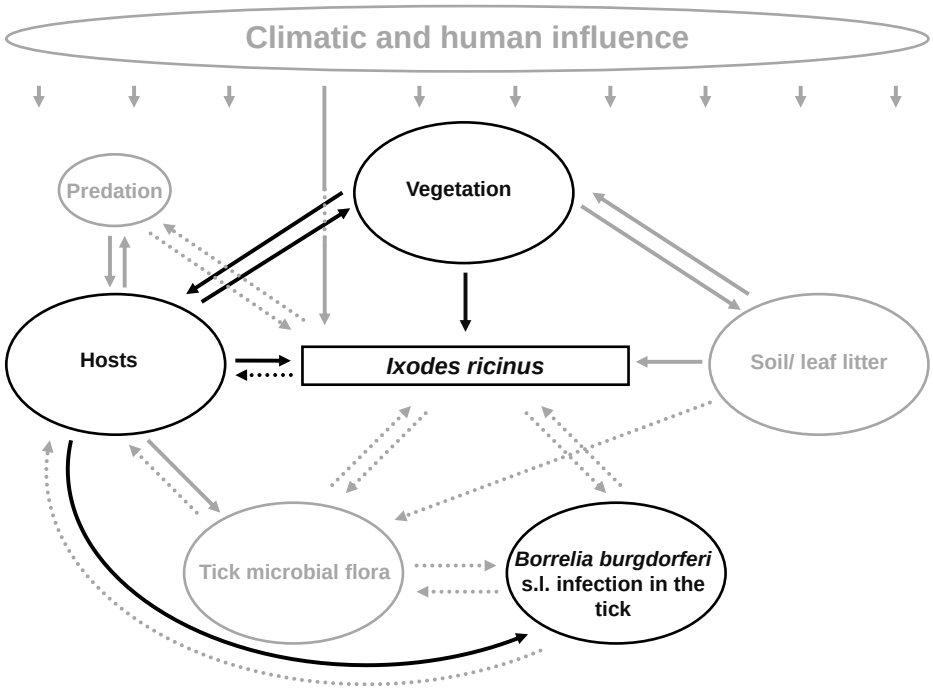
Chapter 5

Variations in *Ixodes ricinus* density and *Borrelia* infections associated with cattle introduced into a woodland in The Netherlands

Fedor Gassner, Patrick Verbaarschot, Renate C. Smallegange, Jeroen Spitzen, Sipke E. Van Wieren and Willem Takken.



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Abstract
The effect of introduced large herbivores on the abundance of *Ixodes ricinus* and their *Borrelia* infections was studied in a natural woodland in The Netherlands. Oak and pine plots either ungrazed or grazed by cattle were selected. Ticks were collected weekly by blanket dragging. *Borrelia* infections were determined by PCR and RFLP. Rodent densities were estimated using mark-release recapture methods. On occasion, the cattle were inspected for tick infestations. Meteorological data were recorded in each habitat.

Significantly more ticks were collected in the ungrazed woodland than in the grazed woodland. Ungrazed oak habitat had higher tick densities than pine habitat, while in the grazed habitats tick densities were similar. *Borrelia* infections ranged from zero in larvae, 26% in nymphs to 33% in adult ticks and consisted of *B. afzelii*, *B. burgdorferi s.s., B. garinii* or *B. valaisiana*. Co-infections were found in five ticks. There was no effect of cattle on *Borrelia* infections in the ticks. In the ungrazed area *Borrelia* infections in nymphs were significantly higher in oak habitat than in pine habitat. More mice were captured in the ungrazed area and these had a significantly higher tick burden than mice from the grazed area. Tick burden on cattle was low.

The results suggest that grazing has a negative effect on small rodents as well as on ticks, but not on *Borrelia* infections. Implications of these results for management of woodland reserves and risk of Lyme disease are discussed.

Introduction

The ecology of Lyme disease is complex and is determined by numerous factors including the vertebrate parasite reservoir, the tick vectors, the blood hosts, the vegetation structure and micro and macro climates (reviewed in Gassner and Van Overbeek 2007). In recent years, a rapid rise in the number of Lyme disease cases has been reported in Germany and The Netherlands (Kampen et al. 2004, Hofhuis et al. 2006) and it has been postulated that this might be due to an expansion of nature reserves, a steady rise in roe deer populations and increased outdoor recreation. For the management of woodlands, wardens of nature reserves increasingly turn to grazing by free ranging bovids, as this can keep the woodlots "open" and helps to keep the vegetation diverse (Olff et al. 1999).

Because large herbivores are often the preferred hosts of adult *Ixodes ricinus*, the principal vector of Lyme disease in Western Europe (Rauter and Hartung 2005), we speculate that tick populations benefit from these measures. However, for tick populations to prosper under increased densities of large herbivores, there must also be a population of smaller vertebrate species as hosts for the larval and nymphal stages (Gray 1998). Several small rodent species and birds constitute the principal hosts for these life stages of *I. ricinus*, in particular for the larval stages (Humair et al. 1993, Kurtenbach et al. 1995, Humair et al. 1999). This implies that the introduction of large herbivores for woodland management should not affect the rodent or bird populations if a rise in tick density is expected. On the other hand, large herbivores might negatively impact local rodent populations (Smit et al. 2001) and thereby alter the hosts composition for immature tick stages as well as the *Borrelia* reservoir composition. Another factor to consider is the fate of the *Borrelia* parasites that *I. ricinus* transmits. Here, high densities of rodents and birds are considered to favour *Borrelia* maintenance, as most larger herbivores are incompetent for *Borrelia* parasites (Jaenson and Tälleklint 1992). If the large herbivores affect the composition of hosts for immature tick stages, *Borrelia* might be maintained in the area by co-feeding on larger herbivores, where the spirochetes are horizontally transmitted through the local bloodstream without a systemic infection (Ogden et al. 1997).

In this paper we report on a study conducted to investigate the impact of grazing from introduced cattle on tick populations and *Borrelia* infections in a woodland reserve in The Netherlands. Data from a grazed woodland were compared with those of an adjacent woodland from which the cattle were excluded as well as from a nearby pasture area.

Materials and Methods

Description of the study site

The study was conducted on the estate "Oostereng" in The Netherlands, 2 km north of the town of Renkum (52°00`27N, 5°45`20E), 40 m above sea level. The area consists of mixed woodland (deciduous and evergreen) and is bordered by pasture and agricultural fields to the south. The soils are sandy as a result of the last glacial ice age covering this part of Europe. The study site covered approximately 1 km^2 of pasture, 2 km^2 of woodlot accessible to cattle and 2 km^2 of woodlot from which cattle were excluded by fencing (Fig. 5.1).

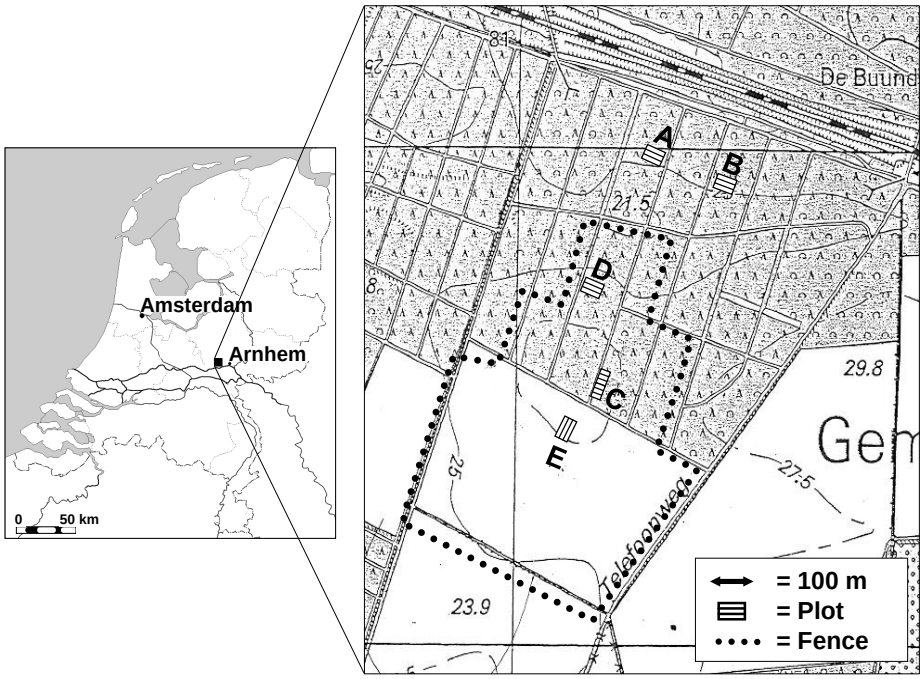


Figure 5.1. Map of The Netherlands showing the study site. Inset: Detail of the study area. The dotted line marks the fence around the area in which cattle could range freely. Blank areas represent pastures, shaded areas represent forest. A: Oak habitat, cattle excluded. B: Pine habitat, cattle excluded. C: Oak habitat, cattle resident. D: Pine habitat, cattle resident. E: Pasture, cattle resident. Plot D and B were also used for rodent captures.

Within the study site, five plots of 400 m^2 each were selected: one on an open pasture (plot E), two within a grazed woodlot (plots C & D), and two in an adjacent forest from which cattle were excluded (plots A & B) (Fig. 5.1). Plots A and C are situated in a habitat that is dominated by oak, plot B and D in a habitat that is dominated by Scots pine. The pasture and adjacent woodlot are grazed by of mix of Groningen white headed oxen and Dutch belted oxen, who were introduced in 2001. The cattle are left on the site throughout the year. During the study, on average 10 animals were present on the study site. In addition to cattle, the study area harbours numerous wildlife such as roe deer, badgers, foxes, rabbits, hares and rodents. These animals can pass through the wire-fence that surrounds the grazed woodlot and pasture. Also, many birds frequent the site. These animals have all been reported previously to act as natural hosts for *I. ricinus* (Gray 1998).

A qualitative vegetation record of the forest plots was made in accordance with the Elton & Miller method, which describes vegetation as percent coverage (Southwood and Henderson 2000). Plot E, which is situated within the pasture at 50 m from the edge of the forest, is mainly covered by perennial ryegrass (*Lolium perenne*), common velvet grass (*Holcus lanatus*) and red fescue (*Festuca rubra*). Dominating herbs are spear thistle (*Cirsium vulgare*), field thistle (*Cirsium arvense*), persicaria (*Persicaria maculosa*), ragwort (*Senecio*

jacobae), and patches of stinging nettle (*Urtica dioica*) and common st. Johnswort (*Hypericum perforatum*). Plot A is dominated by red oak (*Quercus rubra*) and European mountain-ash (*Sorbus aucuparia). The groundcover consists of small patches of wavy hair*-grass (*Deschampsia flexuosa*) in between large areas of oak litter. Plot B contains mostly Scots pine (*Pinus sylvestris*), with a ground cover of *wavy hair*-grass and some patches of blackberry (*Rubus fruticosa*) and blueberry (*Vaccinium myrtillus*). Plot C is dominated by common oak (*Quercus robur*) at a higher density than that of plot A. The ground layer consists mainly of oak litter and small patches of *wavy hair*-grass. Plot D is dominated by Scots pine. Like plot B, the ground layer is dominated by *wavy hair*-grass, but had more litter in between. The plot also includes a patch of blueberry and some blackberry.

A few objects (some fallen branches and blackberry branches) that would obstruct the blanket dragging for tick collection were removed from the study transects prior to the experimental period.

Collection of meteorological data

Temperature (maximum, minimum and average) and relative humidity were recorded throughout the study period using automated data loggers (Gemini Tinytag Plus TPG 1500, INTAB Benelux, Cuijk, The Netherlands).). Data loggers were suspended, using a metal wire, from a livestock-proof metal cage (Lastec B.V., Wageningen, The Netherlands) 5 cm above the litter layer. The data loggers were protected from direct impact of rain water by a PVC cover, which was attached to the metal wire. The data loggers were programmed to take 1 measurement per minute. One data logger was placed in the pasture, one in the grazed woodlot and one in the ungrazed woodlot. Weekly data sets were uploaded to a computer using Easyview software (version 5.5.1.1, 2002. INTAB Benelux).

Collection of ticks

Tick collections were done weekly during a 15-week period from March to July 2005 (week 11 to 25). Sampling started at 09:00 h and ended around 13:00 h. In each plot, an area of 200 m^2 was sampled according to the method described by Wielinga et al. (2006). Ticks were collected by blanket dragging using a white cotton blanket of 1x1 m. A metal chain, sewn in at the lower hem, assured the blanket's contact with the vegetation. The blanket was inspected for ticks at 25 m intervals (Wielinga et al. 2006). Ticks of all three stages were counted and placed in 1.5 cc tubes using forceps. Captured ticks were pooled per 25 m^2 and stored at 4 °C in 70% ethanol until DNA extraction. Sampling was postponed until the next day when the weather was too wet.

In addition to the tick sampling by blanket dragging, ticks were collected on two occasions from nine of the cattle grazing in the study site to obtain information on the average number of ticks per animal. For this purpose, the cattle were gathered in a crush where they could be restrained by two co-workers using a rope harness around the head. Once restrained, the cattle were examined as described in L'Hostis et al. (1994).

Identification of Borrelia *infections*
All life stages of collected *I. ricinus* were examined for infection with *Borrelia* spp.. DNA extraction was performed as described by Schouls et al. (1999), where DNA extracts were produced by boiling individual nymphs and adults in a 4M ammonium hydroxide solution for 20 minutes, followed by 20 seconds of centrifuging at 14.000 rpm, and a final step consisting of 20 minutes at 90 °C in PCR vials with opened caps to evaporate the ammonia. The resulting DNA extracts of approximately 60µl were stored at -80 °C until further analyses. The occurrence of transovarial transmission was examined by extracting DNA from larval *I. ricinus* as described above. Prior to DNA extraction, larvae from two different sampling dates were pooled for each plot into 75 samples of up to 10 larvae.

DNA extracts were analysed for the presence of *Borrelia* spp. using the protocol of Michel et al. (Michel et al. 2003), which uses the Restriction Fragment Length Polymorphism (RFLP) method on PCR products that were amplified from the *B. burgdorferi* s.l. outersurface protein-A (Osp A) gene. Following a nested PCR, each *B. burgdorferi* s.l. positive PCR product was digested separately using 5 different restriction enzymes (*Ssp*I, *Sfu*I, *Bgl*I, *Kpn*21 and *Hind*III) in order to generate genospecies specific digestion products. This RFLP technique can distinguish *B. afzelii*, two different strains of *B. valaisiana*, five different OspA types (type 3 to 7) of *B. garinii*, two strains of *B. burgdorferi s.s.*, and one strain each of *B. lusitaniae* and *Borrelia* A14s.

Mark-recapture of rodent populations
Rodents were trapped in two plots with pine trees, one with and one without cattle, over a 5 day period in June (week 24) of 2005. In each plot, 24 Longworth small mammal traps (Alana Ecology Ltd., Shropshire, U.K.) were placed on the ground in a grid of 4 by 6 and 5 m apart. The rodent grids were 375 m^2 and included the original tick sampling plots.

A pre-baiting period of 4 days was performed prior to the trapping period. The traps were placed on the ground and covered with some litter to minimize excessive heat from the sun. The bait consisted of one mealworm and an oatmeal/peanut butter mixture. The bait was refreshed daily. Rodent traps were inspected at 6-hour intervals (00:00, 06:00, 12:00 and 18:00 h) to reduce stress endured by insectivorous shrews. All animals captured were identified to species and attached ticks were counted. A sample of ticks (as many as practically possible, with a maximum of five ticks) was taken from the rodents, except from shrews, which were set free immediately after inspection. The rodent trapping was approved by the Ethical Animal Experimentation committee of Wageningen University and Research Centre (number 2005056).

Population size estimation
Captured bank voles and wood mice were gently marked with an individual pattern by clipping off some of the top fur with a pair of scissors. The number and frequency of recaptures were used to estimate the rodent population size as described by Lange et al. (1986).

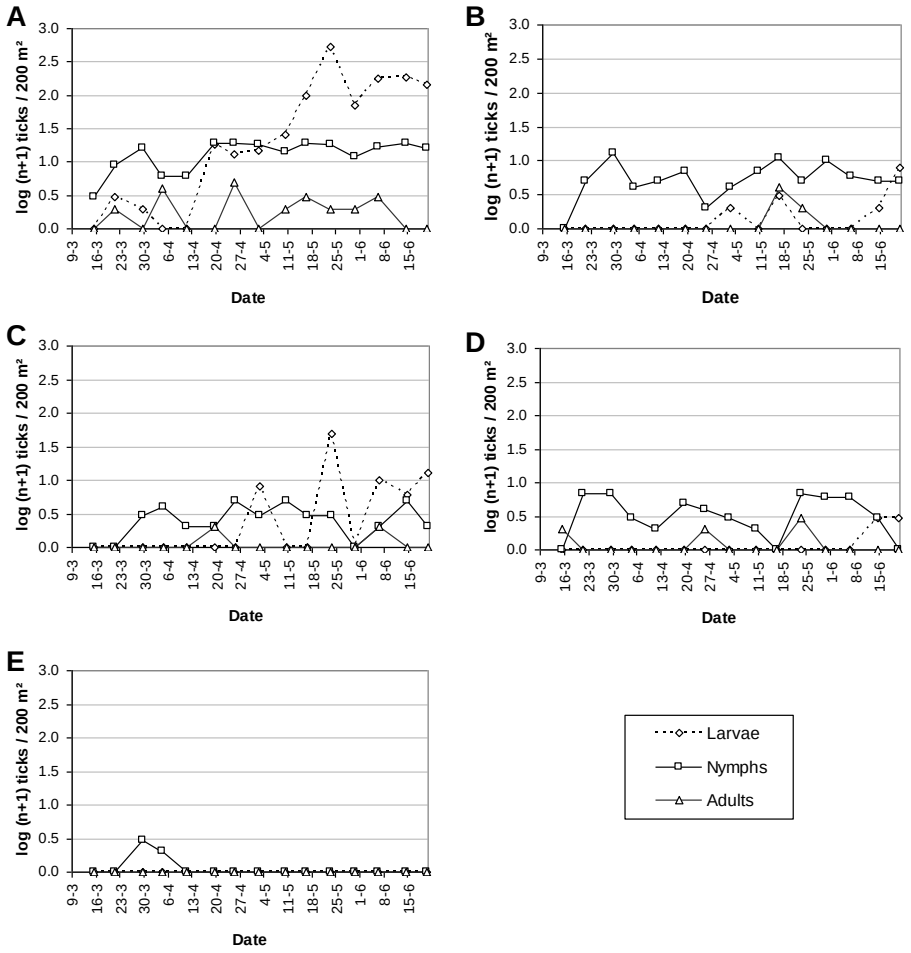


Figure 5.2. Population dynamics of *Ixodes ricinus* over the 5 study plots. A: Oak habitat, cattle excluded. B: Pine habitat, cattle excluded. C: Oak habitat, cattle resident. D: Pine habitat, cattle resident. E: Pasture, cattle resident.

Observation of cattle behaviour and counts of cow pats

The distribution and behaviour (resting or foraging) of cattle grazing in the study area were observed on two different periods by walking the study area and with the use of binoculars, without disturbing the cattle. The first observation was done over a 17-h period, from 06:00 to 23:00 h, distributed over three days. The second observation was conducted during the rodent capture week.

To estimate residence time of cattle in different habitats of the study area, cow pats were counted twice in the pasture and the grazed zones of the pine habitat and the oak habitat, with a one-month interval. Pats were marked with a stick to prevent double counts. The recording of cow pats was done in the same 400 m^2 plots in which ticks were sampled (Bookhout 1994).

Statistical analysis

Statistical analyses were performed in Genstat software (release version 8.11). The data from the pasture were excluded from the analyses. Data on *Borrelia* infections in ticks were analysed using the number of infected ticks as fraction of the total number of infected ticks analysed with the RFLP technique. A Generalized Linear Model (GLM, Binomial distribution, linked in logit) was used to investigate the effect of woodland type and the presence of cattle on these fractions. Two sided t-probabilities were calculated to test pair wise differences in means. Adults and larvae were excluded from the statistical analysis on infections. Data on tick abundance, cow pats and tick burden on rodents were analysed with GLM (Poisson distribution, linked in logarithm) followed by post-hoc t-tests. Effects were considered to be significant at a *P*-value of <0.05.

Results

Meteorological conditions

The study was conducted from March to July 2005. Minimum and maximum temperatures, relative humidity and saturation deficit at ground level during the study period are presented as supplemental data at the end of this chapter. Mean daily minimum temperatures ranged from -4 °C to 16 °C, and mean daily maximum temperatures from 0 °C to 32 °C. The average values of meteorological parameters collected at the three plots were used to calculate mean relative humidity and temperature at 5 cm above the litter layer in the study area. Minimum temperatures varied from -4 °C to 1 °C and maximum temperatures from 21 °C to 32 °C. Corresponding values for relative humidity were 39-100% and 30-100%. Corresponding values of the saturation deficit varied from 0 to 6.4 mmHg, with highest values in June, corresponding with the highest temperatures. On several days saturation deficit values exceeded 4 mm Hg, which causes *I. ricinus* to retreat into the litter layer to avoid dehydration. This behaviour may put the ticks, in particular larvae, out of reach for the blanket dragging method (Randolph and Storey 1999).

Population dynamics of ticks

A total of 1,747 ticks were collected with the blanket dragging method during the study period. *I. ricinus* was the only tick species found. The weekly distribution of the tick collections over the study plots is shown in Figure 5.2 for all three life stages. A few larval ticks were already collected in late March, but the majority of larvae appeared only after mid April, with a peak at the end of May. Nymphal ticks were found for the first time on 10 March 2005 and remained present during the entire study period without a clear peak in population density. Adult ticks were active on nearly each sampling day, albeit in very low numbers.

Only three ticks were found in the pasture at the beginning of the study. GLM analysis revealed that there was no interaction between habitat and grazing status for larvae (P=0.85) and adults (P=0.09). For nymphs, a significant interaction between the factors cattle and habitat was found ($p < 0.001$). Considerable numbers of larvae were captured in the ungrazed oak plot compared to the other areas (Table 5.1). However, there were no significant differences in larval densities between the plots due to high spatial within-plot variation. In some plots larval distribution was significantly biased for one small section, but for the purpose of this paper we did not take this into account. Both

Table 5.1. Distribution of *Ixodes ricinus* per life stage and per study plot [a].

	Tick stage								
	Larvae			Nymphs			Adults		
Plot	n	mean ± SEM		n	mean ± SEM		n	mean ± SEM	
Oak, cattle absent	1,283	321.8 ± 231.7	a	196	49.5 ± 4.9	a	15	3.8 ± 0.9	a
Pine, cattle absent	11	2.8 ± 1.1	a	75	18.8 ± 1.2	b	4	1.0 ± 0.4	b
Oak, cattle present	81	20.3 ± 18.9	a	27	6.8 ± 1.0	c	2	0.5 ± 0.3	b
Pine, cattle present	4	1.0 ± 0.7	a	43	10.3 ± 2.3	c	4	1.0 ± 0.4	b
Pasture, cattle present	0	0	*	3	1.0 ± 0.5	*	0	0	*
Total	1,379			344			25		

[a] Data schow the distribution of *I. ricinus per life stage and per study plot over* 15-week sampling period. Means ± standard error of the mean (SEM) are given for subplots of 50 m^2; n: total number of ticks captured in the 200 m^2 sampling plot. Different letters within one column indicate significant differences between mean values (P≤ 0.04). *: not applicable.

ungrazed plots harboured significantly more nymphs than both grazed plots (p<0.001 for oak without cattle compared to both plots with cattle, P=0.002 for pine compared to the grazed oak plot and P=0.02 for the ungrazed pine plot compared to the grazed one). Within the ungrazed area, significantly more nymphs (p<0.001) were captured in the oak habitat. This difference in nymphal densities between habitats was not found in the grazed area (P=0.16), which explains the significant interaction between habitat and grazing status for nymphs. Adult tick populations were significantly larger in the ungrazed oak plot than in the other plots (P=0.04, P=0.02 and P=0.04 for ungrazed pine plot, grazed oak plot and grazed pine plot respectively). The latter differences can be explained by grazing status (P=0.02) rather than by habitat (P=0.09).There were no significant differences between the other plots (all p>0.44).

Borrelia *infection rates*

Seventy-five larvae (in 21 pools), 340 nymphs and 24 adult ticks were examined for *Borrelia* infections. No *Borrelia* parasites were found in the larvae. On average 25.6% of nymphs and 33.3% of adult ticks were found infected with *Borrelia* spp., *B. afzelii*, *B. garinii*, *B. valaisiana* and *B. burgdorferi s.s.*, with *B. burgdorferi s.s.* being the least abundant (Table 5.2). Five nymphs were co-infected with two *Borrelia* genospecies. The *B. garinii* detected were of 4 different Osp A types: OspA3, OspA5, OspA6 and OspA7 (Table 5.3). There was no effect of the presence of cattle on *Borrelia* infections in nymphs (P=0.70). However, a significant effect of habitat (P=0.01) was found on nymphal *Borrelia* infections: more ticks were infected in the oak plots than in the pine plots. There was no interaction between grazing status and habitat (P=0.71). Nymphs collected in the ungrazed oak plot had a significantly (P=0.04) higher infection rate than those collected in the ungrazed pine plot (Fig. 5.3). Numbers of infected adults were too small for statistical analysis.

Table 5.2. Prevalence of *Borrelia* infections in adult and nymphal *Ixodes ricinus* ticks [a].

		Ticks examined		% of ticks infected by *Borrelia* genotype (total no.)[b]				
Cattle	Stage	No. analysed	% infected (total no.)	*B. afzelii*	*B. garinii*	*Borrelia burgdorferi s.s.*	*B. valaisiana*	*Borrelia* sp. sensu lato[c]
Absent	Nymphs	272	26.1 (71)	8.8 (24)	6.3 (17)	1.1 (3)	9.9 (27)	1.8 (5)
	Adults	18	38.0 (7)	11.1 (2)	5.6 (1)	5.6 (1)	16.7 (3)	0
Present	Nymphs	68	23.5 (16)	5.9 (4)	2.9 (2)	0	5.9 (4)	8.8 (6)
	Adults	6	16.7 (1)	0	0	0	0	16.7(1)

[a] Prevalence of *Borrelia* infections in adult and nymphal *Ixodes ricinus* ticks in a woodland grazed by cattle and a comparable ungrazed woodland. Where applicable, absolute numbers are given between parentheses.

[b] Five co-infections were found in nymphs collected in the ungrazed oak forest (two co-infections of *B. garinii* with *B. vailaisiana*, one of *B. garinii* with *B. burgdorferi s.s.*,one of *B. garinii* with *B. afzelii* and one of *B. afzelii* with *B. valaisiana*).

[c] *Borrelia* sp. sensu lato: genotype could not be determined.

Table 5.3. *Borrelia garinii* OspA types in *Ixodes ricinus* nymphs [a].

		No. of nymphs infected with OspA type:					
Cattle	Total no. of nymphs infected with *B. garinii*	OspA 3	OspA 4	OspA 5	OspA 6	OspA 7	No. of nymphs infected with *B. garinii* only
Absent	17	1	0	4	11[b]	2	1
Present	2	0	0	0	0	0	2

[a] Only one adult (not in table) was found infected with *B. garinii,* which could not be determined to OspA type.

[b] Value indicates two co-infections of OspA5 and OspA6

Rodents

One-hundred and sixty-three wood mice (*Apodemus sylvaticus*) and 6 bank voles (*Clethrionomys glareolus*) were captured in the animal traps, as well as 10 common shrews (*Sorex arraneus*) and 5 pygmy shrews (*Sorex minutus*) (Table 5.4). On one occasion a weasel (*Mustela nivalis*) was caught. Forty percent of the wood mice and 33% of the bank voles were recaptured at least once. Using the mark-recapture method, densities of wood mice were estimated to be 0.2 per m^2 in the grazed woodlot and 0.3 per m^2 in the ungrazed woodlot. Densities of bank voles were zero and 0.02 per m^2, respectively. One male wood mouse, captured in the grazed woodlot, was recaptured 3 days later in the ungrazed woodlot, 500 m from the point where it had been released following first capture.

On 102 rodents that were examined during most of the night and early morning captures, a total of 470 *I. ricinus* ticks were counted of which 460 were larvae and 10 were nymphs (Table 5.4). Most wood mice and bank voles carried larvae despite the fact that during the same period only few larvae were collected in both pine habitats using the blanket dragging method. The mean

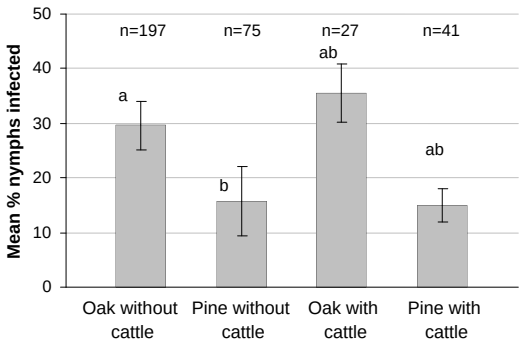


Figure 5.3. Percentage of *Ixodes ricinus* nymphs infected with *Borrelia burgdorferi* s.l. per plot. n = number of nymphs analysed . Different letters above bars indicate significant differences ($p<0.05$). Error bars represent ± SEM.

tick burden on wood mice in the grazed plot (2.8 ticks per mouse) was significantly less than in the ungrazed plot (6.1 ticks per mouse)($P=0.003$). No bank voles were caught in the grazed plot, and the mean number of ticks on bank voles in the ungrazed plot was 1.25.

Cattle

During the cattle observation days the animals were frequently sighted within the forested area, in particular during the night. Occasionally roe deer were also seen. Significantly more cow pats ($p<0.001$) were counted in the grazed oak area (mean=15.63 ± 3.12 SEM) than in the pasture (mean=1.50 ± 0.33 SEM) and pine forest (mean=1.25 ± 0.31 SEM), whereas no differences were found between the pasture and pine area. Nine cattle were inspected on two different occasions (5 weeks apart). Four animals were free of ticks, on the other five the number of ticks collected varied from 1 to 6. With exception of one nymph, these consisted of adult ticks. All ticks were attached to the cows' upper legs.

Table 5.4. Rodent populations and rodent larval tick burdens in areas with and without cattle[a].

Rodent species	Grazed pine forest		Ungrazed pine forest	
	Estimated population (no. per site)	Mean tick burden (n)	Estimated population (no. per site)	Mean tick burden (n)
Wood mouse (*Apodemus sylvaticus)*	76	2.8 (51)	96	6.1 (51)
Bank Vole (*Clethrionomys glareolus*)	0	0	7	1.25 (4)
Common shrew (*Sorex arraneus*)	5[b]	0.2 (5)	5[b]	0.25 (4)
Pygmee shrew (*Sorex minutus*)	0	0	5[b]	0 (3)

[a] The n-values represent the number of animals inspected for presence of ticks. Rodent populations were estimated in plots of 375 m^2.

[b] No recapture data; shrews were not marked.

Discussion

The presence of cattle had a negative impact on tick populations in our study area, in contrast to our initial hypothesis that livestock would favor the growth of tick populations. We consider it unlikely that differences in densities of roe deer might have caused these effects, as deer lairs were equally common in both grazed and ungrazed areas and deer were also frequently sighted in both areas (F. Gassner, unpublished data). This finding, in combination with the observed abundance of rodents, the wooded vegetation and the suitable microclimate in the study area, indicates that the area meets all the requirements of an optimal habitat for *I. ricinus*. The number of ticks collected from the cattle seemed low on both sampling occasions, suggesting that the animals did not encounter questing ticks frequently, even though the animals spent a considerable time of the day in the woodland. In a recent study on 319 cattle in Germany the tick density per animal varied from 1 to 6 in 75% of the animals (Lengauer et al. 2006), suggesting that the tick density on the animals in our study may not have been unusual.

Rodents were abundant at the time of our study, and tick burden on rodents corresponded to burdens found in studies elsewhere in Europe (L'Hostis et al. 1996, Gray 1999, Humair et al. 1999, Hanincova et al. 2003a), with a strong variation between individual rodents and rodent species, as reported by Kurtenbach et al. (1995). Most ticks on rodents were in the larval stage, similar to data from other studies. As only few nymphs were present on the rodents, it is suggested that, in our study area, nymphal stages feed mostly on other host species than wood mice and bank voles.

Our data show that the population density of *I. ricinus* was significantly higher in the ungrazed woodland than in the grazed area, notably in the oak-dominated forest. The dominant factor differentiating both sites was the presence of cattle. The observed interaction between cattle and habitat for nymphal abundance can be explained by our observations from animal behaviour and cow pat counts that cattle were more often present in the oak area than elsewhere in the forest. During the study cattle were frequently observed resting in the oak area, thereby inflicting damage to patches of litter layer, which may negatively affect tick survival by increasing tick exposure and making them more vulnerable to desiccation during periods of high saturation deficit. Deer lairs were present in both sites, so large hosts other than cattle were available to the ticks not only in the ungrazed forest, but also in the grazed area. Small rodents were numerous in both sites, albeit more numerous and carrying more ticks in the ungrazed woodlot than in the grazed one. Cattle may have had a negative effect on the small rodents (Smit et al. 2001).

Whereas tick densities were significantly affected by the presence of cattle, the overall *Borrelia* infection rates in ticks were similar in the grazed and ungrazed woodlots and suggest that the presence of cattle had no effect on infections. Moreover, differences in nymphal infection rate were more explained by habitat type rather than grazing status, where nymphs in the ungrazed oak habitat showed a significantly higher infection rate than nymphs in the ungrazed pine habitat. The observed difference in nymphal infection rates between oak and pine forest may have several explanations. The majority of *Borrelia* infections identified in *I. ricinus* in The Netherlands consists of *B. afzelii* (Rijpkema et al. 1995, Wielinga et al. 2006), suggesting that most larval ticks

feed on small rodents, which are more abundant in oak than in pine forests (Smit et al. 2001, Smit et al. 2003). Hence, infection rates are likely to be higher in ticks present in oak than in pine forests. As *B. garinii* and *B. valaisiana* were also relatively common in the *Borrelia* infections of the nymphal ticks in our study (Table 5.2), birds may also have been common hosts for *I. ricinus* larvae in our study area (Humair et al. 1998, Hanincova et al. 2003b). From the mark-recapture study it was noted that at least one rodent moved freely between both study areas, so the *Borrelia* reservoir may have dispersed between both areas, possibly causing a similar proportion of infected rodents in both areas. Assuming a random, non-selective, host frequenting behaviour, questing larval ticks, therefore, would have a similar chance of becoming infected with *Borrelia*.

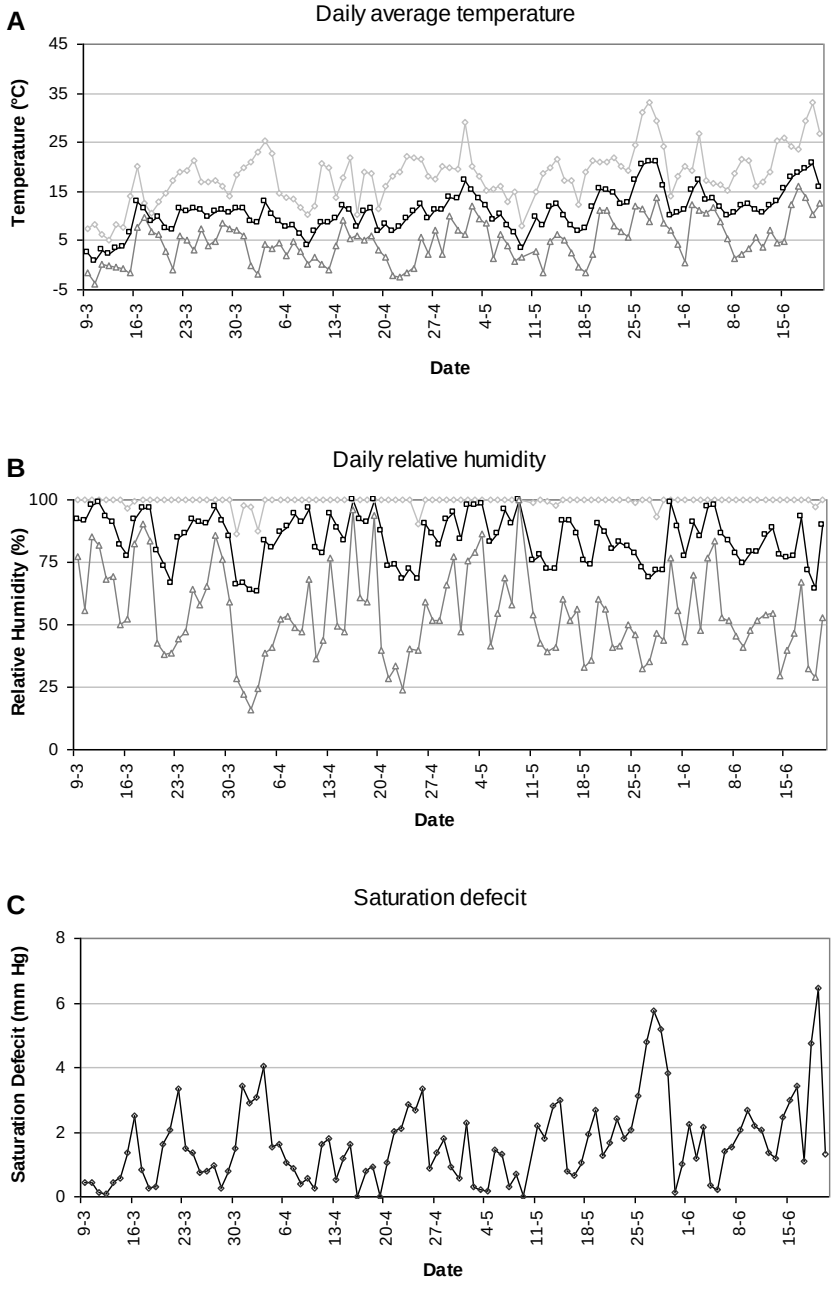
Richter and Matuschka (Richter and Matuschka 2006) recently reported that grazing of cattle had a negative impact on Lyme disease risk. In their study in northern France, however, the effect was caused by significantly different *Borrelia* infection rates in ticks more than by tick densities. Unlike in our study, where tick densities could be compared because of our rigid sampling strategy, Richter and Matuschka do not mention sampling intensity or record tick densities, although they mention that half as many ticks were found in areas where cattle were present compared to areas where cattle were absent. The effect of grazing on Lyme disease risk in their study was therefore likely to be even stronger than in our study. Based on *Borrelia* infection rates in ticks, however, the results of both studies are different. It is possible that differences in vegetation structure and topography between the study areas may have contributed to this as Richter and Matuschka collected ticks in a pasture, whereas in our study ticks were collected from a woodlot where *Borrelia*-infectious mice are likely to be more abundant then in a pasture. As mentioned above, in our study area the rodents could readily move between both sites, as shown by the dispersal behaviour of at least one recaptured mouse, and the probability of ticks becoming infected with *Borrelia* might therefore have been similar in the grazed and ungrazed site. We are not aware of any other study reporting on livestock grazing in woodland areas and risk of Lyme disease but the results of both studies, suggesting that grazing cattle can reduce Lyme disease risk, seem to merit additional studies to understand the reported effects.

It is an interesting observation that the oak forest supported a significantly higher tick population than the pine forest. Previously we found an opposite result in two other areas in The Netherlands, with significantly higher tick densities in pine forest than oak forest (R. Smit, unpublished data). It is likely that this effect is area specific. In the present study we did not estimate the rodent population in the oak forest. However, given the number of larval ticks found on the rodents, ranging from 2.8 to 6.1 ticks per rodent in the grazed and ungrazed pine forest respective,y (Table 5.3), it may be assumed that rodents were equally abundant in the oak forest in order to support such a high density of questing ticks as observed in this study. The ground cover in the oak forest was very different from that in the pine forest, and consisted of dead leaves and blueberries in the former, while of mostly thick grass cover in the latter. This may have resulted in different questing behaviour of ticks between the two sites so that notably larval populations remained “hidden” for our sampling method in the pine forest.

The pasture area just outside the forest obviously did not support a population of *I. ricinus*. This was possibly caused by the exposure of the grass to sunlight, and subsequent higher saturation deficits (SD) in the pasture than in the forest. Especially larval ticks are negatively affected when exposed to SD values exceeding values of above 4 (Randolph and Storey 1999). We assume that small rodents might have used the pasture for foraging, as there were very many wild plants providing good refuge for numerous sources of rodent food. Yet, this did not cause an increase in abundance of ticks. Moreover, the pasture could serve as a lethal sink for ticks that have attached to the cattle in the woodlands and dropped from the cattle in the pasture (Boyard et al. 2007). Although we have no data on the feeding frequency of *I. ricinus* on cattle in our study area, we know from the counts of cow pats that cattle were very often present in the woodlots, and therefore were readily available to ticks as blood hosts. We have no comparative data of tick infestation rates of cattle and other large herbivores sharing the same habitat, but data from Sweden mention a tick burden of 428 to 2,072 on roe deer (Tälleklint and Jaenson 1997), which is much higher than the tick burden on our cattle or cattle studied in Germany (Lengauer et al. 2006) and France (L'Hostis et al. 1994, Boyard et al. 2007). Possibly cattle are less suitable for ticks than roe deer and red deer, which may explain the reduced density of ticks in natural tick habitats grazed by cattle. Our study does not provide an answer to the mechanism that caused the negative impact of cattle on the tick population. Possibly natural immune responses from cattle following tick bites may be detrimental to tick survival and/or fecundity (Willadsen 1999, Kovar 2004).

The results of this study are of significance for the management of nature reserves in The Netherlands and elsewhere, as these are increasingly being stocked with free ranging livestock such as Scottish Highland cattle, Galloway cattle or Limousin cattle in order to prevent the areas from turning into closed forests (Olff et al. 1999). Further studies are needed to elucidate whether livestock has a direct negative impact on the fitness of ticks. If so, a novel tool for the control of Lyme disease is available, which might go hand-in-hand with efficient strategies for the management of nature reserves.

Acknowledgements
We thank Jan Wieringa for giving access to his grazing area and for assistance during the collection of ticks from the livestock. Staatsbosbeheer Oost-Gelderland is thanked for permission to use the natural woodlot as study site. The advice on statistics provided by Saskia Burgers is most appreciated.

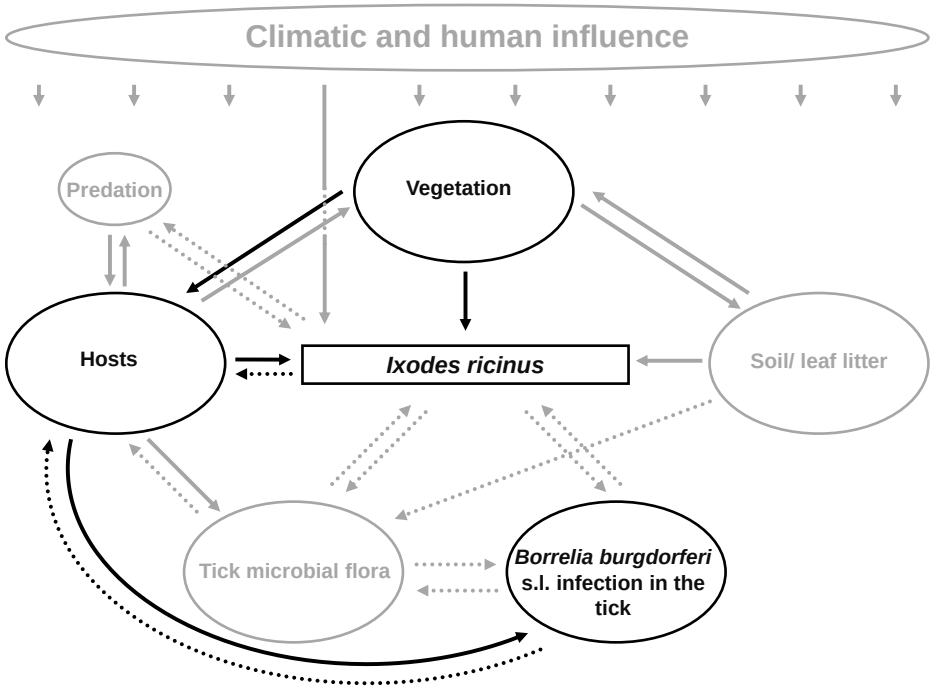


Supplemented figure. Temperature (A), relative humidity (B) and saturation deficit (C) values during the study period. Legend figure A and B: ∆: daily minimum, □: daily mean , ◊: daily maximum.

Chapter 6

High variation in *Ixodes ricinus* infestations and *Borrelia* infections of rodents in The Netherlands

Fedor Gassner, Leo S. van Overbeek, Carin Lombaers van de Plas, Pieter Kastelein, Arno Hoetmer, Maarten Holdinga and Willem Takken



Abstract
The risk of Lyme disease has proven to be difficult to predict, especially since the apparent main driving factors show great spatial as well as temporal variance. The dominant prevalence of rodent-borne *Borrelia afzelii* in the principal vector of Lyme disease, *Ixodes ricinus,* shows that rodents must play a key role in the ecology of Lyme borreliosis in The Netherlands. The aim of this study was to unravel the relationships between tick burden on rodents and *B. burgdorferi* s.l. infections in ticks that are questing the vegetation. Rodent and tick populations were studied in six sites with different vegetation types, and patterns of tick infestation on these rodents were assessed. *Borrelia* infections were determined for rodents, on-host ticks, and questing ticks. Densities of feeding and questing ticks and their *Borrelia* species infections varied between the six sites. Moreover, the abundance of the two dominant rodent species *Apodemus sylvaticus* (wood mouse) and *Myodes glareolus* (bank vole) differed markedly between the sites. Ninety-six percent of the rodents were infested with at least one *I. ricinus* individual. Burdens of host-feeding ticks on rodents (ranges from zero to 241 for larvae, and from zero to 12 for nymphs) varied significantly between rodent species, and larvae : nymph ratios found on these rodents did not match with the ones found among questing ticks in vegetation of the corresponding areas. *Borrelia* species infection rates of rodents varied from zero to 33.3% between sites, whereas those in questing ticks varied from zero to 20.0%. In host-feeding larvae and nymphs, *Borrelia* species infections varied from zero to 29.4% and were correlated with rodent infection level. *Borrelia*-infected rodents had higher nymphal but not larval burdens than uninfected rodents. We conclude that sites where the fraction of the larval and nymphal population engaged in host feeding is high compared to that host-seeking, correspond to sites with high *Borrelia* species infection prevalence in rodents and questing nymphs.

Introduction

Lyme borreliosis is an emerging zoonosis in most temperate regions of the northern hemisphere (Stanek and Strle 2003). The disease is caused by the bacterium *Borrelia burgdorferi* sensu lato that is transmitted by ticks of the *Ixodes ricinus* complex, which can carry numerous other pathogens apart from *Borrelia* species (Gray et al. 2009). In The Netherlands, infectious tick bites faced a nearly four-fold increase over the past two decades to an estimated 22,000 cases in 2009 on a population of over 16 million inhabitants (Hofhuis et al. 2006, Hofhuis et al. 2010). Spatial mapping shows a heterogeneous pattern in the incidence of tick bites and erythema migrans, the early symptoms of infection with *B. burgdorferi* s.l., with some apparent clustering in coastal as well as inland woodlands (Hofhuis et al. 2006). The exact mechanisms behind this spatially heterogeneous increase have yet to be elucidated.

The transmission of the different *Borrelia* species from reservoir hosts to newly-emerged larval ticks is the main step in the circulation of *Borrelia* species in nature (Gray 2002). The primary vector of *Borrelia* species in western Europe, the sheep tick *I. ricinus*, has a four-stage life cycle, consisting of egg, larva, nymph and adult, which is assumed to last between two and six years (Sonenshine 1991, Randolph et al. 2002). After hatching or molting, newly-emerged ticks start questing (i.e., searching for a host in the vegetation) as soon as environmental conditions are suitable (Randolph and Storey 1999). A single blood meal is taken for each transition to the next developmental stage. Larvae preferably feed on smaller hosts such as rodents and smaller bird species, while nymphs feed on small as well as large hosts such as rodents, hedgehogs, wild boar, squirrels, hares, deer and birds. Adults predominantly feed on the larger sized hosts. *Borrelia* species can be acquired by the tick during each blood meal on an infected, reservoir-competent host and once infected, ticks remain infective for life. Different *Borrelia* species have been linked to different hosts. The most dominant species in The Netherlands, *B. afzelii* (Chapter 3, this thesis), is predominantly associated with rodents (Hanincová et al. 2003a, Kurtenbach et al. 2006). Two other common species, *B. valaisiana* and *B. garinii*, are predominantly associated with birds (Hanincová et al. 2003b, Kurtenbach et al. 2006). It is assumed that the woodmouse (*Apodemus sylvaticus*) and the bank vole (*Myodes glareolus*) are the dominant reservoirs for *Borrelia* in The Netherlands (De Boer et al. 1993, Smit et al. 2003, Gassner et al. 2008), since both species are frequently infected with *B. afzelii*.

In a recent study in The Netherlands (Chapter 3, this thesis), large spatial as well as temporal variations in *Borrelia* infection rates of questing ticks within 24 sites were found, suggesting location-dependent variation in parasite transmission. The nature of this variation, however, is incompletely understood. Moreover, the dynamics of *Borrelia* species transmission between *I. ricinus* and *A. sylvaticus* and *M. glareolus* have rarely been studied in field populations in The Netherlands

Under the assumption that rodents are the primary reservoir for *Borrelia* species, we hypothesize that the larval *I. ricinus* burden of rodents can determine the *Borrelia* species infection prevalence in questing *I. ricinus* nymphs. To test this hypothesis, we assessed the density of questing *I. ricinus* and their *Borrelia* species infections in relation to rodent populations, patterns of

tick infestation on these rodents and *Borrelia* species infections in these rodents and on-host ticks in six sites in The Netherlands.

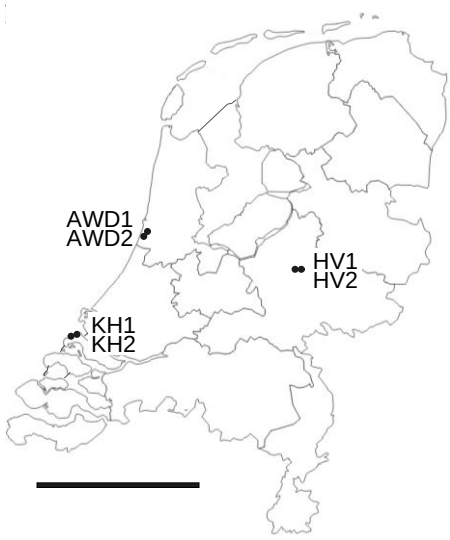


Figure 6.1. Map of The Netherlands, showing the location of the 6 study sites in three locations Kwade Hoek (KH), Hoge Veluwe (HV) and Amsterdam Water supply Dunes (AWD), where ticks and rodents were collected. The black bar indicates a 100 km distance.

Materials and Methods

Description of the study sites and collection of questing ticks

Six sites were selected for tick and rodent captures in three areas in The Netherlands: Kwade Hoek (KH1, KH2), Hoge Veluwe (HV1, HV2) and Amsterdam Water supply Dunes (AWD1, AWD2) (Fig. 6.1). At each site, four representative 50 m^2 sampling transects were laid out and ticks were captured once in June 2007 as described before (Gassner et al. 2008). Corresponding GPS coordinates of the six selected sites were N51º50'17.9" by E03º58'36.9" for KH1; N51º50'06.2" by E03º59'55.3" for KH2; N52º06'20.4" by E05º52'29.6" for HV1; N52º06'20.4" byE05º51'57.5" for HV2; N52º20'23.3" by E04º34'33.1" for AWD1; and N52º20'23.6" by E04º33'46.2" for AWD2. Distances between the sites within each area were 1.5 km for KH, 0.7 km for HV and 0.9 km for AWD.

All areas are characterised by sandy soils, of which the KH and AWD areas are of marine origin and the HV soils are of glacial origin. The areas are regularly visisted by the general public and forestry staff, and are part of municipalities with a high incidence of tick bites and early Lyme disease symptoms in humans (W. van Pelt, personal communication).

The KH is a ~3.8 km^2 coastal nature reserve that is subject to occasional flooding with sea water, which results in a markedly heterogeneous landscape with of meadows and reed fields interspaced with "islands" of dense sea buckthorn (*Hipphae rhamnoides*) and blackberry (*Rubus fruticosus*) shrubs. The more open areas in between the dense shrubs are dominated by reed (*Phragmites australis*) and various grasses such as *Festuca rubra* and *Holcus lanatus.* The area is home to approximately 30 roe deer (*Capreolus capreolus*) and a high variety of bird species, including some pheasants (*Phasianus colchicus*).

The ~50 km^2 HV area has a mixed landscape of drifting sands, heath and woodlands. In HV1 the vegetation is a densely covered deciduous forest dominated by sessile oak (*Quercus petraea*) with an undergrowth dominated of blueberry (*Vaccinicium myrtillus*) and some wavy hairgrass (*Deschampsia flexuosa*). The HV2 site is an evergreen forest, dominated by scots pine (*Pinus sylvestris*) with a similar undergrowth as HV1. The area is home to ~200 red deer (*Cervus elaphus*), ~200 roe deer, at least 50 wild boar (*Sus scrofa*) and ~150 mouflon sheep (*Ovis mousiman*). Songbirds are widely present.

The ~34 km^2 AWD area is dominated by various typical dune vegetations and some oak and pine forests. The AWD1 site is a deciduous forest of mostly sessile oak, with an undergrowth of bracken (*Pteridium aquilinum*). The AWD2 site is an evergreen forest that is dominated by Scots pine with an undergrowth of various grasses and European honeysuckle (*Lonicera periclymenum*). The area is home to ~600 fallow deer (*Dama dama*) and ~600 roe deer. Songbirds as well as seagulls are the dominant birds.

Mark-recapture of rodent populations

Rodent trapping was done at each of the six sites by mark-recapture using the method described by Gassner et al. (2008). All rodent collection grids entirely overlapped the transects used for sampling of questing ticks. Twenty four Longworth small mammal traps (Alana Ecology Ltd., Shropshire, U.K.) were placed in a 4 by 6 grid at 5m intervals. Due to spatial limitations, traps in the two KH sites were placed in two rows of 12 traps at 5m intervals.

A 4-day pre-baiting period preceded the five-day rodent capture. The traps were placed on the ground and covered with some litter to minimize excessive heat from the sun. All traps were provided with an oatmeal/peanut butter mixture, a piece of apple and one mealworm as bait. Baits were replaced daily. Rodent traps were inspected at 6-hr intervals (00:00, 06:00, 12:00 and 18:00 hr) to reduce stress endured by insectivorous shrews. To estimate the rodent population sizes, captured bank voles and wood mice were marked with an individual pattern by clipping off some of their top fur with a pair of scissors. These patterns enabled counts of recaptures, which were used to estimate the rodent population size as described by Lange et al. (1986). Captured shrews were set free immediately upon inspection of the traps.

On the last day of each capture period, all captured rodents were anaesthetised with ether in an induction chamber. After anesthetisation, the captured rodents were weighed and a blood sample was extracted by intracardial penetration with a sterile hypodermic needle. The blood samples were stored at -20 °C in sterile vials containing 20 µl of 10 mM EDTA per 100µl blood. Anesthetised rodents were subsequently euthanised by cervical dislocation. All ticks attached to the animal were removed using sterile forceps and stored as described above. Sex and age (juvenile, subadult or adult) of the ticks were determined. Using sterile scissors, a 3x3 mm sample of ear tissue without attached ticks was cut and stored at 4 °C in 70% ethanol until DNA extraction. The rodent collection procedure was approved prior to the study by the Ethical Animal Experimentation committee of Wageningen University and Research Centre (registration number 2005056).

Identification of Borrelia burgdorferi *s.l. infections*.
Questing nymphs, feeding larvae and nymphs, blood samples and ear biopsies were examined for infection with *Borrelia* species. Larvae removed from the rodents were pooled into groups of up to five individuals of similar level of engorgement, based on a 1 to 5 scale of engorgement. Nymphs were analysed individually. Tick DNA extraction was performed by alkaline lysis as described by Schouls et al. (1999). DNA extraction of rodent blood samples was done using a DNeasy Blood & Tissue Kit (Qiagen, Venlo, The Netherlands) in accordance with the manufacturer's manual, but here using 40 μl proteinase-K and 200 μl 50 mM PBS. DNA extraction from rodent ear biopsies was done using alkaline lysis as described by Gray et al. (2000); the obtained DNA extracts were five-fold diluted in water before PCR-RLB analysis. All DNA samples were analysed for the presence of *B. burgdorferi* sensu stricto*, B. afzelii, B. garinii, B. valaisiana* and *B. lusitaniae* using a PCR Reverse Line Blot essay (Wielinga et al. 2006), which was slightly modified by van Overbeek et al. (2008) with a different reverse primer.

Statistical analyses
Statistical analyses were done with Genstat software (release 12.1). Generalized linear models (GLM) were used to analyse site-to-site differences in questing tick densities and tick burdens on two infected and uninfected rodent species. A Poisson distribution with a logarithm link function was used for site-to-site differences in questing tick counts. Tick densities were used as response variable, month (i.e., May or June 2007) and site (i.e., KH1; KH2; HV1; HV2; AWD1 and AWD2) were used as explanatory variables in the regression models. Data on ticks feeding on rodents were analysed using GLMs with negative binomial distribution, linked in logarithm with an estimated aggregation factor (Wilson 1996, Wilson and Grenfell 1997). In all GLM analyses, overdispersion was taken in account where appropriate by estimating the model dispersion parameter. Chi-square comparisons were made to compare rodent populations and *Borrelia* species infections in rodents between sites. Relations between larval and nymphal tick burdens and between questing and feeding tick populations were assessed using Spearman's rank correlation. Differences in tick abundance at different attachment sites on rodents were assessed with Mann Whitney U-tests. Effects were considered to be significant at $P \leq 0.05$.

Results
Questing ticks and their Borrelia spp. *infections*
A total of 1540 larvae, 270 nymphs and 9 adult questing *I. ricinus* ticks were captured by blanket dragging over the vegetation in the six sites in June 2007. *Ixodes ricinus* was the only tick species found. In KH no larvae were found at both sites. At the AWD and HV sites larvae were found, however, but at both AWD sites larval densities were significantly ($P<0.001$) lower than at the HV sites. Mean larval densities varied from 8 (AWD1) to 362 larvae per 50 m^2 (HV2). Questing nymphs were captured in all six sites (Fig. 6.2), with significant differences between sites (all at $P<0.018$) and a maximum of 39 nymphs per 50 m^2 in HV1. The nymphal densities in both KH sites (1.3 and 2.8 nymphs per 50 m^2) were significantly lower than in both HV sites (resp. 25.3 and 26.8; $P<0.001$). Both HV sites harboured significantly more nymphs than AWD1

(P<0.001) and AWD2 (P<0.003) (Fig. 6.2). Questing adult ticks were captured in all sites, with a maximum of 4 adults / 50 m^2 in AWD2. Adult densities were too low for statistical analysis (data not shown).

A total of 130 questing nymphs, from all study sites, were analysed for the presence of *Borrelia* species. Nymphs from KH2 (n=11), HV2 n=36) and AWD1 (n=6) were not infected (Table 6.1). Of the three sites with infected nymphs, infection prevalence was 20.0% (n=5) (KH1), 3.1% (n=32) (HV1) and 2.5% (n=40) (AWD2).

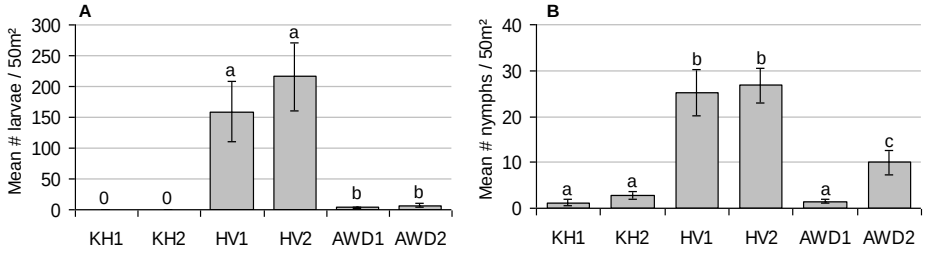


Figure 6.2. Mean no. (+/-1 SEM) of questing *Ixodes ricinus* larvae (A) and nymphs (B) per sampling transect for the six study sites, Kwade Hoek (KH1, KH2), Hoge Veluwe (HV1, HV2) and Amsterdam Water supply Dunes (AWD1, AWD2) in June 2007. Different letters above bars represent significant differences between sites (GLM, P<0.001 for larvae and P≤0.018 for nymphs). 0: no ticks were found.

Rodent populations and Borrelia *infections in rodents.*

In total, 208 wood mouse (*A. sylvaticus*) and 87 bank vole (*M. glareolus*) individuals were captured and marked during the five-day mark-recapture period. In addition, four common voles (*Microtis arvesitis*) were captured in KH1. Rodent population size and species composition varied between sites and between locations (χ^2 P<0.001). *Apodemus sylvaticus* individuals were captured in all sites, whereas *M. glareolus* individuals were not found in KH1 and KH2 (Table 6.1). In the four sites where both rodent species were present, *A. sylvaticus* population estimates were larger than *M. glareolus* population estimates in HV1, HV2 and AWD2. In AWD1 this situation was reversed, with an estimated population of only five *A. sylvaticus* compared to an estimate of 44 *M. glareolus*. The largest population of *A. sylvaticus* was found in KH1 (51 individuals) followed by both HV sites (36 and 43 individuals for HV1 and HV2 resp.).

Among the rodent ear biopsies analysed for *Borrelia* species, on average 17.2% of the *A. sylvaticus* and 9.7% of the *M. glareolus* individuals were infected with *B. afzelii*. One *B. garinii* was identified in an *M. glareolus* in the HV2 site. More *A. sylvaticus* collected in either of the KH sites were infected with *Borrelia* (33.3% and 30.8%) than those collected in the HV sites (9.7% and 10.0%) and AWD (0% and 8.3%) sites (χ^2 P=0.028). *Borrelia*-infected *M. glareolus* were only found in the HV2 (33.3%) and AWD1 (12.5%) sites (Table 6.1). None of the PCR-analysed blood DNA extracts were *Borrelia* positive.

Table 6.1. *Borrelia burgdorferi* sensu lato in questing *Ixodes ricinus,* in rodents and in ticks feeding on these rodents. Single infections with *Borrelia* genospecies other than *B. afzelii* are indicated with a: *B. burgdorferi* s.l. b: *B. garinii* c: *B. valaisiana*.

	Site					
Questing ticks in vegetation	KH1	KH2	HV1	HV2	AWD1	AWD2
Questing nymphs % infected (no. infected/no. analysed)	20.0 (1/5)[a]	0 (0/11)	3.1 (1/32)	0 (0/36)	0 (0/6)	2.5 (1/40)[c]
Questing nymphs % infected 2006-2007 (no. infected/no. analysed)**	39.4 (28/71)	38.0 (19/50)	6.3 (6/96)	7.0 (7/100)	7.2 (46/642)	3.7 (26/711)
Ratio questing nymphs:larvae (June 2007)	1:0	1:0	1:6	1:8	1:2	1:1
***A. sylvaticus* (n=128)**						
Estimated rodent population at the site (number of unmarked rodents captured at day 5)	51 (2)	20 (0)	36 (10)	43 (8)	5 (3)	16 (1)
Rodents % infected (no. infected/no. analysed)	33.3 (11/33)	30.8 (4/13)	9.7 (3/31)	10.0 (3/30)	0 (0/9)	8.3 (1/12)
Feeding larvae % infected (pools analysed/pools infected; total larvae)	4.2 (2/48; 206)	6.3 (1/16; 64)	0 (0/43; 198)	2.5 (1/40; 188)	0 (0/16; 80)	0 (0/20; 90)
Feeding nymphs % infected (no. infected/no. analysed)	5.3 (1/19)	0 (0/12)	12.5 (1/8)[b]	0 (0/2)	0 (0/10)	0 (0/1)
Mean no. of larvae per rodent (range)	9.9 (0-51)	10.6 (0-50)	32.3 (1-189)	33.1 (0-180)	38.4 (5-241)	35.5 (1-188)
Mean no. of nymphs per rodent(range)	0.7 (0-3)	1.7 (0-11)	0.3 (0-3)	0.2 (0-1)	2.0 (0-12)	0.5 (0-3)
Feeding ratio nymphs:larvae	1:15	1:6	1:100	1:166	1:19	1:71
***M. glareolus* (n=41)**						
Estimated rodent population at the site (number of unmarked rodents captured at day 5)	0	0	21 (7)	8 (1)	44 (2)	4 (0)
Rodents % infected (no. infected/no. analysed)	n.a.	n.a.	0 (0/16)	33.3 (2/6)[b]	12.5 (2/16)	0 (0/3)

Table 6.1. Continued from previous page

***M. glareolus* (n=41)**	KH1	KH2	HV1	HV2	AWD1	AWD2
Feeding larvae % infected (pools infected/pools analysed; total larvae)	n.a.	n.a.	0 (0/20; 79)	14.3 (1/7; 28)	29.4* (5/17; 47)	0 (0/3; 8)
Feeding nymphs % infected (no. infected/no. analysed)	n.a.	n.a.	0 (0/0)	0 (0/0)	100.0* (1/1)	0 (0/0)
Mean no. of larvae per rodent (range)	n.a.	n.a.	9.8 (0-83)	5.5 (1-10)	3.8 (0-15)	4.0 (3-5)
Mean no. of nymphs per rodent (range)	n.a.	n.a.	0.06 (0-1)	0	0.06 (0-1)	0
Feeding ratio nymphs:larvae	n.a.	n.a.	1:156	n.a.	1:60	n.a.

* Both rodents and attached tick are infected

**Nymphs were collected on eight occasions from July 2006 to December 2007 nearby the corresponding KH and HV sites of this study (Gassner et al, submitted 2010). For the AWD sites, nymphs were collected in 2002 (W. Takken, unpublished results).

n.a.: not applicable; no specimens found.

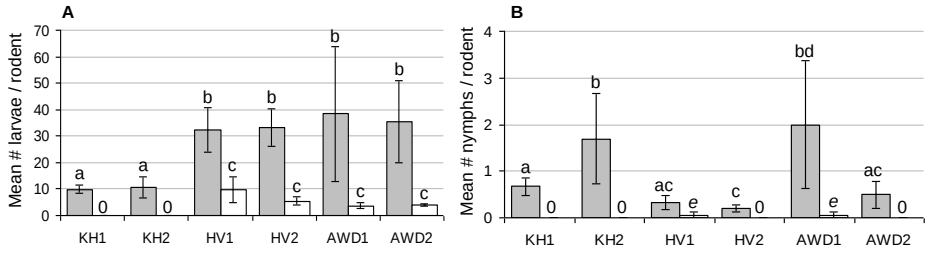


Figure 6.3. Mean larval (A) and nymphal (B) *Ixodes ricinus* infestation level on wood mice (grey bars) and bank voles (white bars) in for the six study sites, Kwade Hoek (KH1, KH2), Hoge Veluwe (HV1, HV2) and Amsterdam Water supply Dunes (AWD1, AWD2). Error bars represent +/-1 SEM. Different letters above bars represent significant differences between sites (GLM, P≤0.009 for larvae and nymphs) and rodent species. 0: no ticks were found.

Feeding ticks and their Borrelia *infections*

The number of parasitising larvae on wood mice (on average 25.3 ± 3.4 larvae per mouse) and bank voles (6.4 ± 2.0 on average) ranged from 0 to 241 per rodent (Table 6.1), with only 4% of rodents free of larvae and 29% of the rodents being parasitised by 80% of the larvae. On-host densities of tick nymphs were much lower than those of larvae (Table 6.1). The mean number of feeding nymphs was 0.7 ± 0.2 on *A. sylvaticus* individuals and 0.04 ± 0.03 nymphs on *M. glareolus* individuals; all nymphs were found feeding simultaneously with at least three larvae. A positive correlation was found between the number of feeding larvae and the number of feeding nymphs (Spearman's rank correlation 0.235, P=0.003). Furthermore, a difference in tick burden was found between the two rodent species. Significantly more larvae were found feeding on *A. sylvaticus* than on *M. glareolus* in the four sites where both rodent species were found (P<0.001) (Fig. 6.3). No adult ticks were found attached to the examined rodents.

In contrast to the absence of questing larvae in the KH, on average 9.9 ± 1.7 (KH1) and 10.6 ± 3.8 (KH2) larvae were found feeding per *A. sylvaticus* specimen in these sites. These mean larval infestation levels were lower compared to all other sites (P≤0.006), which were all similar in number of larvae per individual (between 32.4 and 38.4 larvae per wood mouse) (Fig. 6.3). Mean nymphal infestation for *A. sylvaticus* ranged from 0.2 ± 0.1 nymphs in HV2, to 2.0 ± 1.4 in AWD1. Comparing the sites within the three areas, the mean nymphal infestation on *A. sylvaticus* in KH1 (0.7 ± 0.2) was lower than that in KH2 (1.7 ± 1.0), whereas for AWD, the mean nymphal infestation at site AWD1 (2.0 ± 1.4) was higher than that of site AWD2 (0.5 ± 0.3) (P≤0.009). Nymphal infestations of *A. sylvaticus* were similar in HV1(0.3 ± 0.1) and HV2 (0.2 ± 0.1). Comparing the sites between areas, the differences in mean nymphal infestation between the areas do not show the same pattern as the larval infestation levels. Significantly more nymphs were feeding on individuals of *A. sylvaticus* in KH1 compared to nymphs that were feeding on *A. sylvaticus* in KH2, HV2 and AWD1. The nymphal burdens on *A. sylvaticus* were also higher in the KH2 site compared to the nymphal burdens on *A. sylvaticus* in the HV1, HV2 and AWD2 sites. Moreover, more on-host nymphs were found in the HV1 site compared to AWD1 and in the AWD1 compared to AWD2 respectively (all

P≤0.009). The mean number of feeding nymphs in KH1 was larger than HV1, but this difference was marginally significant (P=0.057). The mean larval infestation levels of *M. glareolus* were similar in all HV and AWD sites, between 3.8 to 9.8 larvae per rodent per site on average (Table 6.1). Only two nymphs were found feeding on bank voles, one in HV1 and one in AWD1.

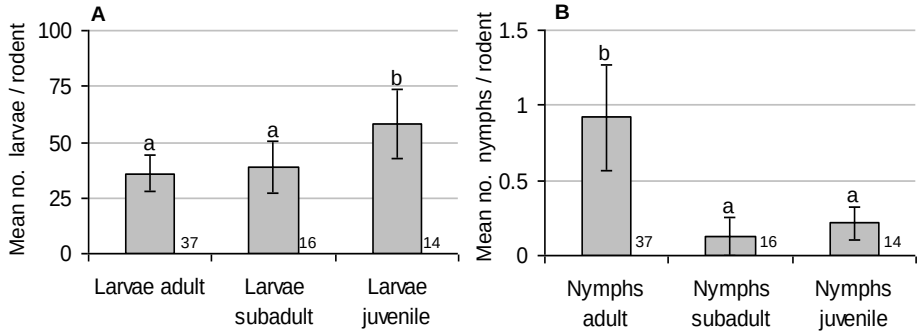


Figure 6.4. Mean (+/-1 SEM) infestation level of larval (A) and nymphal (B) *Ixodes ricinus* on three age groups of *Apodemus sylvaticus*. Numbers next to each bar indicate the number of tested rodents. Different letters above bars represent significant differences between sites. *P=0.023 for larvae and P=0.002 for nymphs.

On both rodent species the distribution of feeding larvae was not homogeneous, larvae being strongly aggregated on the ears compared to the rest of the body (43.1% of the total larvae feeding on *A. sylvaticus* individuals and 52.6% of the total larvae feeding on *M. glareolus*) (P<0.001). Nymphs on *A. sylvaticus* individuals were most frequently found attached to the neck (48.4%) and ears (29.0%) compared to other sections of the body, but no significant differences in nymphal attachment site were found.

The mean tick burden also differed between rodent age classes (Fig.6.4). In *A. sylvaticus*, the mean burden of larvae per juvenile (on average 58.4 ± 15.5 larvae per individual) was higher than that of adult and subadult individuals (on average 36.0 ± 8.1 and 38.9 ± 11.5 larvae per individual respectively, P=0.039). An inverse relation was found for mean nymphal burdens, where on average 0.9 ± 0.3 nymphs were found per adult *A. sylvaticus* individual, compared to 0.1 ± 0.1 and 0.2 ± 0.1 nymphs per subadult and juvenile individuals respectively (P=0.002). These age-dependent tick burdens were absent in *M. glareolus* (data not shown).

Borrelia infection prevalence of the pooled larvae that were feeding on *A. sylvaticus* (Table 6.1) were 4.2% in the KH1 site and 6.3% in the KH2 site. The infection prevalence in feeding larvae was lower in the other four sites (0%, 2.5%, 0% and 0% in HV1, HV2, AWD1 and AWD resp.). All infections in feeding larvae were *B. afzelii. Borrelia* species infections in feeding nymphs were only found in on-host nymphs on *A. sylvaticus* in KH1 (5.3%, *B. afzelii*) and HV2 (12.5%, *B. garinii*). *Borrelia* infections of larvae that were found feeding on *M. glareolus* were only recorded in HV2 (14.3%, *B. afzelii*) and AWD1 (29.4%, *B. afzelii*) (Table 6.1). *Borrelia* infection prevalence in larvae feeding on bank voles

was higher compared to larval infection prevalence in larvae that were feeding on wood mice.

Significantly more nymphs were found feeding on *Borrelia*-infected *A. sylvaticus* individuals (on average 1.1 ± 0.4 per rodent) compared to uninfected individuals (on average 0.5 ± 0.14 per rodent) (P=0.023) (Fig. 6.5). A similar trend was observed for *M. glareolus*, but the number of nymphs found were too few to allow a statistical analysis. For both rodent species, this relation between rodent infection status and tick burden was absent for larvae feeding. There were no significant relations between tick burden and rodent sex or weight.

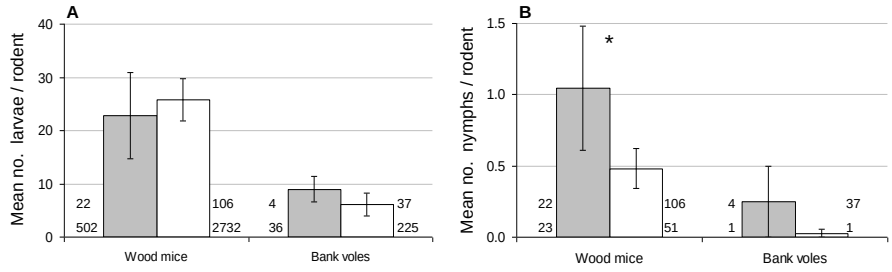


Figure 6.5. Mean (+/-1 SEM) infestation level of larval (A) and nymphal (B) *I. ricinus* on *Borrelia* species infected (grey bars) and uninfected (white bars) *Apodemus sylvaticus* and *Clethrionomys glareolus*. Numbers next to each bar indicate the number of tested rodents (top) and the total no. of ticks on the tested rodents (bottom). *P=0.023 for wood mice.

Relation between feeding and questing ticks

The assumption that higher densities of questing larvae result in higher larval burden on rodents does not hold in our six sites (Spearman rank correlation 0.464, P=0.089). By contrast, a significant and negative relation between questing and on-host nymphs was found; relatively low nymphal densities correspond to high on-host densities, and high nymphal densities in the vegetation resulted in a relatively low nymphal burden on the rodents (Spearman rank correlation -0.829, P=0.008) (Figures 6.3 and 6.4). Furthermore, no relation was found between the infection prevalence of questing nymphs and the infection prevalence in rodents from the corresponding site. The prevalence of on-host nymphs was positively correlated with the prevalence of on-host larvae (Spearman rank correlation 0.418, P=0.003). The *Borrelia* infection rate of on–host larvae was higher at sites with a relatively high *Borrelia* infection prevalence in rodents (Table 6.1).

Discussion

Apodemus sylvaticus (wood mouse) and *Myodes glareolus* (bank vole) are both important hosts for *I. ricinus* larvae, with *A sylvaticus* carrying the bulk of the tick population. Both mouse species play key roles in the circulation of *B. afzelii* in the six studied sites and *M. glareolus* appeared to be a better transmitter of this pathogen than *A. sylvaticus*. Overall, *Borrelia*-infected mice had significantly higher nymphal tick burdens than uninfected mice, which may suggest the existence of a signal between *I. ricinus* ticks and *Borrelia*-infected mice. One of

the assumptions made in this study was that *Borrelia* circulation in habitats can increase if a large proportion of the active larvae and nymphs feeds on rodents. The proposed mechanism behind this assumption is that tick densities vary greatly between sites, which may explain the differences in pathogen loads commonly found in ticks. The nature of these tick-mouse-*Borrelia* interactions will be further discussed.

Interactions between feeding ticks, rodents and Borrelia *species*

In our study, the observed differences in questing tick densities did not correspond with tick numbers found on mice. In the KH and AWD sites, few larvae were found in the vegetation, whereas many were found feeding on the rodents. Two explanations can be given. 1) Questing *I. ricinus* larvae are picked up by the rodents in a microhabitat that is inaccessible to the blanket (Milne 1943), such as burrows or tracks in dense sections of the undergrowth. 2) The feeding larvae represent the majority of the larval population, leaving few questing larvae to be picked up by the blanket. An opposite relationship was observed for nymphs in the HV sites; here many nymphs were found in the vegetation, whereas few were found feeding on the rodents and *I. ricinus* nymphs may feed more frequently on other hosts than mice, or nymphs are more easily picked up by the blanket than by foraging rodents. The proportion of the tick population that successfully finds a blood meal on reservoir hosts will determine the infection prevalence in tick populations.

In all studied sites the infection levels found in questing nymphs were low (max 20% (n=5) infected in KH1) compared to other sites in The Netherlands (Wielinga et al. 2006, Gassner et al. 2008, van Overbeek et al. 2008). However, larger sample sizes of ticks that were collected during separate studies in our study sites over a longer period of time (Smit et al. 2003, Chapter 3, this thesis), showed a high infection prevalence in two nearby sites at KH (39.4% and 38.0%), and infection levels ranging from 3.7% to 7.2% in nearby sites in the HV and AWD area (Table 6.1). These data show that *Borrelia* infection of *I. ricinus* in our study sites is widely present, but with varying infection rates.

Patterns of tick infestation

Overall, rodents were present at each site (Table 6.1), but with marked differences in abundance of the two main species *A. sylvaticus* and *M. glareolus*. Typically, 29% of the rodents harbored 80% of the larvae, which is in line with the findings of Randolph et al. (1999). Moreover, 96% of all rodents was infested by at least one *I. ricinus* larva over all six sites, and all nymphs fed simultaneously with larvae, which can facilitate co-feeding as an additional infection route on top of normal infection of larvae through a systemic infection in the rodents (Gern et al. 1996, Ogden 1997, Richter et al. 2004, Paulauskas et al. 2009). The mean larval infestation level on *A. sylvaticus*, ranging from 9.9 (KH1) to 38.4 (AWD1), was ~10 fold higher than in an earlier study in different woodland areas in The Netherlands (De Boer et al. 1993).

In line with previous studies, *A. sylvaticus* individuals were more frequently used as host by larval and nymphal ticks compared to *M. glareolus* (Randolph 1975, De Boer et al. 1993, Kurtenbach et al. 1995), with most of rodent-feeding ticks aggregated on the ears. Adult *A. sylvaticus* hosted fewer

larvae but more nymphs compared to juvenile *A. sylvaticus* (Fig. 6.4). This age dependency is most likely caused by the rodent immune system, where resistance to tick feeding is built up during continuous exposure to ticks (Levin and Fish 1998). However, this suggestion is contradictory to the higher nymphal infestation of adult mice, which can more likely be explained by the increased chance of active adult rodents to encounter questing nymphs due to their larger home-range, notably in males (Korn 1986). This mechanism indicates that the *Borrelia* circulation may vary depending on the rodent host population demography and therefore on the time of the year.

We observed a significantly higher nymphal burden on infected compared to uninfected *A. sylvaticus*. This finding suggests that *Borrelia* uses a signaling mechanism to enhance its transmission, as has been found for *Plasmodium* parasites and their mosquito vectors (Day and Edman 1983, Lacroix et al. 2005). Moreover, O'Shea et al.(2002) found that *Leishmania*-infected hamsters were more attractive to sandflies than uninfected hamsters, and supported these results by comparing odour profiles of infected and uninfected hamsters. It is interesting to note that this effect was not observed for larvae, suggesting that the signals may are stage specific.

Since the host immune system affects feeding success of *I .ricinus* (Brossard 2004), transmission efficiency of *Borrelia* can also differ between the two rodent species; *M. glareolus* is more likely to transmit *Borrelia* species to *I. ricinus* than *A. sylvaticus*, although the latter is a better host for *I. ricinus* (Talleklint and Jaenson 1997, Gray et al. 1999, Humair et al. 1999, Hanincová et al. 2003a, Sinski et al. 2006). In our study *M. glareolus* indeed carried significantly fewer larvae than *A. sylvaticus*, and a larger fraction of these larvae was found to be infected with *Borrelia* spp. compared to larvae feeding on *A. sylvaticus.*

A remarkable observation was that ticks taken from *Borrelia*-infected mice were not always infected. The best explanation for this is that *Borrelia* parasites can only pass from the host to the tick when the tick is at the final stage of engorgement (Gern et al. 1996, Piesman et al. 2003). Most captured ticks were in early stages of engorgement and had not reached the final state yet, which would explain the relatively low infection rate of ticks collected on *Borrelia*-infected mice. Furthermore, the absence of *Borrelia* DNA in rodent blood might indicate that spirochaetemia and hence infectivity of the rodents was low at the time of collection. Therefore, future studies should preferably allow larvae to feed to repletion and moult to nymphs (Chapter 8, this thesis), which can subsequently be analysed for *Borrelia* infections.

Habitat features that enhance tick - rodent contact

Elevated infection prevalence in questing nymphs and rodents, such as in the KH sites, may be explained by the fact that the landscape is relatively open, with absence of a tree cover and sharp transitions between vegetation types. These heterogeneous habitats seem to facilitate more efficient circulation of vector-borne pathogens (Reisen 2010). Moreover, unsuitable habitats, for example with strong desiccating conditions, may force vectors and hosts to coexist together in refuge zones (Shaman et al. 2005). At KH, hosts may find food, such as blackberry and sea buckthorn fruits, and shelter for predation in the dense shrubs. The dense shrubs will also protect ticks from desiccation and

provide a litter layer for ticks during development or quiescence, and increased abundance of hosts in the shrubs will result in increased feeding and drop off opportunities for ticks. On the other hand, the sheltered continuous woodland areas that dominate the HV and AWD sites provide a microclimate that is suitable for higher questing points for the ticks, which promotes contact with different non-reservoir hosts (Mejlon and Jaenson 1997) and may cause hosts and vectors to be more evenly distributed over the landscape. In these woodland areas, inter- and intraspecies variation in suitability of rodents to feed *I. ricinus* and transmit *Borrelia* species will most likely affect nymphal infection prevalence. Overall, the abundance of ticks will depend on the abundance of reproduction hosts that allow female ticks to feed and lay eggs (Gray et al. 1992, Gray et al. 1998).

In conclusion, this study suggests that the rodent species *A. sylvaticus* and *M. glareolus* play a key role in Lyme disease ecology in the six study sites. We conclude that *Borrelia* infection prevalence in questing nymphs depends on the proportion of the larval and nymphal tick population that is feeding on rodents. We propose that this mechanism is mainly driven by habitat structure. Other factors such as rodent species composition, abundance and population demography, may also affect the rate of *Borrelia* transmission between rodents and ticks. The observed higher nymphal infestation on infected compared to uninfected *A. sylvaticus* deserves further investigation. In addition, future studies should focus on spatial heterogeneity of ticks and rodents on a smaller spatial scale within habitats. Before comparison of sites on a larger spatial scale can be put to value, potentially large local heterogeneity in (infected) tick density (Zeman 1999) needs to be assessed in relation to spatial host activity. The observed spatial heterogeneity in *Borrelia*, tick and rodent populations also stresses the importance of longitudinal studies.

Acknowledgements
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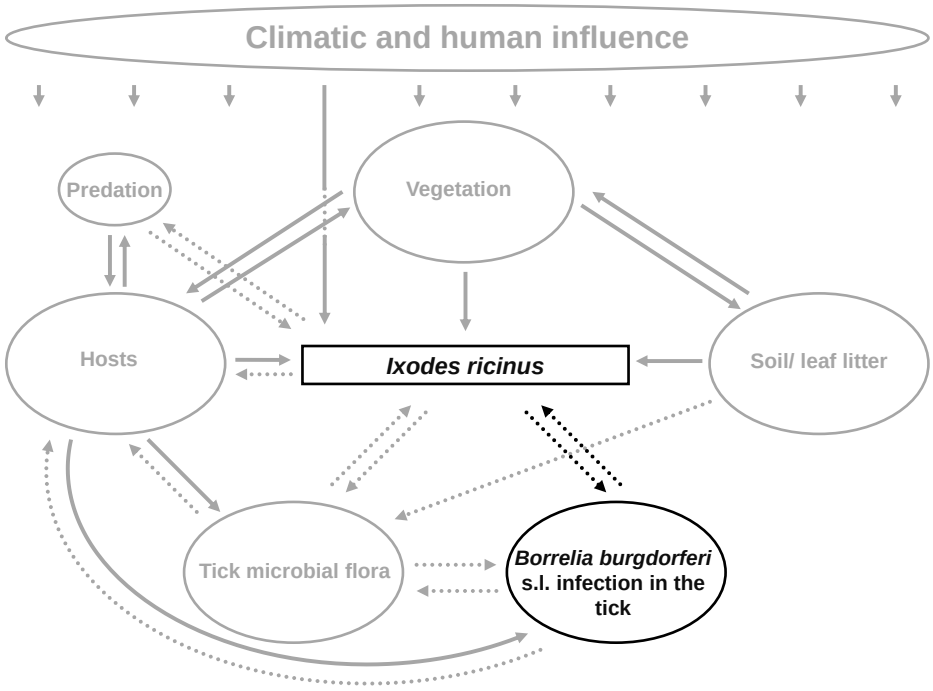
Chapter 7

Behavioural changes in *Ixodes ricinus* ticks mediated by *Borrelia* parasites

Fedor Gassner, Maykel van Gent and Willem Takken



Abstract
Parasite-mediated changes in host behaviour are a common phenomenon that is often regulated by the parasite to increase its dispersal. Here, we investigated whether the infection with the causative agent of Lyme borreliosis, *Borrelia afzelii*, affects the behaviour of the sheep tick *Ixodes ricinus*. By using automated digital tracking we investigated the behaviour of field-collected, naturally-infected, as well as laboratory-reared artificially infected *I. ricinus* nymphs in a vertical walking arena. Field-collected as well as laboratory-reared *B. afzelii*-infected *I. ricinus* nymphs walked faster than uninfected control ticks. Moreover, the laboratory-reared infected ticks walked during longer periods of time. As a result of these behavioural changes, the efficacy of tick host-finding may be higher in *B. afzelii*-infected *I. ricinus*, resulting in increased circulation of *Borrelia* in nature and an increased risk of Lyme borreliosis for humans.

Introduction

Micro-organisms that depend on arthropod vectors for their transmission from one host to another are known to affect their arthropod host's fecundity, mortality, developmental time and behaviour (Dobson 1988, Hurd 2003, Schaub 2006, Lefèvre and Thomas 2008). In some cases, these parasite-driven effects on hosts also affect vectors of medical and/or veterinary importance. For example, mosquito host-searching and blood-feeding behaviour can be affected by malaria parasites (Koella and Packer 1996, Koella et al. 2002) and sandflies show altered feeding behaviour when they are infected with *Leishmania* parasites (Rogers and Bates 2007, Ready 2008). Parasite-driven change of vector behaviour can result in an increased transmission rate of the pathogen, and, therefore, increased human health risks.

Whereas many parasite-driven behavioural changes have been reported for the class Insecta, very few studies describe parasite-mediated effects on behaviour in the Arachnida (Schütte et al. 2006, Schütte and Dicke 2008). For example, few studies have focused on whether *Borrelia* species affect the fitness or behaviour of *Ixodes ricinus* (L.), despite its importance as one of the major vector-borne diseases in the northern hemisphere. By contrast, processes that take place within the tick during and after blood meals have been studied in detail on protein as well as gene expression level (Fingerle et al. 2002, Pal et al. 2004, Hovius et al. 2008), but are lacking with respect to life history traits and the behaviour of *I. ricinus*. The intricate molecular interactions between *Ixodes* and *Borrelia* species suggest that the two organisms have evolved together and, therefore, additional mechanisms required for mutual interactions can be expected.

Ticks of the *Ixodes ricinus* complex are the dominant vectors of *Borrelia burgdorferi* sensu lato, the causative agent of Lyme borreliosis, which occurs throughout the northern hemisphere (Stanek and Strle 2003). Apart from *Borrelia* species, a wide array of other pathogenic and non-pathogenic bacteria, viruses and protozoans can be found in the tick midgut (Halos et al. 2006, Wielinga et al. 2006, van Overbeek et al. 2008, Stanek 2009). *Ixodes ricinus* is predominantly found in the undergrowth of woodlands and has a 2-to-6 year life cycle (Sonenshine 1991, Randolph et al. 2002). After hatching or moulting, newly-emerged ticks start questing (i.e., searching for a host in the vegetation) when environmental conditions are suitable for the specific instars (Lees 1948, Mejlon and Jaenson 1997, Randolph and Storey 1999, Perret et al. 2000). The tick utilises a variety of vertebrate hosts for its three blood meals, which are needed for successive moulting from larva to nymph, from nymph to adult and for the development of eggs by adult females. *Borrelia* species can only be acquired by the tick by a blood meal on a reservoir-competent host such as song birds and mice, and can be maintained trans-stadially and passed to the next host during the second and third blood meal. In turn, vertebrate hosts can only become infected by *Borrelia* species through the bite of an infected tick. Globally, the genus *Borrelia* consists of at least 15 genospecies, commonly named *Borrelia burgdorferi* sensu lato. *Borrelia afzelii*, *B. burgdorferi* sensu stricto, *B. garinii* and *B. valaisiana* circulate commonly in The Netherlands (Chapter 3, this thesis). The first two species and are associated with rodents, whereas the latter two are associated with birds (Hanincová et al. 2003a, Hanincová et al. 2003b, Kurtenbach et al. 2006).

Because *I. ricinus* uses an ambush strategy to find a host and rarely moves far away from the point of emergence after moulting (Gray 1985), any strategy of *Borrelia* species to increase the chance of *I. ricinus* ticks to encounter a host can be evolutionarily adaptive. Therefore, we hypothesized that *Borrelia* species mediate the behaviour of ticks to enhance the chance of transmission to hosts.

In the present study, we aimed to assess whether being infected with *Borrelia afzelii* causes behavioural changes in *I. ricinus*. This hypothesis was tested by digital tracking of field-collected and laboratory-reared nymphal ticks in a walking arena that took into account the natural questing height of nymphs of 50 cm maximum (Lees 1948, Mejlon and Jaenson 1997), including a humidity and temperature gradient that mimicked the conditions that are present in the vegetation. First, we examined the impact of *Borrelia burgdorferi* s. l. infections on walking behaviour of field-collected ticks. Because field-collected ticks are of unknown age and genetic background, and *B. afzelii* was the dominant *Borrelia* genospecies in our field-collected ticks, the experiments were verified by using artificial infection with a single strain of *B. afzelii* in laboratory-reared sibling *I. ricinus* larvae of the same age. All treated ticks were fed simultaneously on mice to eliminate the cohort effect. The results were evaluated with respect to behaviour and metabolic reservoirs.

Materials and methods

Effects of *B. afzelii* on the questing behaviour of *I. ricinus* was assessed using two approaches. In the first approach, field-collected ticks were tested in a behavioural assay. These ticks were subsequently analysed for *Borrelia* infection and for lipid content. In the second approach, larvae of a laboratory colony of *I. ricinus* were capillary-fed with growth medium containing *B. afzelii* (infected group) or with sterile medium (uninfected group). After the capillary feeding step, larvae of the infected group (n=190) and larvae of the uninfected group (n=200) were allowed to blood-feed on Balb-C mice and moult to nymphs, which were then used in the behavioural assay and subsequently analysed for *Borrelia* species infections.

Field collection of ticks

Nymphal *I. ricinus* were collected in the Syselt forest near the town of Ede, The Netherlands (in a 500m radius around 50º1’44.64”N by 5º41’38.74”E) in the fall of 2008. All nymphs were stored per 20 individuals in a glass tube containing moist paper. All captured ticks were stored at 4ºC under humid conditions in the dark until the start of the experiment, at most 3 weeks after capture. At least 2 h before the start of the experiments, ticks were placed in transparent glass vials containing moist filter paper inside the experimental chamber to allow them to acclimatize to the temperature and light.

Laboratory-reared ticks and artificial infection

Recently-emerged *I. ricinus* larvae were kindly provided by the University of Neuchâtel, Switzerland, and dispatched under near 100% RH to Wageningen by mail. A *B. afzelii* strain DT-1, originally isolated from *I. ricinus* from Hilversum, The Netherlands (G. A. Oei, pers. comm.) was grown from cryo-preserved cultures at passage 6 for artificial infection of *I. ricinus* larvae. The

spirochetes were grown in BSK medium at 33 ºC, free of antibodies, until the stationary phase was reached after approximately 1.5 weeks. Prior to capillary feeding, the cultures were resuspended and motility and cell density of the spirochetes were evaluated using a dark field microscope under 400x magnification. Using glass microcapillaries, where the medium was sucked into by capillary force, larvae were either fed with BSK medium containing the *B. afzelii* strain (~ 5.3 x 10^3spirochetes.μl^{-1}) or fed using blanco sterile BSK medium that was incubated under the same conditions as those containing the spirochetes. The capillary feeding procedure was originally developed for feeding nymphs (Broadwater et al. 2002) and was adjusted for larvae in our procedure. Larvae were gently placed upside down on double-sided tape and heat-pulled glass capillaries containing the treatment medium were fitted around the hypostome of the larvae, making sure that the palps were not inserted into the capillary. The capillaries were fixed at a ~20º angle relative to the tick body using a piece of clay. Trays containing six capillary-fitted ticks were placed under a 50Watt light bulb at 100% RH for 30 min. The feeding procedure was verified by adding red stain (1% amaranth) to the medium in some ticks, which were not included in the experiment after feeding. Active swallowing of the medium by the dye-fed larvae was observed under a 90x stereo microscope, resulting in a coloured midgut after 10 min.

After feeding, larvae were gently pulled off the glass capillaries. Clearly swollen larvae were selected and stored at 18 ºC and near 100% RH until further treatment. After 2 days, 190 *B. afzeli*-infected and 200 uninfected, fed larvae were added, separated per treatment, to two anesthetized Balb-C mice using a fine brush. Both mice were housed individually in a Makrolon-2 cage with food and water provided *ad libitum*, containing a 1-cm layer of wood-chips and a PVC tube for housing. The cages were placed in water filled trays to collect engorged larvae, which crawled to the water after detachment from the mice. Engorged ticks were collected daily for 7 days and dried for 12 h in a Petri dish containing five sheets of filter paper. Next, larvae were stored at 18 ºC and near 100% RH for at least 14 weeks, after which eclosed nymphs were used in the behavioural essay. The feeding procedure was performed under sterile conditions. The tick feeding procedure was approved prior to the study by the Ethical Animal Experimentation committee of Wageningen University and Research Centre (registration number 2009037.d).

Experimental conditions and video capture

Ticks were tracked in 21 parallel half round slots (20mm*5mm*400mm), which were milled in a white polyethylene plate (600mm*7mm*400mm) that was clamped to one side of the glass wall of a glass terrarium (www.rainforest-frogs.nl) (Fig. 7.1). Ticks were prevented from escaping by strips of metal gauze on the top and bottom of the slots and polyurethane insulation strips in between the slots. The terrarium was filled with a layer of tap water reaching 5 mm below the lowest point of the slots. A vertical temperature and relative humidity gradient (Fig. 7.2) was created by pumping cold water through copper tubing at the bottom of the terrarium. The whole setup was placed in a climate controlled chamber (20 º C, ~45% RH and continuous illumination at 1400 lux), with a continuous circulation of filtered outdoor air. Temperature and relative humidity were recorded continuously using data loggers fitted with external probes (MSR

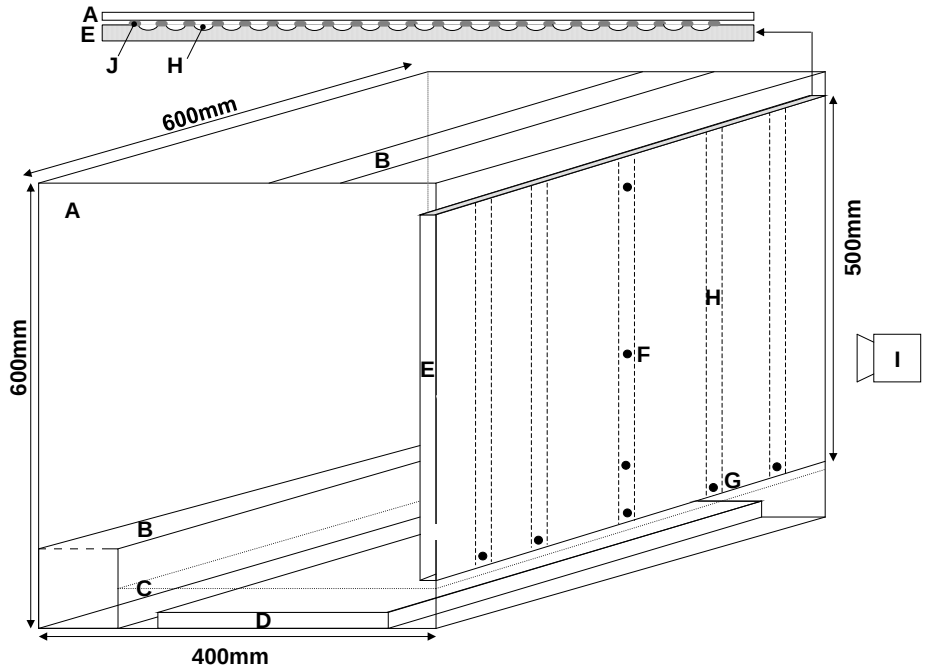


Figure 7.1. Arena for behavioural assay with detailed top view of slots. A: Glass terrarium. B: Metal mesh. C: Water level. D: Temperature regulator. E: White polyethylene board (7mm thick). F: Microclimate measurement opening (4x) in central slot (∅5mm). G: Sealable opening for tick entry (20x) (∅5mm). H: Round slot (21x) (20mm*5mm*40mm). I: webcam attached to PC for frame capture. J: Polyurethane insulation strips (3mm thick* 9mm wide).

electronics GmbH, Henngard, Switserland), which were palced in control holes at three heights in the central slot (Fig. 7.1).

A webcam (Logitech Quickcam Fusion, www.logitech.com) was programmed to shoot a 1280x1024 pixel jpeg frame every 3.54 seconds using Matlab software with an image acquisition toolbox (Mathworks inc. Matlab v.7), (van de Koppel et al. 2008). Reflections from the glass were minimised by fitting white paper sheeting around the camera and adjacent walls. Using a fine brush, the nymphs were introduced into the slots through the sealable entry holes, located at the lower end of the slots, at the start of the experiments. Both treatments of the laborator- reared ticks were randomly distributed over the slots. The ticks were recovered from the arena and preserved in 70% ethanol for further analysis. To prevent contamination of the arena with human odour, the experimenters wore gloves at all times while handling the arena. In between experimental runs, all surfaces in the arena were cleaned using 90% ethanol and dried using compressed air. The tap water in the bottom of the arena was refreshed after each run. Wild ticks were observed during 10 runs, of which four lasted 10 h, three lasted in between 5.5 h and 7 h, one lasted 3.3 h and one lasted 1.8 h. The laboratory-reared ticks were observed during six runs, including one run of 7 h and five runs of 10 h. To avoid a learning effect, each run was done with naïve ticks.

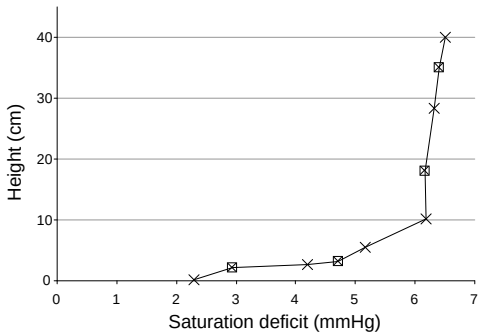


Figure 7.2. Microclimate profile inside the experimental arena (see Figure 7.1) as shown by saturation deficit. Squares indicate were measurements taken through the microclimate measurement openings; crosses are measurements taken centrally in the open area of the arena.

Borrelia *species detection and lipid analyses*

To allow for simultaneous detection of *Borrelia* species and analyses of the lipid fraction, ethanol-stored ticks were cut in half. This was achieved by placing the tick on a droplet of paraffin fixative (Sakura Finetek, Alphen aan den Rijn**,** The Netherlands), which was shortly hardened at -30 ºC. Subsequently, the tick was cut into two equal halves longitudinally using a sterile fine razor blade under a stereomicroscope. The lipid fraction of the ticks was analysed by chloroform extraction (van Es et al. 1998, Randolph and Storey 1999). Presence of *Borrelia* species in the ticks that were subjected to the behavioural analyses was identified by using alkaline lyses as described by Gassner et al. (2008) to extract DNA, and applying a real-time quantitative PCR targeting the *Borrelia* HBB gene (Portnoi et al. 2006).

Data analysis

The captured frames were converted into a grayscale digital video file (AVI) using Virtualdub software (Virtualdub version 1.8.8) at four frames per second. The resulting video files were analysed using tracking software (Ethovision Pro, version 3.1.16, Noldus B.V., Wageningen, The Netherlands), which allows simultaneous tracking of 20 objects in predefined calibrated arenas, which overlapped the slots. The resulting data files contained an x and y coordinate of the presence of a tick in each slot, which allowed calculation of the distance covered per frame by using the Pythagoras theorem. Next, velocity ($mm.s^{-1}$) and time spent in specific zones of the arena could be calculated. The fraction of total time present in a specific zone and the mean velocity per run were compared between the ticks that were infected with *B. afzelii* and ticks in which no *Borrelia* DNA could be detected for the field-collected ticks. For the laboratory-reared ticks, comparisons were made between both treatments. All behavioural data were manually compared to the real position of the tick in the original frames. All data were analysed using non-parametrical Mann-Whitney U-tests in Genstat software (release 12.1).

Results

Of the field-collected *I. ricinus* nymphs, 160 were used in the behavioural assay, of which 71 nymphs (44%) were infected with *B. burgdorferi* s.l. Of these *Borrelia* species infections, 54 (76%) were *B. afzelii*, 4 (5.6%) were *B. garinii* and 7 (9.9%) could not be identified to species by the PCR assay; 8 (11.3%) were double infections (*B. afzelii* + *B. garinii*: n=4; *B. afzelii* + *B. burgdorferi* s.l.:

n=4). For the laboratory-reared ticks, in total, 47 (24.7%) of the infected group and 49 (24.5%) of the uninfected group were recovered from the water trays, after having fed on and being detached from the mice. All but one of the engorged larvae successfully moulted to nymphs within 63 days. In total, 47 nymphs from the infected and 46 ticks from the uninfected group were included in the behavioural assay and PCR analyses. Within the group of infected ticks, 10 (21.3%) of the nymphs were found to be PCR positive for presence of the *Borrelia* species HBB gene.

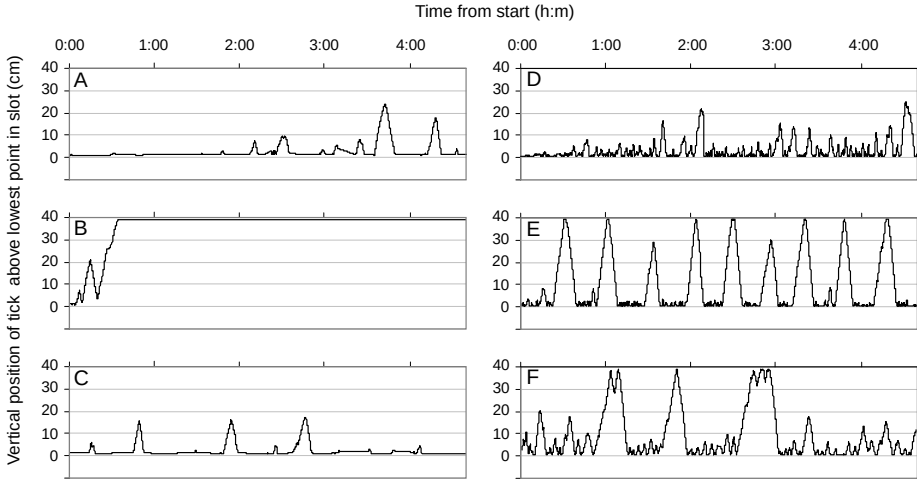


Figure 7.3. Examples of vertical activity profiles of individual *Ixodes ricinus* nymphs in the walking arena. A-B: *Borrelia afzelii*-infected laboratory-reared nymphs. C: *Borrelia*- uninfected laboratory-reared nymph. D-E: Wild *B. afzelii*-infected nymphs. F: Wild *Borrelia*-uninfected nymph.

In both groups of ticks, various patterns of walking behaviour could be observed, which are represented by the examples given in Figure 7.3. Most walking activity was observed in the first 2 h of the experiment, although some ticks showed periods of prolonged activity up to 9 h after the start of the experiment. The mean velocity of both infected (0.16 ± 0.02 $mm.s^{-1}$) and uninfected (0.12 ± 0.01 $mm.s^{-1}$) field-collected nymphs was significantly higher than that of infected (0.05 ± 0.01 $mm.s^{-1}$) and uninfected (0.03 ± 0.01 $mm.s^{-1}$) laboratory-reared nymphs ($P<0.001$). This can also be observed by the higher frequency of walking up and down of field collected ticks (Fig. 7.3). Overall, various behaviours could be observed, such as prolonged periods spent sitting still in the highest dry zone of the arena (at approximately 40cm, Fig. 7.3B). Other ticks consistently avoided the high zone (Fig. 7.3 A, C, D), or walked the whole height of the arena (Fig. 7.3 B, E, F). Figure 3 illustrates the large variation that is present in activity patterns within the infected and uninfected groups.

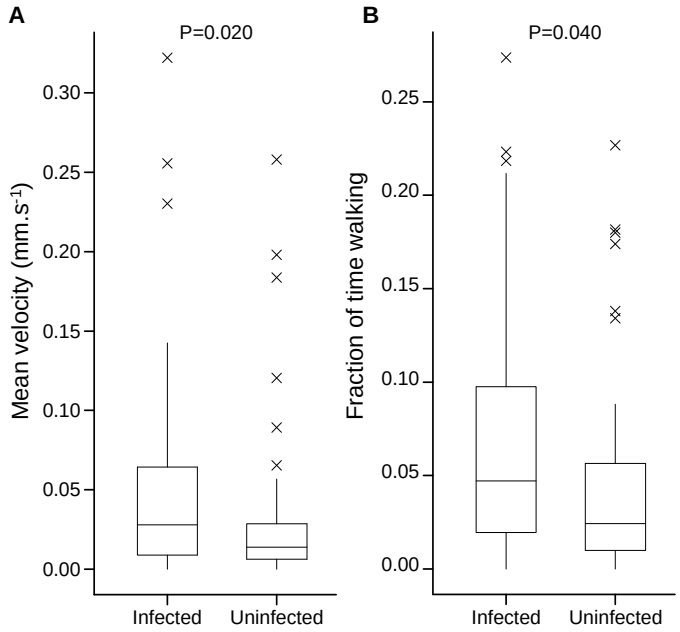


Figure 7.4. Box plot comparing the mean velocity (A) and the fraction of time active (B) of a group of field-collected *Borrelia afzelii*- infected *Ixodes ricinus* nymphs (infected, n=54) and a group of PCR-negative nymphs (uninfected, n=89).

Field-collected I. ricinus *nymphs*

Field-collected, naturally *B. afzelii*-infected *I. ricinus* nymphs walked significantly (P=0.022) faster (median 0.142 mm.s^{-1}) than field-collected *Borrelia*-negative nymphs (0.072 mm.s^{-1}) (Fig. 7.4 A). Although the effect of a *B. afzelii* infection was significant, much variation in velocity was observed (Fig. 7.4A), with the maximum walking speed of 0.578 mm.sec^{-1} for infected and 0.536 mm.s^{-1} for uninfected wild nymphs. Ticks that did not move during the experiment were recorded in both groups. While the median fraction of time spent walking was higher (43.0%) in infected wild nymphs compared to uninfected wild nymphs (36.8%), the overall difference was not significant (Fig. 7.4B). The maximum time that infected ticks spent walking was 94.8%, vs. 97.3% in uninfected nymphs, with nymphs that did not show any activity present in both groups. Differences in maximum walking height in the slots between infected and uninfected nymphs were not significant (all at P>0.12).

The lipid fraction of field-collected nymphs, corrected for dry weight, was significantly larger in *B. afzelii*-infected wild nymphs (median of 6.5%) than of uninfected wild nymphs (median 5.6%) (P=0.035) (Fig. 7.5). The maximum lipid fraction in infected nymphs was 9.3% of the nymphal dry weight, vs. 8.2% of the dry weight in uninfected nymphs. In both groups ticks with lipid fractions below the detection threshold (at ~0.6% of dry weight) of our assay were detected. These were recorded as zero (if these zeros are recorded as missing value, the difference between infected and uninfected nymphs was significant at P=0.010).

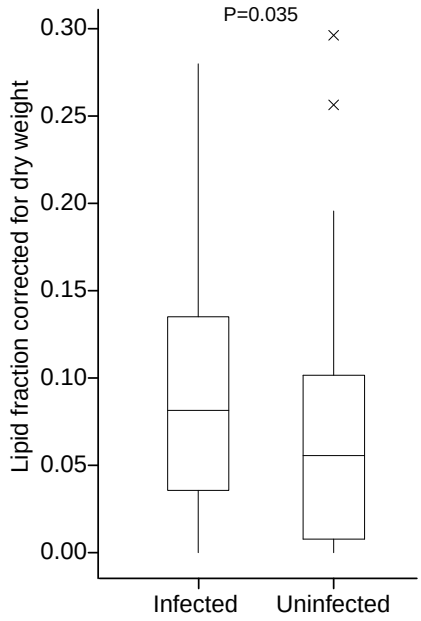


Figure 7.5. Box plot showing the lipid fraction of *Borrelia afzelii* infected field collected *Ixodes ricinus* nymphs (n=54) and field collected nymphs that were PCR negative for *Borrelia* species (n=89).

Laboratory- reared I. ricinus *nymphs*

The laboratory-reared infected nymphs walked significantly faster (P=0.020) (median 0.028 mm.s^{-1}) compared to uninfected laboratory-reared nymphs (median 0.014 mm.s^{-1}, Fig. 7.6 A), (P=0.020). The highest average speed was 0.322 mm.s^{-1} in the infected laboratory-reared nymphs and 0.274 mm.s^{-1} in the uninfected nymphs, while immobile nymphs were observed in both groups. Moreover, the infected nymphs walked during a larger part of the duration of the experiments (medians of 4.7% and 2.4% respectively, Fig. 7.6 B, P=0.040). Infected nymphs walked at most 27.4% of the time compared to a maximum of 22.7% for the uninfected nymphs.

Discussion

Ticks in our arena showed a diverse array of walking patterns. The overall behaviour of walking up and down as observed in other studies (Lees 1948, Perret et al. 2003) was clearly observed, although the frequency of these events was remarkably high in some tick individuals. Our assay discriminated behaviour such as walking or sitting still in different zones in the arena. We observed a significantly higher walking velocity of *B. afzelii*-infected *I. ricinus* nymphs compared to uninfected ones. This was observed in wild as well as laboratory-reared nymphs. Moreover, the fraction of time spent walking was higher in the infected laboratory-reared nymphs, but was not statistically significant in wild nymphs, the latter is most likely caused by the large variation in the data as the wild ticks were of unknown physiological state (Fig. 7.4B). In the field-collected ticks, the presence of other microorganisms (Halos et al. 2006,
Wielinga et al. 2006, van Overbeek et al. 2008, Stanek 2009) may also have

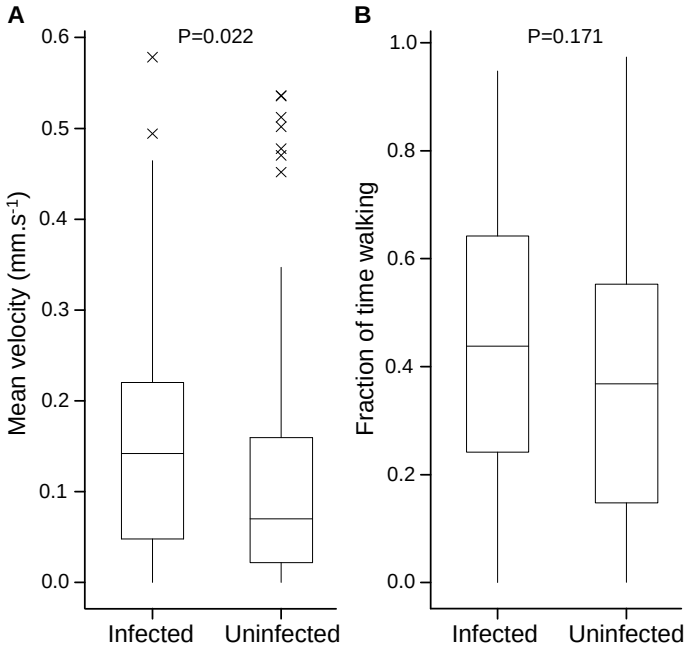


Figure 7.6. Box plots comparing the mean velocity (A) and fraction of total time spent walking (B) of a group of *Ixodes ricinus* nymphs that were artificially fed BSK medium containing *Borrelia afzelii* (infected, n=47), and a group that was fed sterile BSK medium (uninfected, n=46).

affected tick behaviour and physiology, which may add to the variation within the group of infected ticks. Moreover, the physiological age of the field-collected ticks may have been variable, as was supported by the significantly higher lipid fraction in *B. afzelii* infected nymphs. This could have been the result of a cohort difference, where the younger ticks were more frequently infected during their larval blood meal. Another explanation may be that the larval blood meal and subsequent moulting process may have been affected by infection with *Borrelia* species. This process has not yet been established for *I. ricinus* to date, but has been described for other pathogens that may also reside in *I. ricinus* (Randolph 1991, Ross and Levin 2004) and was studied in Chapter 8 (this thesis). These additional effects were avoided in the laboratory-reared and artificially-infected nymphs in our study. These originated from a single female *I .ricinus* and were infected with a single strain of *B. afzelii* and were fed on a single mouse simultaneously. Therefore, the observation of elevated activity in the laboratory-reared *B. afzelii*-infected group excludes the possibility of a cohort or physiological age effect. The observed higher activity of both field-collected and laboratory-reared ticks may lead to a higher probability of host contact and, therefore, an increased transmission of *Borrelia* (Hurd 2009).

Consequences of increased activity of Borrelia- *infected nymphs*

Although beneficial for *Borrelia* species, the higher activity may come at a cost for the tick, because prolonged activity may cause higher exposure to predation, and most importantly to desiccation. However, as *I .ricinus* produces several

thousands of eggs per adult (Sonenshine 1991), the loss of few ticks due to increased activity will not greatly affect the propagation of the genes compared to other causes of larval mortality (e.g., starvation, desiccation, or predation). For *B. afzelii*, only a single transmission event from nymph to a reservoir host will yield an opportunity for hundreds of larvae to become infected through this rodent, because rodents remain infective for the rest of their lives (Gern et al. 1994) and can only become infected through the bite of an infected nymph. Therefore, increasing the chance of host encounter by the tick vector, will increase the basic reproductive number (R0) of the *Borrelia* species. Under humid conditions, i.e., when desiccation risk for ticks is low and ticks need less energy to replenish their water balance, an increased chance of finding a host may have additional advantages for the tick as well, because an increased chance of finding a host benefits tick population dynamics (Randolph et al. 2002). Thus, increased walking speed and prolonged activity are expected to be beneficial for *Borrelia* species and, under humid conditions, also for ticks.

Prolonged activity and an increased walking velocity may also increase the human risk of contracting Lyme borreliosis. Prolonged activity by infected ticks, for example during desiccating conditions as observed by Perret (Perret 2003, Perret et al. 2003), will increase the chance to encounter humans, notably during daytime when humans are more active. The increased walking speed of infected ticks – in our study ~10% increase in the mean velocity – can cause ticks to find a suitable attachment place more efficiently, and lower the chance of detection prior to attachment. These factors may amplify the risk of Lyme disease.

Potential mechanisms of increased activity of Borrelia- *infected nymphs*

The observed increase in activity may be caused by three mechanisms. The fist mechanism involves direct effects due to, for example, resource competition between the tick and the spirochetes. Although most proliferation of *Borrelia* cells occurs during feeding by nymphs (Piesman 2001), some replication may also occur during or after moulting has taken place (Wang et al. 2003), resulting in reduced resources. Reduced resources may trigger the tick to increase its appetitive searching behaviour, a mechanism which has also been described in other examples of parasite-mediated change of vector behaviour (Schaub 2006). However, it is unlikely that the tick lipid reserves were negatively affected by resource competition with *Borrelia* species, as the lipid fraction was significantly larger in infected than in uninfected field-collected nymphs in our study (Fig. 7.5). The second mechanism involves more intricate mechanisms, which are driven by *Borrelia* species gene expression: for example, through manipulation of the water balance maintenance by the tick by reducing the resorption period, by allowing the tick to continue walking during water resorption, or by increasing the resorption threshold at which ticks decide to move to humid zones. Another factor that may lead to increased speed and prolonged walking periods may be manipulation of the genes that control foraging behaviour, such as the cGMP-dependent protein kinase (PKG) encoding gene, which has been found to affect foraging behaviour throughout the animal kingdom (Schafer 2002). A third mechanism of increased activity may be caused by *Borrelia*-species mediated changes of host and/or tick physiology during the larval blood meal, affecting blood quality or leading to

increased blood ingestion when feeding on *Borrelia*-infected hosts as demonstrated in this study. This may explain the observed higher lipid fraction in field collected ticks in our study. To date, the exact mechanism behind the *Borrelia*-induced effects remains speculative and deserves further study.

Other observations of increased activity in Borrelia- *infected ticks*

Effects of infection with *Borrelia* on *I. ricinus* have rarely been examined in the laboratory. Lecfort and Durden (1996) showed some effects of infection with *B. burgdorferi s.s.* on the behaviour of laboratory-reared *I. scapularis*. They found overall decreased activity in infected adult ticks, whereas infected nymphs showed increased phototaxis and increased activity on the vertical surfaces during their experiments, which lasted maximally 5 h. Increased activity in nymphs would potentially increase the transmission potential of *Borrelia*, as nymphs are the only known route of infection to naïve rodents, which are in turn needed for infection of larvae. As adult ticks are less likely to feed on reservoir-competent hosts, behavioral changes in this life stage are less useful for *Borrelia* species transmission. Moreover, it may be beneficial for *Borrelia* species to limit their effects in adult ticks, because egg-to-adult survival is very low and *Borrelia* species may be sporadically vertically transmitted from adult to egg. Alekseev et al. (2000) found reduced activity in *I. ricinus* as well as *I. persulcatus* during 3 -min behavioural assays, whereas increased activity was observed in *Borrelia*-infected adult *I. persulcatus* that also showed morphological anomalies. These contradictory results are likely to be influenced by experimental setup and the short time span of the experiments. Finally, in a behavioural assay that is similar to our approach, Perret showed that *Borrelia*-infected, field-collected *I. ricinus* nymphs display increased activity over prolonged periods of time under desiccating conditions only (Perret 2003). In our experiments, however, ticks were offered the choice to remain active under humid conditions in the lower zone of the arena as well as desiccating conditions higher up in the arena. We observed no effect of *Borrelia*-infection on walking height in our experiments, but velocity and time active were elevated in wild as well as laboratory-reared, *Borrelia*-infected nymphs, regardless of the microclimatic conditions.

Field observations of behavioural anomalies in *Borrelia*-infected members of the *I. ricinus* species complex are few. For example, more *Borrelia*-infected *I. scapularis* ticks were found on vertical objects such as tree trunks compared to the surrounding litter (Lane et al. 2007). In Germany, adult *I. ricinus* females attached to human volunteers were more frequently infected with *Borrelia* species compared to the infection prevalence in females collected by blanket dragging in the same area where the volunteers had walked (Faulde and Robbins 2008). Although field evidence for *Borrelia*-driven behavioural changes in *I. ricinus* is mostly indirect, it supports our observations of increased activity due to *Borrelia*-infections.

Concluding remarks

Our study is the first to describe increased walking velocity and prolonged walking duration in *B. afzelii*-infected *I. ricinus* nymphs as supported by observations on field-collected nymphs as well as laboratory-reared, artificially-infected nymphs. These behavioural changes may lead to a higher probability to find a host for *B. afzelii*-infected *I. ricinus*, leading to increased circulation of *B.*

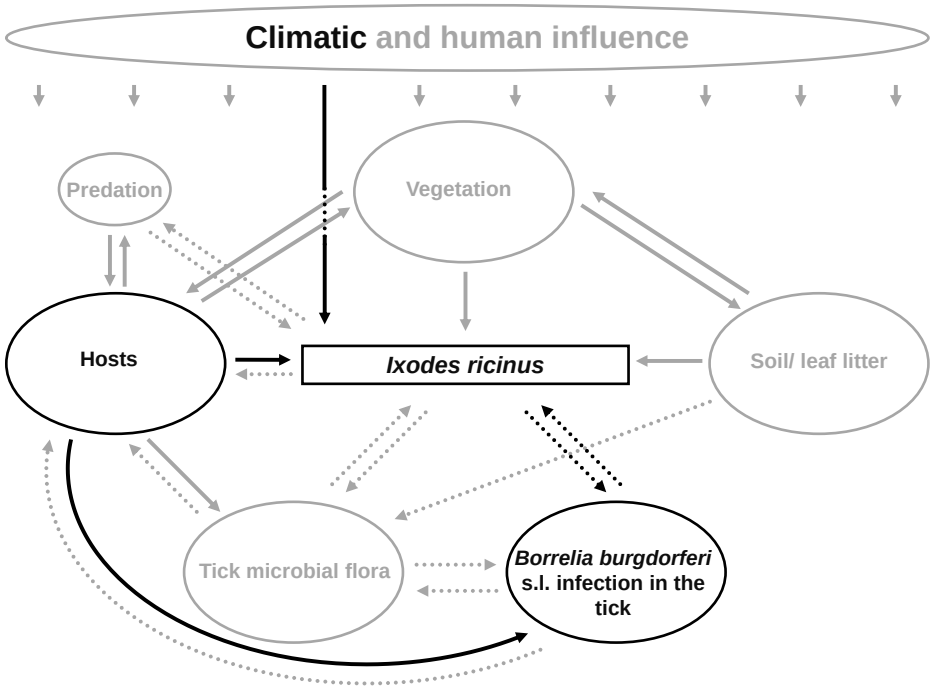
afzelii in nature and a higher *B. afzelii*-associated Lyme disease risk. Indeed, *B. afzelii* is the most prevalent *Borrelia* species in The Netherlands (Gassner et al, Chapter 3, this thesis) and Europe (Rauter and Hartung 2005). The exact mechanism that underlies the *Borrelia* -tick interaction, possibly through *Borrelia* species -mediated gene expression in host-searching *I. ricinus,* deserves further attention. Moreover, confirmation of increased host-finding efficacy by *B. afzelii*-infected *I. ricinus* nymphs should be confirmed experimentally.

Acknowledgements
Lise Gern and Olivier Rais are thanked for providing the *I. ricinus* larvae and advice on rearing ticks. Anneke Oei is thanked for providing the *Borrelia* strain and advice on growing *Borrelia* species. Staatsbosbeheer Oost-Gelderland is thanked for permission to allow rodent captures at the study site.

Chapter 8

Desiccation resistance and development of *Ixodes ricinus* is mediated by *Borrelia burgdorferi* sensu lato parasites

Fedor Gassner, Gilian van Duijvendijk and Willem Takken



Abstract
Parasites are known to be able to manipulate their host's physiology and behaviour to increase the success of dispersal to other hosts. Recently, *Borrelia afzelii*-mediated increased activity and lipid content of *Ixodes ricinus* nymphs was observed. The present study was undertaken to reveal the mechanisms underlying these observations. First, survival of *I. ricinus* nymphs under desiccating (~8.6 mmHg) conditions was studied. Next, blood-feeding duration and blood meal size were studied in wild as well as laboratory-reared *I. ricinus* that were feeding on naturally *Borrelia*-infected and uninfected *Apodemus sylvaticus* under laboratory conditions. Larva–to-nymph developmental time, nymphal size and nymphal weight were quantified in relation to *Borrelia* species infections. We found that *I. ricinus* nymphs infected with *B. burgdorferi* sensu lato were more resistant to desiccation than uninfected ticks. Wild *Borrelia*-infected larvae took larger blood meals than uninfected ones and the newly-emerged nymphs resulting from *Borrelia*-infected fed larvae had a greater body weight. These effects of *Borrelia* infections were not observed in laboratory-reared ticks. The consequences of the parasite-induced changes in relation to the circulation of *B. burgdorferi* s.l. in host vertebrates in nature as well as the differences between wild and laboratory-reared ticks are discussed.

Introduction

Parasitic infections are often harmful or lethal to the host. However, when parasites depend on one host for their dispersal, mortality of the host is often limited or postponed. In this case, the parasite may use some host resources, suppress fecundity, affect developmental time or even affect behaviour (Dobson 1988, Hurd 2003, Schaub 2006, Lefèvre and Thomas 2008). These influences may eventually effect host fitness by increased mortality or reduced fecundity but can benefit the successful transmission of the parasite.

Ticks from the *Ixodes ricinus* species complex are the principal hosts of *Borrelia burgdorferi* sensu lato, the causative agent of Lyme borreliosis. Few studies have focused on the way *Borrelia* species infections affect the physiology and/or behaviour of *I. ricinus*, in spite of the fact that it is one of the major vector-borne diseases in the northern hemisphere (Gray 1998, Stanek and Strle 2003).

The European *I. ricinus* is commonly found in the undergrowth of woodlands and has a 2-6 -year life cycle (Sonenshine 1991, Randolph et al. 2002). The tick requires a blood meal before reaching a new life stage, i.e., from larva to nymph, from nymph to adult and from adult to oviposition. *Borrelia* species can only be acquired through a blood meal on a reservoir-competent host such as song birds and mice. The *Borrelia* species remain in the tick trans-stadially and are passed to the next host during the second and third blood meal of the tick. Each transmission step from host to tick and vice-versa requires a strong adaptation of the *Borrelia* bacteria, especially because *Borrelia* depends strictly on the tick for its transmission.

The specific molecular interactions between *Ixodes* and *Borrelia* species that allow *Borrelia* survival in these different hosts, i.e., the tick and various vertebrate species (Tsao 2009, Schuijt et al. 2010) suggest that the two organisms have evolved together. Therefore, additional mechanisms required for mutual interactions can be expected, especially because transovarial transmission is rare (see review by Rauter and Hartung 2005). The primary route through which *Borrelia* species can disperse to new ticks is through a reservoir-competent host, i.e., a host that develops a systemic infection and can transmit *Borrelia* spirochaetes to feeding ticks. In turn, this host can only become infected through the bite of an infected tick. Because *B. burgdorferi* s.l. strictly depends on transmission by a tick, any mechanism that can increase the host-finding chances of an infected tick may, therefore, be highly adaptive for *Borrelia*.

Gassner et al. (Chapter 7, this thesis) recently showed that *I. ricinus* nymphs that were infected with *B. afzelii* walk faster and during longer time periods compared to uninfected nymphs. Other studies support these results, by showing increased activity (Lefcort and Durden 1996, Perret 2003), changed positions in the vegetation (Lane et al. 2007) or different host-finding frequencies of infected vs. uninfected ticks (Faulde and Robbins 2008). These behavioural changes can lead to increased exposure to desiccating conditions for *Borrelia*-infected ticks, to which they are highly sensitive (Lees 1946, Randolph and Storey 1999). This would imply that infected ticks face increased mortality, unless infection with *B. burgdorferi* s.l. offers the tick protection against desiccation. This mechanism would be most relevant for *I. ricinus* nymphs, which are the only route of infection for many reservoir hosts and

thereby contribute substantially to the circulation of *B. burgdorferi* s.l. in nature. We therefore hypothesize that *Borrelia* species-infected ticks are more resistant to desiccation than uninfected nymphs.

Furthermore, Gassner et al. (Chapter 7, this thesis) showed that lipid contents of infected *I. ricinus* nymphs are larger than of uninfected nymphs. Because *I. ricinus* nymphs have no other means of nutrient uptake apart from their larval blood meal, we hypothesise that *Borrelia* species can affect the blood-feeding process and/or subsequent moulting process.

To test the hypothesis that *Borrelia*-infected ticks are more resistant to desiccation, host-seeking (i.e., questing) *I. ricinus* nymphs were collected in the field. Subsequently, survival of these nymphs under desiccating conditions, which were similar to the dry conditions that ticks can face in nature, was studied in the laboratory. To study the effects of *B. burgdorferi* s.l. on feeding and development, wild-captured *Apodemus sylvaticus* (wood mice), an important larval host and *Borrelia* species reservoir in The Netherlands (Gassner et al., this thesis Chapter 6; De Boer et al. 1993, Gassner et al. 2008), were used. Infected and uninfected mice were kept in the laboratory, where the time until detachment of naturally-attached larvae was measured. After all wild larvae had detached, 250 laboratory reared *I. ricinus* larvae were allowed to blood feed on the mice, allowing measurement of their feeding time. All engorged larvae were weighed and their scutal index was measured. Next, interstadial development time from larva to nymph was monitored and post ecdysis body weight and size of nymphs was measured. Finally, all nymphs were tested for the presence of *B. burgdorferi* s.l.

Materials and methods

Survival of I. ricinus *under desiccating conditions*

Nymphal *I. ricinus* that were used for the desiccation experiment were collected in the Syselt forest near the town of Ede, The Netherlands (in a 500 m radius around 50º1’44.64”N by 5º41’38.74”E) during two consecutive days in July 2008. All nymphs were stored per 20 individuals in a glass tubes containing moist paper. All captured ticks were stored at 4 ºC under humid conditions in the dark until the start of the experiment, maximally 8 days after capture.

The desiccation experiment was conducted in a climate cabinet with a 16 h light (500 lux), 8 h dark cycle. The mean relative humidity during desiccating conditions was 63.0 ± 4.9% at a temperature of 25.0 ± 0.3 ºC, which corresponds with a mean saturation deficit of 8.6 ± 1.2 mmHg. The mean relative humidity during the control experiment was 93.2 ± 7.0% under a temperature of 25.4 ± 1.3 ºC, which corresponds to a saturation deficit of 1.6 ± 1.6 mmHg. All ticks that were used for the experiment were individually transferred to 0.5 ml marked Eppendorf tubes with perforated caps (five punctures made with a needle). The ticks were randomly distributed in the cabinet at each inspection moment. Tick mortality was established by checking the position and posture of the tick in each tube, applying the following four criteria: 1) lack of adherence to the tube surface: dead ticks always fall down to the bottom of the tube; 2) the position of the palps, which are extended sideways and do not cover the hypostome in dead ticks (Fig. 8.1); 3) the tick’s legs are folded inwards (Fig. 8.1); 4) if the first three criteria confirmed mortality,

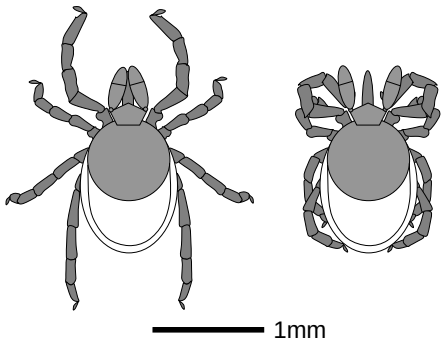


Figure 8.1. *Ixodes ricinus* nymph alive (left) and dead (right).

ticks were exposed to the experimenters' breath and placed at ~100% RH for approximately15 minutes for a final confirmation of death.

Capture and housing of rodents

Rodents were collected on the estate "Oostereng" in The Netherlands, 2 km north of the town of Renkum (52°00`27N, 5°45`20E) according to the same procedure as described by Gassner et al (2008), with the exception that 106 live traps were used here. After a 5 day pre-baiting period in June 2009, all rodents were captured in one night. Captured adult non-lactating *A. sylvaticus* were held in the field in makrolon type 4 cages until the ear tissue samples from these mice had been analysed for the presence of *B. burgdorferi* s.l. in the laboratory. This process took between 12 and 20 h. The next day, of this group of rodents, six uninfected and four infected ones were transported to the laboratory in Wageningen, where they were housed individually in makrolon type 3 cages. The cages were placed on top of a water tray for the collection of engorged larvae. Food and water were provided ad libitum and a 2-cm layer of fine wood chips and a PVC tube were provided as cage enrichments.

Engorged ticks climbed out of the cage and dropped into the water basin, from where they were collected daily at 11:00 hours during a period of 10 days. As soon as all mice were free of ticks, the mice were sedated for approximately 30 minutes. Next, 25 *B. burgdorferi* s.l.-free laboratory-reared *I. ricinus* larvae (kindly provided by Dr. L. Gern, the University of Neuchâtel, Switzerland) were placed between the ears using a brush. All mice were then placed individually in clean cages. During the next 9 days, the engorged larvae were again collected from the water basins as described above. All experiments involving rodents were approved by the animal welfare committee (DEC) of Wageningen University and Research Centre under nr 2009036.

Feeding time and body measurements

The time of detachment was measured from the moment of capture in the field (for the wild larvae) and the moment of release on sedated mice (laboratory-reared larvae) until the moment of collection in the water basins. After collection from the water basin, engorged larvae were placed between sheets of filter paper for 24 hours. Ticks were then weighed on a micro-balance (Sartorius CP2P). Scutal indices were calculated by measuring the width of the scutum and the length of the idiosoma (Gray et al. 2005). These were measured on

calibrated digital photographs, made through a stereo microscope at 50x magnification using Image Focus 2.5 (Euromex microscopen B.V., Arnhem, The Netherlands). After moulting, freshly-emerged nymphs were weighed and the scutal index was determined again.

Tick housing and interstadial development time

All engorged larvae were housed individually in small glass tubes with a piece of paper and perforated lids. The tubes were placed in transparent boxes and were kept at ~100% RH at 20 °C under a 16 h light (500 lux), 8 h dark cycle. Time between collection of larvae from the water basin and emergence of the nymphs was recorded to estimate interstadial development time. Emergence of nymphs was recorded once per day at the same time.

Borrelia *species detection*

Presence of *B. burgdorferi* s.l. was assessed in all ticks resulting from the desiccation experiment, and in all ticks that had successfully moulted to nymphs in the development experiment. DNA from individual ticks was extracted using alkaline lyses as described by Gassner et al. (2008), followed by a conventional PCR procedure using primers targeting the HBB gene of *B. burgdorferi* s.l. (Portnoi et al. 2006).

Data analyses

Differences between the mean survival times *B. burgdorferi* s.l.-infected and uninfected *I. ricinus* under desiccating and under humid conditions were compared using a Student's t-test. Kaplan-Meier curves were generated to visualise the differences and to compare curves of *B. burgdorferi* s.l.-infected and uninfected nymphs using log rank tests. Data from the feeding and development experiment were checked for normal distribution. A nested ANOVA was used to compare differences in feeding time, developmental time, scutal index, and weight of infected and uninfected ticks. The nested ANOVA corrects for pseudo-replication effects caused by the fact that multiple ticks originated from the same mouse. All data were analysed using Genstat statistical software, version 12.1. Results were considered significant at levels of $P \leq 0.05$.

Results

Survival of Borrelia-*infected* I. ricinus *nymphs under desiccation conditions*

A total of 221 field-collected *I. ricinus* nymphs were used to determine the effect of desiccation. As determined after the experiment, 32 (16.9%) of these ticks were infected with *B. burgdorferi* s.l. The average survival time of infected nymphs (64.2 ± 3.2 h, max 95.5 h) was significantly longer (P=0.035) than that of uninfected nymphs (56.3 ± 1.4 h, max 108 h). The Kaplan-Meier survival curves show that uninfected nymphs died faster over the entire experimental period during the desiccation experiment (Fig. 8.2A). The differences in the survival curves were marginally significant (P=0.051). A total of 223 field-collected nymphs were monitored under humid conditions, of which 50 (22.4%) were infected. At the end of the experiment (166.5 h), only 10.6% of the ticks had died under the humid conditions and there was no difference in survial time between infected and uninfected nymphs (Fig. 8.2B).

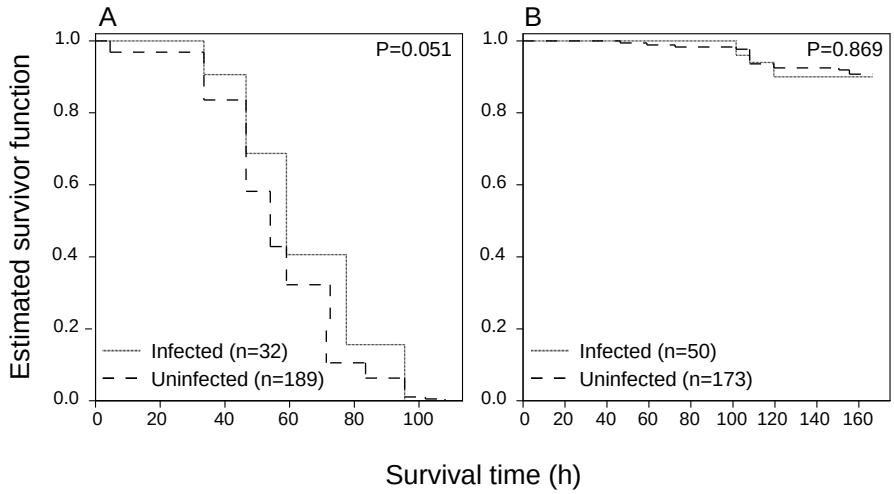


Figure 8.2. Kaplan-Meier survival curves of *Borrelia burgdorferi* sensu lato infected and uninfected field-collected *Ixodes ricinus* nymphs under desiccating (8.6 ± 1.2 mmHg) (A) and humid (1.6 ± 1.6 mmHg) (B) conditions.

Impact of B. burgdorferi *species infections on feeding and development of naturally-attached* I. ricinus*.*

From the four *A. sylvaticus* individuals that were infected with *B. burgdorferi* s.l., 50 engorged, naturally-attached larvae were collected. Twenty-one of these larvae moulted (42%) and 12 of these were infected (57%). From the six mice for which no infection with *B. burgdorferi* s.l. was demonstrated, 64 naturally-attached larvae were collected, of which 23 moulted (36%) and two of these moulted larvae were infected (9%). Scutal index and weight were determined in 44 engorged wild-collected larvae and 44 nymphs that had emerged from these larvae. The mean scutal index of infected engorged larvae (4.41 ± 0.08) was larger than that of uninfected engorged larvae (4.03 ± 0.04), but this difference was not significant (P=0.72) (Fig. 8.3A). The scutal indices of infected and uninfected nymphs were similar (1.96 ± 0.02 and 1.95 ± 0.02, respectively; P=0.88). The mean weight of engorged larvae (0.51 ± 0.02 mg) was significantly higher for infected than that of uninfected larvae (0.44 ± 0.01 mg) (P<0.05). For nymphs that emerged from naturally-attached larvae (Fig. 8.3B), the mean post-ecdysis body weight (0.27 ± 0.01 mg) of infected ones was significantly larger than uninfected ones (0.23 ± 0.01 mg) (P<0.01).

The minimal time to detachment of naturally-attached larvae was at least 1 day and at most 10 days. The mean time to detachment of infected naturally-attached larvae was 4.7 (± 0.5) days and not statistically different from that of uninfected larvae (P=0.756), which detached on average after 3.6 (± 0.2) days. The developmental time from engorged larvae to newly-emerged nymphs lasted at least 35 days, to a maximum of 61 days. The mean developmental time of engorged larvae to moult to nymphs was 48.9 (± 1.3) days for the infected ticks and 45.3 (± 1.2) days for the uninfected ticks. These differences were not significant (P=0.128).

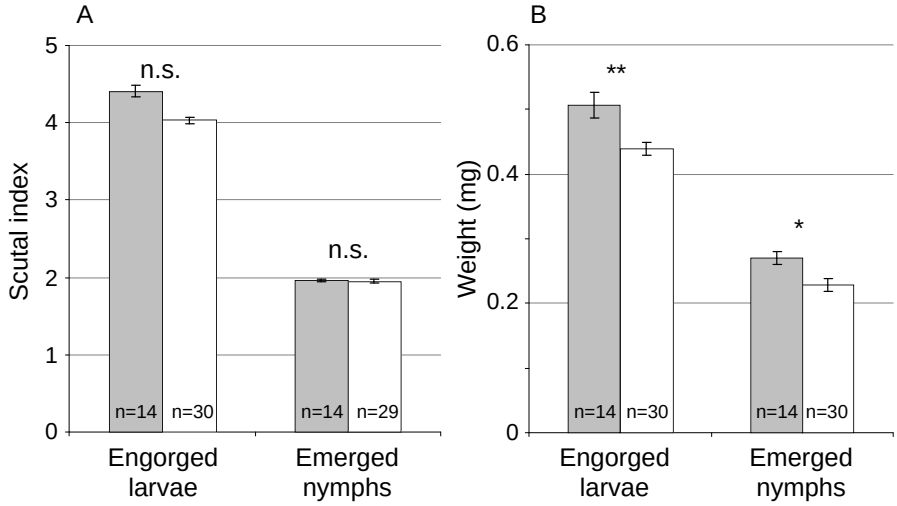


Figure 8.3. Scutal index (A) and wet weight (+/-SEM) (B) of naturally engorged *Ixodes ricinus* larvae and nymphs resulting from these larvae. Grey bars: *Borrelia burgdorferi* sensu lato infected ticks; open bars: uninfected ticks(*: P<0.05; **:P<0.01 n.s.=not significant).

Impact of B. burgdorferi *infections on feeding and development of laboratory-reared* I. ricinus.

Twenty-five laboratory-reared larvae were allowed to feed on each of the 10 *A. sylvaticus* mice that we caught in nature after the naturally-attached ticks had all become detached. From the four infected mice, 12 laboratory-reared engorged larvae were collected (12%). Nine (75%) of these larvae successfully developed into nymphs and six (67%) of these were infected with *B. burgdorferi* s.l. From the six uninfected mice, 28 engorged laboratory reared larvae were collected (19%). Twenty-one (75%) of these larvae developed into nymphs and 10 (36%) of these were infected.

Scutal index and weight were determined of 12 engorged laboratory-reared larvae and 9 newly-emerged nymphs. The scutal indices were similar for infected engorged larvae (4.01 ± 0.10) and uninfected engorged larvae (3.99 ± 0.08). The scutal indices of *B. burgdorferi* s.l.-infected and uninfected nymphs were also similar (1.91 ± 0.02 and 1.86 ± 0.03 resp.). Body weights of engorged infected as well as uninfected laboratory-reared larvae were also similar (0.48 ± 0.02 mg and 0.48 ± 0.01 mg, respectively). No statistical differences were found between the body weights of *B. burgdorferi* s.l. infected and uninfected laboratory-reared nymphs (0.26 ± 0.02 mg and 0.25 ±.0.01 mg, respectively).

The minimal feeding time, from release on the mouse until detachment, in laboratory-reared larvae was at least 3 days and at most 9 days. The mean feeding time of 5.4 (± 0.3) days was not statistically different from that of uninfected laboratory-reared larvae, which fed on average 5.3 (± 0.3) days. The developmental time from engorged larvae to emerged nymphs lasted at least 36 days and maximally 63 days in the laboratory reared group. The mean developmental time of *Borrelia*-infected engorged larvae to moult to nymphs

was 43.3 days (± 1.4). The uninfected ticks fed longer (45.8 days ± 1.4ays), but this difference was not statistically significant (P=0.525).

Discussion

Field-collected questing *Ixodes ricinus* nymphs that were infected with *Borrelia burgdorferi* s.l. survived significantly longer than uninfected nymphs under desiccating conditions. Regardless of parasitic infection, arthropods can prevent dehydration in various ways, e.g., by adjusting the size of their spiracular openings, by actively searching for less desiccating refugia, by quiescence and by direct uptake of water. *Ixodes* ticks do not drink water, but actively take up water from the air (Kahl and Knülle 1988, Needham and Teel 1991). To do so, the tick first uses energy in two ways: to move to a suitable microclimate that is suitable for the uptake process, and second by the process itself, which involves the use of hyperosmotic fluids that are produced by the tick (Rudolph and Knulle 1974).

In our experiment, the ticks were unable to move to moist refugia because the ticks were placed individually in Eppendorf tubes with perforated lids inside a desiccating environment. Therefore, the observed greater survival of *Borrelia*-infected ticks under desiccating conditions must have been a result of physiological differences between infected and uninfected ticks. For example, a higher metabolic rate in infected ticks may have increased the production of metabolic water, a process that has been described for other arthropods (Chapman 1982). Indeed, Gassner et al. (this thesis, Chapter 7) found a higher activity, combined with a higher lipid content, of infected ticks. Hence, we hypothesize that increased dehydration resistance and activity may be a result of a *Borrelia*-mediated increase in lipid content and metabolism. The question remains how *Borrelia* may alter the fat metabolism in *I. ricinus*.

The observed larger blood meal size and post ecdysis body weight of nymphs that were infected with *Borrelia* may have been caused by *Borrelia*-mediated changes in the rodent host, resulting in an increased blood uptake. However, this was not supported by longer time to detachment in naturally-attached and laboratory-reared larvae. Blood meal duration and size effects have been observed in *Babesia microti* infected *I. trianguliceps* (Randolph 1991) and *I. scapularis* (Hu et al. 1997). These observations confirmed our findings with naturally attached larvae, and showed increased engorgement weight of infected larvae. Moreover, extended feeding periods and increased feeding and moulting success were observed by these authors. Other authors describe decreased larva-to-nymph moulting success in *Anaplasma phagocytophilum* infected *I. scapularis* ticks (Ross and Levin 2004). A decreased blood meal size, caused by blockage of the feeding channel and thereby increased biting frequency, was also observed in *Leishmania*-infected sandflies (Beach et al. 1985). Hence, a parasite-mediated change of feeding and development of vectors can be positive as well as negative, but always benefits the fitness of the parasite.

The cause of the larger blood meal size of infected *I. ricinus* larvae in our study remains unknown. Possibly, it may be explained by three hypothetical mechanisms: 1) *Borrelia burgdorferi* parasites mediate changes that lead to a increased blood uptake in the tick mid gut as soon as the first *Borrelia* cells enter the midgut with the infected rodent blood; 2) the increased blood uptake is

regulated through *Borrelia*-infected mice, which possibly have been immuno compromised, due to their *Borrelia* infection or previously feeding ticks; 3) an increased blood meal size, regardless of *Borrelia* influence, lead to increased transmission of *Borrelia*, especially in the later stage of engorgement.

The observed significant effects of *B. burgdorferi* s.l. on blood meal size and nymphal post-ecdysis body weight that were found in ticks attached to field-collected rodents, and absence of significance in these factors in laboratory-reared ticks are inconsistent. Especially because previously we had found (Chapter 7, this thesis) *Borrelia*-induced changes in the behaviour of laboratory-reared ticks of the same origin as those larvae that were used in the present study. This inconsistency may have been caused by loss of, for example, genes or microbial flora that have been involved in *Borrelia*-induced changes. Laboratory colonies of arthropods are known to lose traits such as energy storage and body size during the course of time (Knoppien et al. 1980, Noorman and Den Otter 2001, Huho et al. 2007, Benedict et al. 2009). It is also possible that laboratory-reared ticks respond differently to host-derived immunogens, suppressing the observed effects of *Borrelia* that occur in ticks of natural origin.

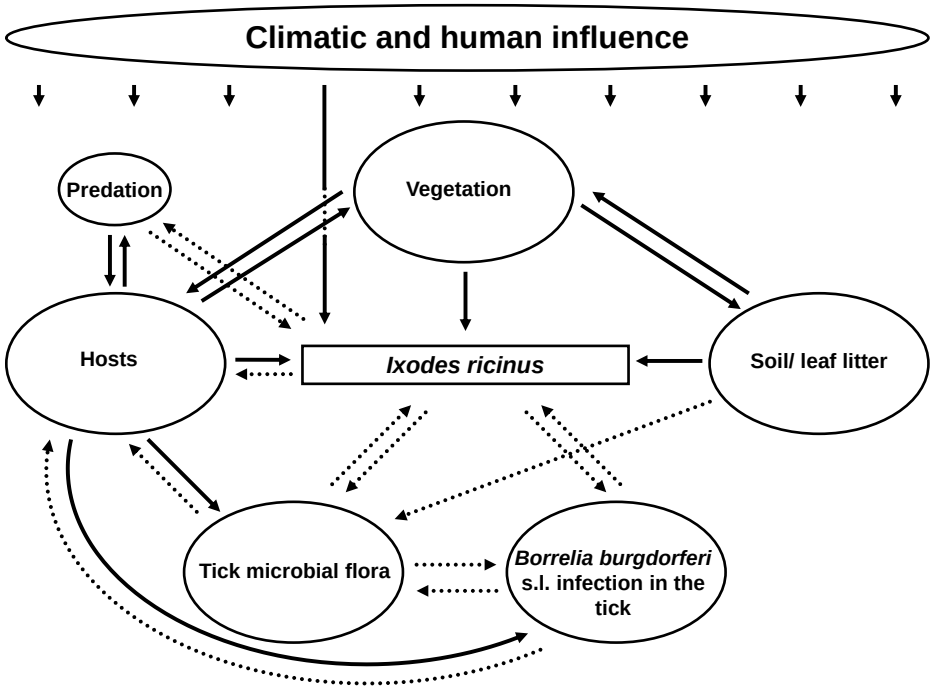
The results of this study support our previous findings reported in Chapter 7 (this thesis), by providing evidence that *B. burgdorferi* s.l.-infected ticks survive longer under desiccating conditions. As *I. ricinus* appears more efficient in finding rodent hosts under humid than under dry conditions (Randolph and Storey 1999), increased activity at a lower humidity may also result in increased host-finding efficiency. The observed increased blood meal size and larger post-ecdysis body weight of nymphs can lead to storage of a larger lipid reservoir in *Borrelia*-infected *I. ricinus* nymphs, as was found previously by us (Chapter 7, this thesis). A larger lipid storage and metabolism in *Borrelia*-infected *I. ricinus* allows for:1) higher activity over prolonged periods of time (Chapman 1982); 2) more active absorption of water from the air (Kahl and Knülle 1988) which, as hypothesised in this study, increases the use of metabolic water. This hypothesis should be assessed by further research. The consequence of the *Borrelia*-mediated changes in *I. ricinus* can be that infection rates further increase as a result of positive selection for *Borrelia*-infected ticks in nature, which results in an increased risk of Lyme borreliosis for humans.

Acknowledgements
Lise Gern and Olivier Rais are thanked for providing the *I. ricinus* larvae and advice on rearing ticks. Ellen Weerman is thanked for her practical assistance and advice on nested ANOVA. Sander Koenraadt is thanked for his advice on survival analysis.

Chapter 9

General discussion and future perspectives

Fedor Gassner



Introduction

Lyme borreliosis is an emerging vector-borne zoonosis throughout the northern hemisphere (Steere 1994, Stanek and Strle 2003, Steere et al. 2004, Smith and Takkinen 2006, Stanek and Strle 2009). The disease is caused by the bacterium *Borrelia burgdorferi* sensu lato and transmitted to humans and animals by ticks of the *Ixodes ricinus* complex (Fig. 1.1, Chapter 1) (Burgdorfer et al. 1982, Burgdorfer 1983). Controlling these ticks in nature has proved to be difficult, as ticks can be transported over longer distances by hosts, ticks can hardly be trapped, and ticks often live in protected nature areas (Tälleklint and Jaenson 1995, Ostfeld et al. 2006, Piesman 2006). Moreover, expansion of suitable habitats for ticks, increased contact with humans, greater host availability, and changing climatic conditions have improved conditions that favour transmission of Lyme borreliosis spirochetes. This has led to the prediction that Lyme borreliosis will be a continuing public health concern in the coming decades (Steere et al. 2004). In The Netherlands, Lyme borreliosis has faced a fourfold linear increase up to 22,000 confirmed cases over the last 15 years (Hofhuis et al. 2006, Hofhuis et al. 2010). Although certain components of the ecology of Lyme borreliosis have been well studied internationally, many interactions between these components require further study (Fig. 1.2, Chapter 1).

In this thesis, I have addressed various aspects of the ecology of Lyme borreliosis and their interactions. I will discuss the findings of this thesis in accordance with the three research questions stated in Chapter 1:

1. What is the spatial and temporal distribution of ticks and their associated microbial communities?

2. Do environmental factors (e.g., large herbivores and rodents) play a role in the spatial distribution of *I. ricinus* and their *B. burgdorferi* s.l. infections?

3. Does infection with *B. burgdorferi* sensu lato affect tick behaviour and development?

Spatiotemporal distribution of ticks and associated microbial communities.

For the first time in The Netherlands, a large scale monitoring programme was initiated to assess spatiotemporal variation in *I. ricinus* and their *B. burgdorferi* s.l. infections (Chapter 3). Previous investigations had included only a few study sites and were of only short duration or small temporal resolution (Nohlmans et al. 1990, De Boer et al. 1993, Rijpkema et al. 1994, Nohlmans et al. 1995, Rijpkema et al. 1995, Rijpkema and Bruinink 1996, Rijpkema et al. 1996, Schouls et al. 1999, Smit et al. 2003, Wielinga et al. 2006, Tijsse et al. 2010). Although these studies yielded valuable ecological information, information on *B. burgdorferi* s.l. strains and methods for detection of pathogens in ticks, the small spatial and temporal scale and discontinuity of the data sets preclude extrapolation of the results to a nationwide perspective. In Chapter 3, we showed that infected ticks were present at all 24 studied sites, which were

spatially distributed over the entire country. There is significant heterogeneity among these 24 sites with respect to tick densities as well as *B. burgdorferi* s.l. infection rates in ticks. The infection rates in *I. ricinus* nymphs were higher than the European average and higher than that recorded in most earlier studies in The Netherlands (Rauter and Hartung 2005). Moreover, apart from seasonal fluctuation in tick density, *B. burgdorferi* s.l. infection rates in ticks showed significant temporal variation. A third scale of variation was observed in *Borrelia* species composition, which differed significantly between sites, with an overall dominance of rodent-associated *B. afzelii*. In spite of the dominance of *B. afzelii* in most sites, several sites contributed a significant number of bird-related *B. valaisiana* and *B. garinii*. These findings indicate that habitat-related host associations are present in the ecology of Lyme borreliosis in The Netherlands.

The observed dynamic heterogeneity in Lyme disease ecology provides opportunities as well as constraints for researchers and policy makers that aim to describe, predict and eventually control Lyme borreliosis. The opportunities lie in the variation in activity of the vector as well as the prevalence of the pathogen, since variation can be the key to unravel factors that determine the variation. A disadvantage of the observed dynamic heterogeneity is that Lyme disease becomes difficult to control, because location-specific factors such as different host species, local habitat characteristics and microclimate may affect disease transmission variously. The dynamic heterogeneity stresses that large scale longitudinal studies are essential in understanding the ecology of Lyme borreliosis.

Combining data on vector and pathogen prevalence in nature (Chapter 3) with high-resolution epidemiological data (Hofhuis et al. 2006, Hofhuis et al. 2010) will help to generate a nationwide spatial risk model for Lyme borreliosis. This model can enable efficient targeting of disease prevention efforts such as tick control and public information, directly at Lyme borreliosis transmission hot spots.

Apart from the observed variation in *I. ricinus* and their *B. burgdorferi* s.l. infections, the diversity of other pathogenic and non-pathogenic bacteria that reside in *I .ricinus* also showed significant variation between habitats (Chapter 4). Although no relation between bacterial diversity and *B. burgdorferi* s.l. infections was found, the recorded diversity opens a new view on interactions between the *I .ricinus* and microbiota, or between bacteria within the same tick. Although not the subject of our study, other studies have shown that co-infections of *Borrelia* species with *Anaplasma* species and *Rickettsia* species occurred more often than was to be expected from coincidental co-infections (Wielinga et al. 2006, Ginsberg 2008). This suggests that transmission and survival of one microorganism may favour transmission and survival of the other, or that both are simultaneously transmitted from the host. The origin and transmission of some of the bacteria that were found remains unresolved. For example, the detection of *Wolbachia* species in ticks (Chapter 4, this thesis; F. Gassner unpblished results; Noda et al. 1997, Benson et al. 2004, Hartelt et al. 2004) deserves future attention. Especially because *Wolbachia* has been described to induce reproductive alterations such as parthenogenesis, male-killing, feminization and cytoplasmic incompatibility in arthropods (reviewed in Werren 1997, Stouthamer et al. 1999).

The observation of DNA from *Rickettsia australis*, the causative agent of Queensland spotted fever in Australia, should be viewed with caution. Although our isolated 16S clone showed nearest matches with this pathogen, recent confirmation targeted at multiple genes showed this clone was more likely *R. helvetica*, which is commonly found in European *I. ricinus* (Christova et al. 2003, Sprong et al. 2009, Tijsse-Klasen et al. 2010). The potentially pathogenic bacterium *Candidatus* Neoehrlichia mikurensis that was detected from ticks collected in all six study sites (Chapter 4) has recently been linked to medical conditions in humans in Europe (Fehr et al. 2010, Von Loewenich et al. 2010, Welinder-Olsson et al. 2010). Future analyses of pathogens in ticks would benefit from specific detection of this pathogen. For example, Wielinga et al. (2006) found *Ehrlichia* that could not be determined to species in 1.8 to 3.8% of ticks at four study sites distributed over The Netherlands. Finally, interactions within the tick microbial communities through quorum sensing (Keller and Surette 2006), niche competition and resource competition in relation to the fitness and behaviour of the tick adds a new and challenging component to the ecology of Lyme borreliosis.

The heterogeneity in tick and pathogen distributions as observed in The Netherlands will form a challenge for future management of and research on tick-borne diseases. At a local scale, such as one forest, or even in the transition zone between habitats (Reisen 2010), hot spots of disease transmission may occur due to local coincidence of factors that promote disease transmission. Insufficient sampling efforts are unlikely to detect such hotspots, which can result in a wrongly-assumed absence of tick borne zoonoses. The future challenge will be to more accurately describe the “underwater “ part of the zoonotic iceberg (Randolph and Šumilo 2007), notably the section that overlaps urban areas, with special emphasis on Lyme borreliosis and other potential tick-borne zoonoses.

Tick – host – pathogen interactions

Over the past few years, it has become evident that deer play a significant role in the amplification of tick populations because they are a blood host to the adult ticks (Wilson et al. 1988, Gray et al. 1998, Rand et al. 2004, Beninati et al. 2006, Ruiz-Fons and Gilbert 2010, Vor et al. 2010). On the other hand, deer cannot transmit *Borrelia* species to feeding ticks (Chapter 1, Fig. 1.1) (Telford III et al. 1988, Jaenson and Tälleklint 1992), and instead may divert ticks from reservoir hosts, such as rodent species, which can infect ticks (LoGiudice et al. 2008, Keesing et al. 2009). It has been proposed that deer management can create an effective management strategy of Lyme disease (Piesman 2006). As various large bovids are increasingly being used in the management of vegetation in natural zones (Olff et al. 1999, Richter and Matuschka 2006, Hancock et al. 2009), concurrently with deer, it was assumed that these grazers might contribute to increased tick populations. In view of this assumption, it was surprising that the role of cattle in the circulation of *Borrelia* species and the growth of *I. ricinus* populations has been scarcely studied in woodland settings.

The data in chapter 5 show that a significantly lower tick density was found in the grazed forests, whereas *B. burgdorferi* s.l. infection rates were similar in the grazed and ungrazed areas. This reduction in tick density was mainly attributed to reduced rodent densities. A similar finding was recorded in

a French forest, where the forest with introduced cattle harboured fewer ticks, but also a lower *B. burgdorferi* s.l. infection rate (Richter and Matuschka 2006). However, little is known about other mechanisms that may promote or inhibit the reducing effect of cattle on tick populations. For example, the resistance of specific breeds of bovines to ticks -and pathogens- may differ (Glass and Jensen 2007) and long-haired cattle such as the Scottish highlander may be more suitable hosts for ticks than short-haired cattle. These species-dependent factors should be taken into account before the introduction of large herbivores into tick-infested habitats. Moreover, differences in the regulatory effect of tick populations may arise from the density and specific terrain used by the cattle. For example, long residence time of cattle in an adjacent field or heath may be detrimental to attached ticks that drop off in these habitats because ticks are less likely to survive in open fields and heathland due to desiccating conditions (Boyard et al. 2007, Tijsse et al. 2010). Finally, the health of the cattle should be carefully monitored, as immuno-compromised or stressed individuals in a population may harbour disproportionally high numbers of parasites (Shaw and Dobson 1995). Ultimately, the use of cattle could aid forest management on the one hand, and aid tick management on the other hand (e.g., Chapter 5, this thesis). Additionally, the possibility of introducing cattle into woodlands, with subsequent treatment of the cattle with synthetic or biological control methods, would enhance the regulatory effect of cattle on ticks.

The regulatory role of rodents in the ecology of Lyme borreliosis (Humair et al. 1999, Hanincová et al. 2003, Kurtenbach et al. 2006) is also suspected in The Netherlands, based on the dominance of rodent-associated *Borrelia* species at most of the locations studied in this country (Chapter 3). The relation between *Borrelia* species, rodents and *I. ricinus* was examined in Chapter 6. Sites with high *B. burgdorferi* s.l. infection rates in rodents corresponded to high infection rates of host-searching ticks in the vegetation. I conclude that the exposure of rodents to host-searching ticks determines infection rates in rodent populations and subsequent transmission of *B. burgdorferi* s.l. to feeding larvae. Such interactions are promoted in habitats where rodents are concentrated in patches of vegetation, especially those were few alternative hosts are available (Allan et al. 2003). This increased contact between ticks and rodent hosts may be promoted by other factors, such as increased attractiveness of infected rodents for ticks (Chapter 6). Similar situations have also been observed in mosquitoes, where *Plasmodium*-infected humans showed increased attractiveness to *Anopheles* species mosquitoes (Day and Edman 1983, Lacroix et al. 2005), in sandflies that exhibit increased attraction to *Leishmania*-infected hamsters (O'Shea et al. 2002) and in virus – aphid interactions, where plants infected with a virus show increased attractiveness for aphids (Eigenbrode et al. 2002, Alvarez et al. 2007). Similar mechanisms could lead to increased transmission potential for *Borrelia* species, as has been proposed by modeling tick disease systems (Brunner and Ostfeld 2008). This combined mechanism of increased host contact and increased attractiveness of infected hosts may amplify the *Borrelia* circulation between ticks and hosts locally, leading to potential transmission hotspots with elevated risk of Lyme borreliosis for humans.

Borrelia mediated changes in *Ixodes ricinus*

Apart from the possible self-amplifying mechanism, where *Borrelia*-infected mice show an increased tick burden as described in Chapter 6, other mechanisms that lead to increased circulation of *B. burgdorferi* s.l. can also be expected. In Chapter 7, I address the question whether ticks that are infected with *Borrelia* species show different behaviour than uninfected ticks. My data show that infected ticks are more active, as expressed by increased walking speed and prolonged activity. Indeed, longer periods of activity can induce the host-finding chance (Randolph et al. 2002), and thereby may contribute to an increased basic reproductive number (R0) for those *Borrelia* species that are able to affect their vector (Hartemink et al. 2008, Matser et al. 2009, Tsao 2009). It is unknown whether all *B. burgdorferi* s.l. genospecies possess the capability of vector manipulation. For example, *B. afzelii* and *B. burgdorferi sensu stricto* are both transmitted by rodents, but the former is by far more dominant in The Netherlands (Chapter 3) and elsewhere in Europe compared to the latter species (Rauter and Hartung 2005). Because of this discrepancy, the manipulative traits may not have evolved in all genospecies.

The question remains, whether the *Borrelia* parasite is truly "selfish", or that the tick may also benefit from the induced changes. The increased survival time under desiccating conditions, increased blood-meal weight and post-ecdysis weight in *Borrelia*-infected ticks that was described in Chapter 8, may also contribute to the fitness of both tick (i.e., through increased survival) and *Borrelia* (i.e., by increasing the chance of transmission to a new host). Moreover, *Borrelia* species can benefit from tick saliva, which provides protection against the host immune system once the spirochetes invade the host body (Fikrig and Narasimhan 2006, Hovius et al. 2008). Therefore, maintaining the health of the tick can benefit *Borrelia* not only by increasing the host-finding chance, but also by saliva-assisted transmission to the host.

Ultimately, R0 modelling by using for example next generation matrices (Hartemink et al. 2008) may form a useful tool to simulate the impact of *Borrelia*-induced changes in tick behaviour and fitness. Combining such an approach with empirical evidence obtained from further behavioural analyses of infected ticks, preferably by assessing host-finding efficiency, can elucidate whether the observed *Borrelia*-mediated changes in ticks are evolutionarily adaptive and can lead to increased risk of Lyme borreliosis for humans.

Future perspectives for prevention of Lyme borreliosis

With currently over 22,000 annual cases of Lyme borreliosis in The Netherlands, it would be valuable to expand prevention efforts from solely public information, as is currently the case, to actual efforts to control ticks, as this is the only effective method for the prevention of Lyme disease (Piesman 2006). The results presented in this thesis show that *Borrelia*-infected *I. ricinus* are widespread in Dutch nature areas. Success of available tick control measures, as reviewed in Chapter 2 of this thesis, may depend on location-specific ecological factors.

Chapter 3 and 6 of this thesis show that, although widely present, tick densities exhibit strong spatial variability. Moreover, multiple hosts may be involved in amplifying tick populations and the circulation of *Borrelia* species. Before tick populations could be targeted by such a measure, an assessment of

host associations would aid in predicting the successful outcome of the control measure. For example, aiming a host based control strategy at rodents in an area where birds play a significant role, would yield little effect on disease prevention. Therefore, future research on tick populations would benefit from discrimination of the *B. burgdorferi* genospecies, possibly in combination with detection of host DNA remains in host-searching ticks for an even higher specificity of host associations (Kirstein and Gray 1996**,** Humair et al. 2007).

Once the responsible hosts are known, tick control can be targeted at the host, for example by application of tick control agents (i.e., chemical or biological) to the bait stations for tick control on deer, or to nesting material for rodents (Eisen et al. 2009). Locations with a dominant role of rodents, such as described in Chapter 6 of this thesis, may benefit from approaches using vaccination of the rodent hosts (Tsao et al. 2004). The tick-reducing effect of cattle that was described in Chapter 5 also shows high potential for future tick control, especially in combination with treatment of the cattle with tick control agents. In addition to gathering more evidence on tick reducing effects of various cattle breeds, future research could assess whether other domestic animals such as sheep, horses and goats show tick-reducing effects when they are released in tick infested habitats. Finally, authorities and the general public would benefit from centrally-available knowledge on tick management strategies. A tick management handbook (Stafford III 2004) for western Europe, and more specifically for The Netherlands would benefit tick control in recreational parks, nature areas and even gardens, where many ticks bites occur (Takken et al. 2007, Takken et al. 2008).

Future perspectives for research on the ecology of Lyme borreliosis

Several lines of research can be proposed based on the results of this thesis. The year round data collected on tick and *Borrelia* infections could be used to create risk maps, which can be used for directing public information and tick-management strategies. In our study, the recruitment and training of volunteers who captured ticks on a monthly basis, has greatly contributed to enlarging the sampling effort. Moreover, such involvement of volunteers will also enhance the awareness of tick-related health problems among the general public. Future analyses of the longitudinal tick monitoring data could be used to appoint variables that are correlated to Lyme borreliosis risk areas, for example by assessing specific plant species and climatic conditions that are related to high Lyme disease risk, on top of those variables that were described in this thesis. Ideally, the explanatory power of these variables would be tested on different spatial scales (Killilea et al. 2008). Finally, our understanding of the tick life cycle duration in The Netherlands could be expanded, for example by introducing ticks into field-based arenas, where they can be continuously monitored in a non-invasive manner (Dautel et al. 2008). Data on seasonal synchrony, in combination with data on the abundance of larval and nymphal *I. ricinus* can be used to predict the establishment of other tick-borne diseases, such as tick borne encephalitis (TBE). The observed co-feeding patterns of larvae and nymphs on *A. sylvaticus* would, in theory, support transmission of TBE, albeit in local foci (Randolph et al. 1999, Randolph et al. 2000, Randolph 2004). It would be very relevant to initiate future research that uses climatic data and data on rodent-tick interactions (as presented in Chapter 5 and 6 of this

thesis) to create predictive risk maps that identify potential TBE foci (Randolph et al. 2000), possibly by making use of the basic reproductive number (R0) of the TBE virus (Hartemink et al. 2008).

B. burgdorferi-induced changes in *I. ricinus* that were elucidated in this thesis (Chapters 7 and 8) raise three main questions. Future research would ideally be directed to answering the following questions:

1. Do the observed *B. burgdorferi* s.l. induced changes in behaviour, desiccation resistance and development of *I. ricinus* lead to an increased host-finding efficacy and accompanied increased *Borrelia* transmission?

2. Do the observed *B. burgdorferi* s.l. induced changes in behaviour, desiccation resistance and development of *I. ricinus* affect the fitness of the tick?

3. What are the mechanisms behind *Borrelia*-induced changes in *I. ricinus*?

An integrated approach of analyses as described in Chapters 7 and 8 of this thesis, combined with a quasi-natural arena setup that includes rodent hosts for *I. ricinus* larvae and nymphs (Randolph and Storey 1999) could be applied to answer the first two questions. Behavioural analyses should preferably be carried out for prolonged periods of time to elucidate the longevity of infected *I. ricinus* under optimal humid conditions. Moreover, behavioural analyses of *Borrelia*-infected and uninfected adult ticks are needed in addition to those of nymphs. A second approach to apply a quasi natural rodent arena, or perhaps dual-choice essay, could be used to test whether *Borrelia*-infected *Apodemus sylvaticus* carry disproportionately high numbers of ticks. This phenomenon, previously suggested by Brunner and Ostfeld (2008), was observed in the current study (Chapter 6, this thesis). Increased activity of *Borrelia*-infected *I. ricinus* on one hand, in combination with potentially increased attractiveness of *Borrelia*-infected rodent hosts, can result in a self-amplifying disease transmission. This can have a significant impact on the risk of Lyme borreliosis for humans.

Concluding remarks

In this thesis, it is shown that the spatial and temporal distribution of ticks and their associated microbial communities, including *B. burgdorferi* s.l., exhibit significant spatial and temporal variation. This variation can be partly attributed to differences in vegetation structure and composition. Furthermore, large herbivores can negatively affect tick populations, but not *B. burgdorferi* s.l. infections in ticks. Moreover, the data show that specific interactions between rodents and ticks can result in differences in infection prevalence between sites. Finally, the data show that *B. burgdorferi* s.l. mediates behavioural and developmental changes in *I. ricinus*. A combination of suitable habitat conditions, *Borrelia*-induced increased attractiveness of reservoir hosts and *Borrelia*-induced host-finding efficacy of *I. ricinus* may lead to self-amplifying

Borrelia transmission hot spots. Such hot spots may account for a disproportional number of Lyme borreliosis cases; therefore prevention efforts through public information and possibly integrated tick management, would ideally be directed at such places.

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Summary
Lyme borreliosis is an emerging vector-borne disease throughout the northern hemisphere. The disease is caused by the bacterium *Borrelia burgdorferi* sensu lato and transmitted between animals and to humans by ticks of the *Ixodes ricinus* complex. The possible causes of its emerging character are to a large extent hypothetical and controlling the tick in nature has proven to be difficult. Next to transmitting *B. burgdorferi* s.l., *I. ricinus* can transmit a range of other pathogens of human and veterinary importance, which makes studying the ecology of *I. ricinus* highly relevant as a tool for understanding, predicting and eventually controlling the risk of Lyme borreliosis for humans.

Ixodes ricinus is a tick species that is dominantly present in woodland areas, where climatic conditions favour tick survival and blood hosts are readily available. The life cycle of *I. ricinus* lasts at least two years, but can take up to six years if hosts are scarce and climatic conditions allow only short periods of activity. All three active life stages of *I. ricinus* – larvae, nymphs and adults - take one blood meal before they moult to the next stage. Egg development, intra-stadial development and diapause all take place in the litter layer. *Ixodes ricinus* finds its host by selecting an ambush position in the lower vegetation, where they wait for a passing host that they can hold on to, and subsequently take their blood meal from. This ambush-based host-searching behaviour is named questing. During questing, ticks may be vulnerable to desiccation, which forces them to retreat to lower zones of the vegetation, were they can take up water vapour from the more humid air. Larval *I. ricinus* emerge from the eggs free of *Borrelia*, which can be picked up during the first blood meal. Not all hosts are able to transmit *Borrelia* to ticks. Hosts that can transmit *Borrelia* to ticks that are feeding on them are referred to as reservoir hosts, and consist dominantly of rodents and song birds. Larger hosts that allow adult female *I. ricinus* to blood feed and produce their several thousands of eggs, are referred to as reproduction hosts. Ultimately, a combination of presence of a suitable, humid climate, availability of reservoir hosts and the availability of reproduction hosts will determine how many infected ticks are present in an area. These factors, in combination with human activity in such areas, determine the risk for Lyme borreliosis.

In this thesis, I studied the ecological determinants of Lyme borreliosis in The Netherlands. To do so, first the manifestation of the Lyme borreliosis in Europe was reviewed (Chapter 2). There, the history and emergence of Lyme Borreliosis is described. Further, it is discussed how climate change alone does not account for the emerging character of Lyme borreliosis, but that changing host populations, changing land use and especially human behaviour can also play a substantial role. Molecular tools for the detection of pathogen DNA in ticks are described, as well as methods that are relevant for controlling ticks in Europe.

It is often assumed, that the observed increase in Lyme borreliosis incidence in The Netherlands can be attributed to increasing tick numbers and increasing infection prevalence in ticks. In Chapter 3, a study is described where the distribution of tick populations and their *Borrelia burgdorferi* s.l. infections was studied in 24 nature areas in The Netherlands between July 2006 and December 2007. This study demonstrated that ticks infected with *Borrelia* species are widespread, but with significant spatial and temporal

variation. The spatial variation could be partly attributed to vegetation characteristics. The study also showed that *Borrelia burgdorferi* s.l infection rates in these ticks were higher than previously assumed, on average 23.7%, in 2006, but dropped substantially to 9.9% in 2007. The causes of such temporal variation in infection rates remain largely unknown. The spatial variation in tick densities and *Borrelia* infection rates in ticks could be partially attributed to the forest floor characteristics and tree cover, respectively and suggest the significance of understanding habitat characteristics for correct interpretation of Lyme disease ecology.

In Chapter 4 , the microbial diversity in ticks was studied in six different habitats and related to *B. burgdorferi* s.l. infections. A strong variation in the bacterial diversity in *I. ricinus* nymphs was found between the studied habitats, which was unrelated to *B. burgdorferi* s.l. infections. Additionally, some potentially pathogenic and symbiotic bacteria were detected, which had so far not been described in ticks from The Netherlands.

In The Netherlands, domestic herbivores are commonly used for management of the vegetation in nature areas. Large herbivores, however, can be a suitable host for ticks and the effects of these herbivores on tick populations were never assessed, in spite of their potential role as tick host. In Chapter 5, it is investigated whether cattle introduced into woodlands can amplify tick populations. It was found that the presence of cattle in woodland resulted in a locally-reduced tick density, whereas *Borrelia* infection prevalence remained the same compared to an ungrazed woodland. Apart from a lower tick density, the population size of small rodents (wood mice and bank voles), which are important hosts for immature ticks, was also found to be reduced with fewer on-host ticks compared to the rodent population of the grazed forest. Hence, it is concluded that cattle negatively affect tick densities in the studied forests, but had no significant effect on *B. burgdorferi* s.l. infection rate. However, the risk for Lyme borreliosis was decreased, as fewer infected ticks were found in the grazed study sites.

Small rodents are assumed to play a key role in the circulation of *B. burgdorferi* s.l. in nature. However, very few studies have focused on the role of rodents in transmitting Lyme disease. In Chapter 6, interactions between rodents and ticks were studied, to find out whether *B. burgdorferi* s.l. infections in questing *I. ricinus* were proportional to infections in rodents in the same area. Here it was shown that rodent populations were abundant, and larval as well as nymphal *I. ricinus* fed abundantly on these rodents. The number of ticks in the vegetation was not in proportion with those feeding on the rodents in some of the studied sites. A high *Borrelia* species infection rate in rodents corresponded with high infection rates in ticks in the vegetation. It is concluded that the contact rate between host-searching ticks and their blood hosts is essential for the circulation of *Borrelia* species, and it is argued that this interaction is largely determined by the habitat structure, which thereby contributes to the heterogeneity of tick densities and *Borrelia* infection rates in ticks.

Since the host contact rate is a very important factor in the ecology of Lyme borreliosis, *Borrelia* species can be expected to manipulate traits of *I. ricinus* that determine the host-finding capability of the tick. Especially since many parasites throughout the animal kingdom manipulate their hosts to promote their own dispersal. In Chapter 7, I studied whether *Borrelia* could

manipulate the behaviour of *I. ricinus*. Here, I showed that *Borrelia*-infected nymphs were more active than uninfected nymphs. Moreover, infected ticks contained a higher lipid fraction, which provides them with an increased energy supply compared to uninfected ticks. This manipulation of *I. ricinus* by *B. burgdorferi* s.l. may result in an increased host-finding chance of the tick, and thereby an increased transmission chance for the parasite. These combined effects are expected to enhance the risk of Lyme borreliosis.

In Chapter 8, the findings of Chapter 7 are reinforced, by showing that *Borrelia* infected ticks can cope better with desiccating conditions than uninfected nymphs. In addition, infected ticks were larger than uninfected ticks. The findings of Chapter 7 and 8 indicate that *Borrelia* species are able to manipulate the behaviour and physiology of *I. ricinus* nymphs, possibly to increase the host-finding chance and thereby the circulation of *Borrelia* in nature.

The findings in my thesis have demonstrated the heterogeneous nature of interactions between ticks, microorganisms and vertebrate hosts, and that this variation can be in part explained by habitat characteristics and host interactions. I have proposed that the application of grazing cattle into woodlands can be a potential tool for tick management. In addition, I have demonstrated that *B. burgdorferi* s.l. can manipulate *I. ricinus* behaviour, desiccation resistance and development. Future applied research based on this thesis would ideally comprise a continuation and expansion of the nationwide monitoring effort towards the establishment of climate, habitat and epidemiology based risk model. Application of cattle for tick management would benefit from additional case studies. Fundamental questions remain on how *B. burgdorferi* changes in *I. ricinus* and whether these changes would affect pathogen transmission, and would thereby be evolutionary adaptive.

Samenvatting
Lyme borreliose is de meest algemeen voorkomende en snelst uitbreidende door vectoren overgedragen aandoening op het Noordelijk halfrond. Deze ziekte, ook wel 'ziekte van Lyme' genoemd, wordt veroorzaakt door de bacterie *Borrelia burgdorferi* sensu lato. Deze wordt overgedragen van dier naar mens via de beet van teken behorende tot het *Ixodes ricinus* complex, wanneer deze een bloedmaaltijd nemen. De mogelijke oorzaken van de toename in ziektegevallen zijn voor een groot deel theoretisch. Naast *Lyme borreliose* kan *I. ricinus* een aantal andere humane en dierlijke parasieten overdragen. De bestrijding van teken in hun natuurlijke leefomgeving is lastig. Onze beperkte kennis tot op heden gecombineerd met het grote medische en veterinaire belang, maken het bestuderen van de ecologie van de teek erg relevant en belangrijk. Door onze kennis van de verspreiding, de fenologie en interacties met pathogenen en gastheren te vergroten, kan gewerkt worden aan een effectievere preventie van tekenbeten en *Borrelia* infecties, of zelfs aan de bestrijding van teken.

Ixodes ricinus leeft voornamelijk in beboste gebieden, maar wordt ook in duingebieden, tuinen en parken gevonden. Een vochtig microklimaat, een beschermende strooisellaag en voldoende gastheren voor de verschillende levensstadia om op te voeden zijn bepalend. De levenscyclus van *I. ricinus* duurt twee á drie jaar, maar ze kunnen tot zes jaar oud worden als de teek moeite heeft met het vinden van bloedmaaltijden. De drie actieve levensstadia van *I. ricinus* (de larve, de nimf en het volwassen stadium) hebben ieder één bloedmaaltijd nodig om te vervellen tot het volgende stadium. Volwassen vrouwtjes gebruiken het bloed voor eiproductie: Eén teek kan ongeveer 2.000 eitjes leggen. De ontwikkeling van de eitjes, de vervelling tussen de levensstadia, de diapauze en het opnemen van vocht uit de lucht vinden in de strooisellaag plaats. De teek verblijft soms kortstondig in de lagere vegetatie, maar deze zone wordt vooral gebruikt voor het wachten op passerende gastheren, waar ze zich vanuit een hinderlaagpositie aan vastgrijpen. Dit specifieke gastheerzoekgedrag vergroot het risico op uitdroging. Om dat te voorkomen, kan een teek tussentijds naar een plaats in de vegetatie lopen waar de luchtvochtigheid hoog is, om daar vocht uit de lucht op te nemen.

De overdracht van *Borrelia* vindt vooral via horizontale transmissie plaats (van gastheer op teek): maximaal 1% van de *I. ricinus* larven wordt via de moederteek met *Borrelia* geïnfecteerd . Maar niet alle besmette gastheren zijn in staat *Borrelia* naar teken over te dragen. *Borrelia* overdracht kan al tijdens de eerste bloedmaaltijd plaatsvinden, welke meestal op kleine knaagdieren of zangvogels wordt genomen, en waarbij de middendarm van de larve wordt geïnfecteerd. Specifieke interacties tussen *Borrelia* en het immuunsysteem van de gastheer zorgen ervoor dat de eerder genoemde zangvogels en knaagdieren ieder specifieke soorten van het geslacht *Borrelia burgdorferi* s.l. op teken kunnen overbrengen. Volwassen teken voeden vooral op grotere gastheren zoals het ree, maar kunnen net als larven en nimfen ook mensen bijten. Kleine gastheren zijn dus van belang voor het voeden van de jonge levensstadia en voor de transmissie van *Borrelia* soorten naar onbesmette teken, en grotere gastheren zijn vooral belangrijk voor de voortplanting van *I. ricinus*. De aanwezigheid van geschikte gastheren gecombineerd met het micro- en macroklimaat bepalen of een habitat geschikt

is voor de vestiging van een (besmette) tekenpopulatie. Voorts bepalen ook het infectiepercentage in teken en de kans dat een mens door een teek gebeten wordt het feitelijke risico voor het oplopen van een besmette tekenbeet.

In dit proefschrift worden de ecologische factoren die een rol spelen in de ecologie van Lyme borreliose in Nederland beschreven. Hoofdstuk 2 begint met een overzicht van de verspreiding van Lyme borreliose in Europa. Er wordt onder andere ingegaan op de geschiedenis en de opmars van Lyme borreliose. Daarnaast wordt beschreven hoe niet alleen klimaatverandering, maar ook veranderingen in landgebruik, gastheerpopulaties en menselijk gedrag een grote rol kunnen spelen in de etiologie van Lyme borreliose. Tevens worden een aantal moleculaire technieken besproken die toegepast kunnen worden voor detectie van pathogenen in teken. Ten slotte worden enkele voor Europa relevante methodes beschreven om teken te bestrijden.

Algemeen wordt aangenomen dat toename in het aantal tekenbeten en in het aantal mensen met *Borrelia* infecties in Nederland komt door toenemende aantallen teken en/of een toename in het *Borrelia* infectie percentage in teken. In hoofdstuk 3 wordt een studie beschreven waarbij de populatie dynamica van teken en hun *Borrelia burgdorferi* sensu lato infecties is geobserveerd in 24 Nederlandse natuurgebieden tussen juli 2006 en december 2007. Deze studie toont aan dat met *Borrelia* geïnfecteerde teken wijdverspreid zijn, maar met een aanzienlijke ruimtelijke en temporele variatie. De ruimtelijke variatie in tekendichtheden, evenals hun infectiegraad met *Borrelia,* kan deels worden toegeschreven aan specifieke kenmerken van de vegetatie. Zo werden hogere tekendichtheden gevonden op locaties met een dikkere strooisellaag, en werden hogere infectiepercentages waargenomen op locaties met een meer open boombedekking.

Verder was het percentage met *Borrelia* geïnfecteerde teken veel hoger dan waargenomen in eerdere studies in Nederland; gemiddeld 23,7% in 2006. Opmerkelijk was de sterke daling in het infectiepercentage naar 9,9% in 2007. De oorzaak van deze temporele variatie blijft grotendeels onbekend.

Deze studie toont duidelijk aan dat een goede kennis van de habitat van de teek essentieel is om de ecologie van de ziekte van Lyme te begrijpen. Vanwege de grote variatie in tekendichtheid en hun *Borrelia* besmettingen, zullen structurele meerjarige waarnemingsreeksen in de toekomst belangrijk zijn om eventuele trends te beschrijven en te verklaren.

In hoofdstuk 4 wordt de algemene microbiële diversiteit in *I. ricinus* nimfen uit zes verschillende habitats beschreven, in relatie tot *B. burgdorferi* s.l. infecties in deze nimfen. De bacteriële diversiteit in *I. ricinus* nimfen toonde veel variatie zowel tussen als binnen de habitats. Er was geen verband tussen *Borrelia* infecties en de bacteriële diversiteit, noch tussen *Borrelia* infecties en de aanwezigheid van specifieke bacteriesoorten. Deze analyse bracht verder enkele potentieel pathogene en potentieel symbiotische bacteriën aan het licht die nog nooit eerder in Nederlandse teken zijn waargenomen.

Bos- en natuurgebieden in Nederland worden vaak via begrazing beheerd. Herbivoren zoals schapen, paarden en runderen zijn echter potentiële gastheren voor teken. Ondanks de grote aantallen recreanten in deze gebieden en de toenemende bezorgdheid voor de ziekte van Lyme, is er nooit onderzoek gedaan in Nederland naar de relatie tussen begrazing en de populatie dynamica van teken en hun infectie percentage. Hoofdstuk 5 beschrijft een

onderzoek in vergelijkbare, door runderen begraasde en onbegraasde, bospercelen gedurende een periode van vijf maanden. De aanwezigheid van runderen in het bos resulteerde in een lagere dichtheid van teken. Ook werden er minder knaagdieren en minder teken per knaagdier aangetroffen in het begraasde bosperceel. Deze knaagdieren, zoals bosmuizen en rosse woelmuizen, kunnen voor de teek een belangrijke rol als gastheer spelen en dienen als een *Borrelia* reservoir. Het *Borrelia* infectie percentage in de aanwezige teken was in beide bospercelen gelijk. Begrazing door runderen heeft dus een negatief effect op de tekendichtheid, maar niet op het *Borrelia* infectiepercentage. Echter, doordat het absolute aantal besmette teken in het begraasde bosperceel lager was, nam het risico voor het oplopen van Lyme borreliose daar af door begrazing.

Van in het bijzonder kleine knaagdieren, zoals bosmuizen en rosse woelmuizen, wordt aangenomen dat ze een belangrijke rol spelen in de circulatie van bepaalde *Borrelia* soorten in de natuur. In Nederland werd hier echter tot op heden zeer weinig onderzoek aan verricht. In Hoofdstuk 6 wordt voor zes locaties beschreven wat de gevolgen zijn van specifieke interacties tussen knaagdieren en teken voor de circulatie van *Borrelia*. Zo is per gebied onderzocht of het infectie percentage in teken met *B. burgdorferi* s.l. overeenkomt met het infectie percentage in knaagdieren. Knaagdieren waren in grote aantallen aanwezig in alle locaties en zowel *I. ricinus* larven als nimfen werden in grote aantallen op de knaagdieren aangetroffen. Het aantal teken dat in de vegetatie werd gevonden was niet op alle locaties gecorreleerd met het aantal teken dat op de knaagdieren werd gevonden. Echter, een hoog infectiepercentage in knaagdieren correspondeerde met een hoog infectiepercentage in teken in de vegetatie. Hieruit blijkt dat de mate van contact tussen teken en knaagdieren belangrijk is voor de circulatie van *Borrelia.* De structuur van de habitat kan hierin een zeer belangrijke rol spelen en draagt daarmee bij aan de geobserveerde heterogeniteit in de dichtheid van met *Borrelia* geïnfecteerde teken.

De mate van contact tussen gastheren en teken een belangrijke factor is in de ecologie van Lyme borreliose. Daarom is het niet onwaarschijnlijk dat *Borrelia* soorten *I. ricinus* kunnen manipuleren, om zo hun transmissie succes te vergroten, iets wat algemeen voorkomt bij interacties tussen gastheren en parasieten. Hoofdstuk 7 laat zien dat met *Borrelia* geïnfecteerde *I. ricinus* nimfen meer actief zijn dan ongeïnfecteerde nimfen. Bovendien hadden geïnfecteerde teken een hoger vetgehalte, waardoor ze een grotere energievoorraad hebben dan ongeïnfecteerde teken. Deze door *B. burgdorferi* geïnduceerde manipulatie van *I. ricinus* kan een verhoogd contact met gastheren ten gevolge hebben, en zo het succes of de transmissie van *Borrelia* bacteriën vergroten. Een consequentie van deze manipulatie kan zijn dat het oplopen van een besmette tekenbeet relatief groter is dan het oplopen van een beet van een onbesmette teek.

In hoofdstuk 8 worden de bevindingen van hoofdstuk 7 ondersteund door een studie naar de droogteresistentie van *I. ricinus* nimfen. Met *Borrelia* besmette nimfen toleren droogte beter dan ongeïnfecteerde nimfen. Bovendien bleek dat geïnfecteerde teken groter waren. De bevindingen van hoofdstuk 7 en 8 samen tonen aan dat *Borrelia* soorten in staat zijn om zowel het gedrag als de

fysiologie van *I. ricinus* te manipuleren, om zo hun transmissie succes te vergroten.

De bevindingen die in dit proefschrift worden beschreven tonen aan dat interacties tussen teken, micro-organismen en gastheren een sterk heterogeen karakter hebben. Een deel van deze heterogeniteit kan verklaard worden door habitat eigenschappen en de interacties tussen teken en hun gastheren. In dit proefschrift wordt begrazing als een mogelijk instrument voor het beheersen van teken beschreven. Bovendien blijkt uit dit proefschrift dat *Borrelia* in staat is het gedrag, de droogteresistentie en de fysiologie van de teek te beïnvloeden. Toekomstig toegepast onderzoek op basis van dit proefschrift zou idealiter bestaan uit het voortzetten en uitbreiden van de landelijke monitoring van tekenpopulaties, om met behulp van klimatologische gegevens, habitatgegevens en epidemiologische gegevens tot risicomodellen van de ziekte van Lyme te komen. Deze modellen kunnen dan gebruikt worden om effectief preventieve maatregelen tegen tekenbeten en overdracht van *B. burgdorferi* s.l. te nemen. Het eventueel toepassen van begrazing als preventief middel zou echter profijt hebben van extra onderzoek waarbij bijvoorbeeld de effecten van andere herkauwers en andere habitattypes op de populatiedynamiek van teken en *Borrelia* worden bestudeerd. In toekomstig meer fundamenteel onderzoek zal de vraag hoe en in welke mate *Borrelia* in staat is de teek te manipuleren centraal moeten staan. Ook zal getest moeten worden of de waargenomen gedragsveranderingen ook daadwerkelijk tot een verhoogde overdracht van *Borrelia* bacteriën leiden, zonder daarmee te grote gevolgen voor de overleving van de teek te hebben.

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times we had and for showing me that great diversity of human culture around the world!

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I've been very fortunate to have been able to meet and work with some great colleague tick researchers worldwide. I would like to thank especially Lise Gern for welcoming me in Neuchâtel to receive training on rearing and feeding ticks. Also, I am very grateful to Sarah Randolph for giving her inspiring key note presentation at the Tick tactics symposium.

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Curriculum vitae

Fedor Gassner[1] was born in Alkmaar, The Netherlands, in 1980. He grew up in the towns of Schagerbrug, Westerland and Hippolytushoef in the north-west of The Netherlands. After finishing secondary school, he participated in a summer course to get his physics degree, which allowed him to start his Bachelor (HBO) degree in Biotechnology at the Noordelijke Hogeschool Leeuwarden. During the last year of this period, he was introduced to biological science during an internship on gene flow in bivalve populations at the NIOZ institute on the island of Texel, The Netherlands. This, together with his experiences during his BSc thesis on extant and fossil microorganisms from an Antarctic ice lake motivated him to continue along the path of biological science. He therefore started a MSc. in biology at Wageningen University, The Netherlands. After 4 years of metamorphosis, including teaching experience, some inspiring entomology essays, a thesis on the ecology of ticks and a thesis on anaerobic microbial methane oxidation, Fedor graduated in 2006 as a biologist. Meanwhile, Fedor was involved in a team of entomologists, led by Prof. Marcel Dicke, which won the "Academische Jaarprijs. This price led to the festival "Wageningen, City of Insects", where he participated as member of the dynamic and creative organizing committee.

In August 2006, Fedor started as junior scientist at the laboratory of Entomology. Supported by the IMPULS grant from the Department of Plant Sciences, Fedor joined Dr. Leo van Overbeek and Prof. Willem Takken to study the microbial diversity and ecology of ticks for a period of six months. A second IMPULS grant allowed Fedor to continue in the field of tick ecology, and also enabled writing research proposals for one additional year. During the first 1,5 years, Fedor was also involved in the analyses of tick densities and *Borrelia* infections, which were determined under the "Nature's calendar" project with Dr. Arnold van Vliet. This led to a PhD project, supported by the Laboratory of Entomology for an additional two and a half years. Here, Fedor continued his work on tick biology and executed various laboratory as well as field based experiments on interactions between hosts, ticks and *Borrelia* bacteria. During this period, Fedor visited Dr. Lise Gern at the University of Neuchâtel, Switserland, to receive training in infecting and rearing ticks. Furthermore, he participated in the training programme of the PE&RC graduate school and visited and presented at several national and international conferences. In 2010, Fedor co-organised a symposium that aimed to bring together Lyme borreliosis experts in The Netherlands and inform them and discuss cutting edge research. As of October 2010, Fedor is employed at the Foundation for Sustainable Development, which is based at Wageningen UR, where he will join the team that investigates the impact of environmental change on phenological processes, including that of ticks.

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PE&RC PhD Education Certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (5.6 ECTS)

- Lyme disease in Europe: facts and no fiction; Gassner F & Van Overbeek LS (2007) Emerging pests and vector-borne diseases in Europe; Takken W & Knols BGJ, Eds, Wageningen Academic Publishers

Writing of project proposal (7 ECTS)

- The effects of variations in habitat characteristics and pathogen infections on the biology of *Ixodes ricinus* in the laboratory and in the field

Post-graduate courses (4.5 ECTS)

- Biodiversity and ecosystem services in a sustainable world; PE&RC (2008)
- Advanced statistics; PE&RC (2009)
- Multivariate analysis; Umetrics (2009)

Laboratory training and working visits (2.3 ECTS)

- Real time PCR / Pathogen culturing; LUMC/AMC (2008)
- Tick rearing; University of Neuchâtel (2008)

Invited review of (unpublished) journal (1 ECTS)

- Stability of local environmental variables for the abundance of questing *Ixodes ricinus* nymphs on pastures; Vector Borne and Zoonotic Diseases (2010)

Deficiency, refresh, brush-up courses (3.5 ECTS)

- Proefdierkunde (2008)
- Basic statistics (2008)

Competence strengthening / skills courses (3.5 ECTS)

- Project planning and time management; PE&RC (2008)
- Scientific writing; WGS (2009)
- PhD Competence assessment; WGS (2009)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.1 ECTS)

- PE&RC Current themes in ecology (2006)
- PE&RC Weekend (2008)
- PE&RC Day (2008-2010)

Discussion groups / local seminars / other scientific meetings (7.5 ECTS)

- Monthly Entomology seminars (2006-2010)
- PhD Meetings Entomology (2006-2010)
- Tick tactics in the Low Lands; Co-initiator and organiser (2010)

International symposia, workshops and conferences (12.1 ECTS

- Annual meeting of The Netherlands Entomological Society; 3 presentations (2006-2009)
- SOVE 2009; Antalya, Turkey; poster presented (2009)
- NAEM Meeting; poster and presentation (2009, 2010)
- EDEN ; Montpellier; oral presentation (2010)

Lecturing / supervision of practical 's / tutorials (3 ECTS)

- Frontiers in medical and veterinary entomology; ENT 51306; 10 half days ex prep (2008, 2009)

Supervision of 3 MSc students; 60 days (9 ECTS)

- Interactions between rodents and ticks in different habitats
- Behaviour of *Borrelia* infected *Ixodes ricinus* ticks
- Survival, bloodmeals and development of *Borrelia* infected *Ixodes ricinus* ticks

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