QUESTING MICROBIOTICKS:

Interactions of microbes, ticks, vertebrates, and the environment

Aleksandra Iwona Krawczyk

1. The influence of external (a)biotic factors on the maintenance of symbionts in tick populations depends much on symbionts' mode of transmission. (this thesis)
2. Ticks choose their symbionts rather than symbionts choose their ticks. (this thesis)
3. An infection that is a burden for an individual can be beneficial for a population.
4. The 16S rRNA amplicon sequencing technique is a powerful tool for forming hypotheses.
5. We are selective about who or what should be a member of the (bio)diversity we want to preserve.
6. Nowadays, it is difficult to distinguish what we really think from what we have read online.

Propositions belonging to the thesis, entitled:

Questioning microbiotics: Interactions of microbes, ticks, vertebrates, and the environment

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Wageningen, 30 March 2021

Questing microbioticks: Interactions of microbes, ticks, vertebrates, and the environment

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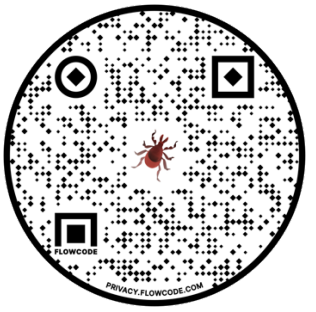


Please click here (for the online version) or scan this QR code to listen to a music track entitled 'Call and Answer'. The music has been inspired by this thesis and composed by Michał Szablowski. The full album is available on: ticksandmicrobes.bandcamp.com

Chapter 1

General introduction

Introduction

In the northern hemisphere, the sheep tick, *Ixodes ricinus,* transmits a plethora of pathogens to humans and animals, posing severe health concerns and economic losses (Heyman et al., 2010; Randolph & Šumilo, 2007; Sprong et al., 2018). For decades, in many European countries, the incidences of tick-borne diseases, most notably Lyme borreliosis and tick-borne encephalitis, have been increasing (Jaenson et al., 2012; Mysterud et al., 2016; Sykes & Makiello, 2017; Walter et al., 2020). The distribution of agents that cause these and many other diseases is changing on a spatiotemporal scale along with their tick vectors, and therefore it is of great importance to understand tick ecology (Beugnet & Marie, 2009; Parola, 2004). The ecology of ticks involves their interactions with biotic and abiotic parts of their natural environment, which impose physiological and evolutionary pressures (Eisen et al., 2017; Hartemink et al., 2019). The abiotic environment includes climatic factors, while the biotic environment consists of vertebrate hosts, vegetation as well as microorganisms inhabiting ticks.

Ixodes ricinus is a hematophagous arthropod species; thus, its survival depends largely on its ability to find and successfully feed on a vertebrate host. It utilizes a multitude of vertebrate species for a blood meal; however, a minimum requirement is animals' presence (mainly deer), on which adult female ticks can feed, meet a male and propagate (Figure 1; Gray et al., 1998b; Hofmeester et al., 2017b). Like their main propagation hosts, *I. ricinus* ticks are predominantly found in forested areas, however, they can also survive in other habitats such as parks and gardens, as long as the leaf litter layer is of suitable thickness, pH, and humidity (Gray, 1998a). Thus, local relative humidity and temperature are substantial determinants for tick survival and behaviour (Gassner et al., 2011; Macleod, 1936; Perret et al., 2000; Randolph, 2004). Further, the climate can also indirectly affect tick survival and activity by affecting tick hosts, vegetation, and soil traits.

Many of the above-mentioned interactions have been extensively studied, and the general mechanisms behind them are well understood. More recently, there is increasing interest in the role of microorganisms in the life cycle of *I. ricinus*, and its vectorial capacity and competence (de la Fuente et al., 2017; Gall et al., 2016; Narasimhan & Fikrig, 2015; Weiss & Aksoy, 2011). Besides known human pathogens such as tick-borne encephalitis virus, *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum*, *Neoehrlichia mikurensis*, and *Babesia* spp. (Heyman et al., 2010; Randolph et al., 2007; Rizzoli et al., 2011), *I. ricinus* harbours many other microorganisms. Some of these microorganisms have developed close and long-term biological interactions with their tick hosts. These symbioses lie on a continuum of interactions that ranges from obligate mutualistic, through commensal to exclusively parasitic (Noda et al., 1997; Sacchi et al., 2004; Scoles, 2004). The functional significance of symbiotic microorganisms for ticks and how they circulate in tick populations remains largely unknown. Lastly, it is poorly understood how tick microorganisms interact with each other, with tick vertebrate hosts and the environment (Figure 2). In the long-term, understanding these interactions can lead to the development of novel and sustainable ways to combat ticks and tick-borne diseases. Recent research on host-symbiont interactions in mosquitoes has been an inspiration for strategies that aim at reducing the mosquito vectorial capacity. For instance, these strategies include paratransgenesis that relies on genetically-modified mosquito symbionts interfering with malaria parasite development and transmission (Mancini et al., 2016; Ricci et al., 2011).

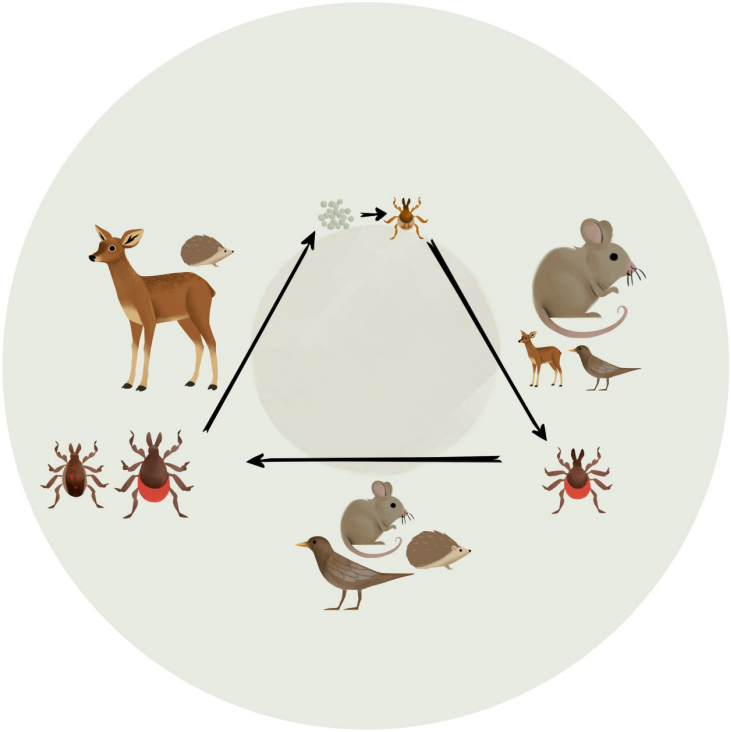


Figure 1. Schematic representation of the life cycle of *I. ricinus*. *Ixodes ricinus* needs from 2 to 4 years to complete its life cycle and consists of four life stages: egg, larva, nymph, and adult. A proportion of female adult ticks mate in the vegetation; however, they also mate on a host (Gray, 1987). After mating and successful feeding, females of *I. ricinus* lay on average 2,000-2,500 eggs in the leaf litter or on the forest floor (Balashov, 1972). During unfavourable environmental conditions, ticks are in so-called diapause, which can delay the hatching of eggs, development of one stage to the next, or delay the onset of host-seeking following the moult (Belozerov, 1982). When the temperature is ≥ 7 °C, larvae, which hatched from the eggs, nymphs, and adult females engage in foraging behaviour called "questing" (Perret et al., 2000; Randolph, 2004; Tagliapietra et al., 2011). Each life stage quests on a different height in the vegetation and feeds (except adult males) on a different range of vertebrates (Hofmeester et al., 2016; Mejlon & Jaenson, 1997). In forested areas, larvae feed predominantly on rodents, nymphs on rodents and birds, and female adults on ungulates, mostly deer (Hofmeester et al., 2017b; Takumi et al., 2019). A tick has to feed once before moulting into the successive life stage. Although females of *I. ricinus* produce many eggs, only one female and one male offspring have to complete their cycles to sustain a stable population.

In 1988, Whipps (Whipps et al., 1988) and colleagues described the collection of microorganisms and their activities within a given environment with the term "microbiome". The word microbiome is a portmanteau of microbe and biome, describing the microbial community in a "reasonably well-defined habitat which has distinct physiochemical properties". With time, the microbiome definition has evolved from ecology-based through interactions with the host, to genomic/methodology-based (Arevalo et al., 2019; Berg et al., 2020; Lederberg & McCray, 2001). Most

researchers agree that the microbiome can comprise bacteria, archaea, fungi, algae, small protists, and viruses, although the latter's integration remains controversial (Berg et al., 2020; Marchesi & Ravel, 2015). Nevertheless, justified by a clear hypothesis and methodology, some researchers focus on a specific group of microorganisms.

Figure 2. Schematic representation of the abiotic and biotic factors, which potentially interact with the microbiome of ticks. To see which factors were studied in individual chapters, see Table 1. 1, climatic conditions; 2, habitat/forest site; 3, ungulates; 4, parasitic wasps; 5, ticks; 6, rodents; 7 and 9, bacterial pathogens; 8, tick microbiome.

In this thesis, when using the term microbiome, I refer to a bacterial community of *I. ricinus* ticks observed with 16s rRNA amplicon sequencing. This sequencing technology is prone to detect the contamination from a sequencing pipeline and the DNA of microorganisms that a tick had contact within the environment. For example, bacteria putatively associated with soil, plants, and vertebrate skin are often amplified in the tick microbiome (Narasimhan et al., 2015). Even when working carefully, it is not always possible to disentangle the contamination from the actual tick-associated microorganisms solely with this technology. Nevertheless, when using the term microbiota, I refer to a set of known and described commensal and mutualistic bacteria inhabiting a tick body. This set of bacteria can be studied independently and out of the microbiome context. In principle, all microorganisms that rely for their survival on ticks can be called symbionts; however,

here, microorganisms that have been shown to cause disease in humans and other vertebrates will be referred to as pathogens.

Over the past two decades, endeavours to understand microbiomes have become a popular topic not only in microbial ecology but also in the context of human and animal health, agriculture, food science, biotechnology, and many more (Berg et al., 2020). Many studies have also highlighted the potential role of the microbiota of arthropod vectors in pathogen transmission processes. It has been shown that microbiota may influence vector competence by affecting essential aspects of the arthropod life cycle, including nutrition supplementation, reproductive fitness, and defence against parasitoids (Manzano-Marín et al., 2015; Weiss et al., 2011). For example, blood-feeding tsetse flies harbour *Wigglesworthia* bacteria, which are thought to compensate flies' nutritionally restricted diet as their genome encodes several vitamin biosynthesis pathways (Akman et al., 2002). Also, *Wigglesworthia* as well as *Wolbachia* influence the reproduction success of tsetse flies (Pais et al., 2008; Weiss et al., 2011). In ticks, such intimate interactions are exemplified by *Rhipicephalus turanicus* and *Ornithodoros moubata* harbouring a *Coxiella*–like symbiont and *Francisell*a–like symbiont, respectively. Genomes of both bacteria were shown to encode major B vitamin synthesizing pathways that are not usually obtainable from a blood-based diet (Duron et al., 2018; Gottlieb et al., 2015; Lalzar et al., 2012; Smith et al., 2015).

Next to affecting essential aspects of the arthropod life cycle, specific microorganisms may modulate the uptake, maintenance, and transmission of pathogenic agents (Cook & McGraw, 2010; Degnan et al., 2009; Weiss et al., 2011). For instance, an *Enterobacter* bacterium isolated from wild *Anopheles gambiae* mosquitoes in Zambia makes 99% of this mosquito population resistant to infection with malaria parasites by interfering with the development of the parasite prior to the invasion of the midgut epithelium (Cirimotich et al., 2011). Similar properties have been assigned to a microsporidian symbiont in another member of the *An. gambiae* complex (Herren et al., 2020). The interactions between arthropod symbiotic bacteria and pathogenic agents can have both a direct and indirect character. Possible direct interactions involve microorganisms secreting compounds, which may affect the growth of a pathogen. Although such specific mechanisms have not yet been described, there are some indications that interference between tick microbiota and tick-borne pathogens may exist. For example, a primary infection by one *Rickettsia* species has been suggested to block (by an unknown mechanism) transovarial transmission of a second *Rickettsia* species (Burgdorfer et al., 1981; Macaluso et al., 2002; Paddock et al., 2015; Sakai et al., 2014). Nevertheless, it has not been documented in *I. ricinus.*

Furthermore, indirect interactions may involve microorganisms inducing the host immune system or altering the integrity of the host gut layer (Gall et al., 2016). Studies have shown that the gut microbiota of black-legged tick, *I. scapularis,* modulates the integrity of the peritrophic matrix that separates the lumen from the digestive cells of the gut, and subsequently affects the establishment of *Borrelia* pathogens (Narasimhan et al., 2014).

Thanks to advanced genetic sequencing technologies, our knowledge of the taxonomic composition and diversity of bacteria in ticks increases. A vast majority of studies focused on bacterial members and consequently revealed that the tick microbiome predominantly consists of Gram-negative bacteria of the phylum Proteobacteria (Andreotti et al., 2011; Azagi et al., 2017; Clay et al., 2008; Duron et al., 2017; Hawlena et al., 2013).

However, little is known about how microorganisms, other than pathogenic agents, are acquired and propagated in *I. ricinus* ticks. **My Ph.D. study's main aim was to describe the microbiome of *I. ricinus* and elucidate how it is generated. For this, I studied the tick microbiome in relation to tick life stages, geographical location, vertebrates, climatic variables, and *Ixodiphagus hookeri*, a natural enemy of *I. ricinus*. As *I. ricinus* is responsible for transmitting pathogenic agents to humans and animals, questions and hypotheses of my studies were framed in the context of tick-borne disease risk.**

Given its biology, there are multiple occasions for microorganisms to enter a tick. It can be postulated that the microbiome of eggs and larvae consists predominantly of symbionts vertically-acquired from the mother and, possibly, from the father. Examples of microorganisms utilizing predominantly this transmission route are *M. mitochondrii* and *R. helvetica* (Sassera et al., 2006; Sprong et al., 2009). Nevertheless, each successive life stage is expected to carry a more complex microbiome. During the off-host phases of ticks, bacteria have an opportunity to enter a tick through openings such as the spiracles, mouth, or anal pore (Andreotti et al., 2011; Narasimhan et al., 2015). Moreover, during each consecutive blood meal, additional microorganisms, including human pathogens such as *B. burgdorferi* s.l. or *A. phagocytophilum* can be acquired (Gern, 2008; Jahfari et al., 2014). Lastly, each of these life stages and sexes faces different challenges, which can extort distinct bacterial communities**.**

In **Chapter 2**, the microbiome of *I. ricinus* in the Netherlands is described, which brings understanding towards the distribution of tick symbionts in tick populations. For the former, with the 16S rRNA amplicon sequencing technique, I explored the microbiome of larvae, nymphs, and female and male adults of *I. ricinus* in six geographical locations (Table 1). For the latter, almost 17,000 individual questing nymphs from 19 forest sites have been screened for the presence of vertically- and horizontally-transmitted tick symbionts.

Table 1. Different abiotic and biotic factors were studied in individual chapters. See also Figure 2.

	Climatic conditions (1)	Habitat/ forest site (2)	Ungulates (3)	Parasitic wasps (4)	Ticks (5)	Rodents (6)	Bacterial pathogens (7 and 9)	Tick microbiome (8)
Chapter								
2		+			+		+	+
3							+	+
4					+	+	+	+
5	+	+					+	+
6			+	+	+	+	+	

Larvae, nymphs, and female adults of *I. ricinus* feed once on a vertebrate host to moult into the following stage or, in the case of females, to oviposit and lay eggs (Figure 1). Next to serving as a blood meal, vertebrates can be amplifying hosts of various pathogens. For example, while feeding, ticks can horizontally acquire *B. afzelii*, and *N. mikurensis* from rodents, *B. garinii* and *B. valaisiana* from birds, and *A. phagocytophilum* predominantly from deer (Coipan et al., 2018; Gern, 2008; Hanincova et al., 2003a and 2003b; Jahfari et al., 2014). Therefore, the vertebrate community strongly affects the bacterial pathogen community in ticks (Takumi et al., 2019). However, it

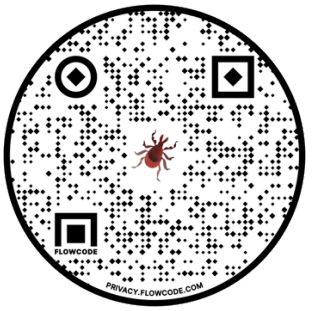
is poorly understood how the underlying microbiome of a tick affects the acquisition of these pathogens and how a blood meal and pathogens contribute to the overall change in the bacterial community. **Chapter 3** describes the study of the microbiome of nymphs infected with distinct pathogens using the 16S rRNA amplicon sequencing technique (Table 1). Also, using the database of Chapter 2, I studied associations within tick microbiota to unravel possible facilitative or adverse effects of vertically-transmitted symbionts on the acquisition of zoonotic pathogens.

Vertebrates are the driving force of the distribution and abundance of ticks, as well as the prevalence of pathogens in questing ticks (Hofmeester et al., 2016; Ostfeld et al., 2006; Takumi et al., 2019). In **Chapter 4,** the dynamics of the prevalences of tick pathogens and symbionts are studied in relation to changes in rodent abundance (Table 1). The rodent population was experimentally manipulated in a forest either by removing rodents from plots to reduce or by adding rodent food (acorns) to increase the density. Rodent densities were monitored for 2.5 years, and tick densities and infection prevalences in ticks were measured for 3.5 years.

Ixodes ricinus spends the vast majority of its life in vegetation, questing for a host, moulting, or diapausing. During these phases, the suitability of the ground-based vegetation and climatic conditions are critical to the tick's off-host survival. For example, to maintain its water balance, *I. ricinus* requires an ambient relative humidity of at least 80% to be active (Lees, 1946; Medlock et al., 2013). Although the environmental requirements for tick survival are well established, it is not known if and how fluctuating climatic conditions influence transmission dynamics of tick symbionts and pathogens. In **Chapter 5**, I studied the spatio-temporal variation of prevalences of tick symbionts *M. mitochondrii* and *R. helvetica*, and pathogens *B. burgdorferi* s.l., *B. miyamotoi*, and *N. mikurensis* in relation to climatic conditions such as evaporation rate, relative humidity, temperature, and precipitation (Table 1). The data analysed for this study included monthly prevalences of tick symbionts and pathogens from 12 Dutch forest sites over four years.

Another possible factor shaping the tick microbiota is the presence and abundance of natural tick enemies such as parasitoid wasps. *Ixodiphagus hookeri* is distributed worldwide and parasitizes a broad range of hard tick species, including *I. ricinus*. This parasitic wasp infests feeding and unfed larvae and nymphs, but the eggs' development only occurs in fully engorged nymphs (Hu & Hyland, 1998b). *Ixodiphagus hookeri* larvae feed on the internal tissue along with the vertebrate blood ingested by their tick host (Hu et al., 1998a). Subsequently, wasps moult into adults and emerge by making a hole in the tick's body (Wood, 1911). Since the emergence of wasps kills the host, a tick cycle is disrupted. In the study presented in **Chapter 6**, we examined if these parasitic wasps affect the transmission dynamics of zoonotic pathogens (Table 1). For that, the ecological interactions among *I. hookeri*, *I. ricinus*, and two vertebrate species groups (ungulates and rodents) were explored.

In **Chapter 7**, the most salient findings of my studies are discussed in light of current scientific developments in arthropod microbiome research. I also discuss the consequences of my findings in the context of risk assessments and potential biocontrol for ticks and tick-borne diseases.



Please click here (for the online version) or scan this QR code to listen to an immersive soundscape of a forest in Amsterdamse Waterleidingduinen, the Netherlands recorded by Vitalij Kuzkin. The Amsterdamse Waterleidingduinen forest is one of the study sites where I have collected samples for this research. Listen with headphones for the best experience. The full album is available on: ticksandmicrobes.bandcamp.com

Chapter 2

Large-scale quantitative microbial population study reveals regional differences in bacterial symbionts of *Ixodes ricinus*

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Abstract

Ixodes ricinus ticks transmit a plethora of pathogens, posing serious health concerns in Europe. These pathogens include horizontally- and vertically-transmitted tick symbionts. It is poorly understood what determines the prevalence of vertically- transmitted symbionts. Here we explored bacterial communities of *I. ricinus* across developmental stages and geographic locations by the 16S rRNA amplicon sequencing combined with accurate quantification of the bacterial load. Secondly, we screened almost 17,000 individual questing nymphs from 19 forest sites for the presence of the most prominent symbionts identified in the microbiome analysis and compared their prevalences and distribution with those of the horizontally-transmitted *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum*, and *Neoehrlichia mikurensis*. There was a surprisingly wide range of total bacterial loads across pools of ticks analysed in our experiment. Accurate identification of low microbial biomass samples permitted comparisons to high biomass samples, despite the significant influence of contaminating DNA. The bacterial communities of ticks were driven by geographical region rather than a life stage, and differences in *Rickettsia* abundance mostly determined the clusters. The most prominent vertically-transmitted tick symbionts included *R. helvetica*, *Rickettsiella* spp., *Midichloria mitochondrii*, and *Spiroplasma ixodetis.* The generalised linear mixed models and relative occurrence analyses performed on data from individually tested nymphs showed that the prevalences of *R. helvetica*, *Rickettsiella* spp., and *M. mitochondrii* differ significantly between the regions and that the proportions in which they occur in tick populations are highly variable. These patterns were contrary to what we observed for horizontally-transmitted *B. burgdorferi* s. l., and *A. phagocytophilum*. Our results show that quantitative techniques for microbiome studies can accurately identify low-biomass samples in data sets with heterogeneous microbial load, even when used in combination with a pooling strategy commonly used to sequence low-biomass arthropod microbiomes. We also showed that geographical regions rather than life stages drive the overall microbiome of *I. ricinus*, and that infection rates of some vertically-transmitted symbionts in *I. ricinus* are affected by selective pressures on a regional scale. These findings can explain regional differences in the risk of disease caused by pathogens such as *R. helvetica.*

Keywords: Tick-borne diseases, Spotted fever Rickettsiosis, Transmission dynamics, Quantitative microbiome analysis, Low-biomass samples, *Ixodes ricinus* microbiome

Introduction

In Europe, *Ixodes ricinus* ticks transmit a plethora of pathogens to humans, posing severe health concerns (Heyman et al., 2010; Randolph & Šumilo, 2007; Sprong et al., 2018). Several studies described a long-lasting increase in the incidences of tick-borne diseases, most notably Lyme borreliosis and tick-borne encephalitis (Jaenson et al., 2012; Mysterud et al., 2016). Furthermore, human infections and diseases involving *Anaplasma phagocytophilum*, *Neoehrlichia mikurensis*, spotted fever group *Rickettsiae*, and *Spiroplasma ixodetis*, are emerging (Matet et al., 2020; Parola, 2004; Parola & Raoult, 2001; Silaghi et al., 2016). Next to human pathogens, a variety of other bacterial microorganisms have evolved symbiotic relationships with *I. ricinus* (Carpi et al., 2011; Duron et al., 2017). These symbionts, presumably, impact their tick host, are dependent on the tick for their survival in nature, and are potentially pathogenic to humans. Therefore, to assess disease risk and formulate possible intervention strategies, it is crucial to identify factors that determine how various microorganisms are acquired and propagated in *I. ricinus* ticks.

Previous studies showed that the infection prevalences of pathogens such as *Borrelia burgdorferi* sensu lato, *A. phagocytophilum*, and *N. mikurensis* are mainly dependent on local vertebrate communities (Takumi et al., 2019; Van Buskirk & Ostfeld, 1995). This dependence reflects that these pathogens are amplified by various animal species and transmitted between ticks and vertebrate hosts during feeding (horizontal transmission; Burri et al., 2014; Hanincova et al., 2003a; Stuen et al., 2013). However, less is known about the factors that determine the infection prevalences of *Rickettsia helvetica* and *S. ixodetis* (Chapter 4; Sprong et al., 2009), which predominantly utilize vertical transmission. Namely, they are transmitted from female ticks to offspring (Parola et al., 1998; Tully et al., 1995).

Symbionts such as *R. helvetica* and *S. ixodetis* can provide fitness benefits but may not be required for tick survival per se, indicating a facultative relationship with ticks. Nevertheless, their role in *I. ricinus* ecology is poorly understood (Bonnet et al., 2017; Duron et al., 2017). Interestingly, infection prevalences of facultative symbionts often vary across tick populations, which may be driven by costs and benefits associated with harbouring them by ticks (Duron et al., 2017; Oliver et al., 2010). In this study, to elucidate spatial distribution patterns of tick symbiont communities, we performed a microbiome analysis of *I. ricinus* ticks from six locations around the Netherlands. Previous studies on the microbiome of *I. ricinus* detected large bacterial diversities, which, in addition to microorganisms associated with soil, plants, and vertebrates, consisted predominantly of the phylum Proteobacteria, which comprises many arthropod-associated microorganisms (Greay et al., 2018; Narasimhan & Fikrig, 2015). The microbiome of *Ixodes* ticks have been shown to vary on spatial scales (Carpi et al., 2011; Van Treuren et al., 2015) and through the ontogeny (Carpi et al., 2011; Kwan et al., 2017; Swei & Kwan, 2017; Zolnik et al., 2016). Therefore, in our study, next to distinct geographical locations, we included all life stages of *I. ricinus*. *Ixodes ricinus* has a three-host life cycle, feeding once per life stage (larva, nymph, and adult female but not male) on various vertebrates. Each feeding is an opportunity for a tick to acquire microorganisms in addition to acquisition from the vegetation, where it spends most of its life (Needham & Teel, 1991).

Ixodes ticks are hematophagous ectoparasites, and due to their feeding strategy, blood meals are not a regular occurrence. Therefore, each blood meal may cause a massive influx of nutrients, as

documented in mosquitoes and triatomine bugs (Eichler & Schaub, 2002; Oliveira et al., 2011). The influx can drastically increase the biomass of tick-associated symbionts. For example, *M. mitochondrii* has been shown to increase in absolute density after a blood meal (Ninio et al., 2015; Sassera et al., 2008). However, most of these approaches focus on a specific symbiont and do not assess the composition of the entire community. Conversely, studies investigating the complete microbiome of *Ixodes* ticks often do not take a total bacterial load into account (Carpi et al., 2011; Kwan et al., 2017; Swei & Kwan, 2017; Zolnik et al., 2016). This can lead to spurious conclusions on the tick microbiome, as low bacterial biomass might have important biological implications that are overlooked. Hence, quantification of bacterial density can aid in a better understanding of tick physiology.

This study adopted a qPCR approach inspired by studies on accurate quantification of the bacterial microbiome (Bogaert et al., 2011; Jian, Luukkonen, Yki-Järvinen, Salonen, & Korpela, 2020). We aimed to demonstrate that this approach can accurately identify samples with low bacterial loads. Combining a qPCR scaling step with 16S rRNA amplicon sequencing allowed us to detect low abundances of these species, even when contaminants inflate the richness of low bacterial load samples. This microbiome analysis allowed us to gain insight into microbial communities in *I. ricinus* and to form the hypothesis that the prevalence of some vertically-transmitted tick symbionts is determined on a larger spatial scale than it has been observed for horizontally-transmitted pathogens. To test this hypothesis, we screened by qPCR nearly 17,000 individual questing nymphs of *I. ricinus*, collected from 19 forest sites. Lastly, we compared distribution patterns of vertically-transmitted symbionts with horizontally-transmitted tick-borne pathogen communities, which were quantified in a previous study (Takumi et al., 2019).

Materials and methods

Sample collection and preparation for microbiome analyses of *I. ricinus*

For microbiome profiling, we utilized pools of *I. ricinus* larvae, nymphs, and individual adult females and males from six locations across the Netherlands (Figure 1, triangles). These locations were selected based on pre-existing knowledge of *B. burgdorferi* s.l. prevalence, the density of ticks, vegetation profile, and vertebrate community obtained in a cross-sectional study (Takumi et al., 2019). The full names of the sites, their coordinates, and vegetation descriptions are provided in Table S1. After obtaining and interpreting the results from microbiome analysis, the locations were assigned to three regions: western (AW, DK), central (HD, ST, BU), and northern (ZM; Figure 1, colours).

Questing *I. ricinus* were collected in 2016 by blanket dragging. In total, 655 ticks were combined into pools by life stage and location. All ticks were washed three times in 70% ethanol, and DNA from 32 pools of 10 larvae, 18 pools of 5 nymphs, 18 pools of 10 nymphs, 34 individual female, and 31 individual male ticks was extracted using the Qiagen DNeasy Blood & Tissue Kit according to the manufacturer's protocol (Qiagen, Venlo, the Netherlands). A sampling scheme and sample metadata are provided in Table S2 and Table S3.

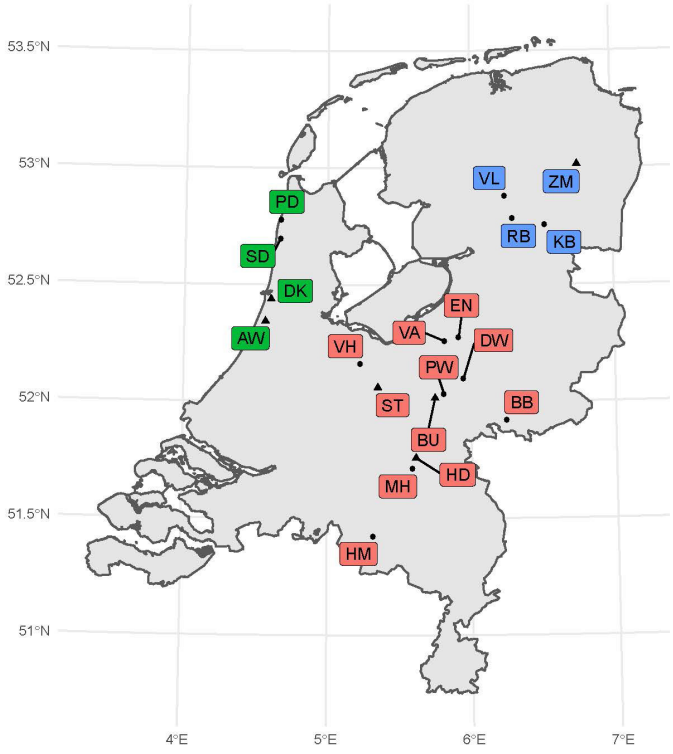


Figure 1. Sampling sites of *I. ricinus* in the Netherlands. Pooled and individual ticks from six forest sites (triangles) were used for a 16s rRNA amplicon sequencing analysis. Individual nymphs from these and 13 (points) other forest sites were tested by qPCR for the presence of tick symbionts. A box marks the sampling site by two letters and the region by colour; red for the central, blue for the northern, and green for the western region. More details on the locations are provided in Table S1.

Microbial profiling and taxonomic clustering

Illumina MiSeq V3-V4 region of 16S rRNA amplicon libraries were generated and sequenced by BaseClear (Leiden, the Netherlands). The description of the method has been published previously (Gommers et al., 2019). Sequenced reads were imported to CLC Genomics Workbench 10.0.1 supplemented with CLC Microbial Genomics Module 3.6.1 (www.clcbio.com). Overlapping pairs of raw reads were merged into single longer reads and trimmed with a quality score limit of 0.05 and 2 ambiguous nucleotides. At this stage, primer sequences were trimmed. Subsequently, reads were fixed-length trimmed (~400 bp). To identify operational taxonomic units (OTUs), reads were clustered using the reference databases SILVA 16S version 128 with 97% identity as the clustering criterion; chimeras were removed.

16S rRNA quantification and total bacterial load

Total bacterial load in all samples was quantified, and proportions were multiplied by the load to convert relative into absolute abundances. Quantification of total bacterial DNA load was

determined by 16S rRNA qPCR as previously described (Bogaert et al., 2011). The total bacterial load is a cost-effective and scalable solution for datasets of this size since quantification methods through flow cytometry are not compatible with the sampling technique. Samples were normalized to control for arbitrary variation in sequencing depth, and the normalized abundances were scaled by the 16S rRNA qPCR values of each sample. For diversity analyses, samples were rarefied to the lowest sequencing depth.

Microbiome analyses

All analyses were carried out in R 3.6.0 (R Core Team, 2019) and RStudio (RStudio Team, 2019). We used the R package *vegan* (version 2.5-6) for ordinations, diversity indices, and fitting of environmental vectors or factors onto ordinations (Oksanen et al., 2007). We also computed silhouette scores from the Bray-Curtis dissimilarities and Sheldon evenness (Sheldon, 1969). All principal coordinate analyses were carried out using Bray-Curtis dissimilarities, and *envfit* correlation to the principal components was corrected for multiple-testing with the Benjamini-Hochberg correction. Additionally, we carried out PERMANOVA with the *adonis* function from R package *vegan* to assess whether the tick life stage, location, or interaction between these two variables significantly affected community variation. We tested for multivariate spread through the *betadisper* function. We classified locations as the western, northern or central region to prevent overfitting.

To test how well different factors explained clusters observed on the PCoA plots, we calculated the Bray-Curtis dissimilarities and evaluated cluster quality as the silhouette score with the factors as cluster labels. The silhouette score, bounded by -1 and 1, takes both cluster cohesion and cluster separation into account. We used k-means clustering of the log-transformed *Rickettsia* abundances to compute silhouette scores for *Rickettsia.*

To investigate correlations between OTUs, bacterial community variation, and regions, we fitted proportional odds models with OTU abundances scaled by the total bacterial load as the dependent variable and used region and tick life stage as independent variables. Details on models are provided in Text S1. We compared these models with the likelihood ratio test, using an implementation of Nagelkerke's pseudo-R^2 from the R package *rcompanion* (version 2.3.7) (Mangiafico & Mangiafico, 2017).

Prevalence of tick symbionts in individual ticks

A total of 1,130 *I. ricinus* ticks of all developmental stages were collected in 2019 from two locations (ST and AW) and tested individually with qPCR for the presence of *S. ixodetis*, *R. helvetica*, *B. burgdorferi* s.l., *A. phagocytophilum*, and *N. mikurensis* for which primers and probes have been developed and published before (Courtney et al., 2004; de Bruin et al., 2015; Heylen et al., 2013b; Jahfari et al., 2012; Chapter 4). In addition, ticks were tested for *Rickettsiella* spp., *M. mitochondrii,* for which primers and probes were designed in this study. All targeted genes and qPCR protocol are provided in Text S2. Subsequently, pathogens and symbionts were assigned the transmission mode based on their presence or absence in the larval stage indicating vertical or horizontal transmission, respectively.

To determine geographic distribution and prevalence of tick symbionts, we analysed a total of 16,555 ticks, which were collected in a previous cross-sectional study (Hofmeester et al., 2017b;

Takumi et al., 2019). Briefly, questing nymphs of *I. ricinus* were collected from 19 locations in forested areas in the Netherlands in 2013 and 2014 (Figure 1, triangles and dots). Details on data collection were described previously (Hofmeester et al., 2017b; Takumi et al., 2019). We tested questing individual nymphs of *I. ricinus* for the presence of *S. ixodetis*, *R. helvetica*, *Rickettsiella* spp., and *M. mitochondrii*. In addition, data generated previously on the prevalence and distribution of tick-borne pathogens from 13, 967 out of 16,555 ticks was incorporated (Takumi et al., 2019). These pathogens included *A. phagocytophilum*, *N. mikurensis*, *B. miyamotoi*, and three genospecies of *B. burgdorferi* s.l. as follows: *B. afzelii*, *B. garinii*, and *B. valaisiana*. Data on B. garinii and B. valaisiana were combined for further analysis as they are considered bird-borne pathogens.

The differences in prevalences of symbionts between regions were explored with generalized linear mixed models with a location as a random effect. The goodness of fit of all models was assessed with a likelihood ratio test, AIC scores, and Nagelkerke's pseudo-R^2 from the R package *rcompanion* (version 2.3.7)(Mangiafico et al., 2017).

Relative occurrence of vertically-and horizontally-transmitted tick symbionts

Based on the presence and absence of symbionts detected with qPCR, we assigned each tick a haplotype, an integer between 0 and 2^{n-1}. Each integer corresponds to one of 2^n distinct outcomes for a series of n qPCR tests performed on the tick. Haplotype frequencies were arranged in a table where rows are the sampling sites and columns are the observed haplotypes. Each row was divided by the row sum. The column mean was subtracted from each column. Principal component analysis of the data table was performed by applying the singular value decomposition (Golub & Van Loan, 1996).

Borrelia afzelii genetic diversity

A (geographic) region is defined here as a (sub)population of ticks from several tick habitats (locations). Geographic distance and physical landscape limit the exchange of ticks between the regions. Genetic information from *B. afzelii*, a horizontally-transmitted symbiont, was used to measure this exchange because genetic information from *I. ricinus* was not available. From 1,389 *B. afzelii* isolates, derived from questing *I. ricinus* from the different regions, a DNA fragment of the intergenic spacer sequences (IGS; Table S4) was generated and compiled (for methodology, see Coipan et al., 2013a). A genotype is a subset of *B. afzelii,* which share an identical sequence. The frequencies of all *B. afzelii* genotypes were counted. A population genetic measure (Fst) was calculated by applying Arlequin to the frequency data (Excoffier & Lischer, 2010), as described previously (Coipan et al., 2018).

Results

Microbial profiling and taxonomic clustering

A total of 131 out of 133 processed samples generated 18,803,386 raw reads on Illumina MiSeq flow cell. Two samples failed at the amplification stage, probably due to low bacterial DNA load. A total of 6,013,524 sequences were assigned taxonomy. A total of 4,978 unique OTUs were identified (Table S5), and the top 10 most abundant taxa consisted of *Rickettsia*, *Rickettsiella*, *Midichloria*, *Pseudomonas*, *Halomonas*, *Rickettsiella*, *Mycobacterium*, *Shewanella*, *Methylobacterium*, and *Williamsia*.

Three taxa, *Pseudomonas*, *Halomonas*, and *Shewanella*, were the most abundant in negative controls, indicating that these microorganisms are contamination from the sequencing pipeline. All counts belonging to these taxa were binned into a synthetic 'Contaminant' taxon (Figure 2). Several tick samples had extremely low biomass, and their community composition was similar to that of sequenced negative controls (Figure S1). These samples lacked tick-associated symbionts, and since the absence of a high-abundance symbiont can be considered a biological phenomenon, we chose to retain these samples while the negative controls were excluded from further analysis. A full list of OTUs identified in the negative controls is provided in Table S5.

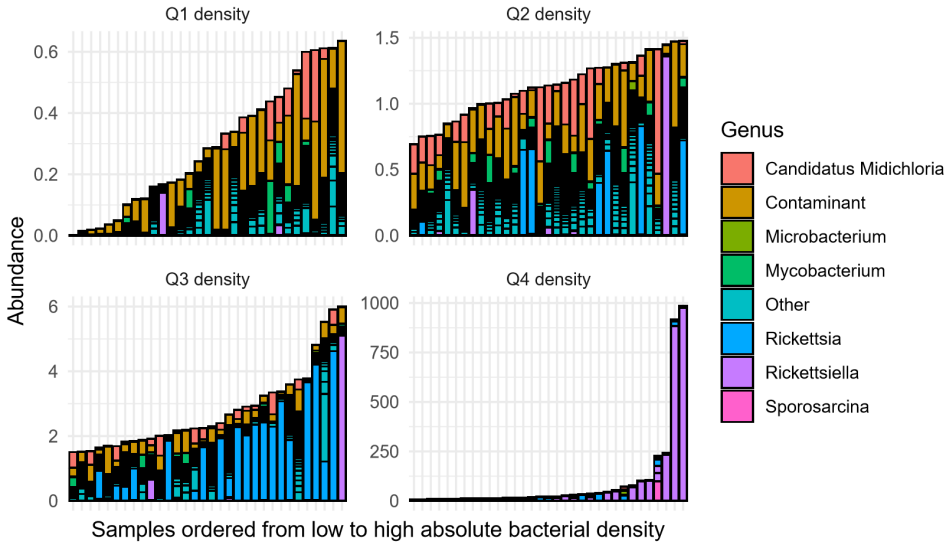


Figure 2. Abundances (number of reads; Y-axis) of the ten most abundant bacterial taxa in individual and pooled *I. ricinus* samples (X-axis). Samples are separated by quartiles of total bacterial load (16S rRNA content in ng/µL). Bacteria present at a minimum threshold in sequenced control samples (blanks) were binned into the artificial 'Contaminant' phylum. Taxa not in the top 10 were labelled 'Other'. A full list of detected bacterial taxa is provided in Table S4.

Rarefaction curves indicated sufficient sequencing coverage for most samples, as demonstrated by most observed OTU accumulation curves having reached a plateau at 2,000 reads (Figure S2). The low amount of total DNA and pooling strategy resulted in highly uneven sequencing depths Figure S3a). We assessed whether sequencing depth correlated to diversity since this would necessitate a rarefaction step. There was no apparent correlation between sequencing depth and diversity (Figure S4a), linear regression model $p = 0.907$, $R^2 = -0.008$), and we chose to avoid rarefaction before additional analyses were carried out.

Abundance of tick-associated symbionts

Known members of the tick microbiome (Duron et al., 2017; Sassera et al., 2006; Sprong et al., 2009), the genera *Rickettsia*, *Midichloria*, and *Rickettsiella* were among 10 of the most abundant taxa in the overall microbiome dataset accounting for 24.4%, 6.3% and 17.1% of all reads, respectively

(Figure 2). Another tick-associated microorganism, *Spiroplasma,* was abundant but only in two locations, ZM and AW, accounting for 0.3% of all reads (Henning et al., 2006). The most abundant tick-borne pathogen in the dataset was *Neoehrlichia* (0.3%). Other pathogenic genera, including *Borrelia* and *Anaplasma,* constituted a small part of the overall tick microbiome accounting for 0.1% and 0.02% of all reads, respectively. Lastly, 0.4% of reads represented *Wolbachia* genus, probably due to endoparasitoid *Ixodiphagus hookeri* eggs in ticks (Tijsse-Klasen et al., 2011; Plantard et al., 2012).

Effect of total bacterial load on the abundance of contaminants

Total bacterial load was weakly but significantly correlated with sequencing depth (Additional file 3: Figure S5a, linear regression model $p = 0.011$, $R^2 = 0.042$). Many samples had low total bacterial loads. Bacterial load was strongly correlated to the total number of uncorrected counts belonging to *Pseudomonas*, *Shewanella*, or *Halomonas* (Figure S5b, linear regression model $p < 0.001$, $R^2 = 0.608$). After scaling normalized sequencing counts by the total bacterial load, the bar plots of bacterial abundances demonstrate that the contaminants make up a large fraction of samples with low total bacterial loads (Figure 2 and Figure S3b). In addition, the lower total bacterial load was correlated to the higher Shannon diversity (Figure S4b, linear regression model, $p < 0.001$, R^2=0.195).

Effect of life stage and region on bacterial community variation

The marginal effects of region, life stage and their interactions on bacterial community variation were significant ($p = 0.001$ for stage, region and stage * region), however the R^2 was small (life stage $R^2 = 0.121$, region $R^2 = 0.146$, life stage * region $R^2 = 0.108$; Table S6).

Our test for multivariate homogeneity of group dispersion was highly significant for both life stage and region ($p < 0.001$ and $p = 0.017$, respectively; Table S6). Silhouette scores from the Bray-Curtis dissimilarities were 0.042 and 0.118 for life stage and region. The low silhouette scores indicate that samples are not tightly grouped by these factors (Figure 3).

Both *Rickettsia* and *Rickettsiella* had significant correlations to the axes ($p = 0.001$ for each), with a silhouette score of 0.122 for *Rickettsia*-based clusters (Figure 4). The models suggest that high *Rickettsia* and *Rickettsiella* abundances could be explained well with a model including only region as a factor (Table S7). For *Rickettsia*, the inclusion of stage and region represented a small but significant improvement in the pseudo-R^2 (pseudo-$R^2 = 0.488$ for the region only, pseudo-$R^2 = 0.523$ for region and stage, likelihood ratio $p = 0.027$), with the Akaike information criterion slightly higher for the model with the only the region as a factor (AIC = 1,289 for the model with the region, AIC = 1,286 for the model with the region and life stage). However, the models for *Rickettsiella* showed no improvement in the pseudo-R^2 (pseudo-$R^2 = 0.445$ for region only, pseudo-$R^2 = 0.446$ for the region and stage, likelihood ratio $p = 0.971$), suggesting that only region could predict high abundance.

Principal Coordinate Analysis of Bray-Curtis dissimilarities overlaid with envfit vectors for tick symbionts. Of these abundance vectors, only *Rickettsia* and *Rickettsiella* correlated significantly to the principal components ($p = 0.001$ for both). Nagelkerke's pseudo-R^2 for different ordinal logistic regression models fitting scaled *Rickettsia* and *Rickettsiella* abundances is provided in Table S7.

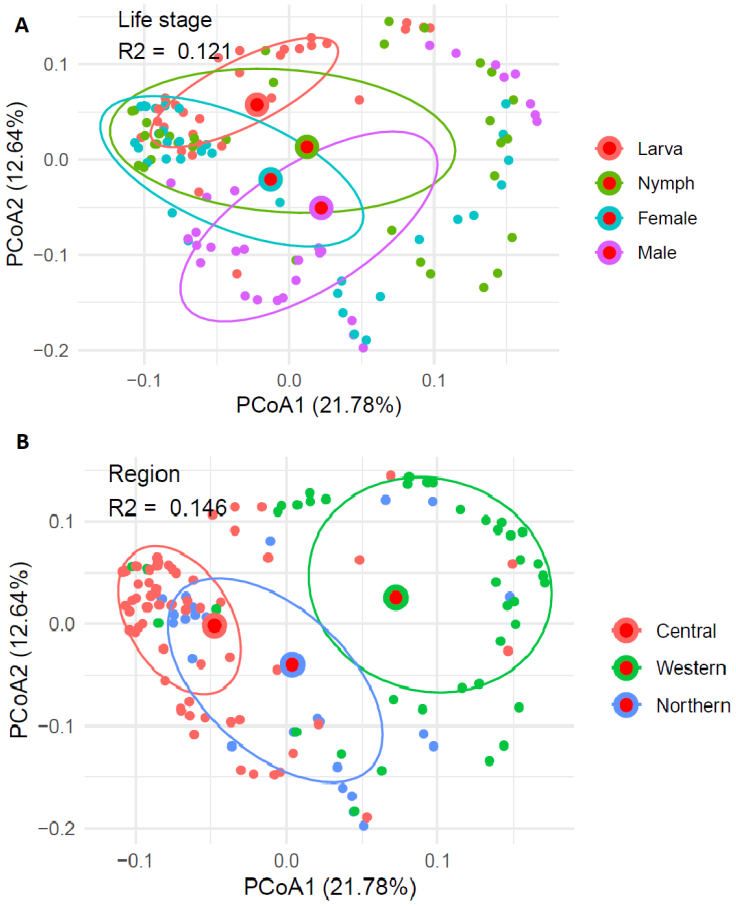


Figure 3. Bacterial community variation in Ixodes ricinus across life stages and regions. (a) Principal Coordinate Analysis of Bray-Curtis dissimilarities overlaid with centroids of tick life stage. Data ellipses contain 50% of the samples belonging to the different life stages. (b) Principal Coordinate Analysis of Bray-Curtis dissimilarities overlaid with centroids of sampling region. Data ellipses contain 50% of the samples found in a specific region. The R^2 values were generated with PERMANOVA with tick life stage and region as factors.

Prevalence and transmission mode of tick symbionts

Rickettsia helvetica, *S. ixodetis*, *Rickettsiella* spp., and *M. mitochondrii* were detected in all life stages (Table S8), indicating vertical transmission. *Borrelia burgdorferi* s.l. (except one larva), *A. phagocytophilum*, and *N. mikurensis* were only detected in nymphal and adult stages (Table S8). Our results corroborate the latter symbionts enter a tick population via horizontal transmission while larvae feed on an infected host (Burri et al., 2014; Hanincova et al., 2003a; Stuen et al., 2013). The vertically-transmitted symbionts were detected to a varying degree in all 19 forest sites (Figure 5). Data on prevalence per symbiont per forest site as well as data generated in the previous study on three *B. burgdorferi* s.l. genospecies including *B. afzelii*, *B. garinii* and *B. valaisiana* (combined),

A. phagocytophilum, and *N. mikurensis* are provided in Table S9. In 12% of the tested nymphs (N = 1,668), none of the nine symbionts were detected, agreeing with the 16S rRNA amplicon sequencing results where many samples had almost no microbiome (Figure S1).

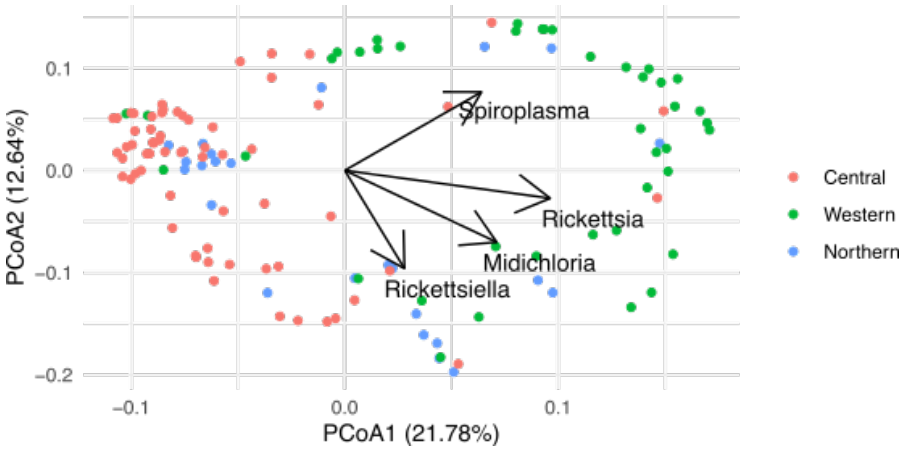


Figure 4. *Rickettsia* abundance covaries with community composition.

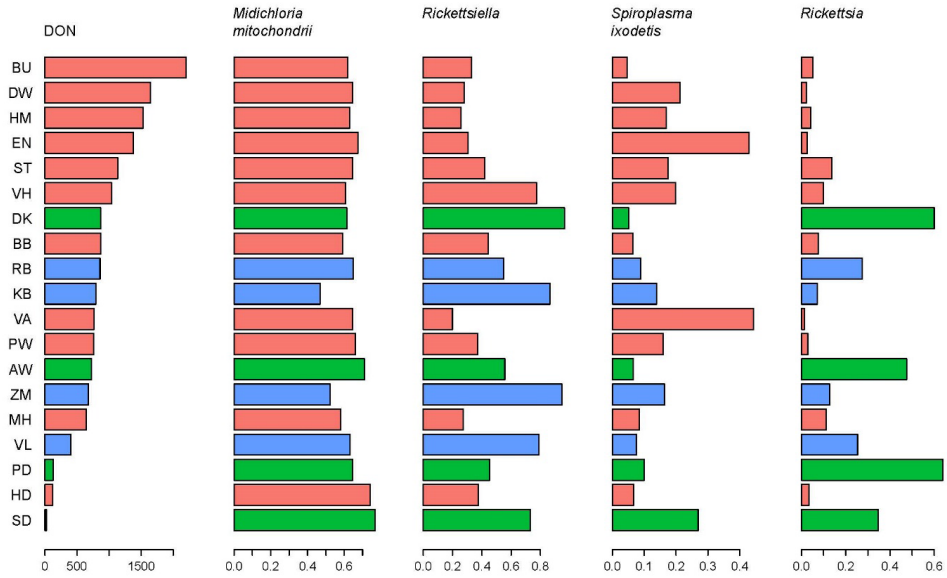


Figure 5. Local and regional occurrence of vertically-transmitted microorganisms. The leftmost column shows the density of nymphs (DON) per forest site (two letter labels). The remaining columns show the prevalence of vertically-transmitted tick symbionts. Colours indicate a region: northern (blue), central (red), and western (green).

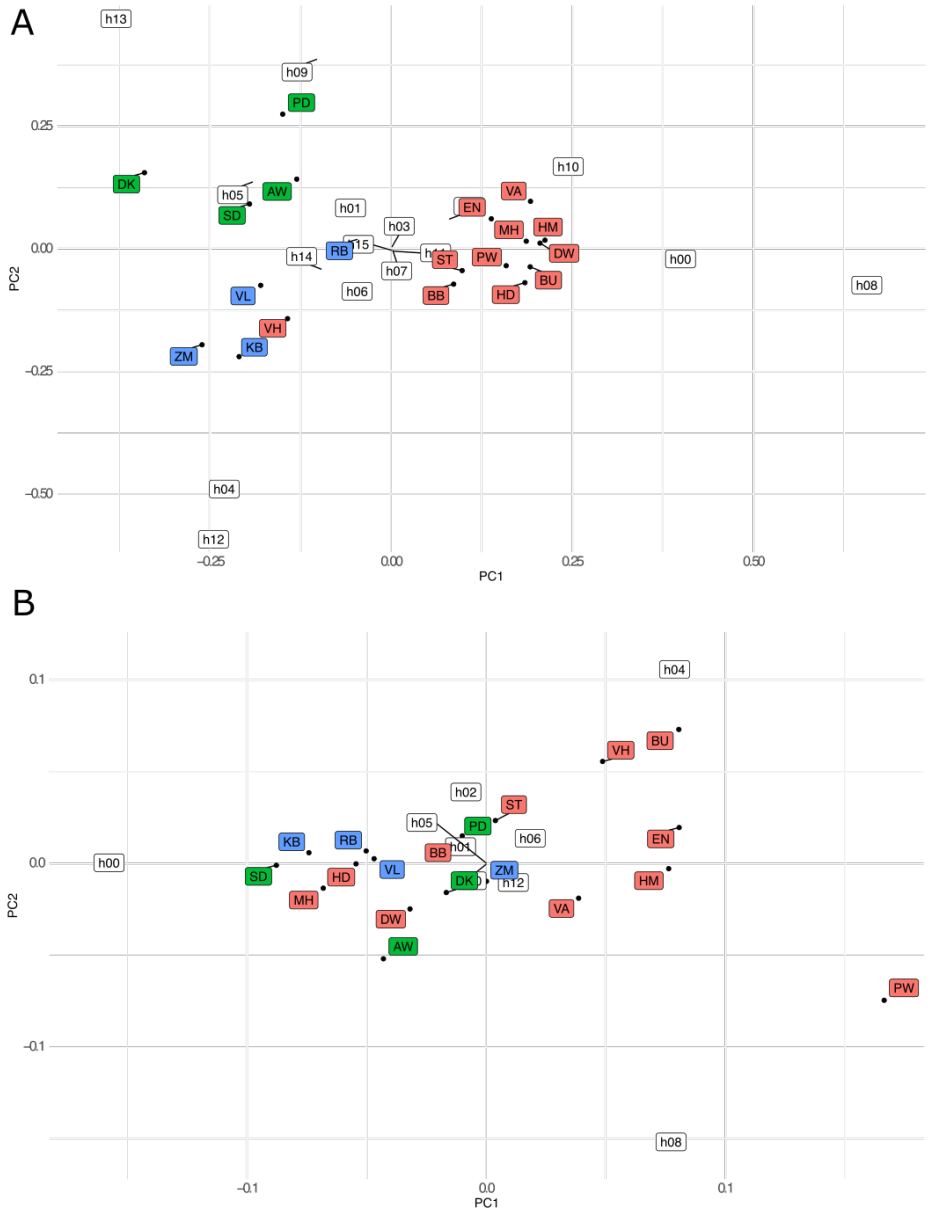


Figure 6. Relative occurrence of symbionts in *I. ricinus* nymphs from 19 forest sites from three geographical regions: northern (blue), central (red), and western (green).
(a) Relative occurrence of vertically-transmitted symbionts such as *R. helvetica*, *M. mitochondrii, S. ixodetis*, and *Rickettsiella* spp. (b) Relative occurrence of horizontally-transmitted symbionts such as *A. phagocytophilum*, *B. afzelii*, *B. garinii* and *B. valaisiana* (combined), and *N. mikurensis*. Tick populations from the same geographical region (see Figure 5) clearly share a similar composition of vertically- but not horizontally-transmitted symbionts. Transparent boxes show haplotype numbers, which correspond to symbiont combinations in

individual ticks; for example, in panel (a), the central region is dominated by nymphs infected only with *M. mitochondrii* (h08) or with none of the symbionts (h00). Please note the different scales of axes between the two figures. A full description of each haplotype is provided in Table S11.

The prevalences of vertically-transmitted *R. helvetica*, *Rickettsiella* spp., and *M. mitochondrii* varied significantly between the regions. Models of region and location as a random effect explained 73%, 52%, and 31% of their variance, respectively (Table S10). The prevalence of *S. ixodetis* did not vary between the regions. From horizontally-transmitted symbionts, only the prevalence of *N. mikurensis* differed between regions. A model of region and location as a random effect explained 34% of its variance. Models for all symbionts, likelihood ratio test results, AIC scores, and Nagelkerke pseudo-R^2 are provided in Table S10.

Relative occurrence of vertically-transmitted symbionts

We found that specific combinations of infections in individual ticks clustered neatly into the three geographic regions: western, central, and northern (Figure 1 and Figure 6a). Questing nymphs were abundant in the central region (red), in which either all symbionts were absent (h00; Table S11 lists all the haplotypes) or in which only *M. mitochondrii* (h08 haplotype) was detected. In the northern region (blue), questing nymphs were abundant, in which *Rickettsiella* spp. was present together with *M. mitochondrii* (h12) or without this species (h04). Three haplotypes dominated the western region (green) as follows: *M. mitochondrii*, *Rickettsiella* spp., and *R. helvetica* (h13), *Rickettsiella* spp. and *R. helvetica* (h05), or *M. mitochondrii* and *R. helvetica* (h09). These three haplotypes (h13, h05, and h09) lacked *S. ixodetis*.

Relative occurrence of horizontally-transmitted symbionts

To contrast these results against symbionts transmitted horizontally, we performed the same analysis on *A. phagocytophilum*, *N. mikurensis, B. afzelii*, *B. garinii,* and *B. valaisiana* (combined). Contrary to the vertically-transmitted symbionts, the forest sites did not cluster neatly into the three geographic regions (Figure 1 and Figure 6b). The principal driver for differentiating the sampling sites was the absence of any horizontally-transmitted symbionts in questing nymphal ticks (h00).

***Borrelia afzelii* genetic diversity**

Observed regional differences are driven possibly by the geographical isolation of tick populations. This possibility could be rejected by applying a population genetic test on *B. afzelii* genospecies. We identified 61 distinct *B. afzelii* genotypes. From these, 39 genotypes (64%) were unique to a particular geographic region (Table S12). Estimated pairwise Fst values were significantly greater than zero for all three pairs from the region: northern, western, and central (Additional file 4: Table S12). Hence, we are unable to reject that the population of *I. ricinus* ticks is subdivided into three geographical regions.

Discussion

Several vertically-transmitted tick symbionts are recognized or potential pathogens for humans (Azad & Beard, 1998; Cisak et al., 2015). Here, we studied determinants of their prevalence by investigating dynamics of *I. ricinus* microbiome across developmental stages and geographical locations and by individually analysing questing nymphs from 19 forest sites on the presence of *R. helvetica*, *Rickettsiella* spp., *M. mitochondrii,* and *S. ixodetis*.

Analysing ticks with next-generation sequencing comes with methodological challenges related to their overall low biomass. Samples with low total bacterial load can suffer from contaminant DNA and cross-contamination (Eisenhofer et al., 2019). Therefore, pooling tick samples can maximize microbial yield and provide a representative sample of microbial taxonomic diversity (Carpi et al., 2011; Tokarz et al., 2019; Zhang et al., 2014). We used a similar approach in our study. Although we expected that the pooling would obfuscate the effects of bacterial biomass on community structure, 16S rRNA quantification demonstrated a surprisingly wide range of total bacterial loads across pools of ticks analysed in our experiment. While we could not determine the total bacterial load for a single tick directly, the negative correlation between established contaminants and total bacterial load suggests that our low microbial biomass pooled samples were more affected by contaminating DNA than high biomass samples (Figure S5b). A similar negative correlation was found for Shannon diversity and total bacterial load, suggesting that diversity metrics are inflated for these low-biomass pools (Figure S4b). However, by scaling microbial abundances through 16S qPCR quantification, the absolute abundances of the contaminants and rare species were reduced while absolute abundances of high-density samples were reflective of the community. Our results show that quantitative techniques for microbiome studies can accurately identify low-biomass samples in data sets with heterogeneous microbial load, even when used in combination with a pooling strategy commonly used to sequence low-biomass arthropod microbiomes.

In fact, the bacterial community composition of some samples did not differ from DNA extraction negative controls (Figure S1). We chose to retain these samples since they may represent a condition of biological interest: the absence of any abundant tick symbionts. For example, an in-depth study on the *I. scapularis* midgut microbiome suggested no stable midgut microbiome (Ross et al., 2018). These results are in line with an increasing body of evidence that many animals, including arthropods, are minimally or facultatively dependent on microbes or do not need a microbiome at all (Hammer et al., 2019). While our methods do not demonstrate the absence of a stable microbiome, the observed similarity between sequenced negative controls and low-biomass samples suggests that some *I. ricinus* individuals harbour a limited microbiome. And whatever microbiome they do harbour, could not be distinguished from negative controls.

As a species, *I. ricinus* has been shown to carry some potential obligate or facultative symbionts such as vertically-transmitted *R. helvetica*, *M. mitochondrii*, *S. ixodetis*, and *Rickettsiella* spp. (Duron et al., 2017). In our microbiome dataset, *R. helvetica*, *M. mitochondrii*, and *Rickettsiella* spp. were especially abundant. By screening questing nymphs individually, we showed that 12% of ticks did not carry any of the mentioned symbionts and any horizontally-transmitted tick-borne pathogens (*B. afzelii*, *B. garinii*, *B. valaisiana*, *A. phagocytophilum*, or *N. mikurensis*). Determinants of these phenomena are not yet understood, as well as the effect of symbionts on ticks' life cycle.

We have observed small but statistically significant differences between microbiome compositions of distinct developmental stages. The developmental stage on its own did not explain the clustering of tick microbiomes. Contrary to what we had expected, bacterial diversity seems to decrease along with tick development, illustrated with a significant difference between larvae and females. However, the opposite trend was observed from nymphs to adult males. It appears that during the off-host phases of ticks, fewer bacterial species enter a tick than has been speculated before (Narasimhan et al., 2015). Alternatively, the decreased bacterial diversity that we observed arose from the technical limitations of next-generation sequencing. It has been shown that abundant bacteria recruit more reads and potentially mask less abundant ones making them less likely to be detected (Gofton et al., 2015).

Previous studies have reported significant differences in *Ixodes* microbiome compositions of distinct life stages. However, they reported contradictory results regarding the dynamics of bacterial diversity. Zolnik et al. (2016) documented increasing, while Kwan et al. (2017), Swei &Kwan (2017), and Carpi et al. (2011) showed decreasing diversity along with tick development. Therefore, the role of the life stage in shaping the tick microbiome is still not elucidated, and it is difficult to subtract this information from the data obtained solely with next-generation sequencing. Thus, in future studies, combining this method with other detection techniques is highly advisable.

Our results demonstrated that (meta)populations of ticks from the western, central, and northern regions of the Netherlands had a different microbiome composition, and these differences are predominantly caused by tick symbiont communities (Figure 3). Although previous studies showed that the microbiome of *Ixodes* ticks varies by geographic location, it has never been shown before that it varies on a larger spatial scale (Carpi et al., 2011; Van Treuren et al., 2015).

These studies have given several probable explanations for differences in bacterial communities suggesting that they arose from the distinct habitats with a dissimilar availability of vertebrate hosts and consequently different environmental and animal host-associated bacteria (Carpi et al., 2011). Interestingly, in our dataset, the strongest determinants of observed differences in microbiome were the abundance and prevalence of *Rickettsia* and *Rickettsiella* (Figure 4). Therefore, based on these results, we hypothesized that the prevalence of *R. helvetica* (the most common *Rickettsia* species in *I. ricinus*) and *Rickettsiella* spp. is determined regionally rather than locally. In addition to these symbionts, we studied *S. ixodetis* and *M. mitochondrii*, which were highly abundant in our microbiome dataset but less responsible for variations in bacterial communities between regions. The prevalences of *R. helvetica*, *Rickettsiella* spp. and *M. mitochondrii* but not *S. ixodetis* were significantly different between three geographical regions, and each of the regions expressed a different proportion of symbionts in tick populations (Figure 1 and Figure 6).

Mechanisms causing such regional heterogenicity in the prevalence of *R. helvetica*, *Rickettsiella* spp., and *M. mitochondrii* remain to be determined. Previous studies on host-symbiont interactions correlated variation in symbiont prevalence with environmental variables: a symbiont is more prevalent in the tick host population when it provides tolerance to a given biotic or abiotic stress, and less prevalent when it is less beneficial for mitigating stress (and therefore, stress is a weaker selective force; Duron et al., 2017; Gundel et al., 2011). Therefore, (meta)populations of ticks from these three regions may be exposed to different stresses. Abiotic stresses may arise from regional differences in soil type, temperature, and humidity related to coastal (the western region)

and inland (the central and northern regions) areas of the Netherlands. Biotic stresses such as the presence and abundance of tick parasites, for example, parasitic wasps, might lead to varying prevalences of facultative defensive symbionts in tick populations as demonstrated for other species of arthropods (Oliver et al., 2008). Alternatively, the varying prevalences of a symbiont between distinct tick (meta)populations may arise from differences in transmission rate. The transmission rate could be potentially affected by tick or symbiont genotypes or the compatibility of particular tick – symbiont genotype pairs (Gundel et al., 2011). This evolutionary divergence within *I. ricinus* and/or a symbiont species could be promoted by a geographical barrier and limited exchange of tick individuals between regions. However, this variation has not yet been studied and documented for arthropod – microbe symbiosis, and here, we did not look at the genetic diversity of studied symbionts.

Nevertheless, our Fst analysis of *B. afzelii* sequences deriving from the same regions revealed distinct genotypes circulating in each region, indicating a limited exchange of ticks between studied (meta) populations. Since all symbionts occur in all regions and even all locations, we do not think that the tick population as such is driving the differences but rather (a)biotic factors, such as parasitoids, climate, or soil type. However, limited exchange of ticks between populations may help maintain the differences in symbiont prevalences and compositions between the regions.

Conclusions

Our study shows that there are regional differences in the symbiont communities of *I. ricinus*. Although all symbionts are found in every location in all regions, the proportions in which they occur in a tick (sub)populations differ. Whereas previous studies showed that the occurrence of horizontally-transmitted pathogens, most notably *B. burgdorferi* s.l. and *A. phagocytophilum*, is greatly determined by vertebrate host communities, this study has demonstrated that the infection prevalence of vertically-transmitted symbionts is determined on a larger spatial scale by currently unknown (a)biotic factors. Consequently, the disease risk of vertically-transmitted pathogens, such as *R. helvetica*, displays regional differences, already in a (small) country like the Netherlands. Whether this phenomenon also holds for other more severe Spotted Fever Group Rickettsia's from other tick species, like *R. conorii* and *R. rickettsii*, is uncharted territory and subject of future studies. Our findings imply that public health investigations and measures for etiological agents like *R. helvetica*, such as risk assessments, implementation of diagnostic modalities, and preventive measures, should consider these regional differences. Lastly, although this study focused on symbiont communities in tick (sub)populations, it already showed that the symbiont composition of individual ticks is highly variable. Future studies investigating the interactions of symbionts in individual ticks and their consequences for tick fitness and pathogen transmission are on their way.

Acknowledgements

We thank Mei Ling Chu (RIVM) for helping with the 16S rRNA quantification.

Supplement

Text S1. Details on models investigating the effect of region and life stage on bacterial community variation.

To investigate correlations between OTUs, bacterial community variation and regions, we fitted proportional odds models with OTU abundances scaled by absolute bacterial density as the dependent variable and used region and tick life stage as independent variables. The model is defined as logit[P(Y_ik≤j)]= θ_j-x_i β_1-x_k β_2, where Yik is the OTU abundance that can fall in j categories, xi and xk are vectors of the region and life stage factors and β is the set of regression parameters for each of these factors. We used the clm function from the R package ordinal (version 2019.4-25) to fit these models (Christensen 2015). With the logit link function, coefficients can be interpreted as the effect that life stage and region factors have on the probability that a taxon reaches a certain level of abundance. We compared these models with the likelihood ratio test, using an implementation of Nagelkerke's pseudo-R^2 from the R package rcompanion (version 2.3.7) (Mangiafico and Mangiafico 2017).

Christensen, R. H. B. (2015). Analysis of ordinal data with cumulative link models—estimation with the R-package ordinal. R-package version, 28.

Text S2. Primers and probes for the detection of *Rickettsiella* spp. and *M. mitochondrii*, target genes for the remaining symbionts and a qPCR protocol.

Rickettsiella spp. qPCR

To detect *Rickettsiella* spp., two regions were targeted: a 196-bp fragment of the glucose-inhibited division gene using primers 5'-TGT AAT CCT TGA GTC TGA TCG T-3', 5'-CAA ACC GAT ATG AAT TTT TCC GG-3' and a probe 5'-ATTO520-TAG TTG GTG TGG TAA CGC AAA TGG GGT-BHQ2-3', and a 76-bp fragment of the dihydrolipoamide succinyltransferase gene using primers 5'- GAT CAA CCC TCT CAA TCA GC-3', 5'- GCC AAA TGG GTG TCA CTA T' and a probe 5'-ATTO647- CAC CCG TCG CAG AAA AAA CTA AAC CTG-BHQ2-3'. A samples positive for at least one marker was considered positive for Rickettsiella spp.

Midichloria mitochondrii qPCR

To detect *M. mitochondrii*, one region was targeted: a 145-bp fragment of the gyrase subunit B gene using primers 5'- CTT GAG AGC AGA ACC ACC TA-3', 5'- CAA GCT CTG CCG AAA TAT CTT-3' and a probe 5'-ATTO424- GAG GGC GGA GTC AAA GAA TTT GTC CAC G-BHQ1-3'.

Targeted genes in qPRCs

Symbiont	Gene	Reference
S. ixodetis	*rpoB*	Chapter 4
R. helvetica	*gltA*	de Bruin et al., 2015
B. burgdorferi s.l	*ospA* and *flaB*	Heylen et al., 2013b
A. phagocytophilum	*ApMSP2*	Courtney et al., 2004
N. mikurensis	*GroEL*	Jahfari et al., 2012

qPCR protocol

All qPCRs were carried out on a LightCycler 480 (Roche Diagnostics Nederland B.V, Almere, the Netherlands) in a final volume of 20 µl with iQ multiplex Powermix, 3 µl of a sample, primers with end concentration of 0.2 µM, and probes. Positive plasmid controls and negative water controls were used on every plate tested. Cycling conditions included an initial activation of the iTaq DNA polymerase at 95°C for 5 min, followed by 60 cycles of a 5 s denaturation at 95°C followed by a 35 s annealing-extension step at 60°C (Ramp rate 2.2°C s^{-1} and a single point measurement at 60°C) and a final cooling cycle of 37°C for 20 s.

Table S1. Metadata for all of the locations including their full names and coordinates as well as information on vegetation and tick density.

Location	Location full name	Coordinates	Habitat	Undergrowth vegetation	Larva/ 1200m2	Nymph/ 1200m2	Adult/ 1200m2	Number of vertebrate species
AW	Amsterdamse Waterleiding Duinen	52°20'36" N 4°33'58"E	Mixed forest	Calamagrostis epigejos	4,186	726	63	12
BB	Bergherbos	51°55'14"N 6°14'30"E	Mixed forest	Deschampsia flexuosa	468	869	32	14
BU	Buunderkamp	52°00'56" N 5°44'50"E	Scots pine forest	Vaccinium myrtillus	2,591	2,200	76	11
DK	Duin en Kruidberg	52°26'16" N 4°36'18"E	Mixed forest	Calamagrostis epigejos	5,274	870	53	6
DW	Deelerwoud	52°05'51" N 5°56'42"E	Scots pine forest	Vaccinium myrtillus	3,178	1,647	161	10
EN	Enkhout	52°16'25" N 5°54'49"E	Scots pine forest	Vaccinium myrtillus	897	1,379	64	13
HD	Herperduin	51°45'33" N 5°36'53"E	Mixed forest	Molinia caerulea	72	120	4	15
HM	Halfmijl	51°25'23" N 5°19'09"E	Mixed forest	Molinia caerulea	1,241	1,531	55	10
KB	Kremboong	52°45'13" N 6°31'16"E	Pedunculate oak forest	Dryopteris dilatata	2,239	794	28	12
MH	Maashorst	51°42'44" N 5°35'24"E	Mixed forest	Deschampsia flexuosa	2,994	640	17	16
PD	Pettemerduin	52°46'33" N 4°40'19"E	Pedunculate oak forest	Polypodium vulgare	0	130	19	10
PW	Planken Wambuis	52°01'54" N 5°48'36"E	Scots pine forest	Vaccinium myrtillus	1,566	760	20	9
RB	Rheebruggen	52°46'60" N 6°17'44"E	Pedunculate oak forest	Dryopteris dilatata	6,203	859	49	13
SD	Schoorlse Duinen	52°41'47" N 4°40'01"E	Mixed forest	Molinia caerulea	0	22	3	6
ST	Stameren	52°03'38" N 5°21'01"E	Mixed forest	Deschampsia flexuosa	1,474	1,134	94	14
VA	Valenberg	52°15'33" N 5°48'47"E	Scots pine forest	Vaccinium myrtillus	2,724	765	92	15
VH	Vijverhof	52°09'43" N 5°13'43"E	Mixed forest	Deschampsia flexuosa	555	1,039	23	11
VL	Vledderhof	52°52'46" N 6°14'25"E	Pedunculate oak forest	Dryopteris dilatata	1,648	403	31	16
ZM	Zwanemeerbos	53°00'46" N 6°45'19"E	Pedunculate oak forest	Pteridium aquilinum	1,225	680	108	11

Table S2. Number of samples collected from different tick life stages at different locations. L10, a pool of ten larvae; N5, a pool of five nymphs; N10, a pool of ten nymphs; AF, adult female; AM, adult male.

	L10	N5	N10	AF	AM	Number of samples
AW	6*	3	3	4	3	19
BU	6	3	3	6	6	24
DK	6	3	3	6	5	23
HD	4	3	3	6	6*	22
ST	4	3	3	6	5	21
ZM	6	3	3	6	6	24
Total number of ticks	320	90	180	34	31	

* one sample from this group was not successfully sequenced

Table S3. Location and tick life stage per sample.

Sample ID	Location	Life stage	Sample ID	Location	Life stage
184L10ZM	ZM	larva	**140L10HD**	HD	larva
185L10ZM	ZM	larva	**141L10HD**	HD	larva
186L10ZM	ZM	larva	**142L10HD**	HD	larva
187L10ZM	ZM	larva	**143L10HD**	HD	larva
188L10ZM	ZM	larva	**147N5HD**	HD	nymph
190L10ZM	ZM	larva	**148N5HD**	HD	nymph
197N5ZM	ZM	nymph	**153N5HD**	HD	nymph
198N5ZM	ZM	nymph	**157N10HD**	HD	nymph
201N5ZM	ZM	nymph	**158N10HD**	HD	nymph
211N10ZM	ZM	nymph	**159N10HD**	HD	nymph
212N10ZM	ZM	nymph	**162AFHD**	HD	female
215N10ZM	ZM	nymph	**163AFHD**	HD	female
221AFZM	ZM	female	**164AFHD**	HD	female
223AFZM	ZM	female	**165AFHD**	HD	female
225AFZM	ZM	female	**166AFHD**	HD	female
228AFZM	ZM	female	**167AFHD**	HD	female
230AFZM	ZM	female	**171AMHD**	HD	male
232AFZM	ZM	female	**172AMHD**	HD	male
234AMZM	ZM	male	**176AMHD**	HD	male
235AMZM	ZM	male	**178AMHD**	HD	male
239AMZM	ZM	male	**180AMHD**	HD	male
243AMZW	ZM	male	**58L10ST**	ST	larva
245AMZW	ZM	male	**59L10ST**	ST	larva
249AMZW	ZM	male	**60L10ST**	ST	larva
61L10ST	ST	larva	**30AFDK**	DK	female
62N5ST	ST	nymph	**1L10DK**	DK	larva
63N5ST	ST	nymph	**20N5DK**	DK	nymph
69N5ST	ST	nymph	**24N10DK**	DK	nymph
72N10ST	ST	nymph	**4L10DK**	DK	larva
74N10ST	ST	nymph	**53AMDK**	DK	male
75N10ST	ST	nymph	**44AMDK**	DK	male
78AFST	ST	female	**40AFDK**	DK	female
79AFST	ST	female	**8L10DK**	DK	larva
82AFST	ST	female	**41AMDK**	DK	male
83AFST	ST	female	**39AFDK**	DK	female
86AFST	ST	female	**22N10DK**	DK	nymph
87AFST	ST	female	**42AMDK**	DK	male
89AMST	ST	male	**6L10DK**	DK	larva
90AMST	ST	male	**43AMDK**	DK	male
92AMST	ST	male	**253L10BU**	BU	larva
96AMST	ST	male	**254L10BU**	BU	larva

table continues

Sample ID	Location	Life stage	Sample ID	Location	Life stage
98AMST	ST	male	**255L10BU**	BU	larva
106L10AW	AW	larva	**256L10BU**	BU	larva
107L10AW	AW	larva	**257L10BU**	BU	larva
109L10AW	AW	larva	**259L10BU**	BU	larva
112L10AW	AW	larva	**266N5BU**	BU	nymph
116L10AW	AW	larva	**268N5BU**	BU	nymph
120N5AW	AW	nymph	**271N5BU**	BU	nymph
124N5AW	AW	nymph	**272N10BU**	BU	nymph
125N5AW	AW	nymph	**274N10BU**	BU	nymph
128N10AW	AW	nymph	**275N10BU**	BU	nymph
130N10AW	AW	nymph	**290AFBU**	BU	female
132N10AW	AW	nymph	**292AFBU**	BU	female
133AFAW	AW	female	**293AFBU**	BU	female
134AFAW	AW	female	**294AFBU**	BU	female
135AFAW	AW	female	**295AFBU**	BU	female
136AFAW	AW	female	**297AFBU**	BU	female
137AMAW	AW	male	**300AMBU**	BU	male
138AMAW	AW	male	**301AMBU**	BU	male
139AMAW	AW	male	**302AMBU**	BU	male
15N5DK	DK	nymph	**303AMBU**	BU	male
19N5DK	DK	nymph	**304AMBU**	BU	male
26N10DK	DK	nymph	**308AMBU**	BU	male
35AFDK	DK	female			
2L10DK	DK	larva			
9L10DK	DK	larva			
29AFDK	DK	female			
28AFDK	DK	female			

Table S4. A list of *B. afzelii* IGS sequencing used for the Fst analysis. Due to its size, the table is available upon request.

Table S5. Read counts for different taxa, including taxonomy per sample. Negative control samples included. Due to its size, the table is available upon request.

Table S6. Results of PERMANOVA testing an effect of tick life stage and region on community variation, and the analysis of variance.

PERMANOVA
Call:
adonis(formula = data ~ stage * region, permutations = 999, method = "bray", by = "margin")
Permutation: free
Number of permutations: 999
Terms added sequentially (first to last)

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
stage	3	5.536	1.8454	7.5192	0.12068	0.001 ***
region	2	6.686	3.3428	13.6202	0.14574	0.001 ***
stage:region	6	4.938	0.8229	3.3530	0.10763	0.001 ***
Residuals	117	28.715	0.2454		0.62595	
Total	128	45.875			1.00000	

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Stage - Multivariate spread
Analysis of Variance Table
Response: Distances

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Groups	3	0.41497	0.138324	19.776	1.484e-10	***
Residuals	125	0.87433	0.006995			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Region - Multivariate spread
Analysis of Variance Table
Response: Distances

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Groups	2	0.14285	0.071427	4.828	0.009543	**
Residuals	126	1.86409	0.014794			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table S7. Results of models of stage and region explaining *Rickettsia* and *Rickettsiella* abundance. Samples k-means clustered by log of *Rickettsia* abundances are listed in a table below.

Envfit results for vectors of symbiont abundances
***VECTORS

	PCoA1	PCoA2	r2	Pr(>r)	
Rickettsia	0.97337	-0.22924	0.5590	0.001	***
Spiroplasma	0.53074	0.84753	0.0221	0.250	
Rickettsiella	0.31317	0.94970	0.2059	0.001	***
Midichloria	0.28293	-0.95914	0.0300	0.161	

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
Permutation: free
Number of permutations: 999

clm model structures (logit link)
Rank-transformed abundance ~ 1
Rank-transformed abundance ~ stage
Rank-transformed abundance ~ region
Rank-transformed abundance ~ stage + region

Nagelkerke model comparisons for Rickettsia
Each model is compared to the model on the above line

Model	Nagelkerke pseudo-R2	p-value	Likelihood	Likelihood.stars
1 Rickettsia ~ 1	0.0000000	1.0000e+00	0.0000e+00	<NA>
2 Rickettsia ~ stage	0.0506633	8.1883e-02	8.1883e-02	0.082 .
3 Rickettsia ~ region	0.4881360	1.7541e-19	4.4286e-19	0 ***

4 Rickettsia ~ stage + region 0.5232070 4.6240e-19 2.7320e-02 0.027 *

Nagelkerke model comparisons for Rickettsiella

Each model is compared to the model on the above line

Model	Nagelkerke pseudo-R2	p-value	Likelihood	Likelihood.stars
1 Rickettsiella ~ 1	0.00869395	7.7079e-01	0.0000e+00	<NA>
2 Rickettsiella ~ stage	0.00869395	7.7079e-01	7.7079e-01	0.771
3 Rickettsiella ~ region	0.44472200	3.3726e-17	5.3958e-18	0 ***
4 Rickettsiella ~ stage + region	0.44576000	5.4882e-15	9.7064e-01	0.971

Samples k-means clustered by log of *Rickettsia* abundances

Sample ID	cluster
184L10ZM_21334_CGAGGCTGTTATGCGA_L001_R1_001_000000000-AYHYK	2
185L10ZM_21335_AAGAGGCATTATGCGA_L001_R1_001_000000000-AYHYK	2
186L10ZM_21336_GTAGAGGATTATGCGA_L001_R1_001_000000000-AYHYK	2
187L10ZM_21337_GCTCATGATTATGCGA_L001_R1_001_000000000-AYHYK	1
188L10ZM_21338_ACTCGCTATCGACTAG_L001_R1_001_000000000-AYRG7	2
190L10ZM_21339_GGAGCTACTCGACTAG_L001_R1_001_000000000-AYRG7	2
197N5ZM_21340_GCGTAGTATCGACTAG_L001_R1_001_000000000-AYRG7	2
198N5ZM_21341_CGGAGCCTTCGACTAG_L001_R1_001_000000000-AYRG7	1
201N5ZM_21342_TACGCTGCTCGACTAG_L001_R1_001_000000000-AYRG7	3
211N10ZM_21343_ATGCGCAGTCGACTAG_L001_R1_001_000000000-AYRG7	2
212N10ZM_21344_TAGCGCTCTCGACTAG_L001_R1_001_000000000-AYRG7	1
215N10ZM_21345_ACTGAGCGTCGACTAG_L001_R1_001_000000000-AYRG7	3
221AFZM_21346_CCTAAGACTCGACTAG_L001_R1_001_000000000-AYRG7	2
223AFZM_21347_CGATCAGTTCGACTAG_L001_R1_001_000000000-AYRG7	1
225AFZM_21348_TGCAGCTATCGACTAG_L001_R1_001_000000000-AYRG7	3
228AFZM_21349_TCGACGTCTCGACTAG_L001_R1_001_000000000-AYRG7	2
230AFZM_21350_ACTCGCTATTCTAGCT_L001_R1_001_000000000-AYRG7	2
232AFZM_21351_GGAGCTACTTCTAGCT_L001_R1_001_000000000-AYRG7	2
234AMZM_21352_GCGTAGTATTCTAGCT_L001_R1_001_000000000-AYRG7	2
235AMZM_21353_CGGAGCCTTTCTAGCT_L001_R1_001_000000000-AYRG7	2
239AMZM_21354_TACGCTGCTTCTAGCT_L001_R1_001_000000000-AYRG7	2
243AMZW_21355_ATGCGCAGTTCTAGCT_L001_R1_001_000000000-AYRG7	1
245AMZW_21356_TAGCGCTCTTCTAGCT_L001_R1_001_000000000-AYRG7	3
249AMZW_21357_ACTGAGCGTTCTAGCT_L001_R1_001_000000000-AYRG7	2
140L10HD_21311_AAGAGGCAAAGGCTAT_L001_R1_001_000000000-AYHYK	2
141L10HD_21312_GTAGAGGAAAGGCTAT_L001_R1_001_000000000-AYHYK	2
142L10HD_21313_GCTCATGAAAGGCTAT_L001_R1_001_000000000-AYHYK	2
143L10HD_21314_ATCTCAGGAAGGCTAT_L001_R1_001_000000000-AYHYK	2
147N5HD_21316_CGTACTAGGAGCCTTA_L001_R1_001_000000000-AYHYK	2
148N5HD_21317_AGGCAGAAGAGCCTTA_L001_R1_001_000000000-AYHYK	2
153N5HD_21318_TCCTGAGCGAGCCTTA_L001_R1_001_000000000-AYHYK	2
157N10HD_21319_GGACTCCTGAGCCTTA_L001_R1_001_000000000-AYHYK	3
158N10HD_21320_TAGGCATGGAGCCTTA_L001_R1_001_000000000-AYHYK	2
159N10HD_21321_CTCTCTACGAGCCTTA_L001_R1_001_000000000-AYHYK	2
162AFHD_21322_CGAGGCTGGAGCCTTA_L001_R1_001_000000000-AYHYK	2
163AFHD_21323_AAGAGGCAGAGCCTTA_L001_R1_001_000000000-AYHYK	3
164AFHD_21324_GTAGAGGAGAGCCTTA_L001_R1_001_000000000-AYHYK	2
165AFHD_21325_GCTCATGAGAGCCTTA_L001_R1_001_000000000-AYHYK	2
166AFHD_21326_ATCTCAGGGAGCCTTA_L001_R1_001_000000000-AYHYK	2
167AFHD_21327_TAAGGCGATTATGCGA_L001_R1_001_000000000-AYHYK	1
171AMHD_21328_CGTACTAGTTATGCGA_L001_R1_001_000000000-AYHYK	2
172AMHD_21329_AGGCAGAATTATGCGA_L001_R1_001_000000000-AYHYK	2
176AMHD_21330_TCCTGAGCTTATGCGA_L001_R1_001_000000000-AYHYK	1
178AMHD_21331_GGACTCCTTTATGCGA_L001_R1_001_000000000-AYHYK	2

table continues

Sample ID	cluster
180AMHD_21332_TAGGCATGTTATGCGA_L001_R1_001_000000000-AYHYK	1
58L10ST_21267_TAAGGCGACCTAGAGT_L001_R1_001_000000000-AYHYK	2
59L10ST_21268_CGTACTAGCCTAGAGT_L001_R1_001_000000000-AYHYK	1
60L10ST_21269_AGGCAGAACCTAGAGT_L001_R1_001_000000000-AYHYK	1
61L10ST_21270_TCCTGAGCCCTAGAGT_L001_R1_001_000000000-AYHYK	2
62N5ST_21271_GGACTCCTCCTAGAGT_L001_R1_001_000000000-AYHYK	2
63N5ST_21272_TAGGCATGCCTAGAGT_L001_R1_001_000000000-AYHYK	1
69N5ST_21273_CTCTCTACCCTAGAGT_L001_R1_001_000000000-AYHYK	2
72N10ST_21274_CGAGGCTGCCTAGAGT_L001_R1_001_000000000-AYHYK	1
74N10ST_21275_AAGAGGCACCTAGAGT_L001_R1_001_000000000-AYHYK	2
75N10ST_21276_GTAGAGGACCTAGAGT_L001_R1_001_000000000-AYHYK	1
78AFST_21277_GCTCATGACCTAGAGT_L001_R1_001_000000000-AYHYK	1
79AFST_21278_ATCTCAGGCCTAGAGT_L001_R1_001_000000000-AYHYK	1
82AFST_21279_TAAGGCGAGCGTAAGA_L001_R1_001_000000000-AYHYK	1
83AFST_21280_CGTACTAGGCGTAAGA_L001_R1_001_000000000-AYHYK	2
86AFST_21281_AGGCAGAAGCGTAAGA_L001_R1_001_000000000-AYHYK	2
87AFST_21282_TCCTGAGCGCGTAAGA_L001_R1_001_000000000-AYHYK	1
89AMST_21283_GGACTCCTGCGTAAGA_L001_R1_001_000000000-AYHYK	1
90AMST_21284_TAGGCATGGCGTAAGA_L001_R1_001_000000000-AYHYK	1
92AMST_21285_CTCTCTACGCGTAAGA_L001_R1_001_000000000-AYHYK	2
96AMST_21286_CGAGGCTGGCGTAAGA_L001_R1_001_000000000-AYHYK	1
98AMST_21287_AAGAGGCAGCGTAAGA_L001_R1_001_000000000-AYHYK	1
106L10AW_21292_CGTACTAGCTATTAAG_L001_R1_001_000000000-AYHYK	1
107L10AW_21293_AGGCAGAACTATTAAG_L001_R1_001_000000000-AYHYK	3
109L10AW_21294_TCCTGAGCCTATTAAG_L001_R1_001_000000000-AYHYK	1
112L10AW_21295_GGACTCCTCTATTAAG_L001_R1_001_000000000-AYHYK	3
116L10AW_21297_CTCTCTACCTATTAAG_L001_R1_001_000000000-AYHYK	3
120N5AW_21298_CGAGGCTGCTATTAAG_L001_R1_001_000000000-AYHYK	3
124N5AW_21299_AAGAGGCACTATTAAG_L001_R1_001_000000000-AYHYK	3
125N5AW_21300_GTAGAGGACTATTAAG_L001_R1_001_000000000-AYHYK	3
128N10AW_21301_GCTCATGACTATTAAG_L001_R1_001_000000000-AYHYK	3
130N10AW_21302_ATCTCAGGCTATTAAG_L001_R1_001_000000000-AYHYK	3
132N10AW_21303_TAAGGCGAAAGGCTAT_L001_R1_001_000000000-AYHYK	3
133AFAW_21304_CGTACTAGAAGGCTAT_L001_R1_001_000000000-AYHYK	2
134AFAW_21305_AGGCAGAAAAGGCTAT_L001_R1_001_000000000-AYHYK	1
135AFAW_21306_TCCTGAGCAAGGCTAT_L001_R1_001_000000000-AYHYK	3
136AFAW_21307_GGACTCCTAAGGCTAT_L001_R1_001_000000000-AYHYK	2
137AMAW_21308_TAGGCATGAAGGCTAT_L001_R1_001_000000000-AYHYK	3
138AMAW_21309_CTCTCTACAAGGCTAT_L001_R1_001_000000000-AYHYK	3
139AMAW_21310_CGAGGCTGAAGGCTAT_L001_R1_001_000000000-AYHYK	3
15N5DK_21249_CTCTCTACTCGACTAG_L001_R1_001_000000000-AYHYK	3
19N5DK_21250_CGAGGCTGTCGACTAG_L001_R1_001_000000000-AYHYK	3
26N10DK_21254_ATCTCAGGTCGACTAG_L001_R1_001_000000000-AYHYK	3
35AFDK_21258_TCCTGAGCTTCTAGCT_L001_R1_001_000000000-AYHYK	3
2L10DK_21244_CGTACTAGTCGACTAG_L001_R1_001_000000000-AYHYK	1
9L10DK_21248_TAGGCATGTCGACTAG_L001_R1_001_000000000-AYHYK	1
29AFDK_21256_CGTACTAGTTCTAGCT_L001_R1_001_000000000-AYHYK	1
28AFDK_21255_TAAGGCGATTCTAGCT_L001_R1_001_000000000-AYHYK	3
30AFDK_21257_AGGCAGAATTCTAGCT_L001_R1_001_000000000-AYHYK	3
1L10DK_21243_TAAGGCGATCGACTAG_L001_R1_001_000000000-AYHYK	1
20N5DK_21251_AAGAGGCATCGACTAG_L001_R1_001_000000000-AYHYK	3
24N10DK_21253_GCTCATGATCGACTAG_L001_R1_001_000000000-AYHYK	3
4L10DK_21245_AGGCAGAATCGACTAG_L001_R1_001_000000000-AYHYK	1
53AMDK_21266_ATCTCAGGTTCTAGCT_L001_R1_001_000000000-AYHYK	3
44AMDK_21264_GTAGAGGATTCTAGCT_L001_R1_001_000000000-AYHYK	3
40AFDK_21260_TAGGCATGTTCTAGCT_L001_R1_001_000000000-AYHYK	3
8L10DK_21247_GGACTCCTTCGACTAG_L001_R1_001_000000000-AYHYK	1
41AMDK_21261_CTCTCTACTTCTAGCT_L001_R1_001_000000000-AYHYK	3
39AFDK_21259_GGACTCCTTTCTAGCT_L001_R1_001_000000000-AYHYK	3
22N10DK_21252_GTAGAGGATCGACTAG_L001_R1_001_000000000-AYHYK	3

table continues

Sample ID	cluster
42AMDK_21262_CGAGGCTGTTCTAGCT_L001_R1_001_000000000-AYHYK	3
6L10DK_21246_TCCTGAGCTCGACTAG_L001_R1_001_000000000-AYHYK	1
43AMDK_21263_AAGAGGCATTCTAGCT_L001_R1_001_000000000-AYHYK	2
253L10BU_21358_CCTAAGACTTCTAGCT_L001_R1_001_000000000-AYRG7	2
254L10BU_21359_CGATCAGTTTCTAGCT_L001_R1_001_000000000-AYRG7	1
255L10BU_21360_TGCAGCTATTCTAGCT_L001_R1_001_000000000-AYRG7	1
256L10BU_21361_TCGACGTCTTCTAGCT_L001_R1_001_000000000-AYRG7	1
257L10BU_21362_ACTCGCTACCTAGAGT_L001_R1_001_000000000-AYRG7	1
268N5BU_21365_CGGAGCCTCCTAGAGT_L001_R1_001_000000000-AYRG7	2
271N5BU_21366_TACGCTGCCCTAGAGT_L001_R1_001_000000000-AYRG7	2
272N10BU_21367_ATGCGCAGCCTAGAGT_L001_R1_001_000000000-AYRG7	2
274N10BU_21368_TAGCGCTCCCTAGAGT_L001_R1_001_000000000-AYRG7	1
275N10BU_21369_ACTGAGCGCCTAGAGT_L001_R1_001_000000000-AYRG7	2
290AFBU_21376_GCGTAGTAGCGTAAGA_L001_R1_001_000000000-AYRG7	2
292AFBU_21377_CGGAGCCTGCGTAAGA_L001_R1_001_000000000-AYRG7	2
293AFBU_21378_TACGCTGCGCGTAAGA_L001_R1_001_000000000-AYRG7	2
294AFBU_21379_ATGCGCAGGCGTAAGA_L001_R1_001_000000000-AYRG7	1
295AFBU_21380_TAGCGCTCGCGTAAGA_L001_R1_001_000000000-AYRG7	2
297AFBU_21381_ACTGAGCGGCGTAAGA_L001_R1_001_000000000-AYRG7	3
300AMBU_21382_CCTAAGACGCGTAAGA_L001_R1_001_000000000-AYRG7	1
301AMBU_21383_CGATCAGTGCGTAAGA_L001_R1_001_000000000-AYRG7	2
302AMBU_21384_TGCAGCTAGCGTAAGA_L001_R1_001_000000000-AYRG7	2
303AMBU_21385_TCGACGTCGCGTAAGA_L001_R1_001_000000000-AYRG7	2
304AMBU_21386_ACTCGCTACTATTAAG_L001_R1_001_000000000-AYRG7	1
308AMBU_21387_GGAGCTACCTATTAAG_L001_R1_001_000000000-AYRG7	1

Table S8. Prevalence (%) of tick symbionts in larvae, nymphs and adults of *I. ricinus* collected in two locations (ST and AW) indicating primary transmission mode (vertical or horizontal).

Symbionts	Larvae (n = 367)	Nymphs (n = 684)	Adults (n = 78)
R. helvetica	34.6 (29.7 - 39.7)	23.7 (20.5 - 27.1)	33.3 (23.1 - 44.9)
S. ixodetis	12.5 (9.3 - 16.4)	10.4 (8.2 - 12.9)	12.8 (6.3 - 22.3)
Rickettsiella spp.	36.8 (31.8 - 41.9)	25.9 (22.6 - 29.3)	37.2 (26.5 - 48.9)
M. mitochondrii	84.2 (80.1 - 87.8)	77.2 (74.0 - 80.4)	73.1 (61.8 - 82.5)
B. burgdorferi s.l.	0.3 (0.0 - 1.5)	12 (9.6 - 14.7)	16.7 (9.2 - 26.8)
A. phagocytophilum	0 (0.0 - 1.0)	2.8 (1.7 - 4.3)	9 (3.7 - 17.6)
N. mikurensis	0 (0.0 - 1.0)	4.4 (3.0 - 6.2)	5.1 (1.4 - 12.6)

Between brackets are the 95% confidence intervals calculated according to Armitage et al. (2001).

Table S9. Prevalence (%) of tick symbionts in nymphs of *I. ricinus* collected in 19 forest sites in the Netherlands. The table continues on the following page.

Location		*M. mitochondrii*			*Rickettsiella* spp.			*S. ixodetis*			*R. helvetica*		
	N tested	N positive	%	CI 95%	N positive	%	CI 95%	N positive	%	CI 95%	N positive	%	CI 95%
Amsterdamse Waterleiding Duinen	718	510	71.0	67.6 - 74.3	400	55.7	52.0 - 59.4	47	6.5	4.7 - 8.4	341	47.5	43.8 - 51.2
Bergherbos	869	513	59.0	55.7 - 62.3	386	44.4	41.1 - 47.8	56	6.4	4.8 - 8.1	66	7.6	5.9 - 9.6
Buunderkamp	2,202	1,364	61.9	59.9 - 64.0	727	33.0	31.1 - 35.0	102	4.6	3.8 - 5.5	113	5.1	4.2 - 6.1
Deelerwoud	1,650	1,065	64.5	62.2 - 66.9	459	27.8	25.7 - 30.0	349	21.2	19.2 - 23.1	36	2.2	1.5 - 3.0
Duin en Kruidberg	858	527	61.4	58.1 - 64.7	827	96.4	94.9 - 97.5	44	5.1	3.7 - 6.6	515	60.0	56.7 - 63.3
Halfmijl	1,531	964	63.0	60.5 - 65.4	396	25.9	23.7 - 28.1	259	16.9	15.0 - 18.8	64	4.2	3.2 - 5.3
Herperduin	120	89	74.2	65.4 - 81.7	45	37.5	28.8 - 46.8	8	6.7	2.2 -11.1	4	3.3	0.9 - 8.3
Kremboong	791	371	46.9	43.4 - 50.4	684	86.5	83.9 - 88.8	110	13.9	11.5 - 16.3	57	7.2	5.5 - 9.2
Kroondomein	1,397	943	67.5	65.0 - 70.0	429	30.7	28.3 - 33.2	599	42.9	40.3 - 45.5	37	2.6	1.9 - 3.6
Maashorst	639	371	58.1	54.1 - 61.9	175	27.4	24.0 - 31.0	54	8.5	6.3 - 10.6	71	11.1	8.8 - 13.8
Pettermerduin	130	84	64.6	55.8 - 72.8	59	45.4	36.6 - 54.3	13	10.0	4.8 - 15.2	83	63.8	55.0 - 72.1
Planken Wambuis	767	507	66.1	62.6 - 69.4	286	37.3	33.9 - 40.8	122	15.9	13.3 - 18.5	22	2.9	1.8 - 4.3
Rheebruggen	863	560	64.9	61.6 - 68.1	474	54.9	51.5 - 58.3	77	8.9	7.0 - 10.8	237	27.5	24.5 - 30.6
Schoorlse Duinen	26	20	76.9	56.4 - 91.0	19	73.1	52.2 - 88.4	7	26.9	9.9 - 44.0	9	34.6	17.2 - 55.7
Stameren	1,129	730	64.7	61.8 - 67.5	475	42.1	39.2 - 45.0	197	17.4	15.2 - 19.7	156	13.8	11.9 - 16.0
Valenberg	763	493	64.6	61.1 - 68.0	152	19.9	17.1 - 22.9	338	44.3	40.8 - 47.8	10	1.3	0.6 - 2.4
Vijverhof	1,039	631	60.7	57.7 - 63.7	804	77.4	74.7 - 79.9	206	19.8	17.4 - 22.3	104	10.0	8.3 - 12.0
Vledderhof	398	251	63.1	58.1 - 67.8	314	78.9	74.6 - 82.8	30	7.5	4.9 - 10.1	101	25.4	21.2 - 30.0
Zwanemeerbos	665	348	52.3	48.5 - 56.2	629	94.6	92.6 - 96.2	109	16.4	13.6 - 19.2	85	12.8	10.3 - 15.6
Total	1,6555	1,0341	62.5	61.7 - 63.2	7740	46.8	46.0 - 47.5	2727	16.5	15.9 - 17.0	2111	12.8	12.2 - 13.3

table continues

Location		*A. phagocytophilum*			*B. afzelii*			*B. garinii + B. valaisiana*			*N. mikurensis*		
	N tested	N positive	%	CI 95%	N positive	%	CI 95%	N positive	%	CI 95%	N positive	%	CI 95%
Amsterdamse Waterleiding Duinen	678	39	5.8	4.1 - 7.8	5	0.7	0.2 - 1.7	1	0.1	0.0 - 0.8	1	0.1	0.0 - 0.8
Bergherbos	742	6	0.8	0.3 - 1.8	19	2.6	1.5 - 4.0	27	3.1	2.4 - 5.3	30	4.0	2.7 - 5.7
Buunderkamp	688	0	0.0	0.0 - 0.5	33	4.8	3.3 - 6.7	3	0.1	0.1 - 1.3	92	13.4	10.9 - 16.1
Deelerwoud	1,650	68	4.1	3.2 - 5.2	19	1.2	0.7 - 1.8	1	0.1	0.0 - 0.3	44	2.7	1.9 - 3.6
Duin en Kruidberg	858	31	3.6	2.5 - 5.1	5	0.6	0.2 - 1.4	12	1.4	0.7 - 2.4	31	3.6	2.5 - 5.1
Halfmijl	624	36	5.8	4.1 - 7.9	29	4.6	3.1 - 6.6	0	0.0	0.0 - 0.6	50	8.0	6.0 - 10.4
Herperduin	120	1	0.8	0.0 - 4.6	3	2.5	0.5 - 7.1	2	1.7	0.2 - 5.9	2	1.7	0.2 - 5.9
Kremboong	791	2	0.3	0.0 - 0.9	2	0.3	0.0 - 0.9	2	0.3	0.0 - 0.9	24	3.0	2.0 - 4.5
Kroondomein	1,397	62	4.4	3.4 - 5.7	21	1.5	0.9 - 2.3	7	0.5	0.2 - 1.0	155	11.1	9.5 - 12.9
Maashorst	639	11	1.7	0.9 - 3.1	7	1.1	0.4 - 2.2	5	0.8	0.3 - 1.8	8	1.3	0.5 - 2.5
Pettermerduin	130	1	0.8	0.0 - 4.2	15	11.5	6.6 - 18.3	1	0.8	0.0 - 4.2	4	3.1	0.8 - 7.7
Planken Wambuis	767	114	14.9	12.4 - 17.6	12	1.6	0.8 - 2.7	7	0.9	0.4 - 1.9	69	9.0	7.1 - 11.2
Rheebruggen	863	7	0.8	0.3 - 1.7	20	2.3	1.4 - 3.6	1	0.1	0.0 - 0.6	30	3.5	2.4 - 4.9
Schoorlse Duinen	26	0	0.0	0.0 - 13.2	1	3.8	0.1 - 19.6	0	0.0	0.0 - 13.2	0	0.0	0.0 - 13.2
Stameren	1,129	9	0.8	0.4 - 1.5	45	4.0	2.9 - 5.3	20	1.8	1.1 - 2.7	70	6.2	4.9 - 7.8
Valenberg	763	42	5.5	4.0 - 7.4	21	2.8	1.7 - 4.2	10	1.3	0.6 - 2.4	43	5.6	4.1 - 7.5
Vijverhof	1,039	4	0.4	0.1 - 1.0	21	2.0	1.3 - 3.1	10	1.0	0.5 - 1.8	121	11.6	9.8 - 13.8
Vledderhof	398	2	0.5	0.1 - 1.8	2	0.5	0.1 - 1.8	15	3.8	2.1 - 6.1	11	2.8	1.4 - 4.9
Zwanemeerbos	665	21	3.2	2.0 - 4.8	11	1.7	0.8 - 2.9	28	4.2	2.8 - 6.0	21	3.2	2.0 - 4.8
Total	1,3967	456	3.3	3.0 - 3.6	291	2.1	1.9 - 2.3	152	0.9	0.9 - 1.3	806	5.8	5.4 - 6.2

Table S10. Results of GLMMs of region and location as a random effect on prevalence of tick symbionts.

		Likelihood ratio test			
		LogLik	Chisq	AIC	Nagelkerke pseudo-R2
A. phagocytophilum	~ 1 + (1|location)	-73.072		150.1446	
	~ region + (1|location)	-72.27	0.4485	152.5408	0.0809828
B. afzelii	~ 1 + (1|location)	-68.845		141.69	
	~ region + (1|location)	-67.226	0.1981	142.4519	0.156805
N. mikurensis	~ 1 + (1|location)	-82.721		169.4416	
	~ region + (1|location)	-78.753	0.01892	165.5065	0.341455
B. garinii + B. valaisiana	~ 1 + (1|location)	-58.077		120.1533	
	~ region + (1|location)	-57.779	0.7424	123.5576	0.0309367
M. mitochondrii	~ 1 + (1|location)	-96.086		196.1718	
	~ region + (1|location)	-92.534	0.02868	193.0688	0.311927
S. ixodetis	~ 1 + (1|location)	-102.41		208.8153	
	~ region + (1|location)	-101.45	0.3824	210.8926	0.0962415
R. helvetica	~ 1 + (1|location)	-107.434		218.8687	
	~ region + (1|location)	-93.729	0.000001116	195.4576	0.763718
Rickettsiella spp.	~ 1 + (1|location)	-121.19		246.382	
	~ region + (1|location)	-114.22	0.0009388	236.4402	0.519909

Table S11. A list of all haplotypes (combinations of infections with symbionts in ticks) used in relative occurrence analyses for Figure 6a and Figure 6b, respectively.

Horizontally-transmitted pathogens:

type	*A. phagocytophilum*	*N. mikurensis*	*B. afzelii*	*B. garinii + B. valaisiana*
h00	0	0	0	0
h01	0	0	0	1
h02	0	0	1	0
h04	0	1	0	0
h05	0	1	0	1
h06	0	1	1	0
h08	1	0	0	0
h10	1	0	1	0
h12	1	1	0	0

Vertically-transmitted symbionts:

type	*M. mitochondrii*	*Rickettsiella* spp.	*S. ixodetis*	*R. helvetica*
h00	0	0	0	0
h01	0	0	0	1
h02	0	0	1	0
h03	0	0	1	1
h04	0	1	0	0
h05	0	1	0	1
h06	0	1	1	0
h07	0	1	1	1
h08	1	0	0	0
h09	1	0	0	1
h10	1	0	1	0
h11	1	0	1	1
h12	1	1	0	0
h13	1	1	0	1
h14	1	1	1	0
h15	1	1	1	1

Table S12. *Borrelia afzelii* Fst analysis results.

Centre	North	West	Genotype count	Percentage
n = 954	n = 154	n = 281		
1	0	0	20	33%
0	1	0	6	10%
0	0	1	13	21%
0	1	1	2	3%
1	0	1	5	8%
1	1	0	7	11%
1	1	1	8	13%
			Total 61	99%

0: Genotype absent. 1: Genotype present. n: Number of *I. ricinus* ticks.

Population pairwise Fst estimates						
Region	Isolate	Haplotype	Locations	North	West	Centre
North	154	23	7			
West	281	28	6	**0.03073**		
Centre	954	40	22	**0.03840**	**0.07780**	

Fst estimate is printed in bold numerals when its p values is less than 0.005.

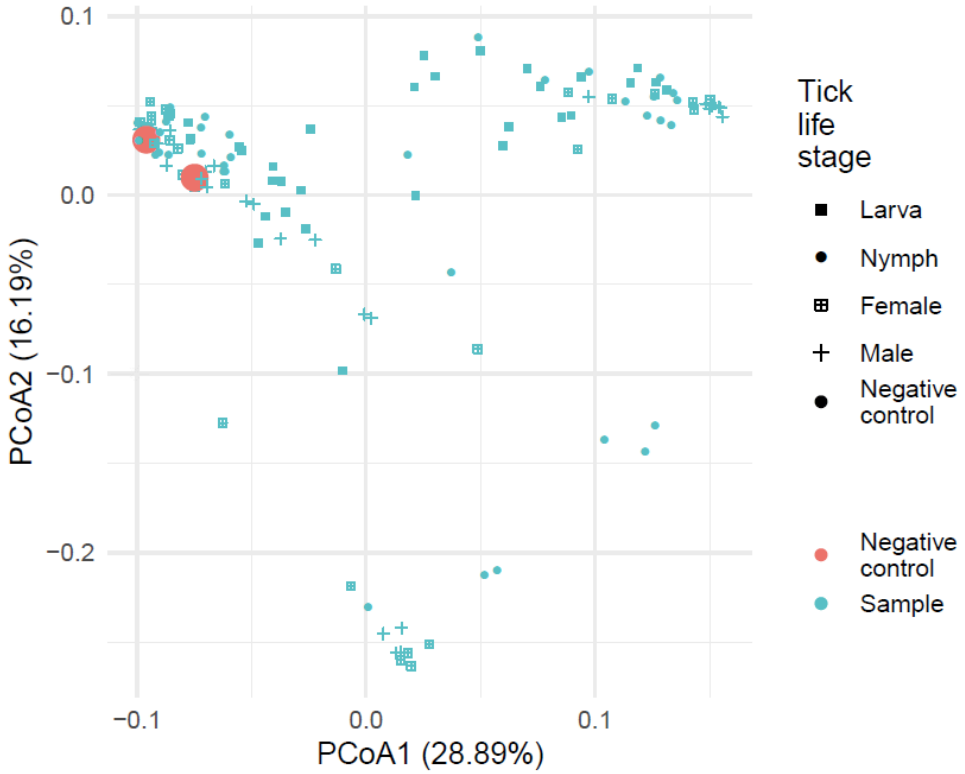


Figure S1. Ordination of community composition with uncorrected abundances.

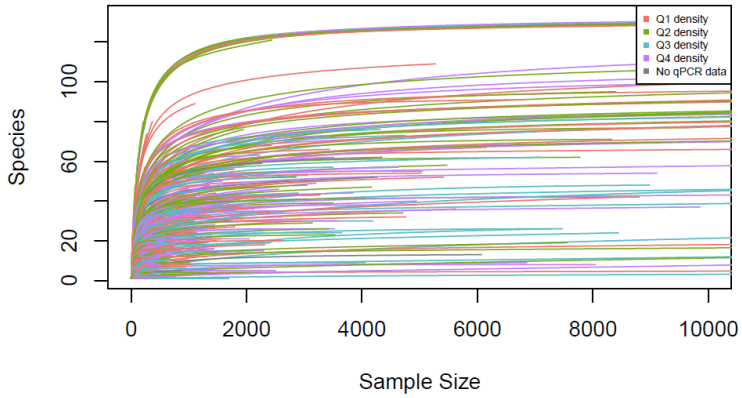


Figure S2. Rarefaction curve of samples coloured by quartile of total bacterial load.

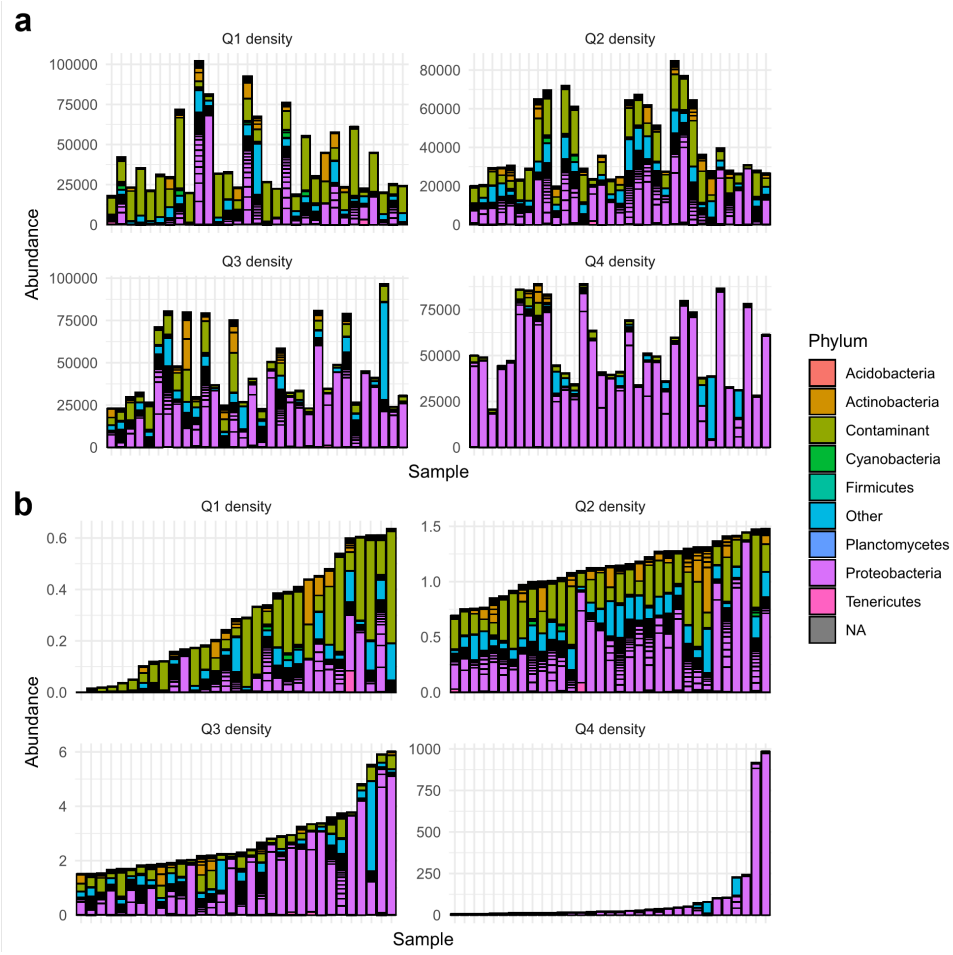


Figure S3. Abundances of bacterial phyla separated by quartiles of total bacterial load (16S rRNA content in

ng/µL)), unscaled (a) or scaled by total bacterial load (b).

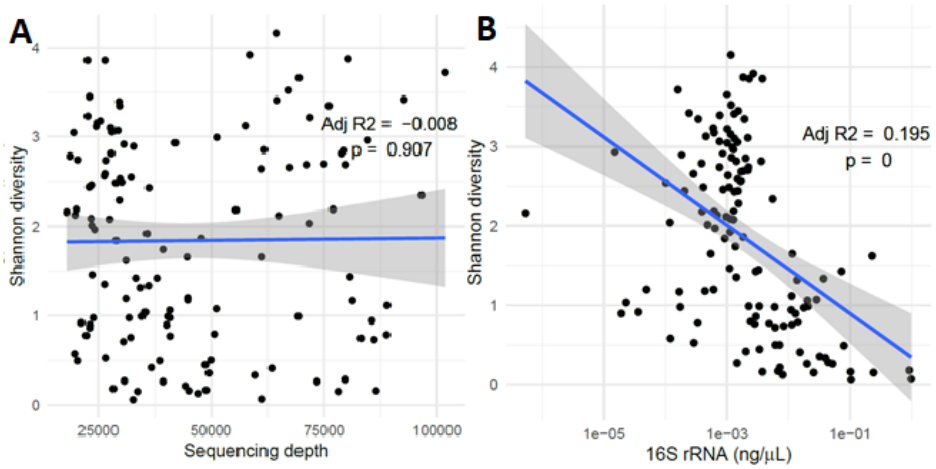


Figure S4. Correlation of Shannon diversity of the unrarefied data to (a) sequencing depth and (b) total bacterial load.

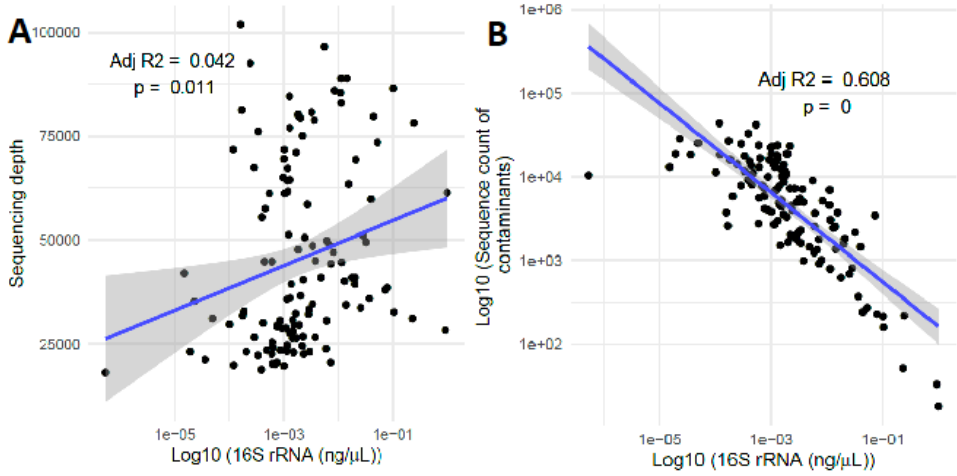
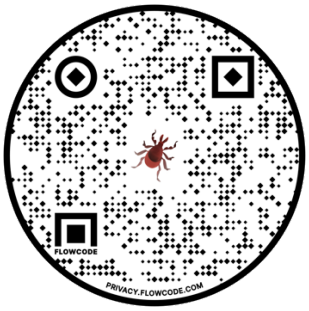


Figure S5. Correlation of total bacterial load to (a) sequencing depth and (b) uncorrected count of contaminant sequences.



Please click here (for the online version) or scan this QR code to listen to an immersive soundscape of a forest in Duin en Kruidberg, the Netherlands recorded by Vitalij Kuzkin. The Duin en Kruidberg forest is one of the study sites where I have collected samples for this research. Listen with headphones for the best experience. The full album is available on: ticksandmicrobes.bandcamp.com

Chapter 3

Co-infection analysis identifies new associations between horizontally- and vertically-transmitted *Ixodes ricinus* microorganisms

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To be submitted in a modified form

Abstract

Microbial communities can affect disease risk by interfering with the transmission or maintenance of pathogens in blood-feeding arthropods. Here, we investigated whether bacterial communities vary between *Ixodes ricinus* nymphs, which are (un)infected with horizontally-transmitted pathogens. Ticks from eight forest sites were tested for the presence of *Borrelia burgdorferi* sensu lato, *Babesia* spp., *Anaplasma phagocytophilum*, and *Neoehrlichia mikurensis* by qPCR, pooled, and their microbiomes were determined by 16S rRNA amplicon sequencing. Tick bacterial communities clustered poorly by pathogen infection status but better by geographic regions. As a second approach, we compared expected and observed co-infections of tick microorganisms. For that, we analysed almost 14,000 nymphs, which were tested for the presence of horizontally-transmitted symbionts (human pathogens) *B. burgdorferi* s.l., *A. phagocytophilum* and *N. mikurensis*, and the vertically-transmitted tick symbionts *Rickettsia helvetica*, *Rickettsiella* spp., *Spiroplasma ixodetis,* and *Midichloria mitochondrii.* We detected positive associations of *M. mitochondrii* and *Rickettsiella* spp. with *B. burgdorferi* s.l. and a significant negative association of *R. helvetica* with *S. ixodetis*. Although a few specific associations do not affect much the entire microbiome composition, they can still be relevant for tick-borne pathogen dynamics. Based on our results, we propose that vertically-transmitted symbionts alter the propensity of ticks to acquire or maintain horizontally-acquired pathogens. The underlying mechanisms for some of these remarkable interactions are discussed and merit further investigation.

Keywords: Lyme borreliosis, *Ixodes ricinus* microbiome, Co-infections, Anaplasmosis, Neoehrlichiosis, Transmission dynamics, Heritable symbionts

Introduction

There is increasing evidence that members of the arthropod microbiome can decrease vector-borne disease risk (Cirimotich et al., 2011; Weiss & Aksoy, 2011). Microbes can modulate the vectorial competence of an arthropod by decreasing its susceptibility to pathogens as well as reducing pathogen transmission, which is necessary for pathogen maintenance in enzootic cycles. For instance, an *Enterobacter* bacterium isolated from wild *Anopheles gambiae* mosquitoes in Zambia makes 99% of this mosquito population resistant to infection with the malaria parasite by interfering with its development prior to the invasion of the midgut epithelium (Cirimotich et al., 2011). Similar properties have been assigned to a microsporidian symbiont in another member of the *An. gambiae* complex (Herren et al., 2020).

Given that ticks transmit a plethora of pathogens causing disease in humans and domestic animals (de la Fuente et al., 2008; Rochlin & Toledo, 2020), an improved understanding of interactions between tick microorganisms is necessary for the development of novel strategies controlling tick-borne diseases. However, it is not well understood whether, let alone how, members of tick microbiomes can impede or facilitate the transmission of pathogens. The microbiome of ticks consists of viruses, bacteria, and protozoa. Their relationships with ticks are often context-dependent and range from obligate mutualistic, through commensal to exclusively parasitic (Noda et al., 1997; Sacchi et al., 2004; Scoles, 2004). In principle, all microorganisms (including tick-borne pathogens) relying for their survival on ticks are called symbionts; here, however, microorganisms that have been shown to cause disease in humans and animals will be called pathogens.

In ticks, bacteria have been the most commonly studied organisms in the microbiome-disease risk context. To date, studies have shown the mutually exclusive occurrence of pathogenic and non-pathogenic *Rickettsia* species in *Amblyomma maculatum* and *Dermacentor andersoni* ticks (Burgdorfer et al., 1981; Noden et al., 2020; Paddock et al., 2015), as well as a negative association between *R. bellii* and *Anaplasma marginale* in *D. andersoni* ticks (Gall et al., 2016). Regarding facilitative interactions, *Candidatus* Midichloria mitochondrii (hereafter *M. mitochondrii*) has been shown to be a successful colonizing partner of pathogenic *R. parkeri,* in *A. maculatum* ticks (Budachetri et al., 2018).

In *I. ricinus*, the most studied symbionts include obligate intracellular bacteria belonging to the genera of *Rickettsia*, *Midichloria*, *Rickettsiella*, and *Spiroplasma*, which are predominantly transmitted vertically from a female tick to eggs (Duron et al., 2017). Nevertheless, little is known about interactions between the tick microbiome members and pathogens such as *Borrelia*, *Anaplasma*, and *Neoehrlichia*. The latter three are transmitted horizontally, meaning ticks are born without and only acquire them as larvae or nymphs while feeding on vertebrates (Breuner et al., 2018; van Duijvendijk et al., 2015). Their colonization of ticks might be potentially inhibited by vertically-transmitted symbionts, which are already present in larvae. As has been observed in other arthropods, several mechanisms are potentially involved. These include direct killing, competition, and enhancement of host immune responses (Pickard et al., 2017; Yordanova et al., 2018). Before describing these mechanisms, we should first identify associations between bacterial species. For this purpose, co-infections analyses are frequently applied. However, until now, studies investigating co-infections of vertically- and horizontally-transmitted symbionts have reported contradictory results. For example, in *Ixodes* ticks, observed associations between *Rickettsia* and

Borrelia have ranged from negative to positive (Moutailler et al., 2016; Raulf et al., 2018; Steiner et al., 2014).

At the same time, horizontally-transmitted pathogens may engage in strategies to promote their replication in the presence of other microbes. Some studies have shown that to increase their colonization success, *Anaplasma* and *Borrelia* may alter the tick gut microbiome by adjusting a tick antibacterial protein expression (Abraham et al., 2017; Narasimhan et al., 2017). However, existing evidence is limited to laboratory experiments, and it remains to be investigated how it relates to the influence of microbiome alteration on infection prevalence under natural conditions.

Based on the above findings, we formulated two hypotheses. The first hypothesis is that ticks infected with pathogenic bacteria such as *B. burgdorferi* sensu lato, possess different bacterial microbiomes than uninfected ticks or ticks infected with pathogenic *Babesia*, *A. phagocytophilum*, and *N. mikurensis*. To test this, we performed 16S rRNA amplicon sequencing on pools of *I. ricinus* nymphs infected with one of these pathogens and compared them with each other. *Ixodes ricinus* is a three-host generalist tick, feeding once per life stage (larva, nymph, and adult) on various vertebrates, which may be amplification hosts of human pathogens (Hofmeester et al., 2016). Questing nymphs feed only once as larva, and therefore, the nymphal microbiome predominantly consists of vertically-transmitted symbionts and potentially of a horizontally acquired pathogen(s). The second hypothesis is that vertically-transmitted members of the tick microbiome either facilitate or impede the maintenance of pathogens. To test this, we used data obtained in the previous studies (Chapter 2; Takumi et al., 2019), where almost 14,000 individual questing nymphs were screened for the presence of the horizontally-transmitted pathogens *B. burgdorferi* s.l., *A. phagocytophilum*, and *N. mikurensis* as well as the predominantly vertically-transmitted symbionts *M. mitochondrii*, *R. helvetica*, *Rickettsiella* spp. and *S. ixodetis* (Duron et al., 2017). Subsequently, we compared the expected and observed co-infections. Previous studies have already shown that positive and negative associations between horizontally-transmitted symbionts can be explained best by a mutual or different amplifying vertebrate host (Coipan et al., 2013b). Therefore, this study aims to examine whether vertically- and horizontally-transmitted symbionts are positively or negatively associated. In a very recent study, we found regional differences in the infection prevalence of vertically-transmitted *M. mitochondrii, R. helvetica*, *Rickettsiella* spp. and the horizontally-transmitted *N. mikurensis,* (Chapter 2). These associations were also analysed at the regional level to prevent negative associations caused by regional differences in symbiont communities.

Materials and methods

Study sites

Questing *I. ricinus* were collected in eight forest sites in the Netherlands (Figure 1). The study sites were selected based on pre-existing knowledge on *Borrelia* genospecies prevalence, the density of ticks, vegetation profile, and vertebrate composition obtained in a previous study (Takumi et al., 2019). The full names of the sites and their coordinates are provided in Table S1.

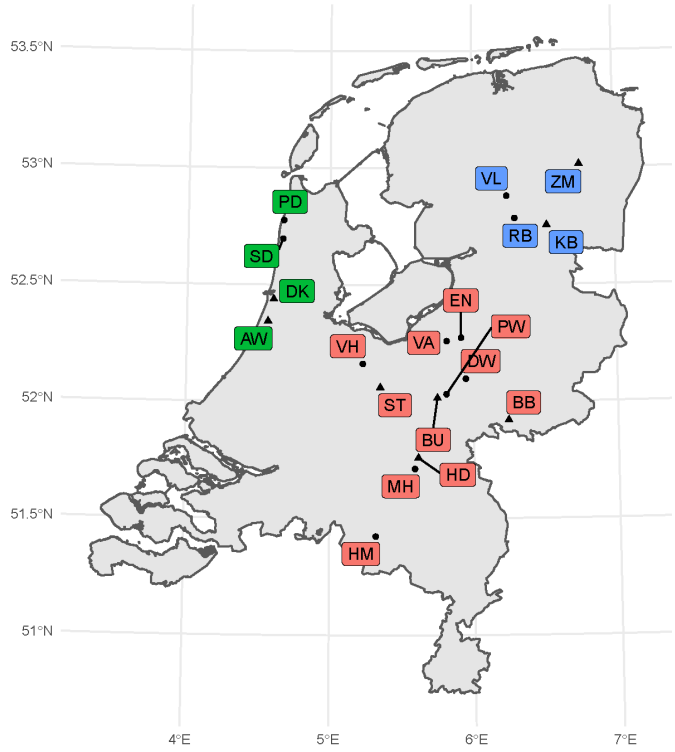

Figure 1. Sampling sites of *I. ricinus* in the Netherlands. Pooled nymphs from eight forest sites (triangles) were used for a 16S rRNA amplicon sequencing analysis. Individual nymphs from these and 13 (points) other forest sites were tested by qPCR for the presence of tick symbionts. A box marks the sampling site by two letters and the region by colour; red for central, blue for northern, and green for the western region. More details on the locations are provided in Table S1.

Sample collection, DNA extraction, and pathogen detection

A total of 7,874 questing *I. ricinus* nymphs was collected in 2016 and 2017. All ticks were washed three times in 70% ethanol, and DNA of individual nymphs was extracted with ammonium hydroxide as described previously (Wielinga *et al.* 2006). The nymphs were analysed individually for the presence of tick-borne pathogens with different (multiplex) real-time PCRs, based on various target genes, as described: *B. burgdorferi* s.l. (Heylen et al., 2013a), *B. miyamotoi* (Hovius et al., 2013), *N. mikurensis* (Jahfari et al., 2012), *A. phagocytophilum* (Courtney et al., 2004), and *Babesia* spp., which has been designed to detect *B. divergens*, *B. venatorum* (formerly called EU1-3), *B. capreoli*, and *B. odocoilei* (Øines et al., 2012). A detailed description of a qPCR protocol and a list of targeted genes are provided in the Chapter 2. Samples positive for *B. burgdorferi* s.l., were subjected to conventional PCR followed by sequencing for genotype identification (Heylen et al., 2013b). After testing for tick-borne pathogens, ticks were pooled and re-extracted using the Qiagen DNeasy Blood & Tissue Kit according to the manufacturer's protocol (Qiagen, Venlo, The Netherlands). Pools consisted of DNA from 5 nymphs, which were positive for one and the

same pathogen and negative for all the others. Sample metadata is provided in Table S2. Pools of nymphs, negative for all pathogens, were also included. For each location, a minimum of six and a maximum of 49 pools were processed (Table S3). Two negative controls in the form of negative extraction and one company internal control were included. DNA concentration in all samples was measured with the Qubit dsDNA HS kit (Thermo Fisher Scientific, Ochten, The Netherlands).

16S rRNA quantification and absolute bacterial density

Absolute bacterial density in all samples was quantified, and proportions were multiplied by a load to convert relative into absolute abundances. Quantification of total bacterial DNA load was determined by 16S rRNA qPCR as previously described (Bogaert et al., 2011). The use of absolute bacterial density is a cost-effective and scalable solution for datasets of this size since quantification methods through flow cytometry are not compatible with the sampling technique. Samples were normalized, and the normalized abundances were scaled by the 16S rRNA qPCR loads (ng/µl) of each sample.

Microbial profiling and taxonomic clustering

Illumina MiSeq V3-V4 region of 16S rRNA amplicon libraries were generated and sequenced by BaseClear (Leiden, the Netherlands). In short, barcoded amplicons from the V3-V4 region of 16S rRNA genes were generated using a 2-step PCR. Between 10 and 25 ng genomic (g) DNA was used as a template for the first PCR with a total volume of 50 ul using the 341F (5'-CCTACGGGNGGCWGCAG-3') and the 785R (5'-GACTACHVGGGTATCTAATCC-3') primers appended with Illumina adaptor sequences. Control PCR reactions were performed alongside each separate amplification without the addition of a template. PCR products were purified, and the size of the PCR products was checked on the Fragment analyzer (Advanced Analytical) and quantified by fluorometric analysis. Purified PCR products were used for the 2nd PCR in combination with sample-specific barcoded primers (Nextera XT index kit, Illumina). Subsequently, PCR products were purified, checked on a Fragment analyzer (Advanced Analytical), and quantified, followed by multiplexing, clustering, and sequencing on an Illumina MiSeq with the paired-end (2x) 300 bp protocol and indexing. The sequencing run was analysed with the Illumina CASAVA pipeline (v1.8.3) with demultiplexing based on sample-specific barcodes. The raw sequencing data produced was processed by removing the sequence reads of too low quality (only "passing filter" reads were selected) and discarding reads containing adaptor sequences or PhiX control with an in-house filtering protocol. A quality assessment on the remaining reads was performed using the FASTQC quality control tool version 0.10.0. Sequenced reads were imported to CLC Genomics Workbench 10.0.1 supplemented with CLC Microbial Genomics Module 3.6.1 (www.clcbio.com). Overlapping pairs of raw reads were merged into single longer reads and trimmed with a quality score limit of 0.05 and 2 ambiguous nucleotides. At this stage, primer sequences were trimmed. Subsequently, reads were fixed-length trimmed (~400 bp). To identify operational taxonomic units (OTUs), reads were clustered using the reference databases SILVA 16S version 128 with 97% identity as the clustering criterion; chimeras were removed with a built-in tool in CLC Genomic Workbench.

Microbiome analyses

All analyses were carried out in R 3.6.3 (R Core Team 2020). We used the R package *vegan* (version 2.5-6) for ordinations and fitting of environmental vectors or factors onto ordinations (Oksanen *et al.* 2007). All principal coordinate analyses were carried out using Bray-Curtis dissimilarities,

and *envfit* was used to correlate metadata to community variation. Results were visualized with ggplot2 (version 3.3.2; Wickham et al., 2020). Only taxa with over 30% prevalence in the data set were included in correlations of taxon abundances to community variation; remaining counts were binned into a taxon labelled 'Other'. Data was permuted 1000 times to test for significance of *envfit* p-values and taxon correlations to community variation; *envfit* correlations were multiple-testing corrected with the Benjamini-Hochberg method for the number of metadata, taxon correlation p-values for the number of taxa above 30% prevalence. Positions of taxon vectors, 16S rRNA vectors, and factors were scaled independently for visualization purposes and are therefore not directly comparable in effect size.

Additionally, we carried out PERMANOVA (Anderson, 2014) with the *adonis* function from R package *vegan* to assess whether pathogen presence, location, or the interaction between these two variables significantly affected community variation. We tested for multivariate dispersion through the *betadisper* function. We classified locations as western, northern, or central regions. Since the variables were significantly overdispersed, we also evaluated cluster quality as the silhouette score with host and region as cluster labels. The silhouette score, bounded by -1 and 1, is a cluster quality index that considers both cohesion (intra-cluster dissimilarities) and separation (nearest-cluster dissimilarities).

Co-infection analyses

To investigate co-infections of tick symbionts, we utilized the qPCR data on the infection prevalences of tick symbionts generated in previous studies (Chapter 2; Hofmeester et al., 2017b; Takumi et al., 2019). Briefly, questing nymphs of *I. ricinus* were collected from 19 forest sites in the Netherlands in 2013 and 2014 (Figure 1). The forest sites were organized in three geographical regions: northern, central, and western (Table S1). A total of 13,968 individual nymphs of *I. ricinus* were screened for *S. ixodetis*, *R. helvetica*, *Rickettsiella* spp., *M. mitochondrii*, *A. phagocytophilum*, *N. mikurensis*, and *B. burgdorferi* s.l. *B. miyamatoi* and *Babesia* spp. data were not included in further investigations due to their low prevalences, which did not allow for performing statistical analyses with confidence.

Co-infections of tick symbionts were investigated by calculating the odds ratio and their 95% confidence intervals (CI). The Fisher's exact test assessed the significance of co-infections, and the results were considered statistically significant at $p < 0.05$. The calculations were performed in R version 3.6.3 (R Core Team 2020) and RStudio (RStudio Team 2019) with the *fisher.test* function from R package *stats.* Subsequently, to correct for multiple testing, the Benjamini-Hochberg procedure was applied (Benjamini & Hochberg, 1995). Odds ratio results were visualized in Cytoscape 3.7.0 (Shannon et al., 2003). The differences between expected and observed numbers of infections were calculated with the exact binomial test. The differences between prevalences of symbionts in populations of nymphs infected and uninfected with pathogens were calculated with z-test.

Results

Microbial profiling and taxonomic clustering

A total of 165 out of 166 processed samples successfully generated 6,590,988 raw reads on Illumina MiSeq flow cell. One sample was excluded because it had an extremely low DNA load, which resulted in no amplification. A total of 4,454,814 reads were assigned taxonomy. A total of 184,682 unique reads were clustered into 8,818 OTUs (Table S4). However, 1,966 OTUs, which accounted for 0.7 % of all reads, could not be assigned to any known taxa and were discarded from further analyses. The top ten of the most abundant genera included *Borrelia*, *Midichloria*, *Neoehrlichia*, *Methylobacterium*, *Mycobacterium*, *Pseudomonas*, *Rickettsia*, *Rickettsiella*, *Sphingomonas*, and *Wolbachia* (Figure S1). Despite being the most abundant taxa in some samples, *Wolbachia* was not considered an *I. ricinus* tick symbiont, and we did not include it in the further qPCR analysis. Its origin in our tick samples is most likely due to the presence of endoparasitoid *Ixodiphagus hookeri* eggs (Plantard et al., 2012; Tijsse-Klasen et al., 2011).

Two types of negative controls were carried out: two extractions without template DNA and one only containing sequencing reagents. Principal coordinates analysis (PCoA) showed that all three negative controls were highly similar, indicating that any errors introduced through reagent contamination were preserved (Figure S2). The ordination also demonstrated that tick samples did not neatly cluster away from the negative controls. The lack of distinction from the negative controls can likely be attributed to low bacterial abundance in some samples, and a high abundance of *Pseudomonas*, a known contaminant that was highly abundant in the negative controls.

The PCoA demonstrated that samples were mostly separated by the geographic region of tick collection (Figure 2). The PCoA did not convincingly show that the variation of the microbial community was different between pathogen-specific pools, neither between the pools with or without pathogens. Also, the effect was not observed within two specific locations analysed separately (ST and BU; Figure S3). Therefore, we tested with PERMANOVA whether communities were significantly different for ticks screened positively for different pathogens. Both pathogen identity and geographic region contributed significantly to the model, with respective R^2 of 0.22 and 0.08 and $p = 0.001$ for both, in addition to a significant interaction term between the two ($R^2 = 0.03$, $p = 0.012$). However, these variables were significantly overdispersed ($p < 0.001$), indicating that the p-values may be overinflated. Therefore, we quantified intra- versus inter-cluster distance for these variables with the silhouette score, a measure for evaluating cluster quality. Indeed, the silhouette scores of -0.04 and 0.07 for pathogen and region respectively, suggest that region, while scoring poorly, can better capture sample clustering compared to pathogen identity. The low silhouette scores combined with the low R^2 of the PERMANOVA led us to conclude that neither region nor pathogen identity can explain bacterial community variation well.

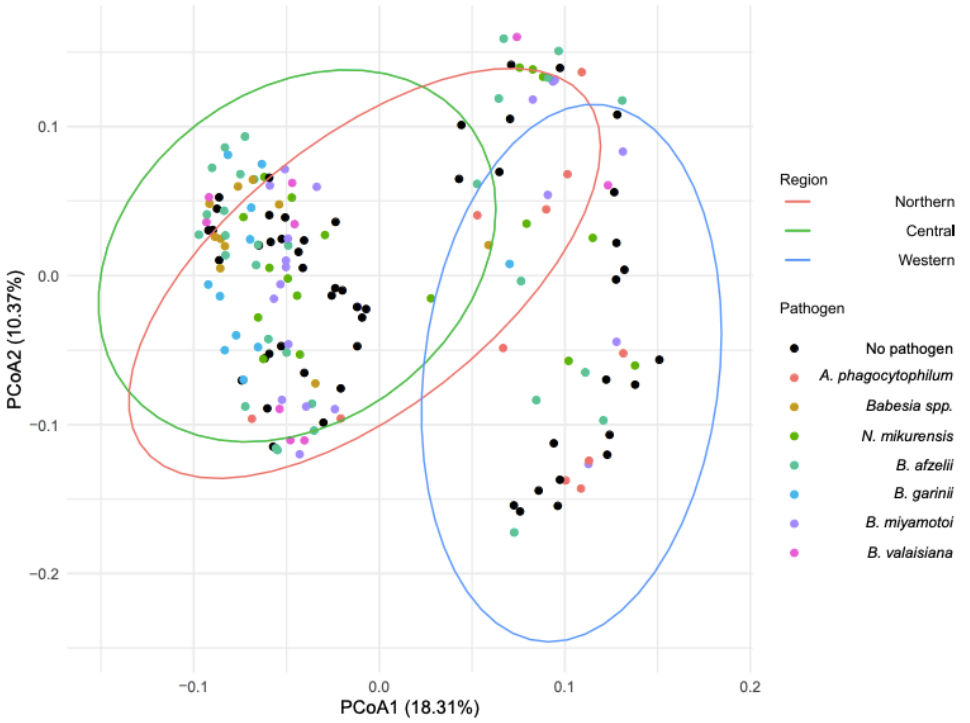

Figure 2. Principal Coordinate Analysis of microbial abundance data scaled by 16S rRNA load. Ticks were screened for the presence of specific pathogens, pooled, and then sequenced. Samples, each containing a pool of five screened ticks, are coloured by a pathogen. The data ellipses contain 90% of samples linked to a specific region in the Netherlands.

Co-infection of vertically- and horizontally-transmitted symbionts

Data on the prevalences of tick symbionts in questing nymphs, generated in previous studies, was analysed for co-infections (Chapter 2; Hofmeester et al., 2017; Takumi et al., 2019). Shortly, a total of 13,967 questing nymphs from 19 forest sites were tested with the qPCR for the presence of *S. ixodetis, R. helvetica, Rickettsiella* spp., *M. mitochondrii, A. phagocytophilum, N. mikurensis,* and *B. burgdorferi* s.l. All symbionts were present in all locations; however, infection rates varied strongly among the bacterium species and locations. Prevalences of the tick symbionts per location and region are provided in Table S5, as well as in Chapter 2 and Takumi et al. (2019).

A total of 1,732 (12.4%) of tested questing nymphs was free of the seven studied symbionts, and 12,225 (87.6%) nymphs were infected with one or more. The exact binomial test showed that infections with at least one, two, three, and four tick symbionts were significantly less frequent than expected (always $p < 0.001$), and infections with at least five, six, and seven symbionts occurred as often as expected (Table S6). A singular infection was detected in 4,791, a double infection in 4,992, a triple infection in 2,177, and a quadruple infection in 307 nymphs. A total of 27 nymphs were infected with five, one with six, and none of the ticks with seven symbionts showing that co-infections of symbionts are not randomly distributed in the tick populations.

Although all possible combinations of two symbionts were found in nymphs, some combinations of symbionts occurred significantly more or less often than expected (Figures 3 and 4). After applying the Benjamini-Hochberg multiple testing correction, we found 14 significant associations in an overall dataset. In the western, northern, and central regions, we detected four, seven, and 13 significant associations, respectively. The most striking observations were a strong negative association of *R. helvetica* with *S. ixodetis*, and positive associations of *M. mitochondrii* and *Rickettsiella* spp. with *B. burgdorferi* s.l. All expected and observed co-infections and odds ratio results for all samples as well as per region are provided in Table 2, Table 7, and Tables S8. Also, populations of *Borrelia*-infected ticks had a higher prevalence of *M. mitochondrii* and *Rickettsiella* spp. than uninfected ticks (Table S9). The only region where we did not observe this association was the western region, which had a very low prevalence of *B. burgdorferi* s.l. in two locations (AW and DK, 1.5% and 5.5%, respectively; Table S5).

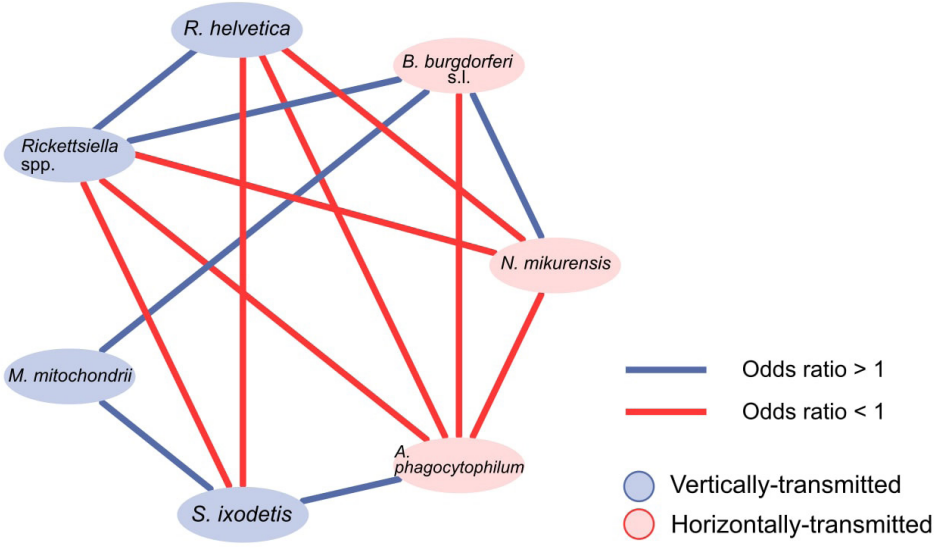

Figure 3. Network visualization of odds ratio results across all locations. Only co-infections with a p-value below 0.05 are shown. The edge colour corresponds to the estimated odds ratio. Odds ratio smaller than 1 indicates that ticks are co-infected by these species significantly more frequently than expected by chance contrast, the odds ratio larger than 1 indicates that ticks are significantly more frequently co-infected. The colours of the nodes indicate whether species are primarily transmitted horizontally or vertically. Details on odds ratio results are provided in Table 1 and Table S7.

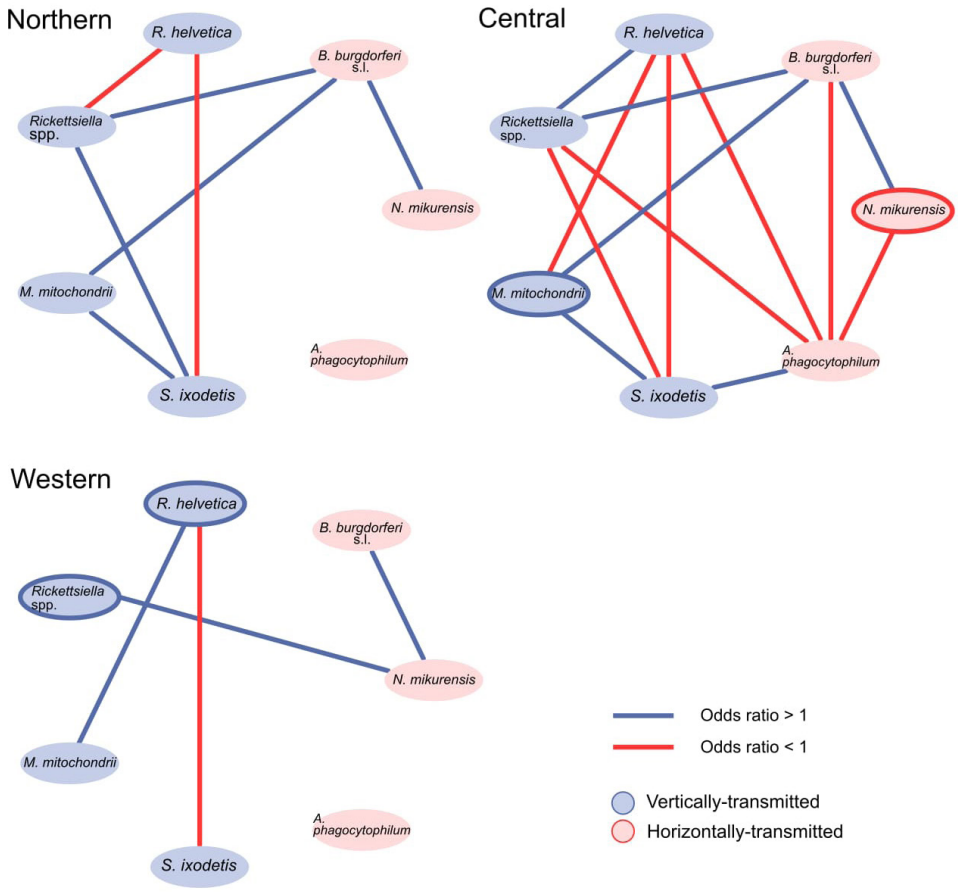

Figure 4. Network visualization of odds ratio results across regions. Only co-infections with a p-value below 0.05 are shown. The edge colour corresponds to the estimated odds ratio. Odds ratio smaller than 1 indicates that ticks are co-infected by these species significantly more frequently than expected by chance. In contrast, odds ratio larger than 1 indicates that ticks are significantly more frequently co-infected. The colours of the nodes indicate whether species are primarily transmitted horizontally or vertically. In the previous study (Chapter 2), the prevalence of some symbionts has been observed to vary significantly between the regions. *Midichloria mitochondrii*, *N. mikurensis* were the most prevalent in the central region, while *R. helvetica*, *Rickettsiella* spp. in the western region what is indicated with a thicker border around the nodes. Details on odds ratio results are provided in Table 1 and Table S8.

Table 1. Details on the odds ratio results only for significantly associated pairs of tick symbionts. The remaining results are provided in Table S7 and TableS8.

Region	Symbiont 1	Symbiont 2	Observed coinfection (n)	Expected coinfection (n)	*p* value	Lower 95% CI	Upper 95% CI	Estimate odds ratio
All	*R. helvetica*	*S. ixodetis*	95	350	5.62E-75	0.16	0.25	0.20
All	*R. helvetica*	*Rickettsiella* spp.	1,308	981	1.05E-57	2.01	2.46	2.22
All	*N. mikurensis*	*B. burgdorferi* s.l.	192	62	2.07E-49	3.64	5.22	4.37
All	*M. mitochondrii*	*S. ixodetis*	1,772	1,557	2.20E-23	1.46	1.76	1.60
All	*Rickettsiella* spp.	*S. ixodetis*	1,062	1,241	2.23E-15	0.64	0.77	0.70
All	*M. mitochondrii*	*B. burgdorferi* s.l.	763	670	5.95E-10	1.33	1.76	1.53
All	*A. phagocytophilum*	*B. burgdorferi* s.l.	9	35	1.32E-07	0.11	0.45	0.24
All	*A. phagocytophilum*	*N. mikurensis*	5	26	3.61E-07	0.06	0.42	0.18
All	*B. burgdorferi* s.l.	*Rickettsiella* spp.	607	534	3.91E-06	1.18	1.53	1.34
All	*N. mikurensis*	*R. helvetica*	74	114	1.79E-05	0.46	0.77	0.60
All	*N. mikurensis*	*Rickettsiella* spp.	351	402	0.000212	0.66	0.88	0.76
All	*A. phagocytophilum*	*Rickettsiella* spp.	199	227	0.007605	0.64	0.93	0.77
All	*A. phagocytophilum*	*S. ixodetis*	102	81	0.012647	1.06	1.69	1.34
All	*A. phagocytophilum*	*R. helvetica*	47	64	0.016579	0.50	0.94	0.69
Northern	*R. helvetica*	*S. ixodetis*	2	46	1.65E-26	0.00	0.09	0.02
Northern	*Rickettsiella* spp.	*S. ixodetis*	285	202	1.38E-06	1.56	3.17	2.20
Northern	*M. mitochondrii*	*S. ixodetis*	221	147	7.53E-06	1.35	2.25	1.74
Northern	*M. mitochondrii*	*B. burgdorferi* s.l.	161	110	0.001445	1.18	2.09	1.56
Northern	*B. burgdorferi* s.l.	*Rickettsiella* spp.	207	151	0.002942	1.18	2.53	1.71
Northern	*N. mikurensis*	*B. burgdorferi* s.l.	16	6	0.003622	1.28	4.27	2.41
Northern	*R. helvetica*	*Rickettsiella* spp.	347	297	0.004694	0.57	0.91	0.72
Central	*N. mikurensis*	*B. burgdorferi* s.l.	166	53	3.54E-44	3.76	5.59	4.59
Central	*M. mitochondrii*	*S. ixodetis*	1,476	1,310	4.47E-18	1.43	1.77	1.59
Central	*M. mitochondrii*	*B. burgdorferi* s.l.	551	475	6.83E-10	1.41	2.00	1.68
Central	*A. phagocytophilum*	*B. burgdorferi* s.l.	4	28	2.15E-08	0.04	0.34	0.13
Central	*A. phagocytophilum*	*N. mikurensis*	4	25	1.39E-07	0.04	0.37	0.14
Central	*Rickettsiella* spp.	*S. ixodetis*	689	771	2.47E-05	0.72	0.89	0.80
Central	*B. burgdorferi* s.l.	*Rickettsiella* spp.	331	279	5.69E-05	1.17	1.59	1.37
Central	*A. phagocytophilum*	*R. helvetica*	6	20	0.000259	0.10	0.61	0.27
Central	*R. helvetica*	*S. ixodetis*	91	119	0.002783	0.55	0.89	0.71
Central	*R. helvetica*	*Rickettsiella* spp.	240	207	0.003741	1.09	1.55	1.30
Central	*M. mitochondrii*	*R. helvetica*	321	352	0.004694	0.65	0.93	0.78
Central	*A. phagocytophilum*	*Rickettsiella* spp.	113	132	0.03314	0.61	0.98	0.78
Central	*A. phagocytophilum*	*S. ixodetis*	93	76	0.02916	1.02	1.69	1.32
Western	*R. helvetica*	*S. ixodetis*	2	59	1.61E-35	0.00	0.05	0.01
Western	*N. mikurensis*	*B. burgdorferi* s.l.	10	2	3.75E-06	3.49	18.93	8.44
Western	*M. mitochondrii*	*R. helvetica*	643	617	0.007262	1.08	1.63	1.32
Western	*N. mikurensis*	*Rickettsiella* spp.	34	27	0.005321	1.40	47.61	5.52

Discussion

This study detected negative and positive associations between tick symbionts, which are utilizing different transmission routes. Populations of *Borrelia*-infected ticks had a higher prevalence of *M. mitochondrii* and *Rickettsia* spp. than ticks without *B. burgdorferi* s.l.. Nevertheless, we did not observe differences in the bacterial microbiome of ticks infected with distinct horizontally-acquired pathogens such as *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. miyamotoi*, *Babesia*, *A. phagocytophilum*, and *N. mikurensis* (Figure 2).

Microbiome of *I. ricinus*

Differences in the bacterial microbiome of ticks with and without pathogens were not detected, even when eliminating the geographical effect (Figure S3). As the presence of a pathogen in nymphs indicates a specific vertebrate host on which it fed as a larva, our findings imply that the vertebrate host does not, or hardly, affect the bacterial community of ticks. Our results are in line with previous findings by Hawlena et al. (2013), but they are in contrast with a study by Swei & Kwan (2017), who found differences in the microbiome of *I. pacificus* ticks feeding on lizards and mice. We are not aware of any of the nymphs studied here fed on lizards, which are relatively uncommon tick hosts in our study areas (Hofmeester et al., 2016). Nevertheless, we cannot rule out a potential influence of reptiles, cold-blooded vertebrates, on the tick microbiome. Reptiles have less stringent requirements for regulating blood biochemical properties, and their blood may have vastly different osmotic pressure and pH compared to mammals and birds (Dessauer 1970). While we could not investigate such effects in this study, this could lead to the absence of host effects compared to the work by Swei & Kwan (2017).

A possible explanation for our finding could be that ticks are genetically equipped to resist opportunistic bacteria, such as host skin commensals. Hayes et al. (2020) has discovered that *I. scapularis* horizontally acquired an antimicrobial toxin gene from bacteria. The gene encodes lytic cell wall-degrading enzymes delivered to the host bite site via saliva and responsible for selectively killing vertebrate's skin-associated bacteria. Interestingly, the enzymes had no intrinsic lytic activity against *B. burgdorferi* (Hayes et al., 2020)(Hayes et al., 2020). Therefore, pathogens might still affect the tick gut microbiome to facilitate their colonization, as previously observed in laboratory experiments (Abraham et al., 2017; Narasimhan et al., 2017). In future work, investigating the individual organs deriving from field-collected ticks may extend our understanding of microbiome-pathogen interactions.

Clustering of tick bacterial communities was best explained by geographical region, which is in accordance with previous studies (Chapter 2; Carpi et al., 2011; Clay et al., 2008; Van Treuren et al., 2015). In our earlier study (Chapter 2), we found a regional distribution of tick symbionts with *Rickettsia* abundance being the most responsible for the clustering of bacterial communities in ticks.

Associations of horizontally- with vertically-transmitted symbionts

Nymphs infected with *M. mitochondrii* and *Rickettsiella* spp. in the Netherlands' northern and central regions were more often infected with *B. burgdorferi* s.l. than expected by chance alone. A third symbiont involvement could explain the lack of significant associations with *B. burgdorferi* s.l. in the western region. It was possibly *R. helvetica*, which was highly prevalent in this region

and was negatively associated with *M. mitochondrii* and *Rickettsiella* spp. (not significant; Figure 4). Alternatively, we may have tested an insufficient number of samples in two out of four locations (Table S5), which probably did not allow for the detection of the actual *B. burgdorferi* s.l. prevalence. Further analyses showed that a positive association with *M. mitochondrii* was found between both *B. afzelii* and *B. garinii* separately (data not shown). These findings imply that ticks infected with *M. mitochondrii* contribute more to the transmission cycle and the acarological risk of Lyme borreliosis than ticks lacking *M. mitochondrii*. Interestingly, *M. mitochondrii* has been shown to be a successful colonizing partner of pathogenic *R. parkeri*, in *A. maculatum* ticks (Budachetri et al., 2018). One possible explanation for the positive association between *B. burgdorferi* s.l. and *M. mitochondrii* is that female nymphs are more often infected with *M. mitochondrii* than male nymphs (Lo et al., 2006). Generally, immature female stages are larger and take a lengthier and larger bloodmeal than male nymphs (Dusbabek, 1996), which might enhance their probability of becoming infected with *B. burgdorferi* s.l.

Little is known about how *M. mitochondrii* and *Rickettsiella* spp. affect ticks' fitness, but both species were proposed to increase tick survival by supplying essential nutrients, enhancing reproductive fitness, or protecting ticks against fungal pathogens (Bonnet et al., 2017; Thu et al., 2019). Therefore, the observed positive associations of these two symbionts with *B. burgdorferi* s.l. could have an indirect character and arise from symbionts providing fitness benefits to ticks, increasing their chances to find vertebrate hosts and acquire horizontally-transmitted pathogens.

Nevertheless, we did not observe any strong association of *M. mitochondrii* and *Rickettsiella* spp. with *N. mikurensis*, which is often coinfected with *B. burgdorferi* s.l., particularly *B. afzelii* (Andersson et al., 2013; Andersson et al., 2014; Chapter 6). We speculate that ticks cannot sustain multiple infections as it comes with fitness costs and that existing infection(s) in ticks impede the colonization of consecutive microorganisms. Given the prevalences of symbionts in our dataset, we have observed much fewer coinfections than expected (Table S6).

The other relationships between horizontally- and vertically-transmitted symbionts were either absent or determined regionally, possibly by significantly varying prevalences of such microorganisms as *R. helvetica*, *N. mikurensis*, and *Rickettsiella* spp. (Chapter 2).

Associations of vertically-transmitted symbionts

We detected a significant negative association between *R. helvetica* and *S. ixodetis* in all three regions. Interestingly, in a study on the microbiome of *I. ricinus*, Aivelo et al. (2019a) have also detected a negative association between *Spiroplasma* and *Rickettsia* sp. It is possible that ticks cannot simultaneously maintain these two symbiont species by vertical transmission, which may affect their distribution in tick populations on different geographical scales. In a previous study, we showed that the prevalence of *R. helvetica* varies significantly between the regions (Chapter 2); nevertheless, whether a negative association with *S. ixodetis* causes it remains unknown. The phenomenon of interference between two vertically-transmitted symbionts in hard ticks has been described before (Burgdorfer et al., 1981; Macaluso et al., 2002; Paddock et al., 2015; Sakai et al., 2014). One *Rickettsia* species' primary infection has been suggested to block (by an unknown mechanism) the transovarial transmission of a second *Rickettsia* species. However, to our knowledge, this so-called rickettsial interference has never been related to any other tick symbiont genus. It would also mean that ticks occasionally acquire *S. ixodetis* from a vertebrate host, which has been suggested, but not undisputedly proven (Binetruy et al., 2019; Chapter 4).

The mutual exclusion may also indicate that both symbionts play a similar role in ticks and that a double infection comes with fitness costs without providing sufficient increases in fitness. For instance, in *Acyrthosiphon pisum*, both *Serratia symbiotica* and *Hamiltonella defensa* provide resistance to parasitoids. Although a double infection provided increased resistance in the laboratory, it was rarely observed under natural conditions (Oliver et al., 2006).

Alternatively, *R. helvetica* and *S. ixodetis* might serve a sex-specific function as male-associated symbionts perish in females and vice versa. Many previous studies observed higher *Rickettsia* prevalence and abundance in females suggesting that male ticks might lose this symbiont through each subsequent moult (Noda et al., 1997; Stańczak et al., 2009; Thapa et al., 2019; Zhu et al., 1992; Zolnik et al., 2016). However, the role of *S. ixodetis* in *I. ricinus* ticks is not known, and since it has been rarely studied in adult ticks, the difference in prevalence (if present) in females and males has never been shown. Interestingly, *S. ixodetis* of *Ixodes* ticks is phylogenetically closely related to male-killing species from the *S. ixodetis* clade, but to-date no evidence of male-killing in ticks has been found (Binetruy et al., 2019; Sanada-Morimura et al., 2013; Tinsley & Majerus, 2006; Van Oosten et al., 2018).

In contrast to the negative association described above, we observed a significant positive association between *R. helvetica* and *Rickettsiella* spp. and between *M. mitochondrii* and *S. ixodetis* in two of the three regions. The associations imply that these pairs of symbionts either serve complementary functions in tick hosts and/or exhibit different tissue tropism in ticks. Different host tissues may constitute distinct microhabitats and be nutritionally favourable, immunotolerant, and easy to colonize for some but not for other microorganisms (Goto et al., 2006).

The association between *R. helvetica* and *M. mitochondrii* varied between the regions from negative to positive (Figure 4). This differential relationship is most likely an effect of regional variation in *R. helvetica* and *M. mitochondrii* prevalences, which we have observed in a previous study (Chapter 2), and not necessarily a direct interaction between the symbionts. Similarly, we think that the observed relationship of *Rickettsiella* spp. with *S. ixodetis* is also an effect of variation in the prevalence of these symbionts between the regions.

Associations of horizontally-transmitted symbionts

Different species of horizontally-transmitted symbionts can be acquired from the same or distinct species of vertebrates (Burri et al., 2014; Coipan et al., 2018; Hanincova et al., 2003a and 2003b; Jahfari et al., 2014). Consequently, it is often reflected in their co-infection patterns in nymphs that fed only once as larvae. For instance, we observed a strong positive association between *B. burgdorferi* s.l. and *N. mikurensis* in all three regions. This result was to be expected given that both *N. mikurensis* and *B. afzelii* (the most prevalent genospecies of *B. burgdorferi* s.l. in *I. ricinus* in the Netherlands) are amplified by rodents, which are often simultaneously infected with both microorganisms (Andersson et al., 2013; Andersson et al., 2014; Chapter 6).

Anaplasma phagocytophilum may be acquired by *I. ricinus* from various vertebrate species and the most commonly from deer (Jahfari et al., 2014). Therefore, the significant negative association of *A. phagocytophilum* with *B. burgdorferi* s.l. and *N. mikurensis* pathogens, as observed in the central region, implies that nymphs that fed on deer had no chance to acquire rodent-borne pathogens and vice versa. However, this association was not observed in the two remaining regions, probably due to the low prevalences of studied pathogens.

Conclusions

With the next-generation sequencing approach used in this study, we did not observe any effect of bacterial pathogens on microbial communities of ticks. The ticks' microbiome predominantly consisted of vertically-transmitted symbionts, which seem to be unaffected by a blood meal and a vertebrate host, on which nymphs fed as larvae. Nevertheless, our more conventional approach showed many significant associations between microorganisms. Although a few specific associations do not affect much the entire microbiome composition, they can still be relevant for tick-borne pathogen dynamics. We showed that *B. burgdorferi* s.l. is more likely to be acquired/maintained by ticks infected with vertically-transmitted symbionts such as *M. mitochondrii* and *Rickettsiella* spp. compared to uninfected ticks.

Additionally, we observed that tick symbionts are heterogeneously distributed across the Dutch tick population. Our current methods revealed novel patterns of associations, and although the mechanisms underlying this distribution are unknown, they are of importance for understanding the role of vertically-transmitted symbionts in the control of tick-borne diseases. For future studies, we recommend investigating the dynamics of symbionts through the ontogeny of ticks and applying bacteria quantifications methods, and identifying microbiomes of ticks during different life events such as moulting, questing, and feeding.

Acknowledgements

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Table S1. Details on studied forest sites. Locations in bold indicate where ticks were sampled for the 16s rRNA amplicon sequencing.

Location	Location full name	Region	Coordinates	Habitat	Undergrowth vegetation	Larva/ 1200m2	Nymph/ 1200m2	Adult/ 1200m2	Number of vertebrate species
AW	**Amsterdamse Waterleiding Duinen**	Western	52°20'36" N 4°33'58"E	Mixed forest	Calamagrostis epigejos	4,186	726	63	12
BB	**Bergherbos**	Central	51°55'14"N 6°14'30"E	Mixed forest	Deschampsia flexuosa	468	869	32	14
BU	**Buunderkamp**	Central	52°00'56" N 5°44'50"E	Scots pine forest	Vaccinium myrtillus	2,591	2,200	76	11
DK	**Duin en Kruidberg**	Western	52°26'16" N 4°36'18"E	Mixed forest	Calamagrostis epigejos	5,274	870	53	6
DW	Deelerwoud	Central	52°05'51" N 5°56'42"E	Scots pine forest	Vaccinium myrtillus	3,178	1,647	161	10
EN	Kroondomein	Central	52°16'25" N 5°54'49"E	Scots pine forest	Vaccinium myrtillus	897	1,379	64	13
HD	**Herperduin**	Central	51°45'33" N 5°36'53"E	Mixed forest	Molinia caerulea	72	120	4	15
HM	Halfmijl	Central	51°25'23" N 5°19'09"E	Mixed forest	Molinia caerulea	1,241	1,531	55	10
KB	**Kremboong**	Northern	52°45'13" N 6°31'16"E	Pedunculate oak forest	Dryopteris dilatata	2,239	794	28	12
MH	Maashorst	Central	51°42'44" N 5°35'24"E	Mixed forest	Deschampsia flexuosa	2,994	640	17	16
PD	Pettemerduin	Western	52°46'33" N 4°40'19"E	Pedunculate oak forest	Polypodium vulgare	0	130	19	10
PW	Planken Wambuis	Central	52°01'54" N 5°48'36"E	Scots pine forest	Vaccinium myrtillus	1,566	760	20	9
RB	Rheebruggen	Northern	52°46'60" N 6°17'44"E	Pedunculate oak forest	Dryopteris dilatata	6,203	859	49	13
SD	Schoorlse Duinen	Western	52°41'47" N 4°40'01"E	Mixed forest	Molinia caerulea	0	22	3	6
ST	**Stameren**	Central	52°03'38" N 5°21'01"E	Mixed forest	Deschampsia flexuosa	1,474	1,134	94	14
VA	Valenberg	Central	52°15'33" N 5°48'47"E	Scots pine forest	Vaccinium myrtillus	2,724	765	92	15
VH	Vijverhof	Central	52°09'43" N 5°13'43"E	Mixed forest	Deschampsia flexuosa	555	1,039	23	11
VL	Vledderhof	Northern	52°52'46" N 6°14'25"E	Pedunculate oak forest	Dryopteris dilatata	1,648	403	31	16
ZM	**Zwanemeerbos**	Northern	53°00'46" N 6°45'19"E	Pedunculate oak forest	Pteridium aquilinum	1,225	680	108	11

Table S2. A list of samples for the 16s rRNA amplicon sequencing and their 16S rRNA load quantified with qPCR. Each sample represent a pool of five questing nymphs.

Sample ID	Location	Pathogen	16S load ng/µl
100STmi	Stameren	*B. miyamotoi*	0.00264713
101STmi	Stameren	*B. miyamotoi*	0.00077266
102STmi	Stameren	*B. miyamotoi*	0.01574441
103STmi	Stameren	*B. miyamotoi*	0.00073893
104STmi	Stameren	*B. miyamotoi*	0.00092463
105STan	Stameren	*A. phagocytophilum*	0.00096711
106STan	Stameren	*A. phagocytophilum*	0.00134633
107STan	Stameren	*A. phagocytophilum*	0.00099743
108STne	Stameren	*N. mikurensis*	0.00168706
109STne	Stameren	*N. mikurensis*	0.00112112
10BUaf	Buunderkamp	*B. afzelii*	0.00024941
110STne	Stameren	*N. mikurensis*	0.00054022
111STne	Stameren	*N. mikurensis*	0.00118861
112STne	Stameren	*N. mikurensis*	0.00052403
113STne	Stameren	*N. mikurensis*	0.00112258
114STne	Stameren	*N. mikurensis*	0.0015093
115STne	Stameren	*N. mikurensis*	0.00062303
116STba	Stameren	*Babesia* spp.	0.00046569
117STba	Stameren	*Babesia* spp.	0.00046798
118STba	Stameren	*Babesia* spp.	0.00050088
119STba	Stameren	*Babesia* spp.	0.00113542
11BUaf	Buunderkamp	*B. afzelii*	0.00029644
120STba	Stameren	*Babesia* spp.	0.00254924
121STnc	Stameren	none	0.00235956
122STnc	Stameren	none	0.00027289
123STnc	Stameren	none	0.01914898
124STnc	Stameren	none	0.00040606
126STnc	Stameren	none	0.00034974
127STnc	Stameren	none	0.00044973
128STnc	Stameren	none	0.00049285
129HDaf	Herperduin	*B. afzelii*	0.00107039
12BUaf	Buunderkamp	*B. afzelii*	0.00298938
130HDaf	Herperduin	*B. afzelii*	0.00032689
131HDaf	Herperduin	*B. afzelii*	0.00041163
132HDaf	Herperduin	*B. afzelii*	0.00078152
133HDnc	Herperduin	none	0.00015876
134HDnc	Herperduin	none	0.00040993
135HDnc	Herperduin	none	0.0002732
136HDnc	Herperduin	none	0.00116692
137HDnc	Herperduin	none	0.00015102
138HDnc	Herperduin	none	0.00080893
139ZMaf	Zwanemeerbos	*B. afzelii*	0.00060624
13BUaf	Buunderkamp	*B. afzelii*	0.00037845
140ZMaf	Zwanemeerbos	*B. afzelii*	0.00150114
141ZMaf	Zwanemeerbos	*B. afzelii*	0.00345234
142ZMaf	Zwanemeerbos	*B. afzelii*	0.00073531
143ZMnc	Zwanemeerbos	none	0.00154407
144ZMnc	Zwanemeerbos	none	0.00084664
145ZMnc	Zwanemeerbos	none	0.00093396
146ZMnc	Zwanemeerbos	none	0.00076696
147ZMnc	Zwanemeerbos	none	0.00159167
148ZMnc	Zwanemeerbos	none	0.00044053
149DKnc	Duin en Kruidberg	none	0.00102886
14BUaf	Buunderkamp	*B. afzelii*	0.00024531
150DKnc	Duin en Kruidberg	none	0.00000267
151DKnc	Duin en Kruidberg	none	0.00111607
152DKnc	Duin en Kruidberg	none	0.00306067

table continues

Sample ID	Location	Pathogen	16S load ng/µl
153DKnc	Duin en Kruidberg	none	0.0032493
154DKnc	Duin en Kruidberg	none	0.00630361
155KBnc	Kremboong	none	0.00479684
156KBnc	Kremboong	none	0.00025908
157KBnc	Kremboong	none	0.00026148
158KBnc	Kremboong	none	0.00029634
159KBnc	Kremboong	none	0.00090243
15BUaf	Buunderkamp	*B. afzelii*	0.00029761
160KBnc	Kremboong	none	0.00037435
161BBnc	Bergherbos	none	0.00285158
162BBnc	Bergherbos	none	0.00085063
163BBnc	Bergherbos	none	0.00025921
164BBnc	Bergherbos	none	0.00015563
165BBnc	Bergherbos	none	0.05635995
166BBnc	Bergherbos	none	0.00015727
167B1	Negative extraction	-	NA
168B2	Negative extraction	-	NA
16BUaf	Buunderkamp	*B. afzelii*	0.00045766
17BUva	Buunderkamp	*B. valaisiana*	0.00039691
18BUva	Buunderkamp	*B. valaisiana*	0.00041469
19BUva	Buunderkamp	*B. valaisiana*	0.00027429
1BUga	Buunderkamp	*B. garinii*	0.00072976
20BUva	Buunderkamp	*B. valaisiana*	0.00396507
21BUva	Buunderkamp	*B. valaisiana*	0.00220184
22BUva	Buunderkamp	*B. valaisiana*	0.00077228
23BUmi	Buunderkamp	*B. miyamotoi*	0.00102838
24BUmi	Buunderkamp	*B. miyamotoi*	0.00017529
25BUmi	Buunderkamp	*B. miyamotoi*	0.00043895
26BUmi	Buunderkamp	*B. miyamotoi*	0.00081642
27BUmi	Buunderkamp	*B. miyamotoi*	0.00110372
28BUmi	Buunderkamp	*B. miyamotoi*	0.0006388
29BUne	Buunderkamp	*N. mikurensis*	0.00051835
2BUga	Buunderkamp	*B. garinii*	0.00023844
30BUne	Buunderkamp	*N. mikurensis*	0.00203323
31BUne	Buunderkamp	*N. mikurensis*	0.00106421
32BUne	Buunderkamp	*N. mikurensis*	0.00188789
33BUne	Buunderkamp	*N. mikurensis*	0.00027913
34BUne	Buunderkamp	*N. mikurensis*	0.00326142
35BUne	Buunderkamp	*N. mikurensis*	0.00045945
36BUne	Buunderkamp	*N. mikurensis*	0.00035626
37BUba	Buunderkamp	*Babesia* spp.	0.00035756
38BUba	Buunderkamp	*Babesia* spp.	0.00027867
39BUba	Buunderkamp	*Babesia* spp.	0.00031108
3BUga	Buunderkamp	*B. garinii*	0.00073395
40BUba	Buunderkamp	*Babesia* spp.	0.00038875
41BUba	Buunderkamp	*Babesia* spp.	0.00047219
42BUnc	Buunderkamp	none	0.00034304
43BUnc	Buunderkamp	none	0.00026822
44BUnc	Buunderkamp	none	0.00073109
45BUnc	Buunderkamp	none	0.00129434
46BUnc	Buunderkamp	none	0.00029463
47BUnc	Buunderkamp	none	0.00011878
48BUnc	Buunderkamp	none	0.00114369
49BUnc	Buunderkamp	none	0.00024895
4BUga	Buunderkamp	*B. garinii*	0.00050148
50AWaf	Amsterdamse Waterleiding Duinen	*B. afzelii*	0.00024949
51AWaf	Amsterdamse Waterleiding Duinen	*B. afzelii*	0.00563775
52AWaf	Amsterdamse Waterleiding Duinen	*B. afzelii*	0.00184653
53AWaf	Amsterdamse Waterleiding Duinen	*B. afzelii*	0.00240908
54AWaf	Amsterdamse Waterleiding Duinen	*B. afzelii*	0.00223412

table continues

Sample ID	Location	Pathogen	16S load ng/µl
55AWmi	Amsterdamse Waterleiding Duinen	*B. miyamotoi*	0.00075361
56AWmi	Amsterdamse Waterleiding Duinen	*B. miyamotoi*	0.00189653
57AWmi	Amsterdamse Waterleiding Duinen	*B. miyamotoi*	0.00330012
58AWmi	Amsterdamse Waterleiding Duinen	*B. miyamotoi*	0.00301268
59AWmi	Amsterdamse Waterleiding Duinen	*B. miyamotoi*	0.00017116
5BUga	Buunderkamp	*B. garinii*	0.0015035
60AWmi	Amsterdamse Waterleiding Duinen	*B. miyamotoi*	0.00332721
61AWmi	Amsterdamse Waterleiding Duinen	*B. miyamotoi*	0.00028158
62AWan	Amsterdamse Waterleiding Duinen	*A. phagocytophilum*	0.00477491
63AWan	Amsterdamse Waterleiding Duinen	*A. phagocytophilum*	0.00188117
64AWan	Amsterdamse Waterleiding Duinen	*A. phagocytophilum*	0.00371986
65AWan	Amsterdamse Waterleiding Duinen	*A. phagocytophilum*	0.00345027
66AWan	Amsterdamse Waterleiding Duinen	*A. phagocytophilum*	0.00030503
67AWan	Amsterdamse Waterleiding Duinen	*A. phagocytophilum*	0.00441214
68AWan	Amsterdamse Waterleiding Duinen	*A. phagocytophilum*	0.00233486
69AWan	Amsterdamse Waterleiding Duinen	*A. phagocytophilum*	0.00090982
6BUga	Buunderkamp	*B. garinii*	0.00140746
70AWne	Amsterdamse Waterleiding Duinen	*N. mikurensis*	0.00176518
71AWne	Amsterdamse Waterleiding Duinen	*N. mikurensis*	0.00592402
73AWnc	Amsterdamse Waterleiding Duinen	none	0.00180493
74AWnc	Amsterdamse Waterleiding Duinen	none	0.00192082
75AWnc	Amsterdamse Waterleiding Duinen	none	0.0075869
76AWnc	Amsterdamse Waterleiding Duinen	none	0.00695538
77AWnc	Amsterdamse Waterleiding Duinen	none	0.00323977
78AWnc	Amsterdamse Waterleiding Duinen	none	0.00133858
79AWnc	Amsterdamse Waterleiding Duinen	none	0.00304138
7BUaf	Buunderkamp	*B. afzelii*	0.00064126
80AWnc	Amsterdamse Waterleiding Duinen	none	0.00172883
81STaf	Stameren	*B. afzelii*	0.00717789
82STaf	Stameren	*B. afzelii*	0.00035027
83STaf	Stameren	*B. afzelii*	0.00036192
84STaf	Stameren	*B. afzelii*	0.00021668
85STaf	Stameren	*B. afzelii*	0.00051808
86STaf	Stameren	*B. afzelii*	0.00333313
87STaf	Stameren	*B. afzelii*	0.00033199
88STaf	Stameren	*B. afzelii*	0.00065231
89STga	Stameren	*B. garinii*	0.00103957
8BUaf	Buunderkamp	*B. afzelii*	0.00115538
90STga	Stameren	*B. garinii*	0.00031645
91STga	Stameren	*B. garinii*	0.00129511
92STga	Stameren	*B. garinii*	0.00106957
93STga	Stameren	*B. garinii*	0.00072671
94STva	Stameren	*B. valaisiana*	0.00041334
95STva	Stameren	*B. valaisiana*	0.00031884
96STva	Stameren	*B. valaisiana*	0.003784
97STmi	Stameren	*B. miyamotoi*	0.00183014
98STmi	Stameren	*B. miyamotoi*	0.00029777
99STmi	Stameren	*B. miyamotoi*	0.00055323
9BUaf	Buunderkamp	*B. afzelii*	0.00063887
BC_blanco	BaseClear internal control	-	NA
72AWne	Amsterdamse Waterleiding Duinen	*N. mikurensis*	0.08884378

Table S3. A scheme of samples for the 16s rRNA amplicon sequencing. Numbers in the table correspond to a number of pools of five questing nymphs, which were shown by qPCR to be infected with the same pathogen.

	A. phagocyto-philum	*B. afzelii*	*B. garinii*	*B. miyamotoi*	*B. valaisiana*	*Babesia* spp.	*N. mikurensis*	none	Total
Buunderkamp	-	10	6	6	6	5	8	8	49
Stameren	3	8	5	8	3	5	8	8*	48
Amsterdamse Waterleiding Duinen	8	5	-	7	-	-	3	8	31
Herperduin	-	4	-	-	-	-	-	6	10
Zwanemeerbos	-	4	-	-	-	-	-	6	10
Bergherbos	-	-	-	-	-	-	-	6	6
Duin en Kruid-berg	-	-	-	-	-	-	-	6	6
Kremboong	-	-	-	-	-	-	-	6	6
Total	11	31	11	21	9	10	19	53	168

* one sample from this category was unsuccessfully sequenced

Table S4. This table shows an OTU table and a number of reads obtained for each OTU for all samples, including blanks. The data was generated with the 16s rRNA amplicon sequencing. Because of its size, the table is available upon request.

Table S5. Prevalence of veritcally- and horizontally-transmitted symbionts per location and region. Formula of 95% exact binomial confidence interval was adapted from: Armitage et al. (2001). The table continues on the following pages.

Location	Region		*A. phagocytophilum*			*B. burgdorferi* s.l.			*N. mikurensis*		
		N tested	N positive	%	CI 95%	N positive	%	CI 95%	N positive	%	CI 95%
Amsterdamse Waterleiding Duinen	Western	678	39	5.8	4.1 - 7.8	10	1.5	0.7 - 2.7	1	0.1	0.0 - 0.8
Bergherbos	Central	742	6	0.8	0.3 - 1.8	92	12.4	10.1 - 15.0	30	4.0	2.7 - 5.7
Buunderkamp	Central	688	0	0.0	0.0 - 0.5	91	13.2	10.8 - 16.0	92	13.4	10.9 - 16.1
Deelerwoud	Central	1,650	68	4.1	3.2 - 5.2	34	2.1	1.4 - 2.9	44	2.7	1.9 - 3.6
Duin en Kruidberg	Western	858	31	3.6	2.5 - 5.1	47	5.5	4.1 - 7.2	31	3.6	2.5 - 5.1
Halfmijl	Central	624	36	5.8	4.1 - 7.9	60	9.6	7.4 - 12.2	50	8.0	6.0 - 10.4
Herperduin	Central	120	1	0.8	0.0 - 4.6	12	10.0	5.3 - 16.8	2	1.7	0.2 - 5.9
Kremboong	Northern	791	2	0.3	0.0 - 0.9	35	4.4	3.1 - 6.1	24	3.0	2.0 - 4.5
Enkhout	Central	1,397	62	4.4	3.4 - 5.7	84	6.0	4.8 - 7.4	155	11.1	9.5 - 12.9
Maashorst	Central	639	11	1.7	0.9 - 3.1	26	4.1	2.7 - 5.9	8	1.3	0.5 - 2.5
Pettermerduin	Western	130	1	0.8	0.0 - 4.2	23	17.7	11.6 - 25.4	4	3.1	0.8 - 7.7
Planken Wambuis	Central	767	114	14.9	12.4 - 17.6	47	6.1	4.5 - 8.1	69	9.0	7.1 - 11.2
Rheebruggen	Northern	863	7	0.8	0.3 - 1.7	45	5.2	3.8 - 6.9	30	3.5	2.4 - 4.9
Schoorlse Duinen	Western	26	0	0.0	0.0 - 13.2	2	7.7	0.9 - 25.1	0	0.0	0.0 - 13.2
Stameren	Central	1,129	9	0.8	0.4 - 1.5	105	9.3	7.7 - 11.1	70	6.2	4.9 - 7.8
Valenberg	Central	763	42	5.5	4.0 - 7.4	56	7.3	5.6 - 9.4	43	5.6	4.1 - 7.5
Vijverhof	Central	1,039	4	0.4	0.1 - 1.0	138	13.3	11.3 - 15.5	121	11.6	9.8 - 13.8
Vledderhof	Northern	398	2	0.5	0.1 - 1.8	33	8.3	5.8 - 11.4	11	2.8	1.4 - 4.9
Zwanemeerbos	Northern	665	21	3.2	2.0 - 4.8	131	19.7	16.7 - 22.9	21	3.2	2.0 - 4.8
Total		1,3967	456	3.3	3.0 - 3.6	1,071	7.7	7.2 - 8.1	806	5.8	5.4 - 6.2

table continues

Location	Region		*M. mitochondrii*			*Rickettsiella* spp.			*S. ixodetis*			*R. helvetica*		
		N tested	N positive	%	CI 95%	N positive	%	CI 95%	N positive	%	CI 95%	N positive	%	CI 95%
Amsterdamse Waterleiding Duinen	Western	678	486	71.7	68.1 - 75.0	379	55.9	52.1 - 59.7	42	6.2	4.5 - 8.3	327	48.2	44.4 - 52.1
Bergherbos	Central	742	432	58.2	54.6 - 61.8	351	47.3	43.7 - 51.0	46	6.2	4.6 - 8.2	60	8.1	6.2 - 10.3
Buunderkamp	Central	688	449	65.3	61.6 - 68.8	221	32.1	28.6 - 35.8	36	5.2	3.7 - 7.2	30	4.4	3.0 - 6.2
Deelerwoud	Central	1,650	1,065	64.5	62.2 - 66.9	459	27.8	25.7 - 30.0	349	21.2	19.2 - 23.2	36	2.2	1.5 - 3.0
Duin en Kruidberg	Western	858	527	61.4	58.1 - 64.7	827	96.4	94.9 - 97.5	44	5.1	3.8 - 6.8	515	60.0	56.7 - 63.3
Halfmijl	Central	624	382	61.2	57.3 - 65.1	186	29.8	26.2 - 33.6	101	16.2	13.4 - 19.3	23	3.7	2.4 - 5.5
Herperduin	Central	120	89	74.2	65.4 - 81.7	45	37.5	28.8 - 46.8	8	6.7	2.9 - 12.7	4	3.3	0.9 - 8.3
Kremboong	Northern	791	371	46.9	43.4 - 50.4	684	86.5	83.9 - 88.8	110	13.9	11.6 - 16.5	57	7.2	5.5 - 9.2
Enkhout	Central	1397	943	67.5	65.0 - 70.0	429	30.7	28.3 - 33.2	599	42.9	40.3 - 45.5	37	2.6	1.9 - 3.6
Maashorst	Central	639	371	58.1	54.1 - 61.9	175	27.4	24.0 - 31.0	54	8.5	6.4 - 10.9	71	11.1	8.8 - 13.8
Pettermerduin	Western	130	84	64.6	55.8 - 72.8	59	45.4	36.6 - 54.3	13	10.0	5.4 - 16.5	83	63.8	55.0 - 72.1
Planken Wambuis	Central	767	507	66.1	62.6 - 69.4	286	37.3	33.9 - 40.8	122	15.9	13.4 - 18.7	22	2.9	1.8 - 4.3
Rheebruggen	Northern	863	560	64.9	61.6 - 68.1	474	54.9	51.5 - 58.3	77	8.9	7.1 - 11.0	237	27.5	24.5 - 30.6
Schoorlse Duinen	Western	26	20	76.9	56.4 - 91.0	19	73.1	52.2 - 88.4	7	26.9	11.6 - 47.8	9	34.6	17.2 - 55.7
Stameren	Central	1,129	730	64.7	61.8 - 67.5	475	42.1	39.2 - 45.0	197	17.4	15.3 - 19.8	156	13.8	11.9 - 16.0
Valenberg	Central	763	493	64.6	61.1 - 68.0	152	19.9	17.1 - 22.9	338	44.3	40.7 - 47.9	10	1.3	0.6 - 2.4
Vijverhof	Central	1,039	631	60.7	57.7 - 63.7	804	77.4	74.7 - 79.9	206	19.8	17.4 - 22.4	104	10.0	8.3 - 12.0
Vledderhof	Northern	398	251	63.1	58.1 - 67.8	314	78.9	74.6 - 82.8	30	7.5	5.1 - 10.6	101	25.4	21.2 - 30.0
Zwanemeerbos	Northern	665	348	52.3	48.5 - 56.2	629	94.6	92.6 - 96.2	109	16.4	13.7 - 19.4	85	12.8	10.3 - 15.6
Total		1,3967	8,739	62.6	61.8 - 63.4	6,968	49.9	49.1 - 50.7	2,488	17.8	17.2 - 18.5	1,967	14.1	13.5 - 14.7

Table S6. A table shows expected and observed co-infections in questing nymphs and results of the exact bionomial test. A column 'Number of infections' indicates at least one, two, three etc infections.

Number of infections	Expected	Observed			p-value	Lower CI	Upper CI
1	13,967	12,235	1	0.87599341	< 2.2e-16	0.8704132	0.8814165
2	13,210	7,444	0.94580082	0.53297057	< 2.2e-16	0.5246545	0.5412729
3	3,587	2,452	0.25681742	0.17555667	< 2.2e-16	0.1692796	0.1819692
4	496	335	0.0354989	0.02398511	< 2.2e-16	0.02151142	0.02665972
5	29	28	0.00208108	0.00200473	0.9246	0.001332526	0.002896093
6	1	1	8.7372E-05	7.1597E-05	1	1,81E+00	3,99E+02
7	0	0	1.1313E-06	0	1	0.0000000000	0.0002640791

Table S7. Pairwise Odd ratio results for all dataset. The Benjamini-Hochberg procedure was applied to correct for multiple testing (Benjamini-Hochberg 1995). Significant positive and negative associations are marked with green and orange colour, respectively.

Loc-ation	Symbiont 1	Symbiont 2	Observed coinfection (n)	Expected coinfection (n)	p-value	rank (i)	(i/m)Q	Lower 95% CI	Upper 95% CI	Estimate odds ratio
All	*R. helvetica*	*S. ixodetis*	95	350	5.62E-75	1	0.002380952	0.16	0.25	0.20
All	*R. helvetica*	*Rickettsiella* spp.	1,308	981	1.05E-57	2	0.004761905	2.01	2.46	2.22
All	*N. mikurensis*	*B. burgdorferi* s.l.	192	62	2.07E-49	3	0.007142857	3.64	5.22	4.37
All	*M. mitochondrii*	*S. ixodetis*	1,772	1,557	2.20E-23	4	0.00952381	1.46	1.76	1.60
All	*Rickettsiella* spp.	*S. ixodetis*	1,062	1,241	2.23E-15	5	0.011904762	0.64	0.77	0.70
All	*M. mitochondrii*	*B. burgdorferi* s.l.	763	670	5.95E-10	6	0.014285714	1.33	1.76	1.53
All	*A. phagocytophilum*	*B. burgdorferi* s.l.	9	35	1.32E-07	7	0.016666667	0.11	0.45	0.24
All	*A. phagocytophilum*	*N. mikurensis*	5	26	3.61E-07	8	0.019047619	0.06	0.42	0.18
All	*B. burgdorferi* s.l.	*Rickettsiella* spp.	607	534	3.91E-06	9	0.021428571	1.18	1.53	1.34
All	*N. mikurensis*	*R. helvetica*	74	114	1.79E-05	10	0.023809524	0.46	0.77	0.60
All	*N. mikurensis*	*Rickettsiella* spp.	351	402	0.000212203	11	0.026190476	0.66	0.88	0.76
All	*A. phagocytophilum*	*Rickettsiella* spp.	199	227	0.007604716	12	0.028571429	0.64	0.93	0.77
All	*A. phagocytophilum*	*S. ixodetis*	102	81	0.012647363	13	0.030952381	1.06	1.69	1.34
All	*A. phagocytophilum*	*R. helvetica*	47	64	0.016578813	14	0.033333333	0.50	0.94	0.69
All	*M. mitochondrii*	*N. mikurensis*	523	504	0.165495876	15	0.035714286	0.96	1.30	1.11
All	*M. mitochondrii*	*R. helvetica*	1,249	1,231	0.365642222	16	0.038095238	0.95	1.16	1.05
All	*B. burgdorferi* s.l.	*R. helvetica*	141	151	0.385313694	17	0.04047619	0.76	1.11	0.92
All	*B. burgdorferi* s.l.	*S. ixodetis*	138	191	0.533309105	18	0.042857143	0.80	1.12	0.95
All	*N. mikurensis*	*S. ixodetis*	146	144	0.812636719	19	0.045238095	0.84	1.23	1.02
All	*M. mitochondrii*	*A. phagocytophilum*	283	285	0.84402954	20	0.047619048	0.80	1.19	0.98
All	*M. mitochondrii*	*Rickettsiella* spp.	4,359	4,360	0.986050514	21	0.05	0.93	1.07	1.00

The largest p value that has p < (i/m)Q is significant, and all of the p-values smaller than it are also significant, even the ones that aren't less than their Benjamini-Hochberg critical value.

Table S8. Pairwise Odd ratio results per region. The Benjamini-Hochberg procedure was applied to correct for multiple testing seperately for each region (Benjamini-Hochberg 1995). The largest p value that has p < (i/m)Q is significant, and all of the p-values smaller than it are also significant, even the ones that aren't less than their Benjamini-Hochberg critical value. Associations excluded based on the Benjamini-Hochberg procedure are coloured with grey. The table continues on the following pages.

Region	Symbiont 1	Symbiont 2	Observed co-infection (n)	Expected co-infection (n)	p-value	rank (i)	**(i/m)Q**	Lower 95% CI	Upper 95% CI	Estimate odds ratio
Northern	*R. helvetica*	*S. ixodetis*	2	46	1.65E-26	1	0.00238	0.00	0.09	0.02
Northern	*Rickettsiella* spp.	*S. ixodetis*	285	202	1.38E-06	2	0.00476	1.56	3.17	2.20
Northern	*M. mitochondrii*	*S. ixodetis*	221	147	7.53E-06	3	0.00714	1.35	2.25	1.74
Northern	*M. mitochondrii*	*B. burgdorferi* s.l.	161	110	0.001445098	4	0.00952	1.18	2.09	1.56
Northern	*B. burgdorferi* s.l.	*Rickettsiella* spp.	207	151	0.002942418	5	0.01190	1.18	2.53	1.71
Northern	*N. mikurensis*	*B. burgdorferi* s.l.	16	6	0.003622177	6	0.01429	1.28	4.27	2.41
Northern	*R. helvetica*	*Rickettsiella* spp.	347	297	0.004693906	7	0.01667	0.57	0.91	0.72
Northern	*M. mitochondrii*	*A. phagocytophilum*	13	14	0.08	8	0.01905	0.24	1.13	0.53
Northern	*B. burgdorferi* s.l.	*S. ixodetis*	38	23	0.08	9	0.02143	0.94	2.03	1.40
Northern	*N. mikurensis*	*R. helvetica*	21	12	0.11	10	0.02381	0.88	2.56	1.53
Northern	*M. mitochondrii*	*Rickettsiella* spp.	1,200	947	0.13	11	0.02619	0.96	1.39	1.15
Northern	*M. mitochondrii*	*R. helvetica*	285	216	0.14	12	0.02857	0.95	1.43	1.16
Northern	*A. phagocytophilum*	*B. burgdorferi* s.l.	5	2	0.2	13	0.03095	0.56	5.05	1.89
Northern	*A. phagocytophilum*	*Rickettsiella* spp.	28	20	0.21	14	0.03333	0.72	8.14	2.07
Northern	*A. phagocytophilum*	*R. helvetica*	3	5	0.35	15	0.03571	0.09	1.55	0.48
Northern	*A. phagocytophilum*	*S. ixodetis*	5	3	0.58	16	0.03810	0.41	3.63	1.36
Northern	*A. phagocytophilum*	*N. mikurensis*	0	1	0.62	17	0.04048	0.00	3.77	0.00
Northern	*M. mitochondrii*	*N. mikurensis*	46	39	0.66	18	0.04286	0.56	1.40	0.89
Northern	*B. burgdorferi* s.l.	*R. helvetica*	41	34	0.79	19	0.04524	0.64	1.34	0.94
Northern	*N. mikurensis*	*Rickettsiella* spp.	68	53	0.79	20	0.04762	0.65	2.00	1.11
Northern	*N. mikurensis*	*S. ixodetis*	9	8	0.87	21	0.05000	0.37	1.73	0.85

table continues

Region	Symbiont 1	Symbiont 2	Observed co-infection (n)	Expected co-infection (n)	p-value	rank (i)	**(i/m)Q**	Lower 95% CI	Upper 95% CI	Estimate odds ratio
Central	*N. mikurensis*	*B. burgdorferi* s.l.	166	53	3.54E-44	1	0.00238	3.76	5.59	4.59
Central	*M. mitochondrii*	*S. ixodetis*	1,476	1,310	4.47E-18	2	0.00476	1.43	1.77	1.59
Central	*M. mitochondrii*	*B. burgdorferi* s.l.	551	475	6.83E-10	3	0.00714	1.41	2.00	1.68
Central	*A. phagocytophilum*	*B. burgdorferi* s.l.	4	28	2.15E-08	4	0.00952	0.04	0.34	0.13
Central	*A. phagocytophilum*	*N. mikurensis*	4	25	1.39E-07	5	0.01190	0.04	0.37	0.14
Central	*Rickettsiella* spp.	*S. ixodetis*	689	771	2.47E-05	6	0.01429	0.72	0.89	0.80
Central	*B. burgdorferi* s.l.	*Rickettsiella* spp.	331	279	5.69E-05	7	0.01667	1.17	1.59	1.37
Central	*A. phagocytophilum*	*R. helvetica*	6	20	0.000259	8	0.01905	0.10	0.61	0.27
Central	*R. helvetica*	*S. ixodetis*	91	119	0.002783	9	0.02143	0.55	0.89	0.71
Central	*R. helvetica*	*Rickettsiella* spp.	240	207	0.003741	10	0.02381	1.09	1.55	1.30
Central	*M. mitochondrii*	*R. helvetica*	321	352	0.004694	11	0.02619	0.65	0.93	0.78
Central	*A. phagocytophilum*	*Rickettsiella* spp.	113	132	0.03314	12	0.02857	0.61	0.98	0.78
Central	*A. phagocytophilum*	*S. ixodetis*	93	76	0.02916	13	0.03095	1.02	1.69	1.32
Central	*B. burgdorferi* s.l.	*S. ixodetis*	140	160	0.06	14	0.03333	0.68	1.01	0.83
Central	*N. mikurensis*	*R. helvetica*	30	40	0.11	15	0.03571	0.49	1.07	0.73
Central	*M. mitochondrii*	*N. mikurensis*	454	436	0.14	16	0.03810	0.96	1.34	1.13
Central	*B. burgdorferi* s.l.	*R. helvetica*	52	43	0.14	17	0.04048	0.91	1.68	1.24
Central	*N. mikurensis*	*S. ixodetis*	131	147	0.36	18	0.04286	0.74	1.10	0.91
Central	*N. mikurensis*	*Rickettsiella* spp.	249	256	0.57	19	0.04524	0.81	1.12	0.95
Central	*M. mitochondrii*	*Rickettsiella* spp.	2,294	2,284	0.66	20	0.04762	0.94	1.11	1.02
Central	*M. mitochondrii*	*A. phagocytophilum*	224	225	0.91	21	0.05000	0.79	1.24	0.99

table continues

Region	Symbiont 1	Symbiont 2	Observed co-infection (n)	Expected co-infection (n)	p-value	rank (i)	**(i/m)Q**	Lower 95% CI	Upper 95% CI	Estimate odds ratio
Western	*R. helvetica*	*S. ixodetis*	2	59	1,61E-35	1	0,00238	0,00	0,05	0.01
Western	*N. mikurensis*	*B. burgdorferi* s.l.	10	2	3,75E-06	2	0,00476	3,49	18,93	8.44
Western	*M. mitochondrii*	*R. helvetica*	643	617	0,007262	3	0,00714	1,08	1,63	1.32
Western	*N. mikurensis*	*Rickettsiella* spp.	34	27	0,005321	4	0,00952	1,40	47,61	5.52
Western	*M. mitochondrii*	*Rickettsiella* spp.	865	848	0,04	5	0,01190	1,01	1,62	1.28
Western	*A. phagocytophilum*	*B. burgdorferi* s.l.	0	3	0,05	6	0,01429	0,00	1,02	0.00
Western	*Rickettsiella* spp.	*S. ixodetis*	88	80	0,08	7	0,01667	0,94	2,85	1.59
Western	*B. burgdorferi* s.l.	*Rickettsiella* spp.	69	62	0,08	8	0,01905	0,93	3,44	1.73
Western	*R. helvetica*	*Rickettsiella* spp.	721	709	0,17	9	0,02143	0,93	1,47	1.17
Western	*N. mikurensis*	*S. ixodetis*	0	2	0,17	10	0,02381	0,00	1,61	0.00
Western	*N. mikurensis*	*R. helvetica*	23	20	0,31	11	0,02619	0,70	3,13	1.45
Western	*A. phagocytophilum*	*Rickettsiella* spp.	58	54	0,32	12	0,02857	0,77	2,89	1.44
Western	*M. mitochondrii*	*S. ixodetis*	75	70	0,34	13	0,03095	0,81	2,01	1.26
Western	*M. mitochondrii*	*B. burgdorferi* s.l.	51	54	0,47	14	0,03333	0,52	1,37	0.84
Western	*B. burgdorferi* s.l.	*R. helvetica*	48	45	0,57	15	0,03571	0,72	1,87	1.15
Western	*M. mitochondrii*	*A. phagocytophilum*	46	47	0,8	16	0,03810	0,56	1,62	0.94
Western	*A. phagocytophilum*	*R. helvetica*	38	39	0,81	17	0,04048	0,56	1,55	0.93
Western	*M. mitochondrii*	*N. mikurensis*	26	24	0,86	18	0,04286	0,44	1,97	0.91
Western	*A. phagocytophilum*	*N. mikurensis*	1	2	1	19	0,04524	0,02	3,97	0.65
Western	*B. burgdorferi* s.l.	*S. ixodetis*	5	5	1	20	0,04762	0,30	2,44	0.97
Western	*A. phagocytophilum*	*S. ixodetis*	4	4	1	21	0,05000	0,23	2,46	0.89

Table S9. A table shows prevalences of vertically-transmitted symbionts in ticks infected and uninfected with horizontally-transmitted pathogens. Differences between the prevalences were calculated with the z test. Significant p-values are in bold.

		N. mikurensis negative	*N. mikurensis* positive	X-squared	DF	p-value	Lower CI	Upper CI
Central	*M. mitochondrii*	0.635339193	0.66374269	2.095466	1	0.147737	-0.06598	0.009177
	S. ixodetis	0.216249718	0.200292398	0.865591	1	0.352179	-0.01602	0.047937
	R. helvetica	0.058936218	0.043859649	2.378764	1	0.122995	-0.00182	0.031974
	Rickettsiella spp.	0.375704305	0.364035088	0.320897	1	0.571069	-0.02656	0.049897
Western	*M. mitochondrii*	0.660628019	0.638888889	0.008948	1	0.924636	-0.151	0.194481
	S. ixodetis	0.064009662	0	1.48916	1	0.222347	0.03803	0.089989
	R. helvetica	0.550120773	0.638888889	0.792429	1	0.373367	-0.26168	0.084144
	Rickettsiella spp.	0.754830918	0.944444444	5.92529	1	**0.014925**	-0.28145	-0.09778
Northern	*M. mitochondrii*	0.56404409	0.534883721	0.181513	1	0.670076	-0.08395	0.14227
	S. ixodetis	0.120486507	0.104651163	0.076232	1	0.78247	-0.05605	0.087719
	R. helvetica	0.174458381	0.244186047	2.324895	1	0.127319	-0.16768	0.028223
	Rickettsiella spp.	0.772709996	0.790697674	0.068215	1	0.793953	-0.11145	0.075474
All locations	*M. mitochondrii*	0.62426867	0.64888337	1.861108	1	0.172497	-0.05925	0.010019
	S. ixodetis	0.17795	0.18114144	0.033287	1	0.855231	-0.03123	0.024847
	R. helvetica	0.14383406	0.09181141	16.56015	1	**4.71E-05**	0.030547	0.073498
	Rickettsiella spp.	0.50277335	0.43548387	13.48768	1	**0.00024**	0.031352	0.103227
		A. phagocytophilum negative	*A. phagocytophilum positive*					
Central	*M. mitochondrii*	0.637479631	0.634560907	0.003084	1	0.955714	-0.04974	0.055575
	S. ixodetis	0.213253666	0.263456091	4.781763	1	**0.028763**	-0.09838	-0.00202
	R. helvetica	0.059424226	0.016997167	10.4616	1	**0.001219**	0.026633	0.058221
	Rickettsiella spp.	0.376969039	0.320113314	4.450048	1	**0.0349**	0.005722	0.10799
Western	*M. mitochondrii*	0.66070327	0.647887324	0.009056	1	0.924185	-0.108	0.133631
	S. ixodetis	0.062924121	0.056338028	3.77E-30	1	1	-0.05492	0.068092
	R. helvetica	0.552745219	0.535211268	0.028523	1	0.865885	-0.10833	0.143396
	Rickettsiella spp.	0.756323257	0.816901408	1.053144	1	0.304784	-0.16028	0.039127
Northern	*M. mitochondrii*	0.564990689	0.40625	2.625943	1	0.10513	-0.02827	0.345748

table continues

		A. phagocytophilum negative	*A. phagocytophilum positive*	X-squared	DF	p-value	Lower CI	Upper CI
	S. ixodetis	0.119553073	0.15625	0.130643	1	0.717767	-0.17891	0.105514
	R. helvetica	0.177653631	0.09375	1.008032	1	0.315375	-0.03393	0.201735
	Rickettsiella spp.	0.772067039	0.875	1.369084	1	0.241969	-0.23442	0.028558
All locations	*M. mitochondrii*	0.62586041	0.62061404	0.03186	1	0.858335	-0.04117	0.051658
	S. ixodetis	0.17659685	0.22368421	6.362754	1	**0.011654**	-0.08701	-0.00717
	R. helvetica	0.14210643	0.10307018	5.237349	1	**0.022107**	0.009382	0.068691
	Rickettsiella spp.	0.50099919	0.43640351	7.106263	1	**0.007682**	0.017169	0.112022
		B. burgdorferi s.l. negative	*B. burgdorferi s.l. positive*					
Central	*M. mitochondrii*	0.62873028	0.73959732	36.05306	1	**1.92E-09**	-0.14468	-0.07705
	S. ixodetis	0.2174061	0.18791946	3.365018	1	0.066595	-0.00058	0.059558
	R. helvetica	0.05684784	0.06979866	1.882738	1	0.170023	-0.0326	0.006702
	Rickettsiella spp.	0.36900034	0.4442953	16.29884	1	**5.41E-05**	-0.1131	-0.03749
Western	*M. mitochondrii*	0.6621118	0.62195122	0.396214	1	0.529051	-0.07371	0.154034
	S. ixodetis	0.06273292	0.06097561	1.34E-30	1	1	-0.05313	0.056643
	R. helvetica	0.55031056	0.58536585	0.258933	1	0.610854	-0.15083	0.080718
	Rickettsiella spp.	0.75465839	0.84146341	2.756091	1	0.096885	-0.17501	0.001403
Northern	*M. mitochondrii*	0.55357865	0.65983607	9.765144	1	**0.001779**	-0.1711	-0.04142
	S. ixodetis	0.11645774	0.1557377	2.883905	1	0.089469	-0.08875	0.010193
	R. helvetica	0.17751719	0.16803279	0.07988	1	0.77746	-0.04204	0.061008
	Rickettsiella spp.	0.76587141	0.84836066	8.155596	1	**0.004293**	-0.13274	-0.03224
All locations	*M. mitochondrii*	0.61848635	0.7124183	36.85436	1	**1.27E-09**	-0.12281	-0.06505
	S. ixodetis	0.17873759	0.17086835	0.366249	1	0.545056	-0.01613	0.031867
	R. helvetica	0.14159429	0.13165266	0.727677	1	0.393637	-0.01169	0.031572
	Rickettsiella spp.	0.49325372	0.56676004	21.07936	1	**4.41E-06**	-0.10492	-0.04209

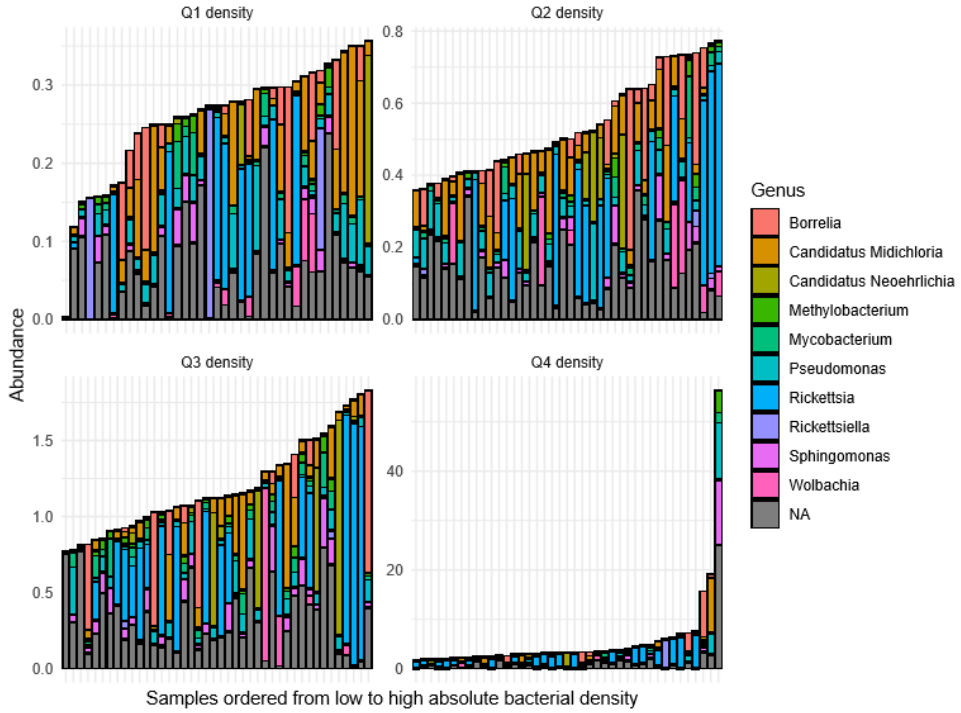


Figure S1. Abundances of the most abundant taxa, separated by quartiles of absolute bacterial density (16S rRNA content in ng/μL). The remaining taxa were binned in the synthetic 'Other' taxon. All abundances are scaled by the bacterial density. The genus Pseudomonas was also highly abundant in negative controls.

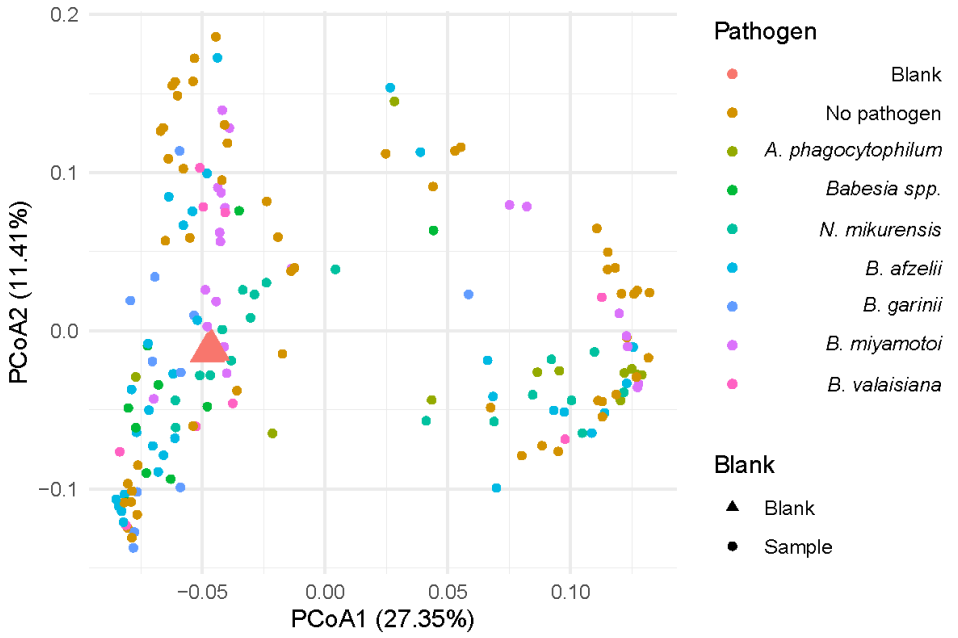


Figure S2. Principal Coordinate Analysis of Bray-Curtis dissimilarities for tick microbiomes compared to blanks. Counts were not corrected for 16S rRNA load. Individual ticks were screened for pathogens, pooled per pathogen, and subsequently subjected to microbiome analysis. Each sample colour reflects a pathogen. Three negative controls (blanks) are shown as a red triangle and group with many tick samples due to their similarity.

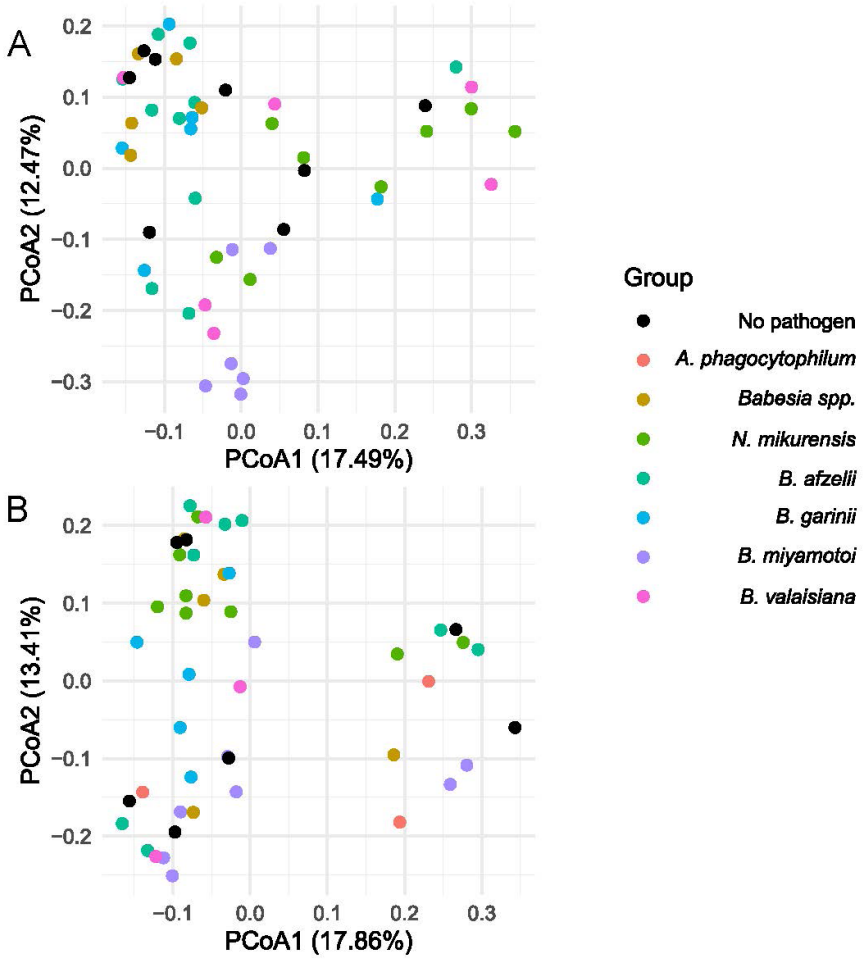
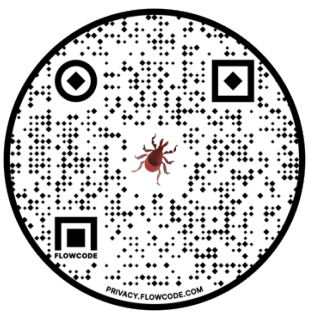


Figure S3. Principal Coordinate Analysis of microbial abundance data scaled by 16S rRNA load. Ticks were screened for the presence of specific pathogens, pooled, and then sequenced. Samples, each containing a pool of five screened ticks, are coloured by a pathogen. (A) Principal Coordinate analysis for the BU location. (B) Principal Coordinate analysis for the ST location.



Please click here (for the online version) or scan this QR code to listen to an immersive soundscape of a forest in Buunderkamp, the Netherlands recorded by Vitalij Kuzkin. The Buunderkamp forest is one of the study sites where I have collected samples for this research. Listen with headphones for the best experience. The full album is available on: ticksandmicrobes.bandcamp.com

Chapter 4

Effect of rodent density on tick and tick-borne pathogen populations: consequences for infectious disease risk

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Abstract

Rodents are considered to contribute strongly to the risk of tick-borne diseases by feeding *Ixodes ricinus* larvae and by acting as amplifying hosts for pathogens. Here, we tested to what extent these two processes depend on rodent density and for which pathogen species rodents synergistically contribute to the local disease risk, i.e. the density of infected nymphs (DIN). In a natural woodland, we manipulated rodent densities in plots of 2,500 m^2 by either supplementing a critical food source (acorns) or by removing rodents for two years. Untreated plots were used as controls. Collected nymphs and rodent ear biopsies were tested for the presence of seven tick-borne microorganisms. Linear models were used to capture associations between rodents, nymphs, and pathogens. Investigation of data from all plots, irrespective of the treatment, revealed a strong positive association between rodent density and nymphal density, nymphal infection prevalence (NIP) with *Borrelia afzelii,* and *Neoehrlichia mikurensis,* and hence DIN's of these pathogens in the following year. The NIP, but not the DIN, of the bird-associated *B. garinii,* decreased with increasing rodent density. The NIPs of *B. miyamotoi* and *Rickettsia helvetica* were independent of rodent density, and increasing rodent density moderately increased the DINs. In addition, NIPs of *Babesia microti* and *Spiroplasma ixodetis* decreased with increasing rodent density, which had a non-linear association with DINs of these microorganisms. A positive density dependence for all rodent- and tick-associated tick-borne pathogens was found, despite the observation that some of them decreased in prevalence. The effects on the DINs were variable among microorganisms, more than likely due to contrasts in their biology (including transmission modes, host specificity, and transmission efficiency). The strongest associations were found in rodent-associated pathogens that most heavily rely on horizontal transmission. Our results draw attention to the importance of considering transmission mode of a pathogen while developing preventative measures to successfully reduce the burden of disease.

Keywords: Disease risk, *Ixodes ricinus*, Tick-borne pathogens, Transmission dynamics, Rodent density, Tick symbionts

Introduction

Lyme borreliosis is the most prevalent tick-borne disease in the northern hemisphere with increasing incidence and expanding endemic regions (Rizzoli et al., 2011; Schwartz et al., 2017). The risk of acquiring Lyme borreliosis is partially determined by the density of questing ticks infected with its causative agent, *Borrelia burgdorferi* sensu lato (Glass et al., 1994; Glass et al., 1995). Particularly, the density of infected nymphs (DIN) is of interest because humans are predominantly exposed to and infected with Lyme spirochetes, as well as other pathogens, by nymphs (Robertson et al., 2000). The density of infected questing ticks is a product of the density of questing ticks and infection prevalence of a pathogen, which both express high temporal variations, presumably attributed to changes in weather conditions and fluctuations in the abundance of vertebrate hosts (Coipan et al., 2013b; Mannelli et al., 2012; Takken et al., 2017). The mechanisms underlying these variations are complex, as climatic conditions, vertebrate hosts and their food source, ticks, and tick-borne microorganisms form biological networks with multiple direct and indirect interactions (Jones et al., 1998). Therefore, quantifying these interactions will help us to understand changes in the distribution and incidence of Lyme borreliosis and other tick-borne diseases.

The most common vectors of tick-borne diseases in the northern hemisphere are ticks of the *Ixodes ricinus* complex. Their survival primarily depends on their ability to find a vertebrate host, which may vary between life stages. In forested areas, larvae of the *I. ricinus* complex feed predominantly on rodents, nymphs on rodents and birds, and adults on ungulates, mostly deer (Hofmeester et al., 2017b; Takumi et al., 2019). Although the presence of deer is generally responsible for the high abundance of ticks (Hofmeester et al., 2017b), variations in the density of nymphs (DON) has been associated with the density of rodents (Ostfeld et al., 2001). For instance, the density of host-seeking *I. scapularis* nymphs was correlated with the abundance of white-footed mice in the previous year. White-footed mice are the main hosts for larval *I. scapularis*; the high abundance of these mice provides more opportunities for larvae to feed successfully, and subsequently emerge as nymphs in the following year.

The abundance of rodent species is affected by many different factors, such as predation, vegetation cover, and food availability (Bogdziewicz et al., 2016; Hofmeester et al., 2017a; Ostfeld et al., 1996). A key food supply for rodents is, acorns and its seasonal availability has been shown to be responsible for the fluctuations in rodent densities between years and geographical locations (Ostfeld et al., 1996; Ostfeld et al., 2000; Wolff, 1996). In general, acorn availability increases the length of the breeding season and facilitates winter survival of forest rodents resulting in a higher rodent density in the following spring (Clotfelter et al., 2007; Jensen, 1982; Jones et al., 1998; McShea, 2000; Pucek et al., 1993). As a consequence, in the temperate zone, an increased rodent density has been shown to cause upsurges in Puumala hantavirus disease in humans (Swart et al., 2017; Tersago et al., 2009). In addition, several North American studies have suggested that acorns and rodents are good predictors for Lyme-disease risk because rodents are reservoir hosts of *B. burgdorferi* (s.l.) (Jones et al., 1998; Ostfeld et al., 2006; Schauber et al., 2005). However, the causal relationship between rodent fluctuations and Lyme disease incidence remains unresolved as this has not been investigated in experimental settings, enabling the exclusion of confounding factors.

In the Netherlands, the wood mice (*Apodemus sylvaticus* Linnaeus) and bank voles (*Myodes glareolus* Schreber) are amplifying hosts of several tick-borne pathogens, including *B. afzelii*, *B.*

miyamotoi, *Babesia microti,* and *Neoehrlichia mikurensis* (Burri et al., 2014; Gassner et al., 2013; Hu et al., 1997a; Karbowiak, 2004; Silaghi et al., 2016), and the most common hosts of larval *I. ricinus* (Hofmeester et al., 2016). Apart from the rodent-borne pathogens mentioned above, *I. ricinus* carries many other microorganisms, including *B. garinii*, *Spiroplasma ixodetis,* and *Rickettsia helvetica* (Stanek, 2009). Most, if not all, of the pathogens, are transmitted between ticks *via* a vertebrate host (horizontally), which can be broadly divided into co-feeding and systemic transmission (Table 1). Co-feeding relies on localized and temporal infection in the vertebrate skin and occurs when infected and uninfected ticks feed close to each other (Gern & Rais, 1996; Voordouw, 2015). Systemic transmission depends more on a persistent infection in a host, which can be local (e.g., skin) or systemic (e.g., blood; Voordouw, 2015). Amplifying hosts are responsible for producing infected ticks and, therefore, for increased risk of human exposure. In addition, ticks maintain microorganisms such as *S. ixodetis via* vertical transmission, with different efficiency (Table 1). Some bacteria, such as *R. helvetica* and *B. miyamotoi*, can utilize both horizontal and vertical transmission routes (Heylen et al., 2016; Hornok et al., 2014). It is unclear how variations in rodent densities affect the disease risk of tick-borne pathogens with different transmission modes, particularly in the European setting.

Table 1. Transmission modes and amplification hosts of tick-borne microorganisms

Microorganism	Transmission mode	Proposed amplification host
B. microti	Horizontal (Rudzinska et al., 1979)	Rodents (Hersh et al., 2012)
B. afzelii	Horizontal (Talleklint & Jaenson, 1994)	Rodents (Hanincova et al., 2003a)
B. garinii	Horizontal (Talleklint et al., 1994)	Birds (Hanincova et al., 2003b)
B. miyamotoi	Horizontal/vertical (van Duijvendijk et al., 2016)	Rodents (van Duijvendijk et al., 2016)
N. mikurensis	Horizontal (Burri et al., 2014)	Rodents (Burri et al., 2014)
R. helvetica	Horizontal/vertical (Burgdorfer et al., 1979)	Birds (Hornok et al., 2014)
S. ixodetis	Vertical/horizontal (Bell-Sakyi et al., 2015)(this study)	Rodents (this study)

The goal of the present study was to investigate how rodent densities, the density of *I. ricinus* nymphs, and transmission dynamics of tick-borne pathogens interact in order to generate the density of infected ticks. To our knowledge, this is the first European study experimentally investigating these relationships in the field. In addition, no prior study has assessed the influence of rodent density on the prevalence and density of tick-borne microorganisms other than rodent-borne. Our approach was to artificially manipulate the rodent densities by either acorn addition or rodent removal for two consecutive years in a natural habitat. We measured and quantified the rodent, nymph, and pathogen population responses to these treatments, as well as performed regression analysis. Using this approach, we aimed to learn whether rodent densities play a major role in shaping the density of questing ticks and transmission dynamics of tick-borne microorganisms, which in turn, will help assess and potentially predict disease risk and formulate possible intervention strategies.

Given that rodents are locally the most substantial hosts for larvae (Hofmeester et al., 2016) and high rodent density results in high larval encounter rates, an increase of the rodent density at a given year$_t$ is expected to lead to a rise in the density of nymphs in the following year (DON$_{t+1}$). Along with the higher rodent densities, transmission events of tick-borne microorganisms are

expected to increase. We anticipate that differences in the microorganisms' modes of transmission as well as host amplification potential are the main determinants in the change after manipulation. Our hypothesis is that the NIP_{t+1} (nymphal infection prevalence) of tick-borne pathogens, such as *B. afzelii*, *N. mikurensis,* and *B. microti*, which are amplified by rodents, is dependent on the density of rodents. Consequently, we expect a synergistic effect of rodent densities on the density of infected nymphs one year later (DIN_{t+1}). Also, we hypothesise that rodent densities will not alter the NIP_{t+1} of tick-associated microorganisms, such as *R. helvetica*, *B. miyamotoi*, and *S. ixodetis*, which predominantly rely on vertical transmission. Further, we expect that $DIN_{t+1\ R.\ helvetica}$, $DIN_{t+1\ B.\ miyamotoi}$ and $DIN_{t+1\ S.\ ixodetis}$ will be only moderately affected by increasing rodent density. In the case of *B. garinii*, a tick-borne pathogen amplified by birds (Kurtenbach et al., 1998a; Taragel'ova et al., 2008), we expect that increasing rodent density will increase the proportion of larvae feeding on rodents and, therefore, have a negative effect on $NIP_{t+1\ B.\ garinii}$. Lastly, we hypothesize that a higher rodent density will have no effect on $DIN_{t+1\ B.\ garinii}$.

Methods

Study sites

The study was conducted at the forest reserves Planken Wambuis (52°01'45" N, 5°48'49" E) and Noord Ginkel (52°02'23" N, 5°45'09" E) near Wageningen, The Netherlands. Both forests are dominated by Scots pine (*Pinus sylvestris*) and harbour a diversity of bird and mammal species, including wood mice (*A. sylvaticus*), bank voles (*M. glareolus*), common shrews (*Sorex araneus*), wild boar (*Sus scrofa*), roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*) and a few free-ranging cattle and horses.

Manipulation of rodent density and estimation of nymphal density

In both forests, six plots of 50 × 50 m were selected with at least 350 m between plots (Figure S1). Each plot was assigned to one of three treatments (rodent removal, control, or acorn addition). In rodent removal plots, rodents were trapped for one night a month with Heslinga live traps (Heslinga Traps, Groningen, The Netherlands) in a 5 × 5 grid with 10 m inter-trap distance. Captured rodents were euthanized by cervical dislocation. The first rodent removal event was directly after the mark-recapture trapping in September 2012. Thereafter, rodents that accidentally found their way into the plots were removed monthly until December 2014 using the same grid with traps. Four control plots received no treatment. To increase rodent density, acorns were added to four plots (Sunyer et al., 2016). Acorns were provided beneath feeding stations, which were made of 60 × 60 cm plates kept 5 cm above the ground to prevent acorn predation by birds and large mammals. In each plot, 16 of these feeding stations were placed in a 4 × 4 grid with 15 m between feeding stations. Each feeding station was provided with 6.25 kg of acorns in November and January of 2012 and 2013 (1600 kg in total). Control feeding stations without acorns were also placed in the control and rodent removal plots. A plastic screen, 40 cm high and dug 10 cm into the ground, was placed as a barrier around the four rodent removal plots to prevent immigration of rodents (Figure S1). To overcome a possible bias in large vertebrate community caused by a visual effect, screens were also placed around the control and acorn addition plots. However, the lowest 10 cm of these screens was left open to enable rodents to walk in and out freely.

Tick density was estimated monthly in each plot by blanket dragging over the vegetation. At each plot, a 1 m^2 blanket was dragged over four transits of 50 m and inspected at 25 m intervals. All attached nymphs were counted. Dragging was performed in the afternoons (12:00–18:00 h CET) when the vegetation was dry. Given that nymphs have been shown to quest when the weekly mean daily maximum temperature exceeds 7 °C (Perret et al., 2000; Randolph, 2004; Sprong et al., 2012), we included temperature data from September 2012 to December 2015 to investigate the relationship between temperature and onset of tick activity. Daily measurements were collected from the nearest weather station (Deelen, KNMI, the Netherlands; Table S1).

Rodent samples and nymph collection

Rodents were sampled at three-month intervals (March, June, September, and December) from September 2012 until December 2014. At each plot, 25 Heslinga live traps were placed in a 5 × 5 grid. Traps were pre-baited with oats for three days, after which they were rebaited with grain, carrot and mealworms and set at 9:00 h CET. Traps were then inspected four times at 12-h intervals. Trapped rodents were marked by shaving a patch of fur from their side (Panzacchi et al., 2010). Rodent density was calculated per species according to the Schnabel method (multiple marking; (Leirs et al., 1997; Schnabel, 1938). During the morning trappings, newly captured rodents were screened for ticks, and larvae were counted. A small ear biopsy was taken with sterile scissors from each newly captured rodent and stored in 70% ethanol at -20 °C until further analysis.

Questing nymphs were collected during monthly density estimation. All nymphs attached to the blanket were collected and stored individually in 70% ethanol at -20 °C until further analysis.

DNA extraction and pathogen detection

Ear biopsies and nymphs were analysed individually. DNA from a maximum of 40 nymphs per plot per month was extracted with ammonium hydroxide as described previously (Wielinga et al., 2006). DNA from the ear biopsies was extracted using the Qiagen DNeasy Blood & Tissue Kit according to the manufacturer's protocol (Qiagen, Venlo, The Netherlands). The lysates were stored at 4 °C. Samples were analysed with different (multiplex) real-time PCRs, based on various target genes depending on microorganism of interest such as *B. burgdorferi* s.l. (Heylen et al., 2013b), *B. miyamotoi* (Hovius et al., 2013), *N. mikurensis* (Jahfari et al., 2012), *R. helvetica* (Stenos et al., 2005), *B. microti,* and *S. ixodetis* (this study; Text S1). A detailed description of the qPCR protocol is provided in Text S1. Samples positive for *B. burgdorferi* s.l. were subjected to conventional PCR followed by sequencing to identify a genotype (Heylen et al., 2013b).

Data analysis and modelling

Data analysis and model building were performed in R version 3.5.1 (R Core Team, 2018) and RStudio (RStudio Team, 2015). To evaluate whether rodent removal and acorn addition treatments were successful, we compared the means of densities of rodents (data from 2013 and 2014) between the treatments using the non-parametric Wilcoxon signed-rank test. The same test was used to evaluate whether the treatments influenced the DON, and density of rodent-associated pathogens, *B. afzelii* and *N. mikurensis* (data from 2014 and 2015). Since monthly at each plot 200 m^2 were inspected for questing nymphs, we combined these measurements into a yearly DON per 2400 m^2 (by summing up all nymphs from 12 months). The differences in the prevalence of microorganisms and tick burdens between two rodent species were compared with the Chi-square test and non-parametric Wilcoxon signed-rank test, respectively.

To investigate how well the density of rodents from 2013 and 2014 predicts DON_{t+1}, NIP_{t+1}, and DIN_{t+1}, we performed regression analyses. Several linear models for DON_{t+1} (the annual median) were assessed with different interactions between rodent density, year, and treatment. For NIP_{t+1}, binomial generalized linear models were assessed with different interactions between rodent density and year. Because NIP is represented by fraction data, we choose a binomial generalized linear model taking into account sample size with the logit link transform. For DIN_{t+1}, linear models were assessed with different interactions between rodent density and year. DIN data were calculated by multiplying DON and NIP, which are both potentially influenced by rodent density and therefore, we have also included (rodent density)^2 as a covariate.

Year (2013, 2014) and treatment (acorn addition, control, and rodent removal) were categorical variables, while DON, NIP, and DIN were numerical variables. The ranges of DON, NIP, and rodent density are provided in Table S2 and Table S3, respectively. For all models, best-fitting models were compared on the basis of a likelihood ratio test, R2 (linear models), and AIC (linear and generalized models). Model selection was performed using histograms to evaluate the normality of the residuals visually. If there clearly was no best model, the simpler model was selected.

Results

Effect of treatment on rodent density, DON, $DIN_{B.\ afzelii}$, and $DIN_{N.\ mikurensis}$

Rodent density was affected by treatment (Figure 1). With bank voles the effects were apparent throughout the intervention period, while with wood mice addition of acorns led to a strong increase in density in the second year of the study. The removal of rodents led to a lower ($p = 0.0031$) rodent density and the addition of acorns led to a higher ($p = 0.042$) rodent density than in the control plots in years 2013, and 2014 (Figure 1).

The DON fluctuated over the years and was the highest from May until October (Figure 2). We observed that the moment that nymphs started to quest was in the first month of the year with a mean temperature above 7 °C. The number of months with a mean temperature below 7 °C varied between the years (Figure 2 and Figure S2). In 2013, five months had mean temperatures below 7 °C, whereas both 2014 and 2015 had three months with mean temperatures below 7 °C, but these were spread differently throughout the year. The mean DON of all plots in 2013, 2014 and 2015 were 581, 272, and 257 per 2,400 m^2 (200 $m^2 \times$ 12 months), respectively. Mean nymphal density in 2014 and 2015 was significantly lower than in 2013 ($p = 0.0083$ and $p = 0.013$, respectively), whereas the mean nymphal densities of 2014 and 2015 were not significantly different ($p = 0.63$; not shown). There was no effect ($p = 0.27$) of acorn addition and a negative effect ($p = 0.043$) of rodent removal on the DON in the same (not shown) or following years. Nevertheless, when the density of nymphs from 2013 served as a baseline to measure the effect of a treatment on the DON in 2014 and 2015, there was no significant effect (Figure 2).

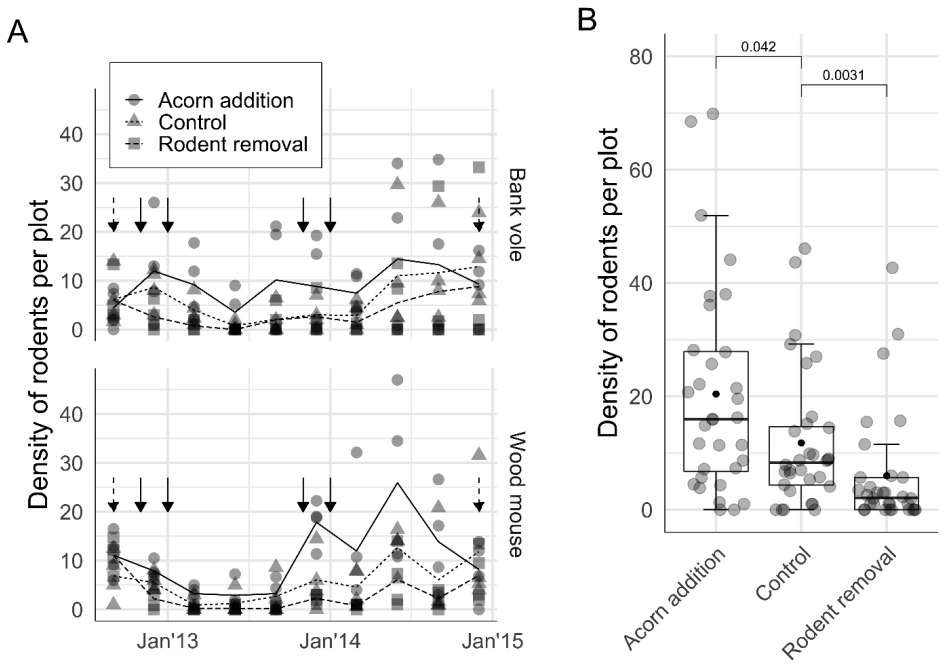


Figure 1. a Mean density of two rodent species, bank vole and wood mouse per plot. Solid arrows indicate events of acorn supplementation (November and January); dashed arrows indicate when monthly removal of rodents started (September 2012) and ended (December 2014). **b** Box plots of rodent density per plot for each treatment (data from 2013 and 2014). The lower and upper hinges correspond to the first and third quartiles (the 25th and 75th percentiles). The upper whisker shows the largest value no further than 1.5 * IQR from the hinge (where IQR is the inter-quartile range, or distance between the first and third quartiles) and the lower whisker shows the smallest value at most 1.5 * IQR of the hinge. The differences in the rodent density between the treatments were calculated based on the mean (black dot) with the Wilcoxon test and the overall difference is statistically significant ($p < 0.0001$). The diagram shows also the median observation (solid horizontal line).

To investigate the effect of treatment on the dynamics of tick-borne pathogens amplified by rodents, we compared the mean $DIN_{B.\ afzelii}$ and $DIN_{N.\ mikurensis}$ between the treatments in 2014 and 2015. Our analyses before and after a correction for a baseline DIN from 2013 showed that there was no effect of either acorn addition or rodent removal on the density of nymphs infected with *B. afzelii* and *N. mikurensis* in the following years (Figure 3 and Figure S3).

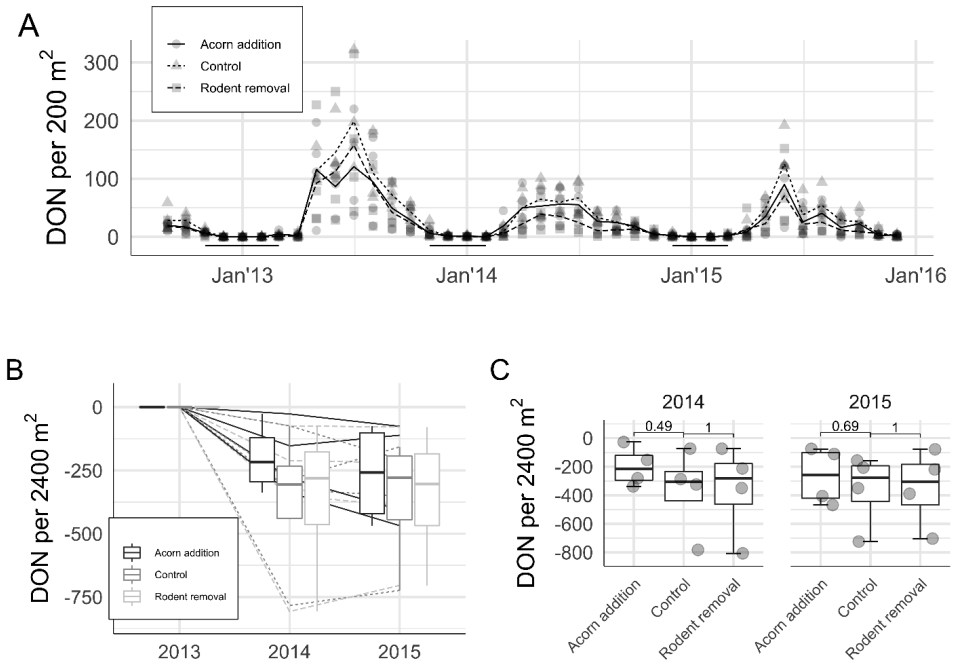


Figure 2. a Mean density of questing nymphs (DON) per 200 m². Horizontal solid lines just above the x-axis depict months with average temperature below 7 °C. In winter 2012/2013, the number of months with the mean temperature below 7 °C was five, while in both 2013/2014 and 2014/2015 was four, however, different months. **b** Density of nymphs (DON) in 2014 and 2015 in all three treatments in comparison to 2013 (baseline year). **c** Differences in DON between the treatments in two separate years calculated with the Wilcoxon test with a correction for a baseline year (2013). The overall differences between the treatments were not significant either in 2014, or 2015 ($p > 0.59$ and $p > 0.87$, respectively).

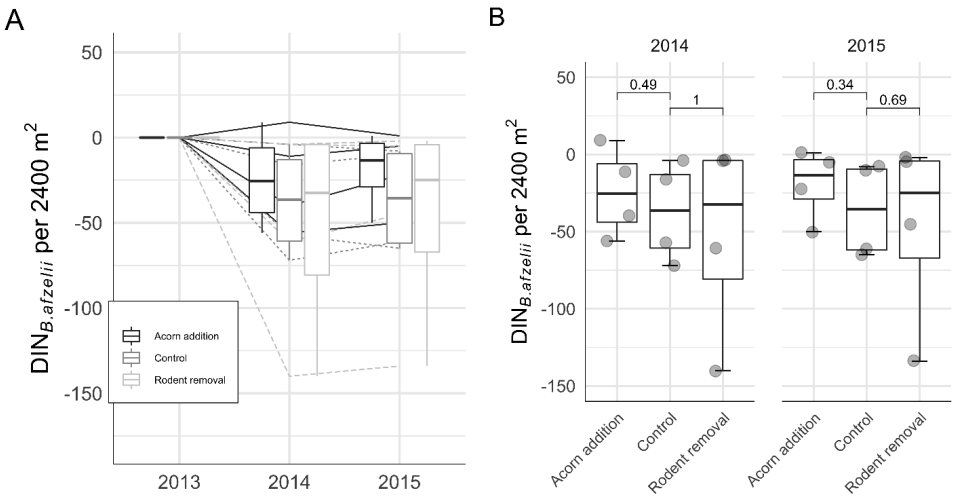


Figure 3. a Density of nymphs infected with *B. afzelii* ($DIN_{B.\ afzelii}$) in 2014 and 2015 in all three treatments in comparison to 2013 (baseline year). **b** Differences in $DIN_{B.\ afzelii}$ between the treatments in two separate years calculated with the Wilcoxon test with a correction for a baseline year (2013). The overall differences between the treatments were not significant either in 2014, or 2015 ($p = 0.69$ and $p = 0.53$, respectively).

Rodent sample and nymph collection

A total of 2,386 rodents was caught in the experiment. From these, 345 bank voles and 547 wood mice were inspected for ticks, from which 155 and 346 were infested with larvae, respectively. The average number of larvae found on wood mice (9.0; 95% CI: 7.6–10.4) was significantly higher ($W = 118{,}520$, $p < 0.0001$) than the average in bank voles (4.2; 95% CI: 3.0–5.4). None of the bank voles and 97 wood mice were infested with nymphs and the average nymphal burden was 0.2 (95% CI: -0.2–0.6).

A total of 772 ear biopsies was taken (478 from wood mice and 294 from bank voles) and subjected to pathogen detection. In addition, 13,916 nymphs were collected by dragging, from which 7,609 were tested for the presence of tick-borne pathogens. A detailed overview of rodent densities, number of analysed rodents, tick density and analysed ticks per treatment, month, and year are provided in Table S2 and Table S3.

Pathogen detection

In the rodent ear biopsies and the collected questing nymphs we detected DNA of *B. burgdorferi* s.l., *B. miyamotoi*, *N. mikurensis*, *B. microti*, *R. helvetica* and *S. ixodetis* (Figure 4). The sequencing success of qPCR-positive ticks (n = 1,017) for *B. burgdorferi* s.l. was 64%, and four genospecies were identified: *B. afzelii*, *B. garinii*, *B. valaisiana,* and *B. burgdorferi* s.s. *Borrelia*-positive rodent biopsies were not sequenced and were treated as *B. afzelii* in further analysis. A justification for this assumption derives from previous studies, which have shown that, in the Netherlands, more than 99% of the positive rodents infected with *B. burgdorferi* s.l. carried *B. afzelii* (Coipan et al., 2018; Mysterud et al., 2019). The prevalence of *B. afzelii* as well as *N. mikurensis* was higher in bank voles

than in wood mice ($\chi^2 = 3.296$, $df = 1$, p = 0.0694 and $\chi^2 = 4.234$, $df = 1$, p = 0.0396, respectively). Interestingly, *S. ixodetis* was almost exclusively detected in wood mice with prevalence significantly higher than in bank voles ($\chi^2 = 14.264$, $df = 1$, p = 0.0002), whereas *B. microti* was almost exclusively found in bank voles with prevalence significantly higher than in wood mice ($\chi^2 = 27.012$, $df = 1$, p < 0.0001). The prevalence of *R. helvetica* was not significantly different between two rodent species ($\chi^2 = 0.803$, $df = 1$, p = 0.3703). A complete overview of infection prevalence of all pathogens in ticks and rodent biopsies is provided in Table S4.

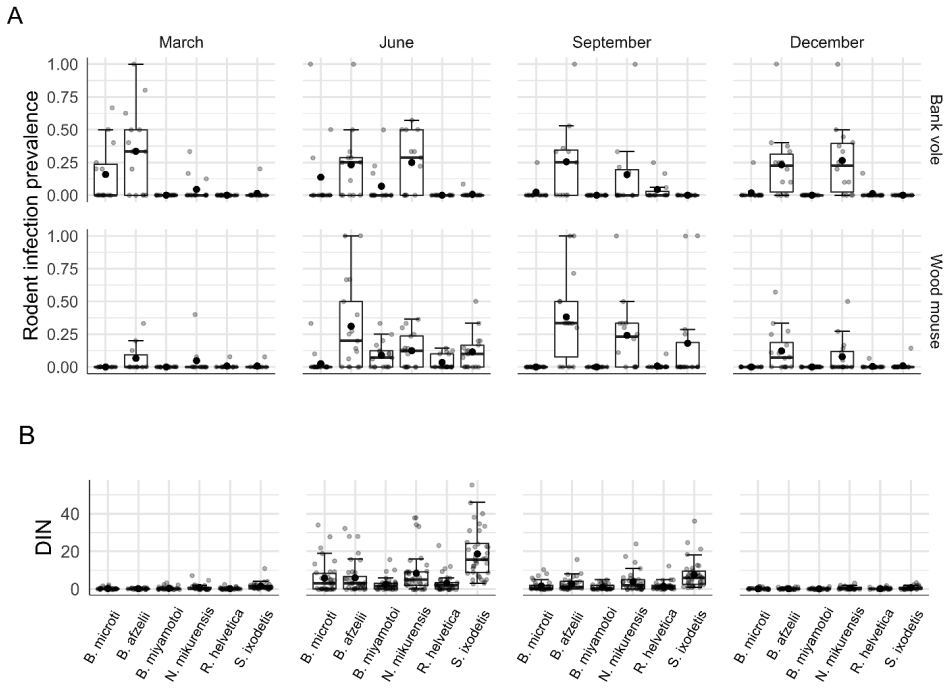


Figure 4. Overview of tick-borne microorganism infections in rodents and nymphs. **a** Rodent infection prevalence separately for each collection month and rodent species. **b** Density of infected nymphs (DIN) separately for each collection month (data combined from 2013 and 2014).

The only pathogen consistently present in both rodent species throughout the year was *B. afzelii* (Figure 4). The infection in rodents persisted despite infected nymphs not being active in months below 7 °C. Other pathogens, such as *N. mikurensis*, *B. miyamotoi*, *R. helvetica,* and *S. ixodetis*, were present in the rodent population mostly when the activity of rodents and (infected) nymphs overlapped (Figure 4).

Rodent density *versus* DON_{t+1}

Equations of all tested models investigating the association between rodent density and DON_{t+1}, NIP_{t+1}, and DIN_{t+1}, their R^2, AIC values, and results of a likelihood test are provided in Table S5. Full equations of the best-fitting models are provided in Table S6, while Table 2 shows significant

interactions incorporated in the models as well as the type of effect rodent density had on all responses (DON_{t+1}, NIP_{t+1} and DIN_{t+1}). Because our treatments affected bank voles and wood mice simultaneously, rodent density data used in the models consist of rodent species added together.

Table 2. Best models for prediction of the density of nymphs (DON), nymphal infection prevalence (NIP), and density of infected nymphs (DIN).

Eq.no.	Response	Equation	Type	Year	Trend
1	DON_{t+1}	$= 1.28 \times \text{rodent density} - 8.75 \times I_{year=2014} + 11.77 \times I_{treatment=control}$	LM	–	↑***
2	NIP_{t+1} *B. afzelii*	$= -3.25 + 0.02 \times \text{rodent density}$	GLM, binomial	–	↑***
3	DIN_{t+1} *B. afzelii*	$= 5.33 + 0.75 \times \text{rodent density}$	LM	–	↑***
4	NIP_{t+1} *N. mikurensis*	$= -2.85 + 0.03 \times \text{rodent density}$	GLM, binomial	–	↑***
5	DIN_{t+1} *N. mikurensis*	$= 6.84 + 1.52 \times \text{rodent density}$	LM	–	↑***
6	NIP_{t+1} *B. miyamotoi*	$= -3.33 + 0.03 \times \text{rodent density} \times I_{year=2014}$	GLM, binomial	2013	↓
				2014	↑**
7	DIN_{t+1} *B. miyamotoi*	$= 4.88 + 0.34 \times \text{rodent density}$	LM	–	↑*
8	NIP_{t+1} *B. microti*	$= -2.93 - 0.02 \times \text{rodent density}$	GLM, binomial	–	↓***
9	DIN_{t+1} *B. microti*	$= 2.64 - 0.04 \times (\text{rodent density})^2 + 1.62 \times \text{rodent density}$	LM	–	↑↓*
10	NIP_{t+1} *B. garinii*	$= -4.28 - 0.04 \times \text{rodent density} + 0.83 \times I_{year=2014}$	GLM, binomial	–	↓***
11	DIN_{t+1} *B. garinii*	$= 3.00$ (*null*)	LM	–	→
12	NIP_{t+1} *R. helvetica*	$= -3.52 + 0.03 \times \text{rodent density} \times I_{year=2014}$	GLM, binomial	2013	→
				2014	↑***
13	DIN_{t+1} *R. helvetica*	$= 3.21 + 0.70 \times \text{rodent density}$	LM	–	↑*
14	NIP_{t+1} *S. ixodetis*	$= -1.04 - 0.01 \times \text{rodent density}$	GLM, binomial	–	↓***
15	DIN_{t+1} *S. ixodetis*	$= 24.94 - 0.12 \times (\text{rodent density})^2 + 5.88 \times \text{rodent density}$	LM	–	↑↓***

Notes: Only significant interactions are shown in the equations; full equations can be found in Table S6. Arrows indicate whether an effect of rodent density was positive, negative or none. Two arrows, one going up and one going down indicate non-linear association (parabola). Asterisks denote significance of an effect (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

The model that fit the data best indicated that rodent density and DON_{t+1} were significantly positively associated ($p = 0.000631$). The best model was a linear model of rodent density incorporating year and treatment as covariates explaining 61% of the variance (Table 2, Eq. 1; Figure 5).

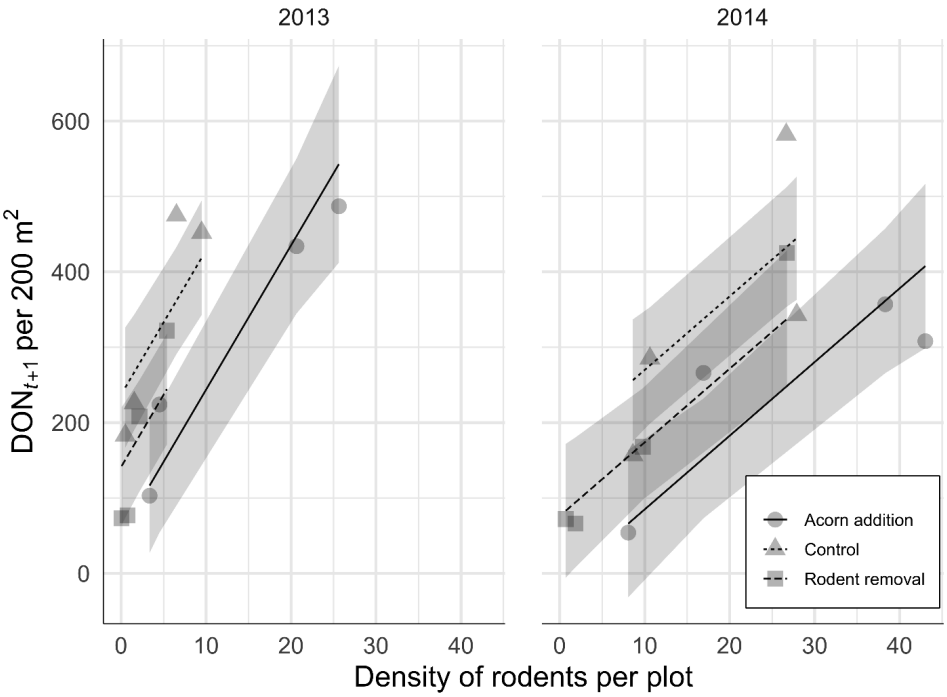


Figure 5. Effect of rodent density on DON_{t+1}. The plot shows the relationships between the number of rodents per plot in year *t* and DON (number per 200 m^2 per plot) in the following year (*t*+1). Rodent density had a significant positive effect on DON in all treatments and years.

Rodent density *versus* rodent-associated pathogens

Regarding *B. afzelii* and *N. mikurensis*, there was a significant positive association between rodent density and NIP_{t+1} ($p < 0.0001$ and $p < 0.0001$), and rodent density and DIN_{t+1} ($p = 0.000187$ and $p < 0.0001$; Figure 6). The best model for both $NIP_{t+1\ B.\ afzelii}$ and $NIP_{t+1\ N.\ mikurensis}$ was a simple generalized linear model of rodent density (Table 2, Eq. 2 and Eq. 4). In the case of DIN_{t+1}, a simple linear model of rodent density was the best and explained 45% and 56% of the variance in $DIN_{t+1\ B.\ afzelii}$ and $DIN_{t+1\ N.\ mikurensis}$, respectively (Table 2, Eq. 3 and Eq. 5). Regarding another pathogen amplified by rodents, *B. microti*, there was a negative effect ($p < 0.0001$) of rodent density on NIP_{t+1} and the best model was a simple generalized linear model of rodent density (Table 2, Eq. 8; Figure 7). In the case of $DIN_{t+1\ B.\ microti}$, the best model was a linear model of rodent density and (rodent density)^2, Table 2, Eq. 9), which explained 20% of the variance. The model, including a quadratic term, allowed to reveal a significant negative ($p = 0.0141$) non-linear association between rodent density and B. microti (Figure 7).

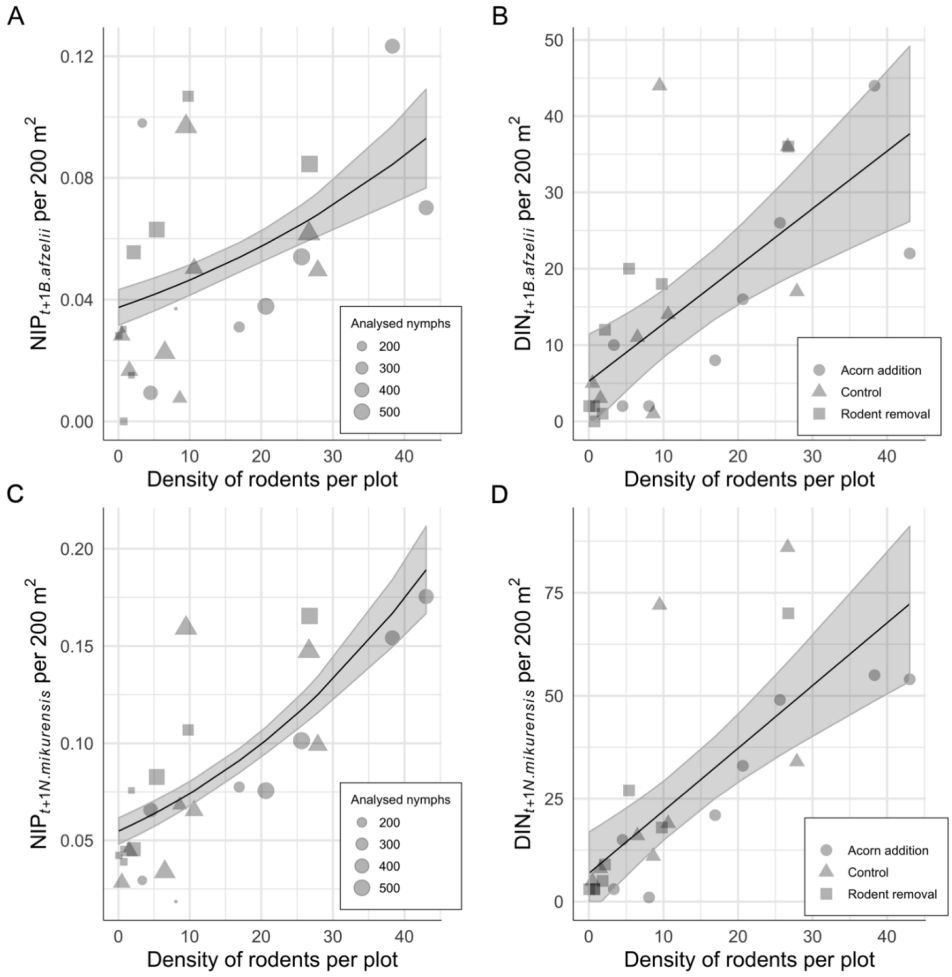


Figure 6. Association between the density of rodents and pathogens amplified by rodents. The graphs show the relationship between the number of rodents per plot in year *t* and NIP and DIN (number per 200 m^2 per plot) in year *t*+1. **a** Effect of rodent density on $NIP_{t+1\ B.\ afzelii}$. Rodent density had a significant positive effect on NIP. **b** Effect of rodent density on $DIN_{t+1\ B.\ afzelii}$. Rodent density has a significant positive effects on DIN. **c** Effect of rodent density on $NIP_{t+1\ N.\ mikurensis}$. Rodent density had a significant positive effect on NIP. **d** Effect of rodent density on $DON_{t+1\ N.\ mikurensis}$. Rodent density had a significant positive effect on DIN.

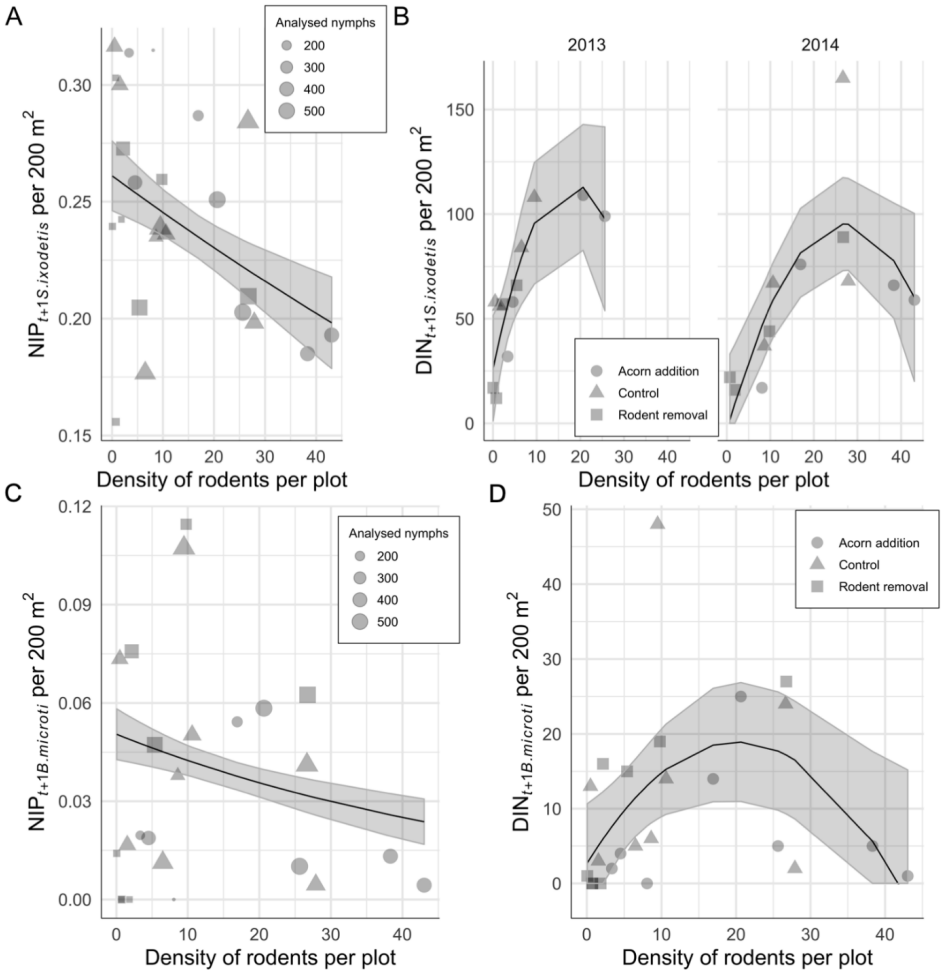


Figure 7. Association between the density of rodents and tick-associated microorganisms. The graphs show the relationship between the number of rodents per plot in year *t* and NIP and DIN (number per 200 m^2 per plot) in year *t*+1. **a** Effect of rodent density on $NIP_{t+1\ S.\ ixodetis}$. Rodent density had significant negative effect on NIP. **b** Effect of rodent density on $DON_{t+1\ S.\ ixodetis}$. Rodent density had a significant non-linear effect on DIN. **c** Effect of rodent density on $NIP_{t+1\ B.\ microti}$. Rodent density had a significant negative effect on NIP. **d** Effect of rodent density on $DIN_{t+1\ B.\ microti}$. Rodent density had a significant non-linear effect on DIN.

Rodent density *versus* a bird-associated pathogen

There was a significant negative association (p = 0.000149) between rodent density and $NIP_{t+1\ B.\ garinii}$ and no association between rodent density and $DIN_{t+1\ B.\ garinii}$ (Figure 8), which remained constant through the experiment. The best model for $NIP_{t+1\ B.\ garinii}$ was a generalized linear model of rodent density and year (Table 2, Eq. 10), while none of the tested models for $DIN_{t+1\ B.\ garinii}$ was better than a null model (Table 2, Eq. 11).

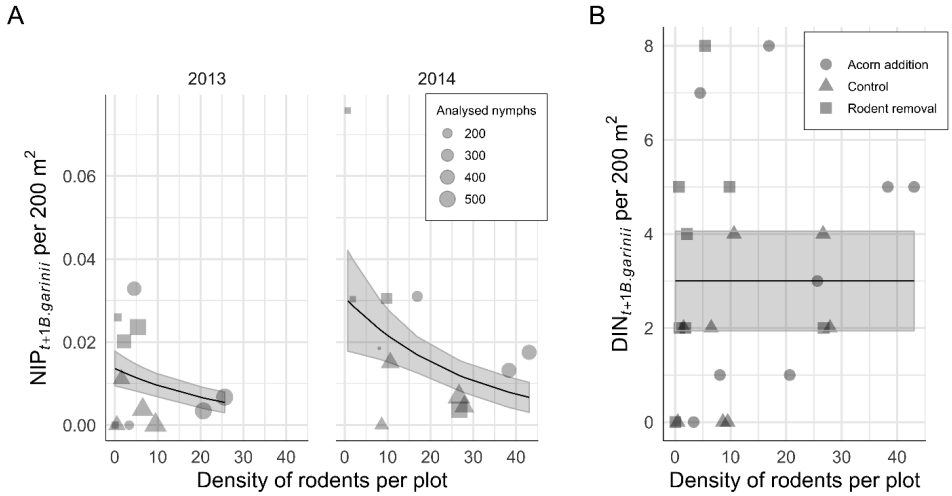


Figure 8. Association between the density of rodents and a pathogen amplified by birds. The graphs show the relationship between the number of rodents per plot in year *t* and NIP and DIN (number per 200 m^2 per plot) in year *t*+1. **a** Effect of rodent density on $NIP_{t+1\ B.\ garinii}$. Rodent density had a significant negative effect on NIP in both years. **b** Effect of rodent density on $DIN_{t+1\ B.\ garinii}$. Rodent density had no effect on DIN.

Rodent density *versus* vertically-transmitted microorganisms

Rodent density had a differential effect on $NIP_{t+1\ R.\ helvetica}$, and a significantly positive effect on $DIN_{t+1\ R.\ helvetica}$ ($p = 0.0143$; Figure 9). In the case of $NIP_{t+1\ R.\ helvetica}$, the best-fitting model was a generalized linear model taking into account the differences in association with respect to year (Table 2, Eq. 12). For $DIN_{t+1\ R.\ helvetica}$, the best model was a simple linear model of rodent density, explaining 21% of the variance (Table 2, Eq. 13).

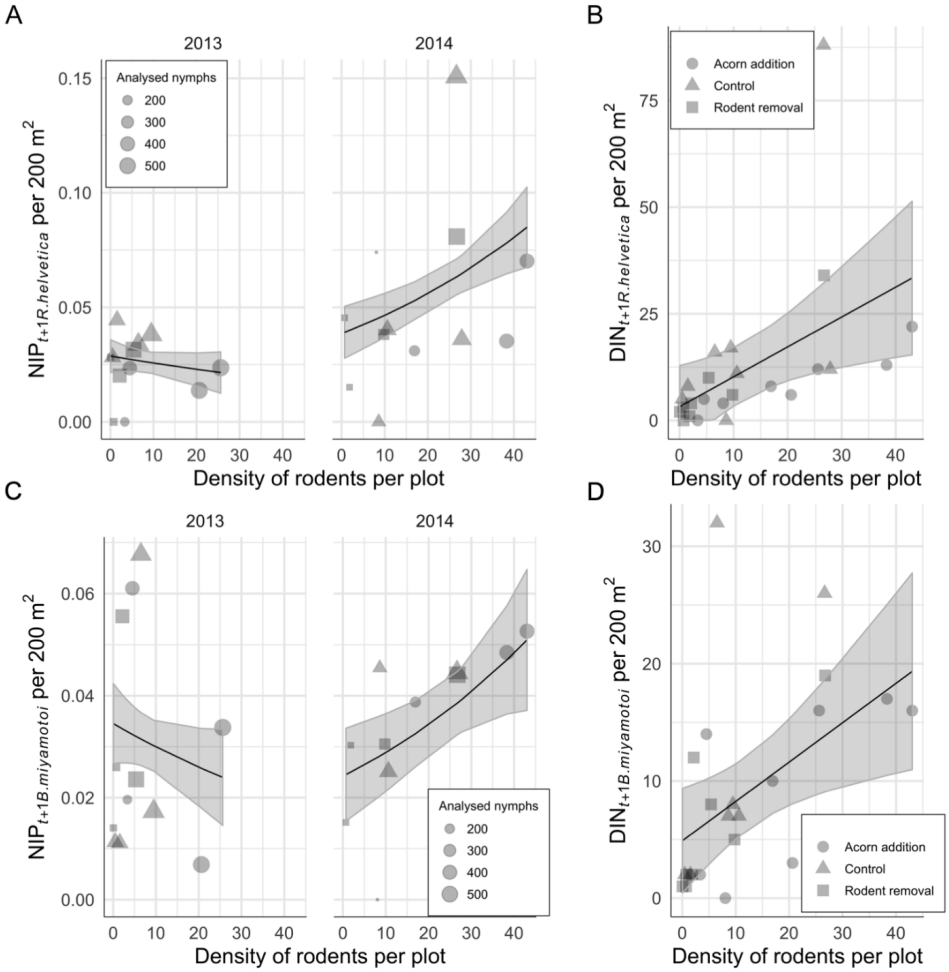


Figure 9. Association between the density of rodents and vertically-transmitted pathogens. The graphs show the relationship between the number of rodents per plot in year t and NIP and DIN (number per 200 m^2 per plot) in year t+1. **a** Effect of rodent density on NIP_{t+1} *R. helvetica*. Rodent density had an inconsistent effect on NIP (no effect in 2013 and significant positive effect in 2014). **b** Effect of rodent density on DON_{t+1} *R. helvetica*. Rodent density had a significant positive effect on DIN. **c** Effects of rodent density on NIP_{t+1} *B. miyamotoi*. Rodent density had an inconsistent effect on NIP (negative but no significant effect in 2013 and significant positive effect in 2014). **d** Effects of rodent density on DON_{t+1} *B. miyamotoi*. Rodent density had a significant positive effect on DIN.

Rodent density had a differential association with $NIP_{t+1\ B.\ miyamotoi}$ between the years (Figure 9). In 2013, the association was negative but not significant ($p = 0.15797$) and in 2014, positive and significant ($p = 0.00862$). The association between rodent density and $DIN_{t+1\ B.\ miyamotoi}$ was significantly positive ($p = 0.0119$; Figure 9). The best model for $NIP_{t+1\ B.\ miyamotoi}$ was a generalized linear model of rodent density, taking into account the differences in association with respect to

year (Table 2, Eq. 6), and for $DIN_{t+1\ B.\ miyamotoi}$, a simple linear model of rodent density explaining only 22% of the variance (Table 2, Eq. 7).

The association between rodent density and $NIP_{t+1\ S.\ ixodetis}$ was significantly negative ($p < 0.0001$) and the best model was a simple generalized linear model of rodent density (Table 2, Eq. 14, Figure 7). In case of $DIN_{t+1\ S.\ ixodetis}$, the best model was a linear model of rodent density and (rodent density)^2, which explained 45% of the variance (Table 2, Eq. 15). The model, including a quadratic term, allowed revealing significant negative ($p = 0.005297$) non-linear association between rodent density and *S. ixodetis* (Figure 7).

Discussion

This study was designed to investigate the association between rodent density and *I. ricinus* nymphs and tick-borne microorganisms. We observed that the densities of rodents affect DON, NIP and DIN in the following year. We found positive associations between rodent density and DON_{t+1} regardless of the year and type of treatment (Figure 5). The NIP_{t+1} and DIN_{t+1} depending on tick-borne pathogens and microorganisms were associated with the rodent density to a different extent, determined by the infection dynamics of the microorganism species (Figs. 6, 7, 9). In addition, although the treatments affected rodent density in the following years, we did not observe any effect on either the DON (Figure 2) or $DIN_{t+1\ B.\ afzelii}$ and $DIN_{t+1\ N.\ mikurensis}$ (Figure 3 and Figure S3).

Rodent density *versus* DON

In all years and plots, we observed a positive association between the DON_{t+1} and rodent density, which as a predictor explained 61% of the variance (Figure 5). Our findings are comparable to previous cross-sectional studies performed in the USA (Ostfeld et al., 2018; Ostfeld et al., 2006) and support that rodents are the main hosts of larval ticks and consistently contribute to a new generation of nymphs in the following year. Regarding the contribution of each rodent species in feeding ticks, wood mice were infested at significantly higher levels with larval ticks than bank voles, which has been reported before (Gassner et al., 2013; Hanincova et al., 2003a; Humair et al., 1993; Kurtenbach et al., 1995; Nilsson & Lundqvist, 1978). The difference in larval tick burden between the two rodent species has been attributed to bank voles acquiring immunity to feeding ticks (Dizij & Kurtenbach, 1995).

Rodent density *versus* pathogens amplified by rodents

As expected, transmission dynamics of *B. afzelii* and *N. mikurensis* were rodent density-dependent. A higher density of rodents increased the probability for larval ticks to feed on an infected rodent and subsequently significantly increased the $NIP_{t+1\ B.\ afzelii}$ and $NIP_{t+1\ N.\ mikurensis}$ (Figure 6). Since DON was also rodent density-dependent, there was a strong synergistic effect of rodent density on $DIN_{t+1\ B.\ afzelii}$ and $DIN_{t+1\ N.\ mikurensis}$ (Figure 6). We observed a significantly higher $NIP_{N.\ mikurensis}$ than $NIP_{B.\ afzelii}$. Possibly, *N. mikurensis*-infected rodents are more infectious than while being infected with *B. afzelii*, which may be due to different tissue tropism of these pathogens in the rodent (Burri et al., 2014; Hanincova et al., 2003a). In addition, *B. afzelii* had a higher infection prevalence in bank voles than in wood mice, which has been reported previously (Coipan et al., 2018; Gassner et al., 2013; Humair et al., 1999; Kybicova et al., 2008; Talleklint et al., 1994). Although one study has reported the opposite, these studies showed that the infectivity of voles was much higher than that

of mice (Gassner et al., 2013; Hanincova et al., 2003a). As mentioned above, the larval infestation was higher in wood mice whilst a greater proportion of bank voles was infected with *B. afzelii*. This indicates that these two rodent species play distinct but complementary roles in *B. afzelii* transmission dynamics.

There was a significantly negative association between rodent density and $NIP_{t+1\ B.\ microti}$ and a non-linear association with $DIN_{t+1\ B.\ microti}$ (Figure 7). We observed a positive association at low and a negative association at high densities of rodents. We detected the parasite almost exclusively in bank voles; thus, our results might be a consequence of the increasing density of wood mouse, which probably is not an amplifying host of *B. microti* (Figure 4). An alternative explanation for this non-linear association might be that *I. ricinus* is not the main vector of this parasite. Previous studies proposed *I. trianguliceps*, a nidicolous rodent tick species as the main vector (Bown et al., 2008; Cayol et al., 2018; Randolph, 1995). It indicates that *B. microti* circulates in the, so called 'cryptic cycle' between specialist ticks and rodents, while *I. ricinus* sporadically becomes infected and perhaps acts as an occasional bridge vector to other host species (Bown et al., 2008).

Rodent density *versus* a pathogen amplified by birds

An increasing density of rodents was negatively associated with $NIP_{t+1\ B.\ garinii}$ (Figure 8). This is probably due to the increased number of nymphs uninfected with *B. garinii*, which fed on the widely abundant rodents, *B. garinii*-incompetent hosts (Kurtenbach et al., 1998b). Our plots were not large enough to cover the territory of birds, *B. garinii*-amplifying hosts (Heylen et al., 2016; Humair et al., 1998), thus, we speculate that all (or the majority) of the collected *B. garinii*-infected nymphs were brought by birds from outside the experimental plots and that these events were more or less constant during the course of the study. The $DIN_{t+1\ B.\ garinii}$ remained unaltered, which suggests that the increase in the DON eliminated the negative effect of rodents on $NIP_{B.\ garinii}$ (Figure 8).

Rodent density *versus* vertically-transmitted tick-borne pathogens and microorganisms

We observed a different association between rodent density and the $NIP_{t+1\ R.\ helvetica}$ depending on the year of study (Figure 9). Although several studies detected *R. helvetica* in rodent blood and skin samples, and in various ectoparasites feeding on rodents, to date, it is not clear which role rodents play in its transmission cycle (Burri et al., 2011; Miťková et al., 2015; Obiegala et al., 2016). Other vertebrates were suggested to be amplifying hosts, for instance, songbirds, which were shown to acquire bacteraemia (Heylen et al., 2016; Hornok et al., 2014). Here, we detected *R. helvetica* in rodent ears of both species; however, it is not possible to infer from our results whether rodents acquire systemic infection. On the other hand, we can speculate that *R. helvetica* causes short-term, localized infection in the skin, which is favourable for co-feeding transmission (Voordouw, 2015), and this transmission route has been attributed to *R. helvetica* on many occasions (Burgdorfer et al., 1979; Heylen et al., 2016; Zemtsova et al., 2010). The infection prevalence in questing nymphs was significantly higher than in rodents, which indicates that ticks are the main amplification hosts of this bacterium. Rodent density was positively associated with $DIN_{t+1\ R.\ helvetica}$, which is unexpected and requires further study (Figure 9).

Rodent density was indifferently associated with $NIP_{t+1\ B.\ miyamotoi}$ (Figure 9). In general, the average NIP in questing ticks was only 3% (CI: 2.6–3.4%), and the fluctuations from year to year were small. *Borrelia miyamotoi* is a predominantly vertically-transmitted bacterium, which means that a

proportion of unfed larvae originating from an infected female tick may also be infected (Scoles et al., 2001; van Duijvendijk et al., 2016). The efficiency of the transmission, in an experimental setting, was shown to vary between 6% and 73% (Scoles et al., 2001). Thus, it is surprising that despite this mode of transmission, the prevalence of *B. miyamotoi* in ticks was not higher. A possible explanation for this could be an inefficient horizontal transmission from infected amplification hosts to naïve ticks as it seems that *B. miyamotoi* does not cause a persistent infection in rodents (Taylor et al., 2013; Wagemakers et al., 2016). Nonetheless, the pathogen can sustain its widespread distribution with only small numbers of ticks being infected. Ultimately, we observed a significant positive effect of rodent density on $DIN_{t+1\ B.\ miyamotoi}$, possibly related to the general increase in DON (Figure 9).

An increasing density of rodents was significantly negatively associated with $NIP_{t+1\ S.\ ixodetis}$ (Figure 7). Decreasing $NIP_{t+1\ S.\ ixodetis}$ and increasing DON along with the increasing rodent density resulted in a non-linear association between rodents and $DIN_{t+1\ S.\ ixodetis}$ (Figure 7). We observed a positive association at low and a negative association at high densities of rodents. Since from the two rodent species investigated in this study we detected *S. ixodetis* almost exclusively in wood mice, a possible explanation is that increasing bank vole populations diluted the prevalence of this bacterium in ticks (Figure 4).

Another tick-borne *Spiroplasma* species has been shown to amplify in rodents only in experimental settings (Tully et al., 1995) and have been reported to cause infections in humans (Aquilino et al., 2015; Lorenz et al., 2002). However, the role of vertebrates in the transmission cycle of S. *ixodetis* in natural conditions is largely unknown. Nevertheless, the detection of *S. ixodetis* in rodent ears indicates that these rodents may facilitate the horizontal transfer of the bacterium to naïve ticks. Our findings are in line with a recent phylogenetic study, which has revealed that horizontal transmission is probably one of the drivers responsible for spreading *S. ixodetis* across the tick community (Binetruy et al., 2019). This transmission mode is proposed in addition to the stable vertical transmission, for which spiroplasmas are known (Fukatsu et al., 2001; Hurst et al., 2003).

Effect of treatments on rodent density, DON, and DIN

The variation in rodent density throughout the season was comparable with studies from other woodland areas (Montgomery, 1989). The density of both rodent species was affected by food resource availability, here acorns, and with our treatment we succeeded to obtain study sites with significantly different densities of rodents (Figure 1). It allowed us to study the effect of rodent density on tick population dynamics and associated pathogen infections. Our results with acorn addition are in accordance with previous findings, where bank vole and wood mouse populations increase after mast years (Clotfelter et al., 2007; Jensen, 1982; McShea, 2000; Ostfeld et al., 1996; Pucek et al., 1993).

Nevertheless, the variation in tick density throughout the years did not follow fluctuations of rodent densities. As a consequence, DON was not affected by our treatment (Figure 2). There was an effect of the treatment on rodents and an effect of rodents on DON; however, the effect of the treatments on rodents was apparently not enough to establish a significant change in DON. This can also be appreciated from considering the size of the confidence bands in Figure 5. It is larger than the vertical distance between the treatment lines. In addition, there was no effect of either acorn addition or rodent removal on $DIN_{B.\ afzelii}$ and $DIN_{N.\ mikurensis}$ in the following years (Figure 3 and Figure S3).

The discrepancy in effect of the treatment indicates that there are additional factors affecting nymphal densities, which expressed high natural variation despite experimental methods. This variation is probably affected by fluctuations in the abundance of other vertebrates and/or meteorological conditions affecting seasonal activity of both rodents and ticks. Although in this study we did not assess abundance of other tick hosts, we observed that nymphal activity was affected by temperature, which has been noticed before (Hartemink et al., 2019; Figure 2 and Figure S2). The onset and annual duration of nymphal activity seemed to be related to a number of months with a mean temperature equal or below 7 °C.

Synchrony in the activity of rodents and ticks and its influence on transmission dynamics of tick-borne microorganisms

In our study, rodent density had differential effects on NIP and DIN depending on the species of tick-borne microorganism, which indicates that there are additional factors playing a role in microorganism dynamics. Some of these factors might be the timing of both activity and infection of rodents and ticks. In temperate European forests, there is a well-documented synchronization between questing larval ticks and rodents, which facilitates the transition of larvae to nymphs (Humair et al., 1999; Randolph, 2002). In addition to driving *I. ricinus* development, rodents contribute to the maintenance of vertically-transmitted microorganisms. However, to propagate horizontally-transmitted tick-borne pathogens, questing larvae have to be synchronized with infected rodents. Depending on the persistence of a pathogen in a rodent population, rodents may infect larvae directly at the onset of the larval activity or after the pathogen has been introduced into the rodent population by infected nymphs. The former situation has been documented for *B. afzelii*, which causes infection in rodents for life, and therefore often persists over winter (Humair et al., 1999). In this study, we observed that *B. afzelii*-infected rodents were, indeed, present throughout the year, also before the onset of ticks (Figure 4).

The latter situation is probably applicable to *N. mikurensis* as the smaller proportion of rodents captured in March was infected with this pathogen than in later months (Figure 4). A possible explanation could be that *N. mikurensis* causes systemic blood infection and decreases the overwintering survival of infected rodents. This phenomenon was observed before in bank voles and Puumala virus (PUUV) despite the expectation that hantaviruses have become well adapted to their rodent hosts during co-evolution (Kallio et al., 2007; Nemirov et al., 2004). Thus, the most favorable scenario for *N. mikurensis* transmission is synchronization in the activity of rodents and infected nymphs right before the onset of larvae (De Boer et al., 1993; Talleklint & Jaenson, 1995). In the Netherlands, nymphs have been shown to start their seasonal activity at least one month before larvae (Hartemink et al., 2019), which seems to be advantageous for zoonotic pathogens overwintering in nymphs rather than in vertebrate hosts.

Study limitations

This study greatly enhanced our understanding about the role of rodents in the dynamics of tick populations and their associated microorganisms. However, we recognize that our semi-experimental approach has logistic limitations on the temporal and spatial extent that must be acknowledged.

First, our results on mechanisms driving the population of nymphs were measured at a relatively small temporal scale, which is only a transition from a larva to a nymph, and do not necessarily hold at a larger scale involving a complete tick life-cycle. A study of many years following all life stages

would have added value and perhaps reveal the robustness of a rodent-tick relationship.

Secondly, the size of the plots was not large enough to cover a territory of other vertebrate species, such as deer and birds, for which we had no data on density fluctuations. Since these vertebrates may substantially contribute to the tick and pathogen cycles, it is advisable to increase a plot size and obtain data on vertebrate abundance/arrival rate by, for instance, camera trapping (Takumi et al., 2019).

In addition, increasing the plot size would be also beneficial for more accurate description of rodent population dynamics. It has been shown that along with growth and maturation, rodents change their home range, and therefore depending on population structure, they might have various effects on tick and pathogen populations (Korn, 1986).

Furthermore, in the first study year (2012), the experiment of acorn addition was already ongoing, thus we have no good baseline density of rodents to compare the effect of treatments to. It is advisable, in future field experiments, to have a longer monitoring period prior to implementation of the intervention, in order to have a solid baseline in place. This would also increase the statistical power to detect the effects of an intervention.

Lastly, it should be borne in mind that there was natural variation between plots, even within the experimental settings. Hence the data are obtained in a complex environment where rodent densities can vary by plot, year or treatment. Tick population and infection dynamics are intricately interwoven with the rodent dynamics, and we realize that a more involved modelling exercise is probably needed to fully understand the ecology. However, in the current approach, our aim was to be 'descriptive' of responses of ticks and their infection, rather than finding the most appropriate mechanistic model.

Conclusions

We demonstrated experimentally that an increase in rodent density positively affects populations of nymphal ticks in the following year. In addition, we show that the prevalence and density of infected ticks with various tick-borne microorganisms are dependent on rodent density to a different extent. These differences probably arose from varying transmission modes of tick-borne microorganisms, and the strongest associations can be observed between rodent density and rodent-associated pathogens that rely on horizontal transmission. Nevertheless, it is not possible to predict disease risk solely on rodent density since we have shown that other factors, independent from our experiment, strongly affected tick density. Our results draw attention to the importance of considering the transmission mode of a pathogen as well as other (spatial and temporal) factors while developing models to predict the tick-borne disease risk.

Acknowledgements

We are grateful to everyone who helped in sample collection during the experiment. We thank all colleagues who provided valuable comments.

Supplement

Text S1. Primers and probes for detection of *B. microti* and *S. ixodetis*, and qPCR protocol.

Detection of pathogens

Babesia microti qPCR

Babesia's from the *B. microti*-like clade were detected using a qPCR, which targets a 104-bp fragment of the Internal Transcribed Spacer region using primers 5'- AAC AGA GGC AGT GTG TAC AAT ACA TTC AGA-3' (Bmicr_ITS_F), 5' CTC ACA CAA CGA TGA AGG ACG CA-3' (Bmicr_ITS_R) and probe 5'-Atto520-GCA+GAA TTT AG+C AAA T+CA ACA GG-BHQ1-3' (Bmicr_ITS_px1).

Spiroplasma ixodetis qPCR

Spiroplasma ixodetis was detected using the primers targeting a 170-bp fragment of the RNA polymerase beta subunit 5'- TGT TGG ACC AAA CGA AGT TG-3' (Spir_rpoB-F), 5'-CCA ACA ATT GGT GTT TGG GG-3' (Spir_rpoB-R) and probe 5'-Atto425-GCT AAC CGT GCT TTA ATG GG-BHQ1-3' (Spri_rpoB-P).

qPCR protocol

All qPCRs were carried out on a LightCycler 480 (Roche Diagnostics Nederland B.V, Almere, the Netherlands) in a final volume of 20 µl with iQ multiplex Powermix, 3 µl of sample, primers with end concentration of 0.2 µM, and probes. Positive plasmid controls and negative water controls were used on every plate tested. Cycling conditions included an initial activation of the iTaq DNA polymerase at 95°C for 5 min, followed by 60 cycles of a 5 s denaturation at 95°C followed by a 35 s annealing-extension step at 60°C (Ramp rate 2.2°C s−1 and a single point measurement at 60°C) and a final cooling cycle of 37°C for 20 s.

Table S1. Daily temperature data from September 2012 to December 2015 collected from the nearest weather station (Deelen, KNMI, the Netherlands). Because of its size, the table is available upon request.

Table S2. Density of nymphs, and number of analysed nymphs for microorganisms per treatment, month and year.

		Density of nymphs per 200 m^2				Analysed for microorganisms			
Treatment	**Month**	**Year**				**Year**			
		2012	**2013**	**2014**	**2015**	**2012**	**2013**	**2014**	**2015**
Control									
	1		0	4	1		0	3	1
	2		0	2	0		0	1	0
	3		5	32	7		5	32	7
	4		14	202	45		14	132	44
	5		454	260	192		160	150	151
	6		581	238	502		160	158	160
	7		795	268	152		160	117	101
	8		440	133	217		160	128	145
	9	115	289	103	113	87	160	98	111
	10	113	173	61	105	113	137	60	94
	11	39	46	20	26	33	43	20	24
	12	0	8	13	7	0	6	13	7
	Total	**267**	**2,805**	**1,336**	**1,367**	**233**	**1,005**	**912**	**845**
Acorn addition									
	1		0	5	0		0	5	0
	2		0	2	0		0	2	0
	3		20	75	8		20	70	8
	4		8	200	33		8	125	33
	5		463	212	139		130	131	113
	6		346	225	361		151	134	136
	7		483	222	103		152	142	84
	8		379	107	162		138	101	101
	9	79	198	99	65	76	125	94	64
	10	68	116	74	90	68	92	71	78
	11	27	25	16	10	17	20	16	9
	12	0	8	11	14	0	8	11	12
	Total	**174**	**2,046**	**1,248**	**985**	**161**	**844**	**902**	**638**
Rodent removal									
	1		0	2	0		0	2	0
	2		0	1	0		0	1	0
	3		4	22	8		3	22	8
	4		7	90	52		7	78	42
	5		369	155	91		139	125	74
	6		450	142	283		147	112	131
	7		631	100	88		157	98	80
	8		365	43	101		136	39	93
	9	74	169	47	47	66	109	45	47
	10	62	97	53	35	61	77	53	34
	11	19	26	22	23	9	19	22	23
	12	0	4	3	3	0	4	3	3
	Total	**155**	**2,122**	**680**	**731**	**136**	**798**	**600**	**535**

Table S3. Density of rodents, and number of analysed individuals for microorganisms per rodent species, treatment, month and year.

		Wood mouse						Bank vole					
		Density per plot *			**Analysed for microorganisms**			**Density per plot ***			**Analysed for microorganisms**		
Treatment	**Month**	**Year**			**Year**			**Year**			**Year**		
		2012	**2013**	**2014**	**2012**	**2013**	**2014**	**2012**	**2013**	**2014**	**2012**	**2013**	**2014**
Control													
	3		3.6	18.4		1	18		15.9	11.6		9	10
	6		5.0	50.6		3	45		3.0	44.0		3	18
	9	27.5	10.8	24.3	19	3	11	24.6	8.5	46.5	9	4	20
	12	22.1	24.2	47.4	14	18	26	34.6	12.0	51.7	5	9	22
	Total	**49.5**	**43.6**	**140.7**	**33**	**25**	**100**	**59.2**	**39.4**	**153.7**	**14**	**25**	**70**
Acorn addition													
	3		13.0	47.9		4	37		36.7	29.8		14	20
	6		11.6	103.8		8	47		14.2	57.7		6	13
	9	44.4	13.0	55.4	32	8	30	17.8	40.6	53.1	5	5	14
	12	31.6	71.4	32.7	10	36	23	47.8	35.3	37.2	8	17	18
	Total	**76.0**	**109.0**	**239.8**	**42**	**56**	**137**	**65.6**	**126.7**	**177.8**	**13**	**42**	**65**
Rodent removal													
	3		1.0	3.0		1	3		3.0	5.9		1	6
	6		1.0	24.4		0	20		0.0	21.9		0	13
	9	44.4	0.6	9.1	27	0	6	23.8	8.0	31.1	4	1	19
	12	8.8	9.2	28.0	3	6	19	10.3	10.5	35.2	3	8	10
	Total	**53.2**	**11.8**	**64.5**	**30**	**7**	**48**	**34.1**	**21.5**	**94.1**	**7**	**10**	**48**

* Rodent density was calculated per species according to the Schnabel method (Schnabel 1938).

Table S4. Prevalences of tick-borne microorganisms in rodents and nymphs. The table continues on the following page.

Microorganism	Wood mouse			Bank vole			Chi-squared
	N = 478	%	95% CI	N = 294	%	95% CI	p value
B. microti	2	0.4	(-0.2-1.0)	20	6.8	(3.9-9.7)	0.0
B. burgdorferi s.l.	107	22.4	(18.6-26.1)	83	28.2	(23.1-33.4)	0.0694
*B. afzelii**	107	22.4	(18.6-26.1)	83	28.2	(23.1-33.4)	0.0694
B. burgdorferi s.s.	-	-	-	-	-	-	-
B. garinii	-	-	-	-	-	-	-
B. valaisiana	-	-	-	-	-	-	-
B. miyamotoi	15	3.1	(1.6-4.7)	5	1.7	(0.2-3.2)	0.2326
N. mikurensis	73	15.3	(12.0-18.5)	62	21.1	(16.4-25.8)	0.0396
R. helvetica	13	2.7	(1.3-4.2)	5	1.7	(0.2-3.2)	0.3703
S. ixodetis	33	6.9	(4.6-9.2)	3	1.0	(-0.1-2.2)	0.0002

* *Borrelia burgdorferi* s.l. in rodent biopsies has been assumed to be *B. afzelii* (Coipan et al., 2018; Jahfari et al., 2017; Mysterud et al., 2019)

Microorganism	Nymphs Total			2012			2013			2014			2015		
	N= 7609	%	95% CI	N = 530	%	95% CI	N = 2647	%	95% CI	N = 2414	%	95% CI	N = 2018	%	95% CI
B. microti	38	0.5	(0.3-0.7)	8	1.5	(0.5-2.5)	10	0.4	(0.1-0.6)	4	0.2	(0.0-0.3)	16	0.8	(0.4-1.2)
B. burgdorferi s.l.	1,015	13.3	(12.6-14.1)	62	11.7	(9.0-14.4)	397	15.0	(13.6-16.4)	273	11.3	(10.0-12.6)	283	14.0	(12.5-15.5)
B. afzelii	489	6.4	(5.9-7.0)	34	6.4	(4.3-8.5)	215	8.1	(7.1-9.2)	110	4.6	(3.7-5.4)	130	6.4	(5.4-7.5)
B. burgdorferi s.s.	9	0.1	(0.0-0.2)	1	0.2	(-0.2-0.6)	1	0.0	(0.0-0.1)	7	0.3	(0.1-0.5)	0	0.0	(0.0-0.0)
B. garinii	109	1.4	(1.2-1.7)	6	1.1	(0.2-2.0)	48	1.8	(1.3-2.3)	25	1.0	(0.6-1.4)	30	1.5	(1.0-2.0)
B. valaisiana	41	0.5	(0.4-0.7)	0	0.0	(0.0-0.0)	17	0.6	(0.3-0.9)	9	0.4	(0.1-0.6)	15	0.7	(0.4-1.1)
B. miyamotoi	227	3.0	(2.6-3.4)	16	3.0	(1.6-4.5)	62	2.3	(1.8-2.9)	74	3.1	(2.4-3.8)	75	3.7	(2.9-4.5)
N. mikurensis	928	12.2	(11.5-12.9)	59	11.1	(8.5-13.8)	456	17.2	(15.8-18.7)	173	7.2	(6.1-8.2)	240	11.9	(10.5-13.3)
R. helvetica	322	4.2	(3.8-4.7)	24	4.5	(2.8-6.3)	112	4.2	(3.5-5.0)	63	2.6	(2.0-3.2)	123	6.1	(5.1-7.1)
S. ixodetis	1,745	22.9	(22.0-23.9)	108	20.4	(16.9-23.8)	584	22.1	(20.5-23.6)	581	24.1	(22.4-25.8)	472	23.4	(21.5-25.2)

Table S5. All tested models for prediction of density of nymphs (DON), nymphal infection prevalence (NIP), and density of infected nymphs (DIN). Best models with highest R2, lowest AIC, and lowest significant LogLik are written in bold. If there was not a best model, the simpler model was selected. None of the models for DINt+1 of B. microti and B. garinii was better than a null model.

Response	Predictor	Covariate	R^2	AIC	Likelihood ratio test	
					LogLik	Chisq
DON_{t+1}	~ 1			190	-93.121	
	~ Rodent density $_{t-1}$		0.27	184	-88.869	**
		+ Year	0.48	176	-84.192	**
		* Year	0.48	177	-83.642	
		+ Year + Treatment	0.58	173	-80.546	*
		*** Year + Treatment**	**0.61**	**172**	**-78.826**	**.**
		* Year * Treatment	0.60	175	-74.368	
NIP_{t+1} *B. afzelii*	~ 1		-	290	-144.064	
	~ Rodent density $_{t-1}$		**-**	**252**	**-123.789**	***
		+ Year	-	253	-123.734	
		* Year	-	254	-123.0.76	
DIN_{t+1} *B. afzelii*	~ 1			197	-96.524	
	~ Rodent density $_{t-1}$		**0.45**	**184**	**-88.750**	***
		+ Year	0.47	184	-87.896	
		* Year	0.44	186	-87.889	
		+ (Rodent density)^2	0.46	184	-88.075	
NIP_{t+1} *N. mikurensis*	~ 1		-	369	-183.60	
	~ Rodent density $_{t-1}$		**-**	**233**	**-114.27**	***
		+ Year	-	233	-113.67	
		* Year	-	235	-113.67	
DIN_{t+1} *N. mikurensis*	~ 1			226	-111.097	
	~ Rodent density $_{t-1}$		**0.56**	**207**	**-100.650**	***
		+ Year	0.56	208	-100.021	
		* Year	0.54	210	-99.978	
		+ (Rodent density)^2	0.59	206	-99.235	*

table continues

Response	Predictor	Covariate	R^2	AIC	Likelihood ratio test	
					LogLik	Chisq
NIP_{t+1} *B. miyamotoi*	~ 1		-	194	-96.145	
	~ Rodent density $_{t-1}$		-	192	-93.927	*
		+ Year	-	193	-93.742	
		*** Year**	**-**	**188**	**-90.119**	**
DIN_{t+1} *B. miyamotoi*	~ 1			173	-84.746	
	~ Rodent density $_{t-1}$		**0.22**	**168**	**-81.216**	**
		+ Year	0.22	169	-80.648	
		* Year	0.19	171	-80.523	
		+ (Rodent density)^2	0.20	170	-80.966	
NIP_{t+1} *B. microti*	~ 1		-	348	-172.94	
	~ Rodent density $_{t-1}$		**-**	**333**	**-164.75**	***
		+ Year	-	335	-164.67	
		* Year	-	335	-163.29	.
DIN_{t+1} *B. microti*	~ 1			190	-92.767	
	~ Rodent density $_{t-1}$		-0.03	191	-92.562	
		+ Year	-0.04	192	-92.202	
		* Year	-0.07	194	-91.879	
		+ (Rodent density)^2	**-0.20**	**186**	**-89.039**	*
NIP_{t+1} *B. garinii*	~ 1		-	178	-88.145	
	~ Rodent density $_{t-1}$		-	175	-85.562	*
		+ Year	**-**	**163**	**-78.658**	***
		* Year	-	165	-78.495	
DIN_{t+1} *B. garinii*	~ 1			115	-55.556	
	~ Rodent density $_{t-1}$		0.01	116	-54.934	
		+ Year	-0.02	117	-54.643	
		* Year	-0.06	119	-54.593	
		+ (Rodent density)^2	-0.04	118	-54.930	

table continues

Response	Predictor	Covariate	R^2	AIC	Likelihood ratio test	
					LogLik	Chisq
NIP_{t+1} *R. helvetica*	~ 1		-	320	-159.01	
	~ Rodent density $_{t-1}$		-	275	-135.40	***
		+ Year	-	249	-121.75	***
		*** Year**	**-**	**245**	**-118.39**	**
DIN_{t+1} *R. helvetica*	~ 1			210	-102.943	
	~ Rodent density $_{t-1}$		**0.21**	**205**	**-99.597**	**
		+ Year	0.17	207	-99.553	
		* Year	0.15	209	-99.279	
		+ (Rodent density)^2	0.22	206	-98.939	
NIP_{t+1} *S. ixodetis*	~ 1		-	234	-116.08	
	~ Rodent density $_{t-1}$		**-**	**219**	**-107.30**	***
		+ Year	-	217	-105.46	·
		* Year	-	219	-105.43	
DIN_{t+1} *S. ixodetis*	~ 1			243	-119.63	
	~ Rodent density $_{t-1}$		0.24	238	-115.83	**
		+ Year	0.31	236	-114.09	·
		* Year	0.32	237	-113.32	
		+ (Rodent density)^2	**0.45**	**231**	**-111.28**	*

Asterisks denote significance of an effect (· = p ≤ 0.1; * = p ≤ 0.05; ** = p ≤ 0.01; *** = p ≤ 0.001).

Table S6. Full equations for the best fitting models for prediction of density of nymphs (DON), nymphal infection prevalence (NIP), and density of infected nymphs (DIN).

No.	Response	Equation
1	DON_{t+1}	$= 6.32 + 1.28 \times \text{rodent density} - 8.75 \times I_{\text{year=2014}} + 11.77 \times I_{\text{treatment=control}} - 5.00 \times I_{\text{treatment=rodent removal}} - 0.54 \times \text{rodent density} \times I_{\text{year=2014}}$
2	NIP_{t+1} *B. afzelii*	$= -3.25 + 0.02 \times$ rodent dens
3	DIN_{t+1} *B. afzelii*	$= 5.33 + 0.75 \times$ rodent dens
4	NIP_{t+1} *N. mikurensis*	$= -2.85 + 0.03 \times$ rodent dens
5	DIN_{t+1} *N. mikurensis*	$= 6.84 + 1.52 \times$ rodent densi
6	NIP_{t+1} *B. miyamotoi*	$= -3.33 - 0.01 \times$ rodent density $- 0.36 \times I_{\text{vear=2014}} + 0.03 \times$ rodent density $\times$
7	DIN_{t+1} *B. miyamotoi*	$= 4.88 + 0.34 \times$ rodent dens
8	NIP_{t+1} *B. microti*	$= -2.93 - 0.02 \times$ rodent den
9	DIN_{t+1} *B. microti*	$= 2.64 - 0.04 \times$ rodent density $^2 + 1.62 \times$ rodent de
10	NIP_{t+1} *B. garinii*	$= -4.28 - 0.04 \times$ rodent density $+ 0.83 \times I_{\text{vea}}$
11	DIN_{t+1} *B. garinii*	$= 3.00$ (*null*)
12	NIP_{t+1} *R. helvetica*	$= -3.52 - 0.01 \times$ rodent density $+ 0.31 \times I_{\text{year=2014}} + 0.03 \times$ rodent density $\times I_{\text{year=2014}}$
13	DIN_{t+1} *R. helvetica*	$= 3.21 + 0.70 \times$ rodent density
14	NIP_{t+1} *S. ixodetis*	$= -1.04 - 0.01 \times$ rodent density
15	DIN_{t+1} *S. ixodetis*	$= 24.94 - 0.12 \times$ rodent density $^2 + 5.88 \times$ rodent density

Figure S1. An example of an experimental plot 50 × 50 m surrounded by screens to study the effect of rodent density on the density of infected nymphs (Photograph: G. van Duijvendijk)

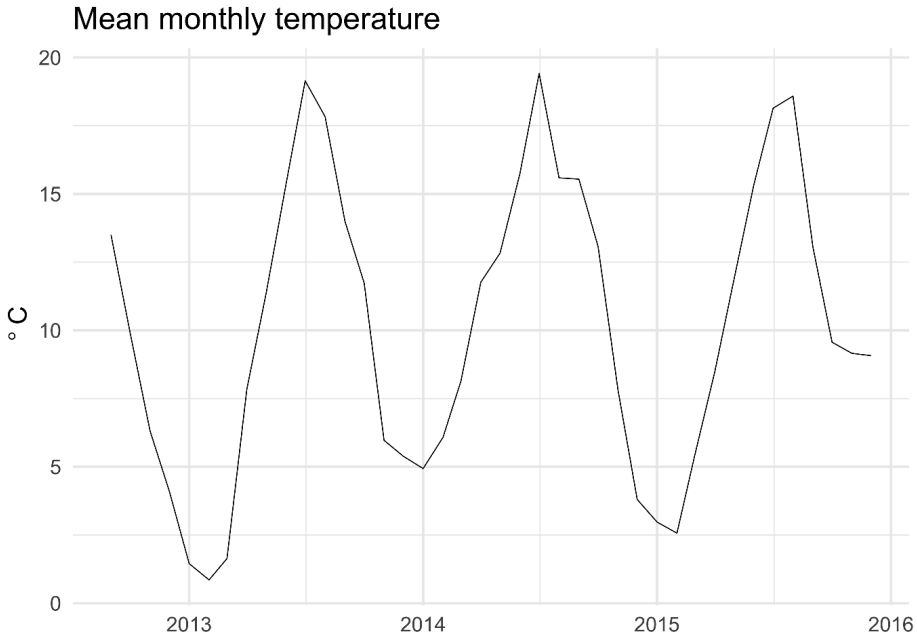


Figure S2. Mean monthly temperature (in °C) from August 2012 to December 2015.

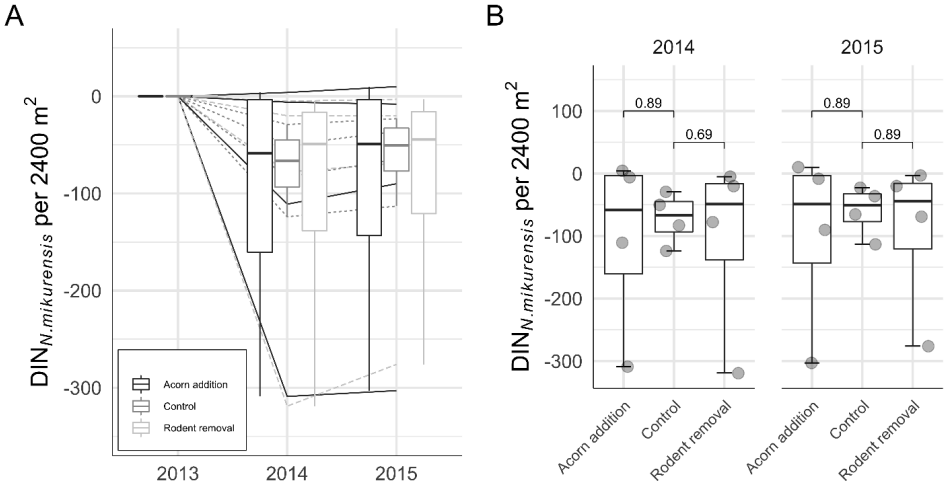
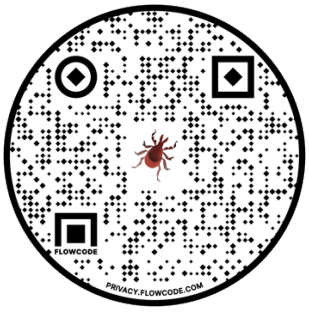


Figure S3. a Density of nymphs infected with *N. mikurensis* (DIN $_{N.\ mikurensis}$) in 2014 and 2015 in all three treatments in comparison to 2013 (baseline year). **b** Differences in DIN $_{N.\ mikurensis}$ between the treatments in two separate years calculated with the Wilcoxon test with a correction for a baseline year (2013). The overall differences between the treatments were not significant either in 2014, or 2015 ($p = 0.87$ and $p = 0.94$, respectively).



Please click here (for the online version) or scan this QR code to listen to an immersive soundscape of a forest in Herperduin, the Netherlands recorded by Vitalij Kuzkin. The Herperduin forest is one of the study sites where I have collected samples for this research. Listen with headphones for the best experience. The full album is available on: ticksandmicrobes.bandcamp.com

Chapter 5

Spatiotemporal variation of heritable *Ixodes ricinus* symbionts is associated with climatic factos

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To be submitted in a modfied form

Abstract

In a longitudinal study, we investigated the dynamics behind the little-understood variation in the infection prevalence of vertically-transmitted pathogens, like *Midichloria mitochondrii* and *Rickettsia helvetica*. We hypothesised that, if present, these variations are associated with climatic factors to a larger degree than in the case of horizontally-transmitted pathogens, such as *Borrelia burgdorferi* sensu lato, and *Neoehrlichia mikurensis*. For that, questing nymphs of Ixodes ricinus from 12 locations in the Netherlands were collected monthly for four years, and the infection prevalences for *B. burgdorferi* s.l., *B. miyamotoi*, *N. mikurensis*, *R. helvetica,* and *M. mitochondrii* determined. The associations of those prevalences with precipitation, relative humidity, temperature, and evaporation rate were investigated. All but *B. miyamotoi* presented spatio-temporal variation. The prevalence of *R. helvetica* was the most variable and positively associated with all climatic factors, except for temperature. *Midichloria mitochondrii* and, surprisingly, *N. mikurensis* were mainly associated with evaporation rate and relative humidity, the former negatively and the latter positively. Concerning *B. miyamotoi* and *B. burgdorferi* s.l., a weak and no association were detected, respectively. We propose that the effect of climatic factors on the infection prevalences of tick bacteria depends on bacterial transmission route and type of symbiosis with Ixodes ricinus. Our findings imply that the infection prevalence of vertically-transmitted pathogens such as *R. helvetica* is affected by climatic factors, which ultimately could explain their regional differences.

Keywords: GLM, Transmission dynamics, Tick-borne diseases, Tick symbionts, Macro climatic conditions, Infection prevalence, *Ixodes ricinus* symbionts

Introduction

Apart from bearing human and animal pathogens, *Ixodes ricinus* ticks may harbour bacterial symbionts suspected to predominantly confer fitness benefits (Bonnet et al., 2017; Duron et al., 2017). Recently, there is an increasing interest in the role of microorganisms in the life cycle of ticks and its vectorial capacity and competence (Gall et al., 2016; Narasimhan & Fikrig, 2015; Weiss & Aksoy, 2011). In the long-term, understanding these roles can lead to the development of novel and sustainable ways to combat ticks and tick-borne diseases as it has been demonstrated for other arthropods (Mancini et al., 2016; Ricci et al., 2011).

Understanding factors driving the density of nymphs (DON) is of interest because humans are predominantly exposed to and therefore infected with tick-borne pathogens transmitted by *I. ricinus* nymphs (Hofhuis et al., 2017; Robertson et al., 2000). *Ixodes ricinus* survival largely relies on finding a vertebrate host, which species may vary between life stages (Gray, 1998a; Hofmeester et al., 2016). However, *I. ricinus* spends the vast majority of its life in vegetation, questing for a host, moulting, or diapausing. During these phases, ticks are challenged by often not optimal climatic conditions. In other arthropods, bacterial symbionts have been shown to provide ecologically contingent benefits; however, it is unclear how unfavourable weather conditions affect nymphal infection prevalence (NIP) of tick symbionts.

Bacteria suspected to be involved in the ecology and physiology of *I. ricinus* include intracellular heritable *Candidatus* Midichloria mitochondrii (hereafter *M. mitochondrii*) and *Rickettsia helvetica* (Duron et al., 2017; Lo et al., 2006; Sprong et al., 2009). They are maintained in tick populations predominantly via vertical transmission route, meaning from a female tick to her offspring. Both symbionts are highly prevalent in *I. ricinus*, and ticks are considered to be their main hosts. However, their functional significance for ticks and what determines their NIP remains mostly unknown. The latter is of particular interest since next to a mutualistic relationship with ticks, *R. helvetica* has been shown to cause disease in humans (Fournier et al., 2000; Nilsson et al., 2010), while *M. mitochondrii* DNA has been detected in several mammalian species, indicating potential pathogenicity (Bazzocchi et al., 2013).

This study analysed the spatio-temporal variation in NIPs of tick symbionts *R. helvetica* and *M. mitochondrii* over four years in 12 locations in the Netherlands. We also explored NIPs' associations with changing climatic factors, including relative humidity, evaporation rate, precipitation, and temperature. The observed spatial and seasonal patterns were contrasted with vertically-transmitted pathogen *Borrelia miyamotoi*, and horizontally-transmitted pathogens *B. burgdorferi* sensu lato, and *Neoehrlichia mikurensis*. The NIPs of these bacteria are to be primarily determined by the abundance and composition of vertebrates (Chapter 4; Hofmeester et al., 2016a; Takumi et al., 2019), and therefore due to the differences in life cycles, we expected to observe differences in NIP variations between symbionts and pathogens. Studying the distribution of tick bacteria across time and space and its associations with climatic factors can contribute to our understanding of tick-symbiont interactions and improve risk assessment of tick-borne diseases.

Materials and methods

Tick collection

The *I. ricinus* tick samples analysed in this study were derived from a more extensive, long-term study, which was performed between 2006 and 2016 in the context of the Dutch phenological network De Natuurkalender (Nature's Calendar; Garcia-Marti et al., 2018; Gassner et al., 2011; Hartemink et al., 2019; Takken et al., 2017). Nymphal ticks of *I. ricinus* were collected at the beginning of each month by trained volunteers in 12 sites of the Netherlands (see Figure 1) between January 2012 and December 2015.

Questing ticks were collected at each site by dragging a white cotton cloth (1m2) over two transects of 100m2 each. The cloth was inspected every 25 meters, and ticks were collected using forceps. All samples were placed in individual containers with 70% ethanol, counted, and stored at -20oC until DNA extraction. The ixodid species and life stage identification were performed by the research team in Wageningen.

Collection sites are mainly forest areas with a mixture of deciduous and non-deciduous trees, characterised by a diversity of ground cover. The exceptions to these general characteristics are Kwade Hoek, Schiermonnikoog, and Wassenaar, that are coast/dune areas; and Twiske, which is a marsh area. Kwade Hoek presents the most marginal habitat of the group, being dominated by coastal shrub. These sites were selected due to the presence of *I. ricinus* ticks and the availability of volunteers to perform the monthly collections.

A total of 18 from the 576 planned collections were not performed due to a lack of proper conditions, which can be consulted in Table S11. The number of collected nymphs per site, month, and year are provided in Table S1. Additional details regarding the habitat's descriptions and geographic coordinates of each site are provided in Table S9.

Climatic data

Weather data was obtained from the data center of the Royal Netherlands Meteorological Institute (KNMI) (available at https://data.knmi.nl/datasets). The data measurements and calculations are described in Hartemink et al. (2019). Climatic factors used in this study included mean temperature, precipitation, evaporation rate, relative humidity, vapour pressure and saturation deficit.

DNA Extraction and Bacteria Detection

Collected nymphs were processed individually. DNA from a subset of nymphs per month per location was extracted with ammonium hydroxide as previously described (Wielinga et al., 2006). The lysates were stored at 4°C prior to molecular analysis. Samples were analysed with different (multiplex) real-time PCRs, based on various target genes depending on microorganism of interest such as *B. burgdorferi* s.l. (Heylen et al., 2013b), *B. miyamotoi* (Hovius et al., 2013), *N. mikurensis* (Jahfari et al., 2012), *R. helvetica* (de Bruin et al., 2015) and *M. mitochondrii* (Chapter 2). Targeted genes are provided in Table S12. All qPCRs were carried out on a LightCycler 480 (Roche Diagnostics Nederland B.V, Almere, The Netherlands) in a final volume of 20 µl with iQ multiplex Powermix, 3 µl of a sample, primers with end concentration of 0.2 µM, and probes. Positive plasmid controls and negative water controls were used on every plate tested. Cycling

conditions included an initial activation of the iTaq DNA polymerase at 95°C for 5 min, followed by 60 cycles of a 5 s denaturation at 95°C followed by a 35 s annealing-extension step at 60°C (ramp rate 2.2°C s−1 and a single point measurement at 60°C) and a final cooling cycle of 37°C for 20 s. To minimise contamination and false-positive samples, the DNA extraction, PCR mix preparation, sample addition, and qPCR analyses were performed in separated air locked dedicated labs.

Spatio-temporal variables

To investigate the spatio-temporal dynamics of infection prevalence in nymphal ticks (NIP), the response variables *M. mitochondrii*, *N. mikurensis*, *R. helvetica*, *B. burgdorferi* s.l. and *B. miyamotoi* prevalence were modelled using the Generalised Linear Model (GLM) approach. The binomial distribution with the logit link function were applied to the "glm" function of the R package "stats" (R Core Team, 2020). NIP values were determined as the fraction of positive nymphs for each bacterium per total of tested nymphs, randomly selected for qPCR screening from the collections per transect and month (0-30 nymphs, depending on availability). The density of questing nymphs (DON) was calculated using the number of collected nymphs per 100m^2. In most cases, the prevalences were initially calculated per 200m2 (two transects); however, since not all locations had two transects for all years, the prevalence was divided by two.

Location was used as the spatial explanatory variable. Given that we wanted to test if climatic factors were associated with the infection prevalence variation of vertically-transmitted *I. ricinus* symbionts, season and year were used to explore the symbionts temporal prevalence variation. Seasonality is a natural phenomenon. However, how we define seasons is less dependent on specific weather conditions, and therefore natural cycles, and more dependent on four equal divisions of the year. Therefore, we redefined seasons monthly variation per year based on temperature data. Winter was defined as the no-tick-activity season (as an approximation due to the lack of data, explained in further detail below). This approach allowed us to define seasons individually for each year and as close as possible to the different activity phases of *I. ricinus* nymphs. However, without an additional detailed study of the nymphal activity in each different period, some bias was certainly introduced. Months with mean temperatures ≥ 15°C were included in summer and months with mean temperatures < 15°C were selected as spring (if included between February and July) and autumn (if included between September and November). Although some nymphal *I. ricinus* activity was detected during winter for this database (Table S1; Gassner et al., 2011; Hartemink et al., 2019), due to the missing data and a low number of obtained and tested nymphs for this season in most locations (see Table S1 and Table S11 for additional details), no prevalence data was used for winter periods to avoid interpretation errors. In other words, we did not include and test winter prevalence data in this study. Considering that previous studies demonstrated that *I. ricinus* nymphs higher onset of questing begin when temperatures are ≥ 7°C (Perret et al., 2000; Randolph, 2004; Tagliapietra et al., 2011), months with maximum temperatures < 7°C were defined as winter. December and January were always considered winter months due to the low number of ticks collected and an abundance of missing data (Table S1 and Table S11).

Global and individual effects on NIP of year, as well as the individual effect of each season and location, were tested by applying these explanatory variables to the models in their factor and discrete forms. Individual effects on NIP of each explanatory variable were tested using "Anova" function and the Wald test from the R package "car" (Fox & Weisberg, 2019), to which the type II sum of squares was applied to models without interaction terms and the type III to models with

interaction terms. Summaries of the models were obtained using the "summary" function of the "base" R package (R Core Team, 2020).

By last, pairwise comparisons of estimated marginal means of the final models were calculated using "emmeans" and the "pair" functions of the "emmeans" package (Lenth et al., 2020). The ps were obtained using the tukey method.

Macro-climatic variables

Climatic data was calculated based on the mean values per season. To test if macro-climatic factors had influence on symbionts NIP variation, two models including climatic data as explanatory variable were constructed by using the same previously described GLM method. In the first model, the climatic variables from the same year and season as the NIP (NIP^{T}) were used as the explanatory variables. In the second model, the climatic variables were added to a model with NIP data from the following season (NIP^{T+1}). This approach allowed to test, for instance, if the spring climatic data influenced the symbiont prevalence variation observed in summer. The summary and analysis of deviance to assess the significance of the individual predictor terms, as previously described to the spatio-temporal model, were also applied in these models. Models predictions, using "plot_model" function of the "sjPlot" package (Lüdecke, 2019), were used to access the effect of individual climatic variables on NIP. Additionally, multiple pairwise comparisons of climatic factors from different locations and seasons were performed based on method "anova".

Goodness of fit

Regarding how well the obtained statistical models fit the data and how much variation is explained, the following parameters were used: (1) analysis of variance using the chi-squared test to assess if there was a significant difference between the residual deviance of the model in comparison to its null model, using "anova" function from R package "stats" (R Core Team, 2020); (2) log-likelihood, AIC and BIC parameters were obtained with "glance" function from "broom" package (Robinson & Hayes, 2020) to compare best fit models of each dataset; (3) pseudo R2 values (ρ2) were obtained with "nagelkerke" function from the "rcompanion" package (Mangiafico, 2020) to calculate the explained variation of each model; and (4) the brier score was obtained with the "BrierScore" function from the "DescTools" package (R Core Team, 2020) to assess the quality of the probabilistic predictions.

Collinearity tests were performed using the "vif" function of the "car" package (Fox et al., 2019). Diagnostic plots were also visualised using the R package "sjPlot" (Lüdecke, 2019) to identify possible points of our dataset which were not well fitted and possible outliers investigated and, if necessary, removed. The *R. helvetica* dataset used for the climatic models was the only one presenting problematic outliers with high residual and leverage values (n = 126 and 123, both points for Wassenaar, in Summer 2014 and Spring 2015). Based on their discrepancy to the remaining data points (diagnostic plots available in Figure S3), these outliers were removed from the dataset to achieve a better fit.

All statistical analyses were performed with R statistical software v.3.6.3 (R Core Team, 2020) in R Studio v.1.2.5033 (RStudio Team, 2020). Data manipulation was performed using the packages "dplyr" and "tidyr" (Wickham et al., 2019a; Wickham & Henry, 2019b); final tables were exported using "broom", "purrr" and "tibble" packages (Henry & Wickham, 2019; Muller & Wickham, 2019;

Robinson et al., 2020); and final figures were obtained using the packages "ggplot2", "ggpubr", "ggthemr" (Kassambara, 2019; Wickham, 2016) and Inkscape software v.0.92.4 (available at https://www.inkscape.org/).

Results

Tick densities and Infection prevalence

The Natuurkalender project team collected a total of 58,027 questing *I. ricinus* ticks between January 2012 and December 2015 in 12 sites in the Netherlands (Figure 1), of which 15,090 were nymphs (for additional details, see Table 1 and Table S1). The highest number of collected nymphs was in 2015 (n = 4,845) and in the location of Wassenaar (n = 2,956). The lowest number of ticks was collected in the year 2014 (n = 2,835) and the location of Kwade Hoek (n = 84).

Figure 1. Overview of the sampling sites along the Netherlands. BI, Bilthoven; DR, Dronten; ED, Ede; GI, Gieten; HB, Hoog Baarlo; KH, Kwade Hoek; MO, Montferland; SC, Schiermonnikoog; TW, Twiske; VA, Vaals; VE, Veldhoven; WA, Wassenaar.

Figure2 shows the seasonal activity of nymphal *I. ricinus*, where spring and summer showed the highest densities, and winter presented the lowest densities. *Midichloria mitochondrii* prevalence showed to be the most stable over the seasons, generally above 50%. *Rickettsia helvetica* prevalence was mainly variable within a year between 10% and 20%, though occasionally reaching 30% to 40% prevalence (March 2015, August 2014, and August 2015). *Borrelia miyamotoi* presented the lowest seasonal prevalence, equal or lower to 5%. *Neoehrlichia mikurensis* showed very irregular annual

prevalence patterns, mainly found between 5% to 10% of nymphs, but sporadically descending to 1% or lower (August 2014) and ascending to 15% or higher (June 2014 and 2015, November 2012 and 2014). Lastly, *B. burgdorferi* s.l. prevalence showed some stability throughout the year, being variable between 10% and 15%. DON variation per site can be consulted in Figure S1.

Table 1. Sample size and qPCR targeting results regarding symbionts of *I. ricinus* nymphs. Additional information per season, year and location is provided in Table S1.. Nticks, number of ticks; Nnymph, number of nymphs; Ntested, tested nymphs; positives, number of qPCR positive nymphs; %, percentage of positive nymphs

Location	N_{nymph}	N_{tested}	*M. mitochondrii*		*R. helvetica*		*B. miyamotoi*		*N. mikurensis*		*B. burgdorferi* s.l.	
			positives	%	positives	%	positives	%	positives	%	positives	%
Bilthoven	410	354	239	68	37	10	11	3	29	8	24	7
Dronten	393	360	263	73	56	16	6	2	18	5	83	23
Ede	993	730	468	64	49	7	17	2	62	8	98	13
Gieten	2817	1208	930	77	228	19	22	2	134	11	142	12
Hoog Baarlo	1440	820	652	80	62	8	20	2	30	4	35	4
Kwade Hoek	84	73	66	90	22	30	1	1	9	12	20	27
Montferland	1340	793	569	72	123	16	15	2	61	8	107	13
Schiermonnikoog	167	163	88	54	32	20	1	1	26	16	25	15
Twiske	987	624	388	62	120	19	15	2	14	2	53	8
Vaals	578	475	304	64	34	7	7	1	7	1	63	13
Veldhoven	2925	1277	918	72	123	10	36	3	126	10	159	12
Wassenaar	2956	1353	831	61	726	54	42	3	87	6	251	19
TOTAL	15090	8230	5716	69.5	1612	19.6	193	2.3	603	7.3	1060	12.9

Effect of Spatio-temporal variables on NIP

Three variables were used to evaluate whether the prevalence of the screened bacteria showed spatio-temporal variation: location as a spatial variable, year and season as temporal variables. The goodness of fit parameters for the best models are presented in Table 2. The best models were selected based on the comparisons between the null models, models with and without interactions (Table 2) and analysis of variance for individual terms (Table 3). Summaries presenting the estimates, standard error, *z*-values and associated *P*s are provided in Table S4. The significance of the variation for the overall time scale (year as a discrete variable) was also assessed (Table S4). After constructing and selecting the best-fitted models, pairwise comparisons of estimated marginal means (EMMs) were calculated. The results are shown in Figure 3 (for additional details, see Table S5 and Table S6).

The three explanatory variables explained a significant NIP variation for four out of the five screened bacteria of *I. ricinus* ticks (McFadden $\rho^2 = 0.13\text{-}0.45$, Cox-Snell, and Nagelkerke $\rho^2 = 0.41\text{-}0.97$ of the prevalence variability). The *R. helvetica* NIP model showed the best fit. The exception was *B. miyamotoi*, which was not significantly associated with either predictor (Figure 3). Nonetheless, the interaction term between year and season shows a significant association with its prevalence variation (Table S3 and Table S4) and 5%-10% of the *B. miyamotoi* NIP variability was explained with the constructed spatio-temporal model (ρ^2, Table 2).

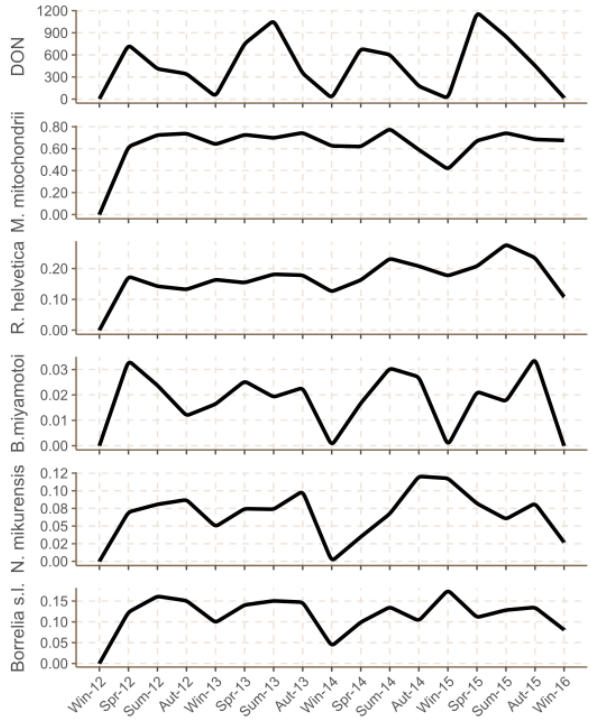

Figure 2. The density of nymphal *I. ricinus* and nymphal infection prevalence per season and year for five bacterial symbionts. Winter 2012 prevalences were pushed to zero to avoid wrong interpretations since only two of the five ticks collected in that season were tested. The density of nymphs (DON) for the two transects per location is presented per 100m^2. Win, Winter; Spr, Spring; Sum, Summer; Aut, Autumn.

Table 2. Goodness of fit parameters for the final spatio-temporal models. Ps were obtained in comparison with the null models. Interaction between year and season, when used, is represented with asterisks. McFadden (MF) and Nagelkerke (NG) are two different algorithms to calculate pseudo R^2 values ($\rho 2$). Note that McFadden ρ^2 values of between 0.20 and 0.40 represent a very good fit of the model (Louviere et al., 2000). Brier score measures the accuracy/calibration of probabilistic predictions, and the lower is, the better the calibration is. For additional details, including Cox Snell pseudo R^2 , see Table S2.

Symbiont (Y)	Model (~X)	MFρ^2	NGρ^2	Brier Score	Pr(>Chi)
M. mitochondrii	year * season + location	0.13	0.55	0.26	0.000
R. helvetica	year * season + location	0.45	0.97	0.11	0.000
B. miyamotoi	year * season + location	0.05	0.10	0.02	0.142
N. mikurensis	year * season + location	0.13	0.42	0.06	0.000
B. burgdorferi s.l.	year + season + location	0.14	0.43	0.10	0.000

Concerning the temporal predictors, for the overall period (2012-2015), only *R. helvetica* and *B. burgdorferi* s.l. showed a significant prevalence tendency, with the former increasing (estimate = 0.18 ± 0.03, z = 6.97) and the latter decreasing (estimate = -0.08 ± 0.03, z = -2.57) (Table S4). For the individual years, only *N. mikurensis* and *B. miyamotoi* NIPs did not show any significant variation (Table S3), although *M. mitochondrii* predictions suggest it to be stable as well (Figure 3, Table S5, and Table S6). In contrast, the prevalences of *R. helvetica* and *B. burgdorferi* s.l. were variable for some years (Figure 3, Table S4 and Table S6). Seasons had a significant effect in all NIP

models, except for *B. miyamotoi* (Table S3, Figure 3, and Table S6).

Spring was consistently the season in the model summaries where most significant variations of NIP variations in comparison to autumn were detected, except for *N. mikurensis* Table S4. *Midichloria mitochondrii* and *B. burgdorferi* s.l. expressed a significant decrease of prevalence, but both associated to low estimates and low z values (estimate = -0.53 and -0.18, z = -3.89 and -2.05). The same was observed regarding *R. helvetica* and *B. miyamotoi* (estimate = 0.45 and 1.11, z = 2.40 and 2.24), although showing a significant increase of prevalence. *Neoehrlichia mikurensis* did not have a significant prevalence variation regarding any season in the model summary (Table S4). Nonetheless, it is necessary to consider that the interaction terms, significant for all bacteria except for *B. burgdorferi* s.l., support that seasons of different years were also associated with prevalence variation (Table S4).

Indeed, the estimated marginal means (EMMs) results evidenced the slightly different effects of the predictors regarding the models (Figure 3 and Table S5). In comparison to the other seasons, both *M. mitochondrii* and *B. burgdorferi* s.l. had the highest prevalence in summer and spring, respectively. Although lower EMMs in spring compared to autumn were also observed, these differences were not significant. Summer was also detected in the EMMs analyses as the season with a higher prevalence for *R. helvetica*. However, spring and autumn did not show significant differences. On the other hand, *B. miyamotoi* NIP was stable over the seasons, which agrees with the analysis of variance of its spatio-temporal model (Table S3). Lastly, *N. mikurensis* showed the highest NIP values in autumn (Figure 3 and Table S6), which could explain the association found with the season predictor detected in the analysis of variance results (Table S3).

Regarding the spatial predictor, location explained a significant NIP variation for all symbionts except for *B. miyamotoi*. This explanatory variable contributed the most to the increase of the goodness of fit of the final models (Chisq and *P*s of Table S3). However, no pattern between different bacteria was found (Table S4). The most positively correlated locations with the NIP variation of *M. mitochondrii* were Hoog Baarlo, Gieten, and Kwade Hoek (z = 4.68, 3.93 and 3.78, respectively; p = <0.001). The location with the highest positive correlation with *R. helvetica* prevalence variation was by far Wassenaar (z = 13.08, p =<0.001), followed by Twiske, Kwade Hoek, and Gieten (z = 4.49, 4.43 and 4.33, respectively; p =<0.001).

Regarding the *N. mikurensis*, Twiske and Vaals were the locations with the strongest negative association with this bacterium prevalence (z = -4.11 and -3.88, respectively; p = <0.001). The only location with a significantly positive correlation with *N. mikurensis* NIP variation was Schiermonnikoog (z = 2.97, p =0.003). *Borrelia burgdorferi* s.l. NIP showed Dronten, Wassenaar, and Kwade Hoek as the most positively associated (z= 5.76, 5.31 and 5.04, respectively; p = <0.001). By last, *B. miyamotoi* did not show a significant association with any of the tested variables. Note that, although the spatio-temporal models show significant interaction effects between year and season for all symbionts, except for *B. burgdorferi* s.l. (Table 2), we interpreted the main effects of the models. Our goal was to understand general patterns, even if they were possibly not repeated every year or season. Taking that into account, we clearly see the main effects on the original dataset (see Figure 2 and Figure 3), meaning that both main effects of year and season are meaningful for NIPs variation. However, we highlight the importance of considering that the main patterns observed in this study can suffer some variations when inspected with higher detail, in different periods or in other locations.

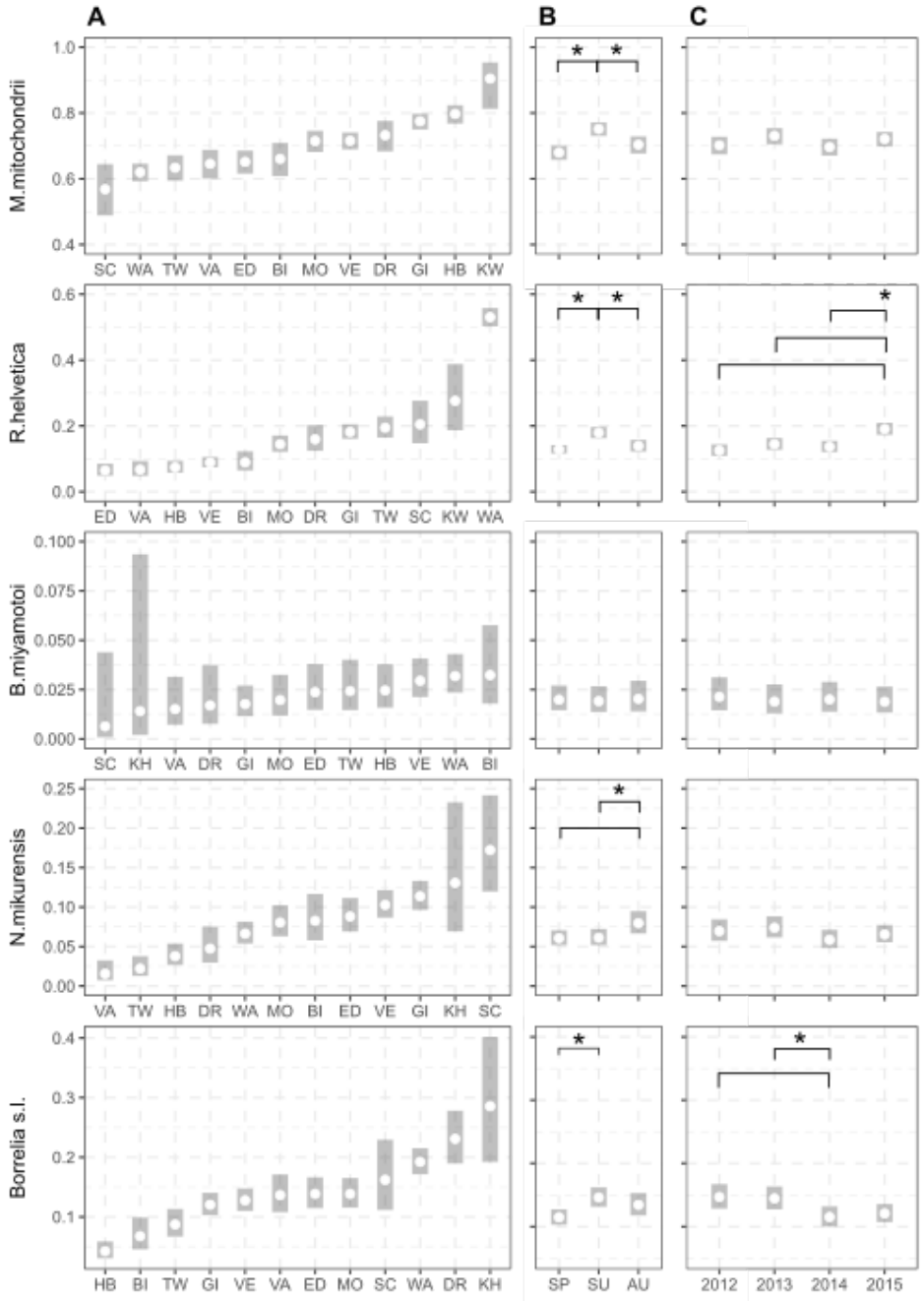

Figure 3. Estimated marginal means and 95% confidence intervals for bacterial symbionts infection prevalence in nymphal Ixodes ricinus regarding spatial and temporal factors. Note that the order of the location, in the x-axis, changes between plots. Y-axis is expressed in probabilities. Pairwise results were performed on the log odds ratio scale. Asterisks indicate significant pairwise differences ($p < 0.05$). Due to the high number of

significative associations between locations, they are provided only in Table S6. Abbreviations of the locations are provided in the caption of Figure 1.

Effect of Climatic variables on NIP

In total, six macro-climatic predictors (evaporation rate, precipitation, relative humidity, mean temperature, vapour pressure, and saturation deficit) were used as explanatory variables to investigate if climatic factors were associated with the dynamics of NIP. For that, all variables were included in two GLM models for each bacterium NIP as the response variable: one with the NIP of the same season as the climatic data (NIP_T), and the other with the following season NIP (NIP_{T+1}). After being tested, both vapour pressure and saturation deficit were excluded from the final models due to multicollinearity (variance inflation factor > 10). The seasonal variation of the selected climatic variables is presented in Figure 4, of which only the relative humidity demonstrated a significant variation between location and season (see Figure 5 and Figure S2).

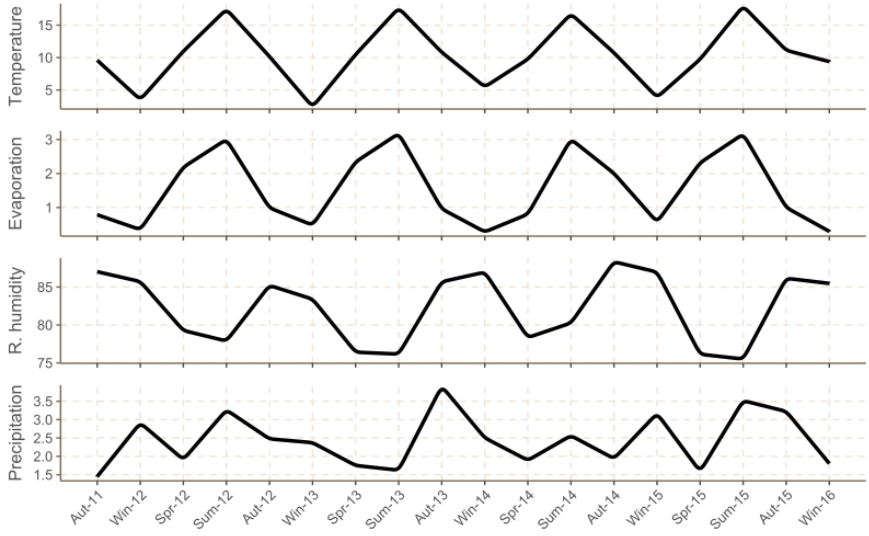

Figure 4. Climatic variables variation per season and year. Win, Winter; Spr, Spring; Sum, Summer; Aut, Autumn.

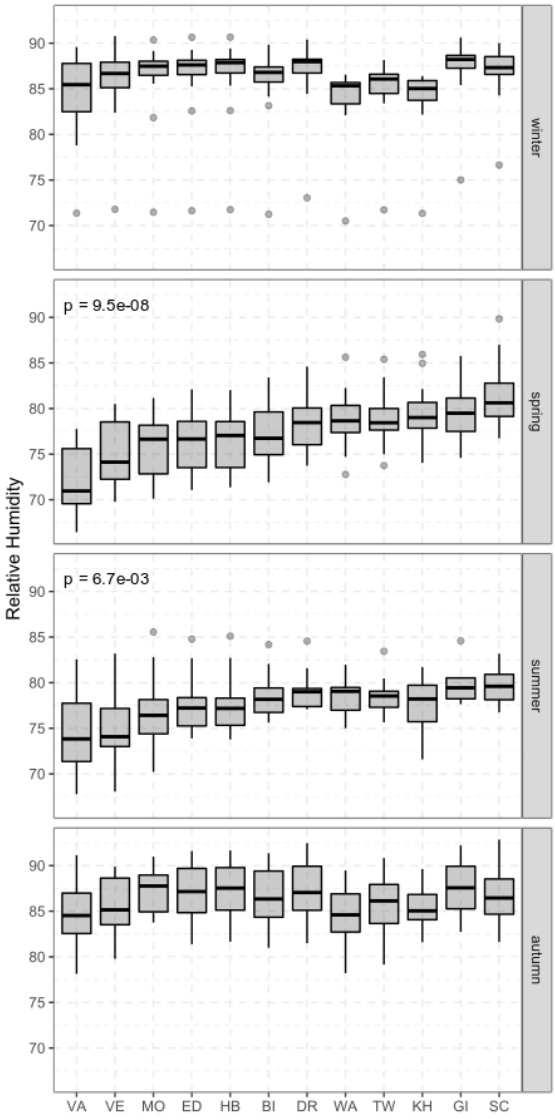

Figure 5. Relative humidity variation per location and season. Significant global Ps based on analysis of variance method are presented (p). Spring and summer present significant variation of relative humidity per location. Abbreviations of the locations are provided in the caption of Figure 1.

Goodness of fit summary concerning the best climatic models is presented in Table 3 (additional details are provided in Table S7). Models summaries presenting estimates, standard error, z-values, and associated Ps outputs are provided in Table S8.

Table 3. Goodness of fit parameters for the final climatic models. Ps were obtained with the chi-squared test in comparison with the null models. The table shows models regarding the climatic data effect on NIPs from the same season (NIP_T) and NIPs from the following season (NIP_{T+1}). McFadden (MF) and Nagelkerke (NG) are two different algorithms to calculate pseudo R^2 (ρ^2) values. Note that McFadden ρ^2 values of between 0.20 and 0.40 represent a very good fit of the model (Louviere et al., 2000). logLik, the logarithm of the likelihood; AIC, Akaike information criterion; BIC, Bayesian information criterion.

Symbiont (Y)	Model (~X)	Response	Deviance	logLik	AIC	BIC	MFρ^2	NGρ^2	Brier Score	Pr(>Chi)
M. mitochondrii	EV + Prec + RH + Tmean	NIP_T	50	-573	1155	1170	0.04	0.30	0.22	0.000
M. mitochondrii	EV + Prec + RH + Tmean	NIP_{T+1}	54	-571	1151	1166	0.04	0.32	0.22	0.000
R. helvetica	EV + Prec + RH + Tmean	NIP_T	108	-752	1515	1529	0.07	0.54	0.13	0.000
R. helvetica	EV + Prec + RH + Tmean	NIP_{T+1}	70	-771	1553	1567	0.04	0.40	0.13	0.000
B. miyamotoi	EV + Prec + RH + Tmean	NIP_T	3	-176	362	377	0.01	0.02	0.02	0.628
B. miyamotoi	EV + Prec + RH + Tmean	NIP_{T+1}	7	-174	358	373	0.02	0.05	0.02	0.139
N. mikurensis	EV + Prec + RH + Tmean	NIP_T	37	-419	847	862	0.04	0.23	0.07	0.000
N. mikurensis	EV + Prec + RH + Tmean	NIP_{T+1}	24	-425	860	875	0.03	0.16	0.07	0.000
B. burgdorferi s.l.	EV + Prec + RH + Tmean	NIP_T	8	-414	837	852	0.01	0.06	0.11	0.093
B. burgdorferi s.l.	EV + Prec + RH + Tmean	NIP_{T+1}	11	-412	835	850	0.01	0.07	0.11	0.032

Overall, the amount of bacteria prevalence variation explained with these models is relatively low (McFadden ρ^2 = 0.01-0.07, Cox-Snell and Nagelkerke ρ^2 = 0.07-0.54 of the prevalence variability). Again, *R. helvetica* NIP models had the best fit (Table 3). The macro-climatic variables of NIP_T and NIP_{T+1} models had an equivalent effect on *M. mitochondrii* prevalence variation. On the other hand, the climatic variables had a stronger effect on *R. helvetica* and *N. mikurensis* NIP_T than NIP_{T+1}. The NIP_{T+1} model was the only one showing an association with both *B. burgdorferi s.l.* and *B. miyamotoi,* although the model was not significant for the latter (Table 3).

Regarding the individual climatic variables, precipitation was positively associated with the *M. mitochondrii* NIP_T (estimate = 0.15, z = 4.00, Figure 6). Relative humidity and evaporation rate variables had a negative association with *M. mitochondrii* NIP_{T+1} (estimate = -0.04 and -0.14, z = -4.13 and -2.25; Figure 6 and Figure 7).

With the *R. helvetica* NIP_T, several climatic variables had a positive association, including the precipitation, relative humidity and the evaporation rate (estimate = 0.29, 0.03, and 0.24; z = 6.90, 3.48, and 3.31; respectively; Figure 6). Concerning the NIP_{T+1} (Figure 7), evaporation rate and relative humidity were still positively associated (estimate = 0.57 and 0.05, z = 6.62 and 4.13), while mean temperature had a weak but negative effect (estimate = -0.06, z = -3.49).

For the *B. miyamotoi* model, only two variables were significantly and negatively associated with NIP_{T+1}: evaporation rate and relative humidity (estimate = -0.37 and -0.06, z = -2.20 and 2.05; see Figure 7). Inconsistently with these results for the individual terms, the climatic model of *B. miyamotoi* did not present a significant difference to the null model (Table 3), which might be related to the low prevalence detected in this study.

In the case of *N. mikurensis*, the climatic variables that showed a significant relationship with NIP_T were the evaporation rate and relative humidity (estimate = 0.34 and 0.07, z = 3.30 and 5.79). The mean temperature had a weak and negative association with the NIP_T (estimate = -0.05, z = -2.13). From the NIP_{T+1} model, again evaporation rate and relative humidity consistently showed positive association (estimate = 0.30 and 0.05, z = 2.67 and 3.35).

For the *B. burgdorferi* s.l model, climatic variables did not show any association with individual terms (Figure 6 and Figure 7), although the climatic model NIP_{T+1} was significantly different from the null model (see Table 3).

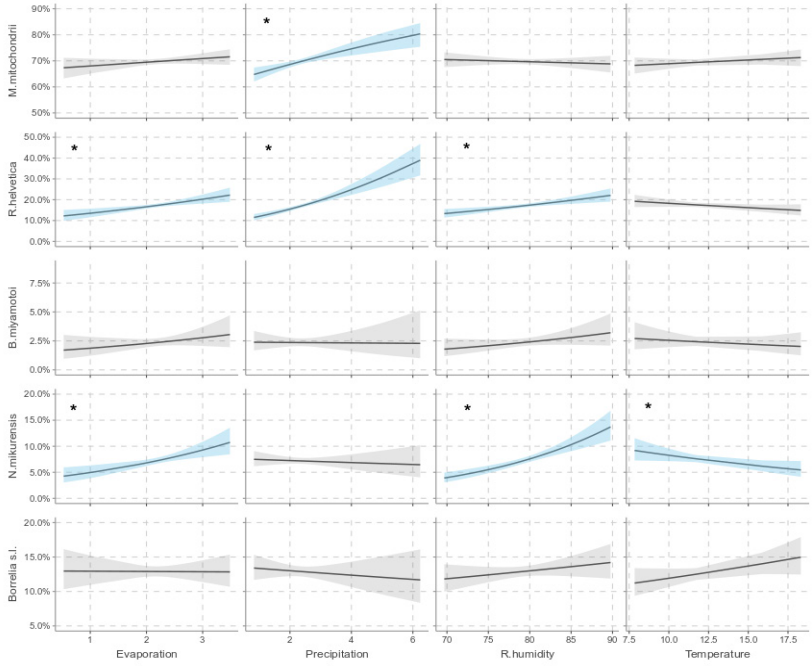

Figure 6. Marginal effects of macro-climatic variables on NIP_T. All values of the climatic variables were used to compute the predictions. The obtained values are presented in the X-axis. Nymphal infection prevalence (NIP) is presented on the Y-axis. Asterisks and confidence intervals in blue instead of grey indicate significant associations.

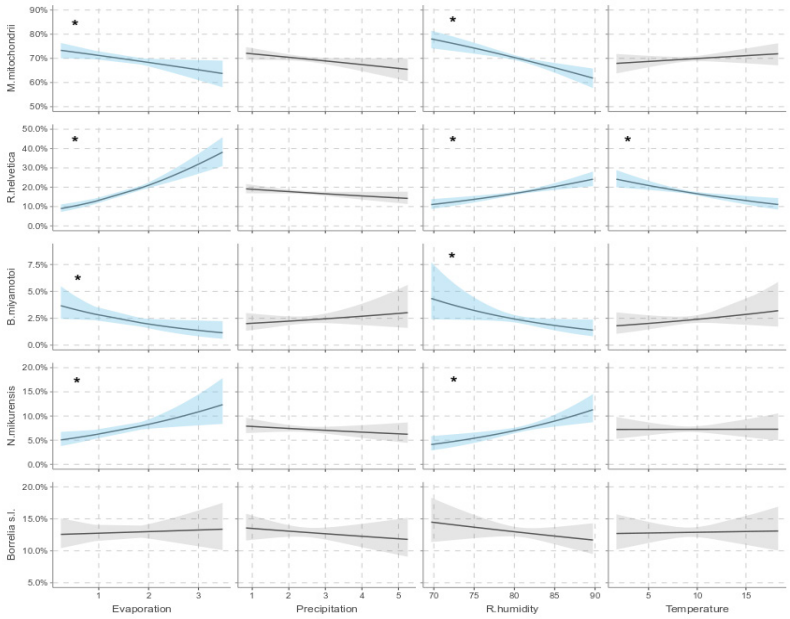

Figure 7. Marginal effects of macro-climatic variables on NIP_{T+1}. All values of the climatic variables were used to compute the predictions. The obtained values are presented in the X-axis. Nymphal infection prevalence (NIP) is presented on the Y-axis. Asterisks and confidence intervals in blue instead of grey indicate significant associations.

Discussion

This study investigated the spatio-temporal variation of nymphal infection prevalences (NIPs) for vertically-transmitted tick symbionts and their associations to climatic factors. The observed seasonal patterns were contrasted with those of vertically- and horizontally-transmitted pathogens. We observed spatio-temporal variation of NIPs for the bacterial symbionts *M. mitochondrii*, *R. helvetica*, and pathogens *N. mikurensis* and *B. burgdorferi* s.l. throughout the study period and locations. The exception to this pattern was *B. miyamotoi*, showing only a small temporal variation, probably due to the low prevalence detected. Additionally, we observed many significant associations of NIP fluctuations and macro-climatic factors.

Although the Netherlands presents relatively similar habitats throughout the country (Table S9), we detected marked differences in tick densities and NIPs from different bacterial symbionts between locations (Table 1 and Figure 3). The location was the primary determinant in the variation of NIPs for all bacteria, with the exception of *B. miyamotoi*. This result is similar to previous studies, although these focused only on *B. burgdorferi* s.l. (Ehrmann et al., 2018; Gassner et al., 2011; Takken et al., 2017; Hartemink et al., submitted). Interestingly, spatial distributions of distinct symbionts in tick populations were independent of each other, with the highest and lowest prevalences for different symbionts being detected in different locations.

Vertically-transmitted symbionts

Midichloria mitochondrii NIP expressed spatial and seasonal variation and was associated with three climatic variables. This bacterium is a heritable tick symbiont with an efficient transmission rate predominantly located in ovaries and salivary glands of *I. ricinus* (Epis et al., 2013; Lo et al., 2006; Sassera et al., 2006). Consequently, it is more prevalent in *I. ricinus* females, with a highly variable prevalence in males and immature stages (Sassera et al., 2008). Therefore, the observed spatial and seasonal variation of its NIP might be partially associated with the proportion of sexes by location (Figure 3). However, due to technical limitations, tick sex of nymphal stages can currently not be assessed.

Another explanation for the observed spatial differences in *M. mitochondrii* NIP could be linked to a variation in DON between the locations (Figure S1). Among all studied microorganisms, *M. mitochondrii* NIP was the only one positively associated with DON (estimate = 0.0003, Pr(>|z|) = 0.01; available on Table S10), although the model did not explain further the NIP variation in comparison to the best-fit spatio-temporal model previously presented (Table 2). This result suggests that *M. mitochondrii* infection benefits tick populations irrespectively to location. Moreover, the observed seasonal fluctuations could result from the mutualistic relationship between this symbiont and *I. ricinus*. The significantly higher NIP in summer than spring and a general decrease of nymphal densities (Figure 2) might be associated with a higher survival rate of ticks infected with *M. mitochondrii*.

Interestingly, it has been hypothesized that this symbiont confers multiple tissue-specific roles such as essential nutrients supplementation, enhancement of reproductive fitness, anti-oxidative defence, energy production, and the maintenance of homeostasis and water balance (Olivieri et al., 2019). Thus, *M. mitochondrii* can assist its tick host to cope with continually changing weather conditions. Here, *M. mitochondrii* NIP_{T} was positively associated with precipitation, and NIP_{T+1} was negatively associated with evaporation and relative humidity. The mechanisms behind these associations are unclear; however, it has been demonstrated before that, for instance, decreasing relative humidity influences negatively *I. ricinus* survival. *Midichloria mitochondrii* infected ticks' survival likely increases under such unfavourable conditions, leading to an increased NIP_{T+1}.

Although we detected seasonal fluctuations (Table S4 and Table S5), *M. mitochondrii* NIP was relatively stable throughout the study, pointing out a possible mechanism that allows its long-term maintenance in *I. ricinus* populations. We propose that the density of *I. ricinus* and its interactions with ambient conditions are primary factors driving *M. mitochondrii* transmission dynamics.

Rickettsia helvetica NIP had the highest spatio-temporal variation and was the most associated with climatic factors from all studied microorganisms. This bacterium is commonly detected in *I. ricinus* populations, and ticks have been suggested as the primary amplification hosts (Boretti et al., 2009; Sprong et al., 2009). The vertical transmission of this symbiont has been reported to be very frequent and effective, suggesting that this is *R. helvetica's* primary route of transmission (Burgdorfer et al., 1979; Perlman et al., 2006). In other arthropods, *Rickettsia* species were shown to manipulate reproduction or confer defence against pathogens; however, its role in ticks is unknown (Duron et al., 2017; Engelstdter & Hurst, 2009; Łukasik et al., 2013).

Here, the distribution of *R. helvetica* in nymphs was heterogeneous, with the highest prevalence in Wassenaar (Figure 3). Previous studies also observed a spatial-temporal NIP variation with the

highest values in summer (Figure 3; Boretti et al., 2009; Nijhof et al., 2007; Sprong et al., 2009). It has been suggested, but never shown, that the prevalence of this symbiont in *I. ricinus* ticks is affected by specific environmental conditions rather than vertebrate composition (Chapter 2; Coipan et al., 2013b; Sprong et al., 2009; Telford, 2009).

Regarding the vertebrate composition, next to vertical transmission, *R. helvetica* can probably utilise horizontal transmission routes, indicating that vertebrates may play a role in *R. helvetica* transmission dynamics. On many occasions, it has been detected in tick hosts such as rodents, roe deer, birds, and domestic animals (Hornok et al., 2014; Nijhof et al., 2007; Obiegala et al., 2016; Tomassone et al., 2017), and it has been shown to be involved in cases of human disease (Azagi et al., 2020; Fournier et al., 2000; Nilsson et al., 2010). Even though this symbiont can survive in warm-blooded animals, and it is unclear whether vertebrates' composition and abundance are responsible for the observed variations in NIP of *R. helvetica* between the locations. Nevertheless, it might contribute to the observed seasonal variation.

In this study, both *R. helvetica* NIP_T and NIP_{T+1} were positively associated with evaporation rate and relative humidity, NIP_T positively with precipitation, and NIP_{T+1} negatively with temperature (Figure 6 and Figure 7). Although it is unclear whether these climatic factors affect *R. helvetica* directly or indirectly via a tick host, our results underline the role of environmental conditions in the transmission dynamics of this symbiont.

From all studied climatic factors, only relative humidity showed a significant variation between locations and seasons, showing that spatial variation in NIP can be associated with spatial variation in climate (Figure 5). Here, five forest sites with the highest *R. helvetica* NIP expressed the highest relative humidity in spring and summer (and often the lowest in autumn and winter; Figure 3and Figure 5): Wassenaar, Twiske, Kwade Hoek, Gieten, and Schiermonnikoog. All these sites but one (Gieten) are located near the Dutch coast (Figure 1). Interestingly, the two sites with the lowest average relative humidity, which are in the southernmost in-land part of the Netherlands, Vaals, and Veldhoven, also expressed one of the lowest prevalences of *R. helvetica*. This spatial distribution of *R. helvetica* in questing nymphs here observed is in accordance with the previous studies (Chapter 2; Sprong et al., 2009). Although we detected a significant, minor short-term effect of relative humidity, we propose that the spatial distribution of *R. helvetica* NIP in Dutch tick populations derives from long-term differences in relative humidity between locations. Nevertheless, it remains unclear whether *R. helvetica* confers fitness benefits to *I. ricinus* in certain environmental conditions or whether certain environmental conditions facilitate propagation and transmission of this symbiont in tick populations.

Climatic factors are highly intertwined with many aspects of tick ecology, making it challenging to unravel mechanisms behind their effects on transmission dynamics of tick symbionts solely with descriptive studies. For instance, precipitation is not only expected to affect the relative humidity of the habitat suitable for *I. ricinus* survival but also vegetation cover variation throughout the year, known to be associated with DON (Gassner et al., 2011; Hartemink et al., 2019; Mannelli et al., 2012). In fact, precipitation was previously positively associated with higher nymphal abundances of *I. scapularis* (Ostfeld et al., 2006) and might have a similar effect in *I. ricinus* with consequences for *I. ricinus* symbionts' transmission dynamics.

Horizontally-transmitted pathogens

The overall prevalence of the pathogen *B. burgdorferi* s.l. slightly decreased over the 4-year study period, which is similar to what has previously been observed in Latvia and Norway (Mysterud et al., 2013; Okeyo et al., 2020; Rosef et al., 2009). This result is probably associated with the increase of the roe deer population, which has been observed in the Netherlands over the last 60 years (Bruinderink & Broekhuizen, 2016). Deer are the most common reproduction host for *I. ricinus*, serving as a food source for female ticks, and an increase in deer abundance has been linked to elevation in the density of ticks (Bouchard et al., 2019; Hofmeester et al., 2016). Simultaneously, this can lead to a decrease in the prevalence of *B. burgdorferi* s.l. in questing ticks since deer are incompetent hosts for this pathogen (Jaenson et al., 1994; Hartemink et al., submitted). Similarly, in Norway, a high abundance of roe and red deer have been associated with a lower prevalence of *B. burgdorferi* s.l. in *I. ricinus* nymphs (Mysterud et al., 2013; Rosef et al., 2009).

In Europe, the *B. burgdorferi* s.l. complex consists of multiple genospecies, many of which cause disease in humans (Rizzoli et al., 2011). Ticks acquire *B. burgdorferi* s.l. via horizontal transmission from various vertebrate host species other than deer (Gern et al., 1998; Strnad & Rego, 2020), while *N. mikurensis* is acquired from rodents (Burri et al., 2014). Although we did not investigate the vertebrate community, it has previously been shown that the prevalence of horizontally-transmitted pathogens is positively affected by local vertebrate presence and abundance (Chapter 4; Hofmeester et al., 2016; Takumi et al., 2019). Therefore, the significant spatial variation of both of these horizontally-transmitted symbionts observed here is expected to be associated with differences in the vertebrate community between locations.

This study observed a distinct seasonal variation in NIP for *B. burgdorferi* s.l. and *N. mikurensis*. The former expressed the highest prevalence in summer, similarly to *M. mitochondrii* and *R. helvetica*, and the latter in autumn (Figure 3). Both *N. mikurensis* and *B. afzelii* (the most prevalent *B. burgdorferi* s.l. genospecies in the Netherlands) are known to be amplified by rodents, meaning infected nymphs acquired these pathogens while feeding as larvae on infected animals (Burri et al., 2014; Gassner et al., 2011; Takken et al., 2017). Nevertheless, due to their biology, *N. mikurensis* and *B. afzelii* have different relationships with their vertebrate host.

Borrelia afzelii is known to cause infection in rodents for life, often persisting in animals over winter (Chapter 4; Humair et al., 1999). Therefore, the summer population of *B. afzelii*-infected nymphs probably consists of individuals that emerged in the preceding spring as larvae, which fed on infected rodents and moulted into nymphs, and of larvae and nymphs which fed and become infected in the previous year. Although both horizontally-transmitted pathogens can overwinter in ticks, this pattern does not appear to apply to *N. mikurensis*. Indeed, this bacterium causes systemic blood infection, which can potentially decrease the overwintering survival of infected rodents, leading to a smaller proportion of *N. mikurensis*-infected rodents in spring than in later months (Chapter 4; Andersson et al., 2014). That implies that *N. mikurensis* must be introduced into the rodent population by infected nymphs before being transmitted to larvae, which probably delays a peak in *N. mikurensis* NIP from summer to autumn. An alternative explanation could be related to the seasonality of the remaining genospecies in *B. burgdorferi* s.l., which was not investigated in this study.

Interestingly, the *B. burgdorferi* s.l. NIP was weakly influenced by the general climate (Table 3), but no individual climatic factors effects were detected (Figure 6 and Figure 7). This result is in accordance

with a previous study by Hartemink et al. (submitted) and contributes to the idea that other factors than climatic variables drive the transmission dynamics of *B. burgdorferi* s.l. in the Netherlands (as described above). Nevertheless, despite being horizontally-transmitted, *N. mikurensis* NIP was associated with several climatic variables, and these associations resembled those observed in *R. helvetica* (Figure 6 and Figure 7). *Neoehrlichia mikurensis* and *R. helvetica* do not share similar spatial distribution on a local scale. However, both have been shown to have regional distribution in the Netherlands (Chapter 2). Probably the differences in climatic conditions between the locations do not drive long-term *N. mikurensis* distribution as we suspect of *R. helvetica.* However, fluctuating weather conditions may have a short-term effect on the persistence *N. mikurensis* in *I. ricinus* ticks. Finally, *B. miyamotoi* did not show any significant association with location, season, or year (Figure 3). *Borrelia miyamotoi* is a predominantly vertically-transmitted bacterium with transmission efficiency in an experimental setting varying between 6% and 73% (Scoles et al., 2001; van Duijvendijk et al., 2016). Horizontal transmission from infected rodent hosts to naïve ticks has been observed, however inefficient as *B. miyamotoi* does not cause a persistent infection in rodents (Taylor et al., 2013; Wagemakers et al., 2016). Nevertheless, the density of *I. ricinus* nymphs infected with *B. miyamotoi* has been associated with the density of questing nymphs and the encounter probability of bank voles (Takumi et al., 2019). Here, although weak, an association of its NIP variability was found between seasons of different years, which explained between 5-10% of the observed variation (Table 2). These results led us to believe that some temporal variation occurs. Regarding climatic factors, B. miyamotoi NIPT+1 was positively associated with evaporation and relative humidity; however, confidence intervals were wide, suggesting that these results are not reliable (Figure 6 and Figure 7). It must be acknowledged that the *B. miyamotoi* NIP observed for all sampling sites was very low, which might have influenced our results. Future studies should increase the statistical power by increasing the sample size per temporal scale.

In addition to the previously published papers based on this dataset (Gassner et al., 2011; Hartemink et al., 2019; Takken et al., 2017), this study's results provided insights concerning the spatio-temporal NIP variations of vertically- and horizontally-transmitted tick microorganisms. Interestingly, some microorganisms, despite having the same transmission route, expressed different spatio-temporal patterns and associations with climatic factors. One explanation lies probably in the nature of symbiosis with *I. ricinus* and their roles in tick ecology and physiology, which remain to be described.

Climate warming has been found to impact tick phenology and geographical expansion of *I. ricinus* species complex in Europe, and predicted to contribute to the increase of tick-host contacts, which ultimately can affect pathogens' transmission (Jore et al., 2014; Levi et al., 2015; Li et al., 2016). Moreover, *I. ricinus* from different climatic regions were suggested to have different temperature resistance range (Gilbert et al., 2014), implying different potentials to adapt to climatic change. In addition, various bacterial symbionts may confer a range of ecologically important traits, including adaptation to the changing abiotic environment (Feldhaar, 2011). Long-term studies using modelling approaches should be applied to a broader area of Europe with distinct habitats and environmental conditions and varying symbionts communities. This approach could improve our understanding of symbionts' roles in tick physiology in the context of tick-borne disease transmission dynamics.

Acknowledgements

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Supplement

Table S1. Number of collected Ixodes ricinus nymphs and positive qPCR results per season, year and location. N, number nymphs; Nt, number of tested nymphs; positive, number of positive nymphs; %, percentage of positive nymphs; CI, 95% confidence intervals (calculated based on Armitage et al. (2001)). NA, nymphs were not tested.

Season	Year	Location	N	Nt	*M. mitochondrii*			*N. mikurensis*			*R. helvetica*			*B. burgdorferi* s.l.			*B. miyamotoi*		
					positive	**%**	**CI**	**positive**	**%**	**CI**	**positive**	**%**	**CI**	**positive**	**%**	**CI**	**positive**	**%**	**CI**
autumn	2012	Bilthoven	13	13	8	61.5	31.6 - 86.1	1	7.7	0.2 - 36.0	2	15.4	1.9 - 45.4	0	0.0	0.0 - 24.7	0	0.0	0.0 - 24.7
autumn	2012	Dronten	21	20	19	95.0	75.1 - 99.9	2	10.0	1.2 - 31.7	2	10.0	1.2 - 31.7	6	30.0	11.9 - 54.3	0	0.0	0.0 - 16.8
autumn	2012	Ede	41	39	31	79.5	63.5 - 90.7	6	15.4	5.9 - 30.5	2	5.1	0.6 - 17.3	10	25.6	13.0 - 42.1	3	7.7	1.6 - 20.9
autumn	2012	Gieten	216	78	58	74.4	63.2 - 83.6	2	2.6	0.3 - 9.0	7	9.0	3.7 - 17.6	5	6.4	2.1 - 14.3	1	1.3	0.0 - 6.9
autumn	2012	HoogBaarlo	73	45	31	68.9	53.4 - 81.8	1	2.2	0.1 - 11.8	4	8.9	2.5 - 21.2	0	0.0	0.0 - 7.9	0	0.0	0.0 - 7.9
autumn	2012	KwadeHoek	2	0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0
autumn	2012	Montferland	31	30	26	86.7	69.3 - 96.2	11	36.7	19.9 - 56.1	1	3.3	0.1 - 17.2	2	6.7	0.8 - 22.1	0	0.0	0.0 - 11.6
autumn	2012	Schiermonnikoog	8	8	4	50.0	15.7 - 84.3	0	0.0	0.0 - 36.9	1	12.5	0.3 - 52.7	0	0.0	0.0 - 36.9	0	0.0	0.0 - 36.9
autumn	2012	Twiske	31	31	22	71.0	52.0 - 85.8	0	0.0	0.0 - 11.2	3	9.7	2.0 - 25.8	4	12.9	3.6 - 29.8	0	0.0	0.0 - 11.2
autumn	2012	Vaals	25	25	12	48.0	27.8 - 68.7	0	0.0	0.0 - 13.7	0	0.0	0.0 - 13.7	4	16.0	4.5 - 36.1	0	0.0	0.0 - 13.7
autumn	2012	Veldhoven	99	64	49	76.6	64.3 - 86.2	4	6.3	1.7 - 15.2	6	9.4	3.5 - 19.3	12	18.8	10.1 - 30.5	1	1.6	0.0 - 8.4
autumn	2012	Wassenaar	128	71	53	74.6	62.9 - 84.2	10	14.1	7.0 - 24.4	28	39.4	28.0 - 51.7	21	29.6	19.3 - 41.6	0	0.0	0.0 - 5.1
autumn	2013	Bilthoven	36	35	25	71.4	53.7 - 85.4	4	11.4	3.2 - 26.7	2	5.7	0.7 - 19.2	1	2.9	0.1 - 14.9	0	0.0	0.0 - 10.0
autumn	2013	Dronten	45	45	39	86.7	73.2 - 94.9	3	6.7	1.4 - 18.3	13	28.9	16.4 - 44.3	13	28.9	16.4 - 44.3	1	2.2	0.1 - 11.8
autumn	2013	Ede	62	62	50	80.6	68.6 - 89.6	5	8.1	2.7 - 17.8	6	9.7	3.6 - 19.9	8	12.9	5.7 - 23.9	1	1.6	0.0 - 8.7
autumn	2013	Gieten	246	89	65	73.0	62.6 - 81.9	9	10.1	4.7 - 18.3	9	10.1	4.7 - 18.3	14	15.7	8.9 - 25.0	0	0.0	0.0 - 4.1
autumn	2013	HoogBaarlo	63	62	54	87.1	76.1 - 94.3	1	1.6	0.0 - 8.7	3	4.8	1.0 - 13.5	1	1.6	0.0 - 8.7	1	1.6	0.0 - 8.7
autumn	2013	KwadeHoek	0	0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0
autumn	2013	Montferland	48	48	33	68.8	53.7 - 81.3	5	10.4	3.5 - 22.7	9	18.8	8.9 - 32.6	7	14.6	6.1 - 27.8	1	2.1	0.1 - 11.1
autumn	2013	Schiermonnikoog	4	4	3	75.0	19.4 - 99.4	0	0.0	0.0 - 60.2	0	0.0	0.0 - 60.2	0	0.0	0.0 - 60.2	0	0.0	0.0 - 60.2
autumn	2013	Twiske	18	18	12	66.7	41.0 - 86.7	1	5.6	0.1 - 27.3	4	22.2	6.4 - 47.6	3	16.7	3.6 - 41.4	0	0.0	0.0 - 18.5
autumn	2013	Vaals	2	1	1	100.0	2.5 - 100.0	0	0.0	0.0 - 97.5	0	0.0	0.0 - 97.5	0	0.0	0.0 - 97.5	0	0.0	0.0 - 97.5
autumn	2013	Veldhoven	66	59	39	66.1	52.6 - 77.9	11	18.6	9.7 - 30.9	6	10.2	3.8 - 20.8	6	10.2	3.8 - 20.8	5	8.5	2.8 - 18.7
autumn	2013	Wassenaar	109	59	38	64.4	50.9 - 76.4	9	15.3	7.2 - 27.0	34	57.6	44.1 - 70.4	18	30.5	19.2 - 43.9	2	3.4	0.4 - 11.7
autumn	2014	Bilthoven	15	15	12	80.0	51.9 - 95.7	1	6.7	0.2 - 31.9	2	13.3	1.7 - 40.5	0	0.0	0.0 - 21.8	0	0.0	0.0 - 21.8
autumn	2014	Dronten	12	12	10	83.3	51.6 - 97.9	0	0.0	0.0 - 26.5	6	50.0	21.1 - 78.9	2	16.7	2.1 - 48.4	0	0.0	0.0 - 26.5
autumn	2014	Ede	19	19	9	47.4	24.4 - 71.1	0	0.0	0.0 - 17.6	2	10.5	1.3 - 33.1	2	10.5	1.3 - 33.1	0	0.0	0.0 - 17.6
autumn	2014	Gieten	41	41	25	61.0	44.5 - 75.8	6	14.6	5.6 - 29.2	3	7.3	1.5 - 19.9	4	9.8	2.7 - 23.1	2	4.9	0.6 - 16.5
autumn	2014	HoogBaarlo	38	38	34	89.5	75.2 - 97.1	1	2.6	0.1 - 13.8	1	2.6	0.1 - 13.8	0	0.0	0.0 - 9.3	1	2.6	0.1 - 13.8
autumn	2014	KwadeHoek	6	6	5	83.3	35.9 - 99.6	0	0.0	0.0 - 45.9	1	16.7	0.4 - 64.1	0	0.0	0.0 - 45.9	0	0.0	0.0 - 45.9
autumn	2014	Montferland	25	25	16	64.0	42.5 - 82.0	4	16.0	4.5 - 36.1	5	20.0	6.8 - 40.7	3	12.0	2.5 - 31.2	1	4.0	0.1 - 20.4
autumn	2014	Schiermonnikoog	0	0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0

table continues

Season	Year	Location	N	Nt	*M. mitochondrii*			*N. mikurensis*			*R. helvetica*			*B. burgdorferi* s.l.			*B. miyamotoi*		
					positive	**%**	**CI**	**positive**	**%**	**CI**	**positive**	**%**	**CI**	**positive**	**%**	**CI**	**positive**	**%**	**CI**
autumn	2014	Twiske	38	38	20	52.6	35.8 - 69.0	1	2.6	0.1 - 13.8	16	42.1	26.3 - 59.2	6	15.8	6.0 - 31.3	1	2.6	0.1 - 13.8
autumn	2014	Vaals	7	7	5	71.4	29.0 - 96.3	0	0.0	0.0 - 41.0	0	0.0	0.0 - 41.0	0	0.0	0.0 - 41.0	0	0.0	0.0 - 41.0
autumn	2014	Veldhoven	69	65	26	40.0	28.0 - 52.9	26	40.0	28.0 - 52.9	4	6.2	1.7 - 15.0	8	12.3	5.5 - 22.8	1	1.5	0.0 - 8.3
autumn	2014	Wassenaar	66	66	34	51.5	38.9 - 64.0	1	1.5	0.0 - 8.2	29	43.9	31.7 - 56.7	9	13.6	6.4 - 24.3	3	4.5	0.9 - 12.7
autumn	2015	Bilthoven	26	25	14	56.0	34.9 - 75.6	1	4.0	0.1 - 20.4	2	8.0	1.0 - 26.0	2	8.0	1.0 - 26.0	1	4.0	0.1 - 20.4
autumn	2015	Dronten	4	4	2	50.0	6.8 - 93.2	0	0.0	0.0 - 60.2	0	0.0	0.0 - 60.2	1	25.0	0.6 - 80.6	0	0.0	0.0 - 60.2
autumn	2015	Ede	29	29	15	51.7	32.5 - 70.6	1	3.4	0.1 - 17.8	1	3.4	0.1 - 17.8	4	13.8	3.9 - 31.7	1	3.4	0.1 - 17.8
autumn	2015	Gieten	280	129	92	71.3	62.7 - 78.9	7	5.4	2.2 - 10.9	21	16.3	10.4 - 23.8	15	11.6	6.7 - 18.5	1	0.8	0.0 - 4.2
autumn	2015	HoogBaarlo	33	31	22	71.0	52.0 - 85.8	7	22.6	9.6 - 41.1	0	0.0	0.0 - 11.2	3	9.7	2.0 - 25.8	2	6.5	0.8 - 21.4
autumn	2015	KwadeHoek	0	0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0
autumn	2015	Montferland	70	64	47	73.4	60.9 - 83.7	8	12.5	5.6 - 23.2	17	26.6	16.3 - 39.1	4	6.3	1.7 - 15.2	2	3.1	0.4 - 10.8
autumn	2015	Schiermonnikoog	7	6	3	50.0	11.8 - 88.2	1	16.7	0.4 - 64.1	0	0.0	0.0 - 45.9	2	33.3	4.3 - 77.7	0	0.0	0.0 - 45.9
autumn	2015	Twiske	11	11	2	18.2	2.3 - 51.8	0	0.0	0.0 - 28.5	5	45.5	16.7 - 76.6	0	0.0	0.0 - 28.5	1	9.1	0.2 - 41.3
autumn	2015	Vaals	34	34	20	58.8	40.7 - 75.4	0	0.0	0.0 - 10.3	1	2.9	0.1 - 15.3	4	11.8	3.3 - 27.5	0	0.0	0.0 - 10.3
autumn	2015	Veldhoven	199	147	115	78.2	70.7 - 84.6	13	8.8	4.8 - 14.6	25	17.0	11.3 - 24.1	26	17.7	11.9 - 24.8	8	5.4	2.4 - 10.4
autumn	2015	Wassenaar	191	128	84	65.6	56.7 - 73.8	12	9.4	4.9 - 15.8	71	55.5	46.4 - 64.3	21	16.4	10.5 - 24.0	5	3.9	1.3 - 8.9
spring	2012	Bilthoven	23	23	11	47.8	26.8 - 69.4	1	4.3	0.1 - 21.9	2	8.7	1.1 - 28.0	0	0.0	0.0 - 14.8	2	8.7	1.1 - 28.0
spring	2012	Dronten	24	24	21	87.5	67.6 - 97.3	0	0.0	0.0 - 14.2	6	25.0	9.8 - 46.7	3	12.5	2.7 - 32.4	1	4.2	0.1 - 21.1
spring	2012	Ede	110	89	57	64.0	53.2 - 73.9	13	14.6	8.0 - 23.7	6	6.7	2.5 - 14.1	16	18.0	10.6 - 27.5	4	4.5	1.2 - 11.1
spring	2012	Gieten	255	104	59	56.7	46.7 - 66.4	4	3.8	1.1 - 9.6	22	21.2	13.8 - 30.3	8	7.7	3.4 - 14.6	1	1.0	0.0 - 5.2
spring	2012	HoogBaarlo	200	85	61	71.8	61.0 - 81.0	2	2.4	0.3 - 8.2	3	3.5	0.7 - 10.0	4	4.7	1.3 - 11.6	2	2.4	0.3 - 8.2
spring	2012	KwadeHoek	3	3	3	100.0	29.2 - 100.0	0	0.0	0.0 - 70.8	0	0.0	0.0 - 70.8	1	33.3	0.8 - 90.6	0	0.0	0.0 - 70.8
spring	2012	Montferland	76	75	39	52.0	40.2 - 63.7	2	2.7	0.3 - 9.3	11	14.7	7.6 - 24.7	11	14.7	7.6 - 24.7	2	2.7	0.3 - 9.3
spring	2012	Schiermonnikoog	39	37	24	64.9	47.5 - 79.8	7	18.9	8.0 - 35.2	10	27.0	13.8 - 44.1	7	18.9	8.0 - 35.2	0	0.0	0.0 - 9.5
spring	2012	Twiske	153	71	37	52.1	39.9 - 64.1	3	4.2	0.9 - 11.9	12	16.9	9.0 - 27.7	8	11.3	5.0 - 21.0	7	9.9	4.1 - 19.3
spring	2012	Vaals	56	56	29	51.8	38.0 - 65.3	1	1.8	0.0 - 9.6	1	1.8	0.0 - 9.6	4	7.1	2.0 - 17.3	0	0.0	0.0 - 6.4
spring	2012	Veldhoven	214	70	42	60.0	47.6 - 71.5	9	12.9	6.1 - 23.0	3	4.3	0.9 - 12.0	9	12.9	6.1 - 23.0	2	2.9	0.3 - 9.9
spring	2012	Wassenaar	322	108	78	72.2	62.8 - 80.4	10	9.3	4.5 - 16.4	54	50.0	40.2 - 59.8	21	19.4	12.5 - 28.2	4	3.7	1.0 - 9.2
spring	2013	Bilthoven	38	33	26	78.8	61.1 - 91.0	10	30.3	15.6 - 48.7	1	3.0	0.1 - 15.8	3	9.1	1.9 - 24.3	1	3.0	0.1 - 15.8
spring	2013	Dronten	90	63	46	73.0	60.3 - 83.4	3	4.8	1.0 - 13.3	5	7.9	2.6 - 17.6	11	17.5	9.1 - 29.1	2	3.2	0.4 - 11.0
spring	2013	Ede	79	38	17	44.7	28.6 - 61.7	5	13.2	4.4 - 28.1	1	2.6	0.1 - 13.8	5	13.2	4.4 - 28.1	0	0.0	0.0 - 9.3
spring	2013	Gieten	219	69	62	89.9	80.2 - 95.8	3	4.3	0.9 - 12.2	11	15.9	8.2 - 26.7	9	13.0	6.1 - 23.3	2	2.9	0.4 - 10.1
spring	2013	HoogBaarlo	182	47	43	91.5	79.6 - 97.6	2	4.3	0.5 - 14.5	1	2.1	0.1 - 11.3	1	2.1	0.1 - 11.3	0	0.0	0.0 - 7.5
spring	2013	KwadeHoek	14	14	11	78.6	49.2 - 95.3	2	14.3	1.8 - 42.8	2	14.3	1.8 - 42.8	4	28.6	8.4 - 58.1	0	0.0	0.0 - 23.2
spring	2013	Montferland	90	60	54	90.0	79.5 - 96.2	3	5.0	1.0 - 13.9	5	8.3	2.8 - 18.4	15	25.0	14.7 - 37.9	1	1.7	0.0 - 8.9
spring	2013	Schiermonnikoog	16	16	7	43.8	19.8 - 70.1	2	12.5	1.6 - 38.3	4	25.0	7.3 - 52.4	1	6.3	0.2 - 30.2	0	0.0	0.0 - 20.6
spring	2013	Twiske	147	70	43	61.4	49.0 - 72.8	0	0.0	0.0 - 5.1	8	11.4	5.1 - 21.3	5	7.1	2.4 - 15.9	2	2.9	0.3 - 9.9
spring	2013	Vaals	44	30	23	76.7	57.7 - 90.1	0	0.0	0.0 - 11.6	9	30.0	14.7 - 49.4	4	13.3	3.8 - 30.7	1	3.3	0.1 - 17.2
spring	2013	Veldhoven	303	60	46	76.7	64.0 - 86.6	4	6.7	1.8 - 16.2	2	3.3	0.4 - 11.5	7	11.7	4.8 - 22.6	1	1.7	0.0 - 8.9

table continues

Season	Year	Location	N	Nt	*M. mitochondrii*			*N. mikurensis*			*R. helvetica*			*B. burgdorferi* s.l.			*B. miyamotoi*		
					positive	**%**	**CI**	**positive**	**%**	**CI**	**positive**	**%**	**CI**	**positive**	**%**	**CI**	**positive**	**%**	**CI**
spring	2013	Wassenaar	285	90	51	56.7	45.8 - 67.1	10	11.1	5.5 - 19.5	42	46.7	36.1 - 57.5	18	20.0	12.3 - 29.8	5	5.6	1.8 - 12.5
spring	2014	Bilthoven	6	6	4	66.7	22.3 - 95.7	0	0.0	0.0 - 45.9	1	16.7	0.4 - 64.1	0	0.0	0.0 - 45.9	0	0.0	0.0 - 45.9
spring	2014	Dronten	39	37	21	56.8	39.5 - 72.9	0	0.0	0.0 - 9.5	8	21.6	9.8 - 38.2	5	13.5	4.5 - 28.8	0	0.0	0.0 - 9.5
spring	2014	Ede	130	82	54	65.9	54.6 - 76.0	3	3.7	0.8 - 10.3	2	2.4	0.3 - 8.5	12	14.6	7.8 - 24.2	1	1.2	0.0 - 6.6
spring	2014	Gieten	112	103	82	79.6	70.5 - 86.9	3	2.9	0.6 - 8.3	15	14.6	8.4 - 22.9	17	16.5	9.9 - 25.1	0	0.0	0.0 - 3.5
spring	2014	HoogBaarlo	61	60	38	63.3	49.9 - 75.4	0	0.0	0.0 - 6.0	3	5.0	1.0 - 13.9	2	3.3	0.4 - 11.5	2	3.3	0.4 - 11.5
spring	2014	KwadeHoek	0	0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0
spring	2014	Montferland	113	86	51	59.3	48.2 - 69.8	3	3.5	0.7 - 9.9	7	8.1	3.3 - 16.1	7	8.1	3.3 - 16.1	1	1.2	0.0 - 6.3
spring	2014	Schiermonnikoog	16	16	6	37.5	15.2 - 64.6	10	62.5	35.4 - 84.8	0	0.0	0.0 - 20.6	6	37.5	15.2 - 64.6	0	0.0	0.0 - 20.6
spring	2014	Twiske	140	81	46	56.8	45.3 - 67.8	0	0.0	0.0 - 4.5	10	12.3	6.1 - 21.5	5	6.2	2.0 - 13.8	2	2.5	0.3 - 8.6
spring	2014	Vaals	114	72	41	56.9	44.7 - 68.6	0	0.0	0.0 - 5.0	2	2.8	0.3 - 9.7	7	9.7	4.0 - 19.0	1	1.4	0.0 - 7.5
spring	2014	Veldhoven	282	143	111	77.6	69.9 - 84.2	8	5.6	2.4 - 10.7	7	4.9	2.0 - 9.8	5	3.5	1.1 - 8.0	2	1.4	0.2 - 5.0
spring	2014	Wassenaar	295	151	64	42.4	34.4 - 50.7	2	1.3	0.2 - 4.7	81	53.6	45.4 - 61.8	17	11.3	6.7 - 17.4	5	3.3	1.1 - 7.6
spring	2015	Bilthoven	77	55	37	67.3	53.3 - 79.3	1	1.8	0.0 - 9.7	5	9.1	3.0 - 20.0	5	9.1	3.0 - 20.0	3	5.5	1.1 - 15.1
spring	2015	Dronten	39	39	32	82.1	66.5 - 92.5	1	2.6	0.1 - 13.5	3	7.7	1.6 - 20.9	11	28.2	15.0 - 44.9	1	2.6	0.1 - 13.5
spring	2015	Ede	189	168	94	56.0	48.1 - 63.6	12	7.1	3.7 - 12.1	15	8.9	5.1 - 14.3	11	6.5	3.3 - 11.4	4	2.4	0.7 - 6.0
spring	2015	Gieten	192	152	119	78.3	70.9 - 84.6	40	26.3	19.5 - 34.1	40	26.3	19.5 - 34.1	11	7.2	3.7 - 12.6	4	2.6	0.7 - 6.6
spring	2015	HoogBaarlo	112	100	68	68.0	57.9 - 77.0	7	7.0	2.9 - 13.9	4	4.0	1.1 - 9.9	9	9.0	4.2 - 16.4	1	1.0	0.0 - 5.4
spring	2015	KwadeHoek	31	24	23	95.8	78.9 - 99.9	5	20.8	7.1 - 42.2	5	20.8	7.1 - 42.2	9	37.5	18.8 - 59.4	1	4.2	0.1 - 21.1
spring	2015	Montferland	159	98	71	72.4	62.5 - 81.0	4	4.1	1.1 - 10.1	15	15.3	8.8 - 24.0	8	8.2	3.6 - 15.5	3	3.1	0.6 - 8.7
spring	2015	Schiermonnikoog	26	25	14	56.0	34.9 - 75.6	3	12.0	2.5 - 31.2	4	16.0	4.5 - 36.1	3	12.0	2.5 - 31.2	1	4.0	0.1 - 20.4
spring	2015	Twiske	251	149	95	63.8	55.5 - 71.5	1	0.7	0.0 - 3.7	19	12.8	7.9 - 19.2	7	4.7	1.9 - 9.4	1	0.7	0.0 - 3.7
spring	2015	Vaals	84	60	40	66.7	53.3 - 78.3	2	3.3	0.4 - 11.5	6	10.0	3.8 - 20.5	6	10.0	3.8 - 20.5	1	1.7	0.0 - 8.9
spring	2015	Veldhoven	496	142	91	64.1	55.6 - 72.0	11	7.7	3.9 - 13.4	14	9.9	5.5 - 16.0	17	12.0	7.1 - 18.5	1	0.7	0.0 - 3.9
spring	2015	Wassenaar	652	208	137	65.9	59.0 - 72.3	13	6.3	3.4 - 10.5	123	59.1	52.1 - 65.9	38	18.3	13.3 - 24.2	5	2.4	0.8 - 5.5
summer	2012	Bilthoven	9	8	7	87.5	47.3 - 99.7	1	12.5	0.3 - 52.7	2	25.0	3.2 - 65.1	2	25.0	3.2 - 65.1	1	[12.5]	0.3 - 52.7
summer	2012	Dronten	19	19	8	42.1	20.3 - 66.5	1	5.3	0.1 - 26.0	2	10.5	1.3 - 33.1	1	5.3	0.1 - 26.0	0	0.0	0.0 - 17.6
summer	2012	Ede	41	41	22	53.7	37.4 - 69.3	3	7.3	1.5 - 19.9	2	4.9	0.6 - 16.5	7	17.1	7.2 - 32.1	0	0.0	0.0 - 8.6
summer	2012	Gieten	268	60	49	81.7	69.6 - 90.5	4	6.7	1.8 - 16.2	8	13.3	5.9 - 24.6	5	8.3	2.8 - 18.4	1	1.7	0.0 - 8.9
summer	2012	HoogBaarlo	92	60	52	86.7	75.4 - 94.1	6	10.0	3.8 - 20.5	4	6.7	1.8 - 16.2	6	10.0	3.8 - 20.5	1	1.7	0.0 - 8.9
summer	2012	KwadeHoek	7	7	7	100.0	59.0 - 100.0	1	14.3	0.4 - 57.9	1	14.3	0.4 - 57.9	0	0.0	0.0 - 41.0	0	0.0	0.0 - 41.0
summer	2012	Montferland	24	24	17	70.8	48.9 - 87.4	0	0.0	0.0 - 14.2	1	4.2	0.1 - 21.1	6	25.0	9.8 - 46.7	0	0.0	0.0 - 14.2
summer	2012	Schiermonnikoog	9	9	8	88.9	51.8 - 99.7	2	22.2	2.8 - 60.0	4	44.4	13.7 - 78.8	0	0.0	0.0 - 33.6	0	0.0	0.0 - 33.6
summer	2012	Twiske	43	41	29	70.7	54.5 - 83.9	2	4.9	0.6 - 16.5	10	24.4	12.4 - 40.3	8	19.5	8.8 - 34.9	0	0.0	0.0 - 8.6
summer	2012	Vaals	24	24	15	62.5	40.6 - 81.2	0	0.0	0.0 - 14.2	0	0.0	0.0 - 14.2	3	12.5	2.7 - 32.4	2	8.3	1.0 - 27.0
summer	2012	Veldhoven	198	85	63	74.1	63.5 - 83.0	10	11.8	5.8 - 20.6	5	5.9	1.9 - 13.2	20	23.5	15.0 - 34.0	3	3.5	0.7 - 10.0
summer	2012	Wassenaar	87	43	28	65.1	49.1 - 79.0	4	9.3	2.6 - 22.1	21	48.8	33.3 - 64.5	10	23.3	11.8 - 38.6	2	4.7	0.6 - 15.8
summer	2013	Bilthoven	80	56	33	58.9	45.0 - 71.9	9	16.1	7.6 - 28.3	5	8.9	3.0 - 19.6	6	10.7	4.0 - 21.9	3	5.4	1.1 - 14.9
summer	2013	Dronten	50	50	30	60.0	45.2 - 73.6	4	8.0	2.2 - 19.2	4	8.0	2.2 - 19.2	14	28.0	16.2 - 42.5	0	0.0	0.0 - 7.1

table continues

Season	Year	Location	N	Nt	*M. mitochondrii*			*N. mikurensis*			*R. helvetica*			*B. burgdorferi* s.l.			*B. miyamotoi*		
					positive	**%**	**CI**	**positive**	**%**	**CI**	**positive**	**%**	**CI**	**positive**	**%**	**CI**	**positive**	**%**	**CI**
summer	2013	Ede	176	60	51	85.0	73.4 - 92.9	6	10.0	3.8 - 20.5	7	11.7	4.8 - 22.6	12	20.0	10.8 - 32.3	1	1.7	0.0 - 8.9
summer	2013	Gieten	436	60	55	91.7	81.6 - 97.2	2	3.3	0.4 - 11.5	32	53.3	40.0 - 66.3	13	21.7	12.1 - 34.2	1	1.7	0.0 - 8.9
summer	2013	HoogBaarlo	376	90	76	84.4	75.3 - 91.2	0	0.0	0.0 - 4.0	6	6.7	2.5 - 13.9	1	1.1	0.0 - 6.0	1	1.1	0.0 - 6.0
summer	2013	KwadeHoek	3	2	1	50.0	1.3 - 98.7	0	0.0	0.0 - 84.2	0	0.0	0.0 - 84.2	0	0.0	0.0 - 84.2	0	0.0	0.0 - 84.2
summer	2013	Montferland	334	90	64	71.1	60.6 - 80.2	9	10.0	4.7 - 18.1	11	12.2	6.3 - 20.8	21	23.3	15.1 - 33.4	1	1.1	0.0 - 6.0
summer	2013	Schiermonnikoog	21	21	10	47.6	25.7 - 70.2	1	4.8	0.1 - 23.8	5	23.8	8.2 - 47.2	2	9.5	1.2 - 30.4	0	0.0	0.0 - 16.1
summer	2013	Twiske	23	23	9	39.1	19.7 - 61.5	1	4.3	0.1 - 21.9	2	8.7	1.1 - 28.0	0	0.0	0.0 - 14.8	0	0.0	0.0 - 14.8
summer	2013	Vaals	71	71	49	69.0	56.9 - 79.5	1	1.4	0.0 - 7.6	4	5.6	1.6 - 13.8	14	19.7	11.2 - 30.9	0	0.0	0.0 - 5.1
summer	2013	Veldhoven	384	90	74	82.2	72.7 - 89.5	12	13.3	7.1 - 22.1	14	15.6	8.8 - 24.7	10	11.1	5.5 - 19.5	5	5.6	1.8 - 12.5
summer	2013	Wassenaar	182	65	21	32.3	21.2 - 45.1	5	7.7	2.5 - 17.0	33	50.8	38.1 - 63.4	9	13.8	6.5 - 24.7	1	1.5	0.0 - 8.3
summer	2014	Bilthoven	25	25	12	48.0	27.8 - 68.7	0	0.0	0.0 - 13.7	0	0.0	0.0 - 13.7	1	4.0	0.1 - 20.4	0	0.0	0.0 - 13.7
summer	2014	Dronten	36	34	25	73.5	55.6 - 87.1	3	8.8	1.9 - 23.7	6	17.6	6.8 - 34.5	13	38.2	22.2 - 56.4	1	2.9	0.1 - 15.3
summer	2014	Ede	59	46	41	89.1	76.4 - 96.4	2	4.3	0.5 - 14.8	3	6.5	1.4 - 17.9	8	17.4	7.8 - 31.4	1	2.2	0.1 - 11.5
summer	2014	Gieten	289	207	174	84.1	78.3 - 88.8	42	20.3	15.0 - 26.4	23	11.1	7.2 - 16.2	28	13.5	9.2 - 19.0	6	2.9	1.1 - 6.2
summer	2014	HoogBaarlo	177	170	149	87.6	81.7 - 92.2	1	0.6	0.0 - 3.2	32	18.8	13.2 - 25.5	7	4.1	1.7 - 8.3	9	5.3	2.4 - 9.8
summer	2014	KwadeHoek	2	2	1	50.0	1.3 - 98.7	0	0.0	0.0 - 84.2	1	50.0	1.3 - 98.7	0	0.0	0.0 - 84.2	0	0.0	0.0 - 84.2
summer	2014	Montferland	47	47	43	91.5	79.6 - 97.6	3	6.4	1.3 - 17.5	6	12.8	4.8 - 25.7	6	12.8	4.8 - 25.7	0	0.0	0.0 - 7.5
summer	2014	Schiermonnikoog	9	9	4	44.4	13.7 - 78.8	0	0.0	0.0 - 33.6	3	33.3	7.5 - 70.1	2	22.2	2.8 - 60.0	0	0.0	0.0 - 33.6
summer	2014	Twiske	74	43	31	72.1	56.3 - 84.7	3	7.0	1.5 - 19.1	11	25.6	13.5 - 41.2	4	9.3	2.6 - 22.1	0	0.0	0.0 - 8.2
summer	2014	Vaals	30	20	13	65.0	40.8 - 84.6	0	0.0	0.0 - 16.8	2	10.0	1.2 - 31.7	1	5.0	0.1 - 24.9	0	0.0	0.0 - 16.8
summer	2014	Veldhoven	132	129	90	69.8	61.1 - 77.5	6	4.7	1.7 - 9.8	8	6.2	2.7 - 11.9	12	9.3	4.9 - 15.7	3	2.3	0.5 - 6.6
summer	2014	Wassenaar	284	218	159	72.9	66.5 - 78.7	4	1.8	0.5 - 4.6	126	57.8	50.9 - 64.4	47	21.6	16.3 - 27.6	9	4.1	1.9 - 7.7
summer	2015	Bilthoven	57	55	45	81.8	69.1 - 90.9	0	0.0	0.0 - 6.5	11	20.0	10.4 - 33.0	4	7.3	2.0 - 17.6	0	0.0	0.0 - 6.5
summer	2015	Dronten	10	10	8	80.0	44.4 - 97.5	0	0.0	0.0 - 30.8	1	10.0	0.3 - 44.5	3	30.0	6.7 - 65.2	0	0.0	0.0 - 30.8
summer	2015	Ede	48	48	23	47.9	33.3 - 62.8	6	12.5	4.7 - 25.2	1	2.1	0.1 - 11.1	2	4.2	0.5 - 14.3	1	2.1	0.1 - 11.1
summer	2015	Gieten	245	99	81	81.8	72.8 - 88.9	11	11.1	5.7 - 19.0	35	35.4	26.0 - 45.6	12	12.1	6.4 - 20.2	2	2.0	0.2 - 7.1
summer	2015	HoogBaarlo	29	28	23	82.1	63.1 - 93.9	2	7.1	0.9 - 23.5	1	3.6	0.1 - 18.3	1	3.6	0.1 - 18.3	0	0.0	0.0 - 12.3
summer	2015	KwadeHoek	16	15	15	100.0	78.2 - 100.0	1	6.7	0.2 - 31.9	12	80.0	51.9 - 95.7	6	40.0	16.3 - 67.7	0	0.0	0.0 - 21.8
summer	2015	Montferland	304	128	94	73.4	64.9 - 80.9	9	7.0	3.3 - 12.9	33	25.8	18.5 - 34.3	16	12.5	7.3 - 19.5	3	2.3	0.5 - 6.7
summer	2015	Schiermonnikoog	5	5	4	80.0	28.4 - 99.5	0	0.0	0.0 - 52.2	0	0.0	0.0 - 52.2	2	40.0	5.3 - 85.3	0	0.0	0.0 - 52.2
summer	2015	Twiske	43	43	38	88.4	74.9 - 96.1	1	2.3	0.1 - 12.3	20	46.5	31.2 - 62.3	1	2.3	0.1 - 12.3	1	2.3	0.1 - 12.3
summer	2015	Vaals	80	68	54	79.4	67.9 - 88.3	3	4.4	0.9 - 12.4	9	13.2	6.2 - 23.6	16	23.5	14.1 - 35.4	2	2.9	0.4 - 10.2
summer	2015	Veldhoven	440	180	139	77.2	70.4 - 83.1	10	5.6	2.7 - 10.0	26	14.4	9.7 - 20.4	23	12.8	8.3 - 18.6	4	2.2	0.6 - 5.6
summer	2015	Wassenaar	329	123	72	58.5	49.3 - 67.3	5	4.1	1.3 - 9.2	74	60.2	50.9 - 68.9	17	13.8	8.3 - 21.2	1	0.8	0.0 - 4.4
winter	2012	Bilthoven	0	0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0
winter	2012	Dronten	0	0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0
winter	2012	Ede	0	0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0
winter	2012	Gieten	2	1	1	100.0	2.5 - 100.0	0	0.0	0.0 - 97.5	0	0.0	0.0 - 97.5	0	0.0	0.0 - 97.5	0	0.0	0.0 - 97.5
winter	2012	HoogBaarlo	0	0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0

table continues

Season	Year	Location	N	Nt	*M. mitochondrii*			*N. mikurensis*			*R. helvetica*			*B. burgdorferi* s.l.			*B. miyamotoi*		
					positive	**%**	**CI**	**positive**	**%**	**CI**	**positive**	**%**	**CI**	**positive**	**%**	**CI**	**positive**	**%**	**CI**
winter	2012	KwadeHoek	0	0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0
winter	2012	Montferland	2	2	2	100.0	15.8 - 100.0	0	0.0	0.0 - 84.2	1	50.0	1.3 - 98.7	0	0.0	0.0 - 84.2	0	0.0	0.0 - 84.2
winter	2012	Schiermonnikoog	2	2	1	50.0	1.3 - 98.7	0	0.0	0.0 - 84.2	0	0.0	0.0 - 84.2	0	0.0	0.0 - 84.2	0	0.0	0.0 - 84.2
winter	2012	Twiske	0	0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0
winter	2012	Vaals	0	0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0
winter	2012	Veldhoven	0	0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0
winter	2012	Wassenaar	4	2	1	50.0	1.3 - 98.7	1	50.0	1.3 - 98.7	2	100.0	15.8 - 100.0	1	50.0	1.3 - 98.7	0	0.0	0.0 - 84.2
winter	2013	Bilthoven	3	3	3	100.0	29.2 - 100.0	0	0.0	0.0 - 70.8	2	66.7	9.4 - 99.2	0	0.0	0.0 - 70.8	0	0.0	0.0 - 70.8
winter	2013	Dronten	4	3	2	66.7	9.4 - 99.2	1	33.3	0.8 - 90.6	0	0.0	0.0 - 70.8	0	0.0	0.0 - 70.8	0	0.0	0.0 - 70.8
winter	2013	Ede	3	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
winter	2013	Gieten	7	7	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
winter	2013	HoogBaarlo	1	1	0	0.0	0.0 - 97.5	0	0.0	0.0 - 97.5	0	0.0	0.0 - 97.5	0	0.0	0.0 - 97.5	0	0.0	0.0 - 97.5
winter	2013	KwadeHoek	0	0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0
winter	2013	Montferland	13	12	9	75.0	42.8 - 94.5	0	0.0	0.0 - 26.5	1	8.3	0.2 - 38.5	1	8.3	0.2 - 38.5	0	0.0	0.0 - 26.5
winter	2013	Schiermonnikoog	1	1	0	0.0	0.0 - 97.5	0	0.0	0.0 - 97.5	0	0.0	0.0 - 97.5	0	0.0	0.0 - 97.5	0	0.0	0.0 - 97.5
winter	2013	Twiske	11	1	1	100.0	2.5 - 100.0	0	0.0	0.0 - 97.5	0	0.0	0.0 - 97.5	1	100.0	2.5 - 100.0	0	0.0	0.0 - 97.5
winter	2013	Vaals	3	3	1	33.3	0.8 - 90.6	0	0.0	0.0 - 70.8	0	0.0	0.0 - 70.8	0	0.0	0.0 - 70.8	0	0.0	0.0 - 70.8
winter	2013	Veldhoven	23	23	17	73.9	51.6 - 89.8	2	8.7	1.1 - 28.0	3	13.0	2.8 - 33.6	2	8.7	1.1 - 28.0	0	0.0	0.0 - 14.8
winter	2013	Wassenaar	5	5	2	40.0	5.3 - 85.3	0	0.0	0.0 - 52.2	1	20.0	0.5 - 71.6	1	20.0	0.5 - 71.6	0	0.0	0.0 - 52.2
winter	2014	Bilthoven	0	0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0
winter	2014	Dronten	0	0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0
winter	2014	Ede	2	2	2	100.0	15.8 - 100.0	0	0.0	0.0 - 84.2	1	50.0	1.3 - 98.7	1	50.0	1.3 - 98.7	0	0.0	0.0 - 84.2
winter	2014	Gieten	3	3	3	100.0	29.2 - 100.0	0	0.0	0.0 - 70.8	0	0.0	0.0 - 70.8	0	0.0	0.0 - 70.8	0	0.0	0.0 - 70.8
winter	2014	HoogBaarlo	0	0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0
winter	2014	KwadeHoek	0	0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0
winter	2014	Montferland	1	1	1	100.0	2.5 - 100.0	0	0.0	0.0 - 97.5	0	0.0	0.0 - 97.5	0	0.0	0.0 - 97.5	0	0.0	0.0 - 97.5
winter	2014	Schiermonnikoog	2	2	0	0.0	0.0 - 84.2	0	0.0	0.0 - 84.2	1	50.0	1.3 - 98.7	0	0.0	0.0 - 84.2	0	0.0	0.0 - 84.2
winter	2014	Twiske	1	1	1	100.0	2.5 - 100.0	1	100.0	2.5 - 100.0	0	0.0	0.0 - 97.5	1	100.0	2.5 - 100.0	0	0.0	0.0 - 97.5
winter	2014	Vaals	1	1	0	0.0	0.0 - 97.5	0	0.0	0.0 - 97.5	0	0.0	0.0 - 97.5	0	0.0	0.0 - 97.5	0	0.0	0.0 - 97.5
winter	2014	Veldhoven	10	10	8	80.0	44.4 - 97.5	0	0.0	0.0 - 30.8	0	0.0	0.0 - 30.8	1	10.0	0.3 - 44.5	0	0.0	0.0 - 30.8
winter	2014	Wassenaar	7	7	3	42.9	9.9 - 81.6	0	0.0	0.0 - 41.0	3	42.9	9.9 - 81.6	0	0.0	0.0 - 41.0	0	0.0	0.0 - 41.0
winter	2015	Bilthoven	2	2	2	100.0	15.8 - 100.0	0	0.0	0.0 - 84.2	0	0.0	0.0 - 84.2	0	0.0	0.0 - 84.2	0	0.0	0.0 - 84.2
winter	2015	Dronten	0	0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0
winter	2015	Ede	5	5	2	40.0	5.3 - 85.3	0	0.0	0.0 - 52.2	0	0.0	0.0 - 52.2	0	0.0	0.0 - 52.2	0	0.0	0.0 - 52.2
winter	2015	Gieten	6	6	4	66.7	22.3 - 95.7	1	16.7	0.4 - 64.1	1	16.7	0.4 - 64.1	0	0.0	0.0 - 45.9	0	0.0	0.0 - 45.9
winter	2015	HoogBaarlo	3	3	1	33.3	0.8 - 90.6	0	0.0	0.0 - 70.8	0	0.0	0.0 - 70.8	0	0.0	0.0 - 70.8	0	0.0	0.0 - 70.8
winter	2015	KwadeHoek	0	0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0	0	0.0	0.0 - 100.0
winter	2015	Montferland	3	3	2	66.7	9.4 - 99.2	0	0.0	0.0 - 70.8	0	0.0	0.0 - 70.8	0	0.0	0.0 - 70.8	0	0.0	0.0 - 70.8
winter	2015	Schiermonnikoog	2	2	0	0.0	0.0 - 84.2	0	0.0	0.0 - 84.2	0	0.0	0.0 - 84.2	0	0.0	0.0 - 84.2	0	0.0	0.0 - 84.2

table continues

Season	Year	Location	N	Nt	*M. mitochondrii*			*N. mikurensis*			*R. helvetica*			*B. burgdorferi* s.l.			*B. miyamotoi*		
					positive	**%**	**CI**	**positive**	**%**	**CI**	**positive**	**%**	**CI**	**positive**	**%**	**CI**	**positive**	**%**	**CI**
winter	2015	Twiske	3	3	2	66.7	9.4 - 99.2	0	0.0	0.0 - 70.8	0	0.0	0.0 - 70.8	0	0.0	0.0 - 70.8	0	0.0	0.0 - 70.8
winter	2015	Vaals	3	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
winter	2015	Veldhoven	10	10	8	80.0	44.4 - 97.5	0	0.0	0.0 - 30.8	0	0.0	0.0 - 30.8	1	10.0	0.3 - 44.5	0	0.0	0.0 - 30.8
winter	2015	Wassenaar	10	9	6	66.7	29.9 - 92.5	1	11.1	0.3 - 48.2	4	44.4	13.7 - 78.8	3	33.3	7.5 - 70.1	0	0.0	0.0 - 33.6

Table S2. Goodness of fit parameters for the spatio-temporal models. Deviance and p were obtained in comparison with (1) the null models and with (2) the model without interactions. Interaction between year and season, when used, is represented with asterisks. McFadden, Cox-Snell and Nagelkerke are three different algorithms to calculate pseudo R^2 values (ρ^2). logLik, logarithm of the likelihood; AIC, Akaike information criterion; BIC, Bayesian information criterion.

Symbiont (Y)	Model (~X)		Deviance	Pr(>Chi)	logLik	AIC	BIC	McFadden ρ2	Cox.Snell ρ2	Nagelkerke ρ2	Brier.Score
M. mitochondrii	NULL				-1126	2254	2258				0.25
M. mitochondrii	year + season + location	1	233	0.000	-1009	2052	2121	0.10	0.48	0.48	0.26
M. mitochondrii	year * season + location	1	287	0.000	-982	2010	2103	0.13	0.55	0.55	0.26
M. mitochondrii	year * season + location	2	54	0.000	-982	2010	2103	0.13	0.55	0.55	0.26
N. mikurensis	NULL				-736	1474	1478				0.06
N. mikurensis	year + season + location	1	162	0.000	-655	1344	1412	0.11	0.36	0.37	0.06
N. mikurensis	year * season + location	1	190	0.000	-641	1328	1421	0.13	0.41	0.42	0.06
N. mikurensis	year * season + location	2	28	0.000	-641	1328	1421	0.13	0.41	0.42	0.06
R. helvetica	NULL				-1373	2748	2752				0.13
R. helvetica	year + season + location	1	1212	0.000	-767	1568	1636	0.44	0.97	0.97	0.11
R. helvetica	year * season + location	1	1231	0.000	-758	1561	1654	0.45	0.97	0.97	0.11
R. helvetica	year * season + location	2	19	0.004	-758	1561	1654	0.45	0.97	0.97	0.11
B. burgdorferi s.l.	NULL				-700	1402	1406				0.10
B. burgdorferi s.l.	year + season + location	1	199	0.000	-600	1235	1303	0.14	0.42	0.43	0.10
B. burgdorferi s.l.	year * season + location	1	202	0.000	-599	1243	1336	0.14	0.43	0.44	0.10
B. burgdorferi s.l.	year * season + location	2	4	0.709	-599	1243	1336	0.14	0.43	0.44	0.10
B. miyamotoi	NULL				-305	612	616				0.02
B. miyamotoi	year + season + location	1	15	0.549	-298	629	697	0.02	0.04	0.05	0.02
B. miyamotoi	year * season + location	1	29	0.142	-290	627	719	0.05	0.08	0.10	0.02
B. miyamotoi	year * season + location	2	14	0.025	-290	627	719	0.05	0.08	0.10	0.02

1 - comparison with the null model
2 - comparison with the no interaction model

Table S3. Analysis of variance for individual terms regarding the best fit spatio-temporal models for nymphal *Ixodes ricinus* infection prevalence. Interaction between year and season, when used, is represented with asterisks.

Symbiont (Y)	Model (~X)	term	Chisq	Pr(>Chi)
M. mitochondrii	year * season + location	(Intercept)	429.384	0.000
		year	11.366	0.010
		season	28.574	0.000
		location	157.981	0.000
		year:season	53.506	0.000
N. mikurensis	year * season + location	(Intercept)	1604.059	0.000
		year	4.294	0.231
		season	14.519	0.001
		location	116.258	0.000
		year:season	26.575	0.000
R. helvetica	year * season + location	(Intercept)	1464.978	0.000
		year	42.524	0.000
		season	22.361	0.000
		location	1043.572	0.000
		year:season	18.858	0.004
B. miyamotoi	year * season + location	(Intercept)	751.639	0.000
		year	0.112	0.990
		season	0.253	0.881
		location	12.448	0.331
		year:season	13.125	0.041
B. burgdorferi s.l.	year + season + location	year	12.477	0.006
		season	13.006	0.001
		location	153.912	0.000

Table S4. Summary results for the final spatio-temporal models. year[discrete] term refers to the model where the year variable was applied as a discrete variable. Spatio-temporal model regards the model with year, season and location as predictors. An interaction between year and season was used in all the spatio-temporal models, with exception for *B. burgdorferi* s.l. SE, standard error.

Model (~X)	term	*M. mitochondrii*				*R. helvetica*				*B. miyamotoi*				*N. mikurensis*				*B. burgdorferi* s.l.			
		estimate	**SE**	**z.value**	**Pr(>|z|)**	**estimate**	**SE**	**z.value**	**Pr(>|z|)**	**estimate**	**SE**	**z.value**	**Pr(>|z|)**	**estimate**	**SE**	**z.value**	**Pr(>|z|)**	**estimate**	**SE**	**z.value**	**Pr(>|z|)**
year	(Intercept)	-12.88	43.57	-0.30	0.768	-362.75	51.82	-7.00	**0.000**	25.58	131.45	0.19	0.846	57.32	76.48	0.75	0.454	150.54	59.37	2.54	**0.011**
	year[discrete]	0.01	0.02	0.31	0.753	0.18	0.03	6.97	**0.000**	-0.01	0.07	-0.22	0.824	-0.03	0.04	-0.78	0.434	-0.08	0.03	-2.57	**0.010**
Spatio-temporal	(Intercept)	0.88	0.16	5.50	**0.000**	-2.87	0.24	-12.10	**0.000**	-4.11	0.54	-7.55	**0.000**	-2.28	0.26	-8.74	**0.000**	-2.46	0.23	-10.68	**0.000**
	year[2013]	-0.02	0.15	-0.10	0.918	0.58	0.20	2.86	**0.004**	0.67	0.54	1.23	0.219	0.11	0.23	0.48	0.634	-0.02	0.10	-0.18	0.854
	year[2014]	-0.69	0.16	-4.34	**0.000**	0.53	0.21	2.47	**0.013**	0.78	0.56	1.39	0.165	0.41	0.24	1.67	0.096	-0.28	0.10	-2.75	**0.006**
	year[2015]	-0.27	0.14	-1.91	**0.056**	0.72	0.19	3.84	**0.000**	1.06	0.50	2.10	**0.036**	-0.19	0.23	-0.81	0.416	-0.23	0.10	-2.37	**0.018**
	season[spring]	-0.53	0.14	-3.89	**0.000**	0.45	0.19	2.40	**0.016**	1.11	0.49	2.24	**0.025**	-0.23	0.23	-0.99	0.322	-0.18	0.09	-2.05	**0.040**
	season[summer]	-0.11	0.16	-0.71	0.475	0.34	0.22	1.56	0.118	0.72	0.55	1.30	0.195	-0.07	0.25	-0.27	0.786	0.10	0.09	1.09	0.277
	location[Dronten]	0.31	0.17	1.84	0.066	0.69	0.23	2.99	**0.003**	-0.64	0.52	-1.25	0.211	-0.59	0.32	-1.85	0.064	1.42	0.25	5.76	**0.000**
	location[Ede]	-0.05	0.14	-0.35	0.727	-0.33	0.23	-1.41	0.158	-0.30	0.40	-0.76	0.447	0.08	0.24	0.32	0.751	0.80	0.24	3.34	**0.001**
	location[Gieten]	0.53	0.13	3.93	**0.000**	0.84	0.20	4.33	**0.000**	-0.65	0.38	-1.72	0.086	0.38	0.22	1.75	0.080	0.64	0.23	2.76	**0.006**
	location[HoogBaarlo]	0.68	0.15	4.68	**0.000**	-0.18	0.22	-0.80	0.426	-0.32	0.38	-0.83	0.406	-0.85	0.27	-3.12	**0.002**	-0.49	0.27	-1.79	0.074
	location[KwadeHoek]	1.57	0.42	3.78	**0.000**	1.40	0.32	4.43	**0.000**	-0.83	1.06	-0.79	0.432	0.41	0.41	1.01	0.311	1.71	0.34	5.04	**0.000**
	location[Montferland]	0.26	0.14	1.87	0.061	0.54	0.21	2.64	**0.008**	-0.50	0.40	-1.23	0.219	0.02	0.24	0.10	0.923	0.80	0.24	3.37	**0.001**
	location [Schiermonnikoog]	-0.38	0.20	-1.91	**0.055**	0.97	0.27	3.58	**0.000**	-1.69	1.05	-1.61	0.107	0.87	0.29	2.97	**0.003**	0.98	0.31	3.21	**0.001**
	location[Twiske]	-0.13	0.14	-0.93	0.354	0.93	0.21	4.49	**0.000**	-0.27	0.41	-0.67	0.500	-1.41	0.34	-4.11	**0.000**	0.28	0.26	1.07	0.282
	location[Vaals]	-0.04	0.15	-0.24	0.810	-0.30	0.25	-1.17	0.240	-0.74	0.49	-1.50	0.133	-1.66	0.43	-3.88	**0.000**	0.78	0.25	3.09	**0.002**
	location[Veldhoven]	0.26	0.13	1.95	**0.051**	0.01	0.20	0.05	0.959	-0.09	0.35	-0.25	0.803	0.28	0.22	1.30	0.195	0.70	0.23	3.06	**0.002**
	location[Wassenaar]	-0.21	0.13	-1.63	0.104	2.47	0.19	13.08	**0.000**	-0.04	0.35	-0.12	0.906	-0.22	0.23	-0.98	0.329	1.19	0.22	5.31	**0.000**
	year[2013]: season[spring]	0.50	0.20	2.55	**0.011**	-0.84	0.26	-3.27	**0.001**	-0.97	0.64	-1.53	0.127	0.01	0.32	0.03	0.973				
	year[2014]: season[spring]	0.70	0.19	3.68	**0.000**	-0.73	0.26	-2.80	**0.005**	-1.54	0.66	-2.35	**0.019**	-1.13	0.34	-3.30	**0.001**				
	year[2015]: season[spring]	0.52	0.17	3.02	**0.003**	-0.56	0.23	-2.45	**0.014**	-1.58	0.58	-2.73	**0.006**	0.38	0.29	1.29	0.196				
	year[2013]: season[summer]	-0.08	0.21	-0.40	0.688	-0.15	0.27	-0.55	0.579	-0.87	0.69	-1.25	0.211	-0.17	0.33	-0.52	0.604				
	year[2014]: season[summer]	0.99	0.21	4.73	**0.000**	-0.24	0.28	-0.87	0.386	-0.56	0.68	-0.82	0.410	-0.62	0.33	-1.88	0.060				
	year[2015]: season[summer]	0.42	0.20	2.13	**0.033**	0.12	0.26	0.45	0.650	-1.41	0.65	-2.15	**0.031**	-0.20	0.33	-0.62	0.533				

Table S5. Estimated marginal means for nymphal infections regarding the spatio-temporal factors season, year and location. Prob, probability; SE, standard error; LCL, lower confidence interval; UCL, upper confidence interval.

Term	*M. mitochondrii*				*R. helvetica*				*B. miyamotoi*				*N. mikurensis*				*B. burgdorferi* s.l.			
	prob	**SE**	**LCI**	**UCI**	**prob**	**SE**	**LCI**	**UCI**	**prob**	**SE**	**LCI**	**UCI**	**prob**	**SE**	**LCI**	**UCI**	**prob**	**SE**	**LCI**	**UCI**
Season																				
spring	0.68	0.01	0.66	0.70	0.13	0.01	0.12	0.14	0.02	0.00	0.01	0.03	0.06	0.00	0.05	0.07	0.11	0.01	0.10	0.13
summer	0.75	0.01	0.73	0.77	0.18	0.01	0.16	0.20	0.02	0.00	0.01	0.03	0.06	0.01	0.05	0.07	0.15	0.01	0.13	0.16
autumn	0.70	0.01	0.68	0.73	0.14	0.01	0.12	0.16	0.02	0.00	0.01	0.03	0.08	0.01	0.07	0.09	0.13	0.01	0.12	0.15
Year																				
2012	0.70	0.01	0.67	0.73	0.13	0.01	0.11	0.14	0.02	0.00	0.01	0.03	0.07	0.01	0.06	0.08	0.15	0.01	0.13	0.17
2013	0.73	0.01	0.71	0.75	0.14	0.01	0.13	0.16	0.02	0.00	0.01	0.03	0.07	0.01	0.06	0.09	0.14	0.01	0.13	0.16
2014	0.70	0.01	0.67	0.72	0.14	0.01	0.12	0.15	0.02	0.00	0.01	0.03	0.06	0.01	0.05	0.07	0.12	0.01	0.10	0.13
2015	0.72	0.01	0.70	0.74	0.19	0.01	0.17	0.21	0.02	0.00	0.01	0.03	0.07	0.01	0.06	0.08	0.12	0.01	0.11	0.14
Location																				
Bilthoven	0.66	0.03	0.61	0.71	0.09	0.01	0.06	0.12	0.03	0.01	0.02	0.06	0.08	0.01	0.06	0.12	0.07	0.01	0.05	0.10
Dronten	0.73	0.02	0.68	0.78	0.16	0.02	0.12	0.20	0.02	0.01	0.01	0.04	0.05	0.01	0.03	0.08	0.23	0.02	0.19	0.28
Ede	0.65	0.02	0.62	0.69	0.06	0.01	0.05	0.09	0.02	0.01	0.01	0.04	0.09	0.01	0.07	0.11	0.14	0.01	0.11	0.17
Gieten	0.77	0.01	0.75	0.80	0.18	0.01	0.16	0.20	0.02	0.00	0.01	0.03	0.11	0.01	0.10	0.13	0.12	0.01	0.10	0.14
Hoog Baarlo	0.80	0.01	0.77	0.82	0.07	0.01	0.06	0.09	0.02	0.01	0.02	0.04	0.04	0.01	0.03	0.05	0.04	0.01	0.03	0.06
Kwade Hoek	0.90	0.03	0.81	0.95	0.28	0.05	0.19	0.39	0.01	0.01	0.00	0.09	0.13	0.04	0.07	0.23	0.29	0.05	0.19	0.40
Montferland	0.71	0.02	0.68	0.75	0.14	0.01	0.12	0.17	0.02	0.01	0.01	0.03	0.08	0.01	0.06	0.10	0.14	0.01	0.12	0.17
Schiermonnikoog	0.57	0.04	0.49	0.64	0.20	0.03	0.15	0.28	0.01	0.01	0.00	0.04	0.17	0.03	0.12	0.24	0.16	0.03	0.11	0.23
Twiske	0.63	0.02	0.59	0.67	0.19	0.02	0.16	0.23	0.02	0.01	0.01	0.04	0.02	0.01	0.01	0.04	0.09	0.01	0.07	0.11
Vaals	0.65	0.02	0.60	0.69	0.07	0.01	0.05	0.09	0.02	0.01	0.01	0.03	0.02	0.01	0.01	0.03	0.14	0.02	0.11	0.17
Veldhoven	0.72	0.01	0.69	0.74	0.09	0.01	0.07	0.11	0.03	0.00	0.02	0.04	0.10	0.01	0.09	0.12	0.13	0.01	0.11	0.15
Wassenaar	0.62	0.01	0.59	0.65	0.53	0.01	0.50	0.56	0.03	0.00	0.02	0.04	0.07	0.01	0.05	0.08	0.19	0.01	0.17	0.22

Table S6. Odds ratios for the estimated marginal means pairwise comparisons regarding the spatio-temporal models. Interaction between year and season, when used, is represented with asterisks. OR, odds ratio; SE, standard error.

Model (~X)	Pairwise comparison terms	*M. mitochondrii*				*R. helvetica*				*B .miyamotoi*				*N. mikurensis*				*B. burgdorferi* s.l.			
		OR	SE	z.ratio	p.value	OR	SE	z.ratio	p.value	OR	SE	z.ratio	p.value	OR	SE	z.ratio	p.value	OR	SE	z.ratio	p.value
season																					
year + season + location	autumn / spring	1.119	0.071	1.758	0.184	1.095	0.090	1.102	0.513	1.022	0.194	0.113	0.993	1.341	0.145	2.715	0.018	1.200	0.107	2.049	0.101
year + season + location	autumn / summer	0.785	0.053	-3.573	0.001	0.740	0.062	-3.615	0.001	1.060	0.209	0.298	0.952	1.326	0.147	2.539	0.030	0.908	0.081	-1.088	0.522
year + season + location	spring / summer	0.702	0.040	-6.147	0.000	0.676	0.048	-5.492	0.000	1.038	0.178	0.217	0.974	0.989	0.103	-0.110	0.993	0.757	0.059	-3.566	0.001
year																					
year + season + location	2012 / 2013	0.869	0.067	-1.816	0.266	0.849	0.087	-1.596	0.381	1.129	0.260	0.527	0.953	0.938	0.123	-0.485	0.962	1.019	0.103	0.184	0.998
year + season + location	2012 / 2014	1.025	0.075	0.331	0.987	0.906	0.088	-1.018	0.739	1.067	0.231	0.300	0.991	1.202	0.160	1.386	0.508	1.323	0.135	2.748	0.031
year + season + location	2012 / 2015	0.911	0.064	-1.333	0.542	0.610	0.055	-5.440	0.000	1.134	0.235	0.605	0.931	1.067	0.130	0.536	0.950	1.253	0.120	2.367	0.084
year + season + location	2013 / 2014	1.180	0.086	2.255	0.109	1.066	0.099	0.692	0.900	0.945	0.206	-0.259	0.994	1.281	0.165	1.923	0.218	1.298	0.129	2.635	0.042
year + season + location	2013 / 2015	1.049	0.074	0.679	0.905	0.718	0.062	-3.840	0.001	1.004	0.212	0.020	1.000	1.138	0.134	1.097	0.692	1.230	0.115	2.222	0.117
year + season + location	2014 / 2015	0.889	0.058	-1.808	0.269	0.673	0.054	-4.928	0.000	1.063	0.206	0.313	0.989	0.888	0.105	-1.003	0.748	0.948	0.088	-0.582	0.937
location																					
year + season + location	Bilthoven / Dronten	0.711	0.118	-2.050	0.659	0.518	0.120	-2.839	0.164	1.934	0.996	1.281	0.982	1.808	0.572	1.871	0.777	0.241	0.060	-5.756	0.000
year + season + location	Bilthoven / Ede	1.041	0.145	0.288	1.000	1.416	0.331	1.488	0.944	1.378	0.544	0.812	1.000	0.932	0.220	-0.296	1.000	0.450	0.108	-3.337	0.040
year + season + location	Bilthoven / Gieten	0.566	0.076	-4.234	0.001	0.443	0.086	-4.193	0.002	1.853	0.703	1.625	0.900	0.702	0.152	-1.634	0.897	0.529	0.122	-2.757	0.199
year + season + location	Bilthoven / HoogBaarlo	0.495	0.072	-4.860	0.000	1.228	0.274	0.921	0.999	1.326	0.509	0.736	1.000	2.286	0.617	3.061	0.092	1.631	0.447	1.785	0.827
year + season + location	Bilthoven / KwadeHoek	0.206	0.085	-3.820	0.007	0.258	0.081	-4.323	0.001	2.338	2.463	0.806	1.000	0.599	0.244	-1.259	0.984	0.181	0.061	-5.045	0.000
year + season + location	Bilthoven / Montferland	0.777	0.109	-1.808	0.814	0.583	0.119	-2.637	0.259	1.665	0.671	1.266	0.983	1.030	0.243	0.124	1.000	0.451	0.107	-3.372	0.036
year + season + location	Bilthoven / Schiermonnikoog	1.479	0.294	1.967	0.716	0.382	0.103	-3.553	0.020	5.206	5.469	1.571	0.920	0.432	0.126	-2.872	0.151	0.375	0.114	-3.213	0.059
year + season + location	Bilthoven / Twiske	1.124	0.160	0.821	1.000	0.407	0.084	-4.332	0.001	1.343	0.546	0.726	1.000	3.973	1.361	4.028	0.003	0.757	0.196	-1.075	0.996
year + season + location	Bilthoven / Vaals	1.068	0.161	0.440	1.000	1.362	0.344	1.220	0.988	2.177	1.067	1.587	0.914	5.654	2.420	4.047	0.003	0.459	0.116	-3.094	0.084
year + season + location	Bilthoven / Veldhoven	0.774	0.102	-1.956	0.723	1.010	0.206	0.050	1.000	1.100	0.387	0.271	1.000	0.786	0.171	-1.107	0.994	0.495	0.114	-3.064	0.091
year + season + location	Bilthoven / Wassenaar	1.194	0.153	1.379	0.968	0.087	0.016	-12.949	0.000	1.017	0.353	0.048	1.000	1.266	0.286	1.047	0.997	0.304	0.068	-5.306	0.000
year + season + location	Dronten / Ede	1.463	0.210	2.650	0.252	2.735	0.574	4.789	0.000	0.712	0.343	-0.705	1.000	0.516	0.146	-2.338	0.449	1.866	0.313	3.719	0.011
year + season + location	Dronten / Gieten	0.796	0.111	-1.638	0.895	0.855	0.141	-0.950	0.999	0.958	0.450	-0.092	1.000	0.389	0.104	-3.540	0.021	2.193	0.343	5.026	0.000
year + season + location	Dronten / HoogBaarlo	0.696	0.104	-2.435	0.382	2.372	0.469	4.366	0.001	0.686	0.323	-0.800	1.000	1.264	0.394	0.753	1.000	6.757	1.450	8.907	0.000

table continues

Model (~X)	Pairwise comparison terms	*M. mitochondrii*				*R. helvetica*				*B .miyamotoi*				*N. mikurensis*				*B. burgdorferi* s.l.			
		OR	SE	z.ratio	p.value	OR	SE	z.ratio	p.value	OR	SE	z.ratio	p.value	OR	SE	z.ratio	p.value	OR	SE	z.ratio	p.value
year + season + location	Dronten / KwadeHoek	0.289	0.120	-2.985	0.113	0.498	0.148	-2.350	0.441	1.209	1.317	0.174	1.000	0.332	0.145	-2.531	0.321	0.751	0.220	-0.977	0.998
year + season + location	Dronten / Montferland	1.092	0.158	0.609	1.000	1.125	0.200	0.664	1.000	0.861	0.421	-0.306	1.000	0.570	0.161	-1.986	0.704	1.867	0.308	3.788	0.008
year + season + location	Dronten / Schiermonnikoog	2.079	0.420	3.626	0.015	0.738	0.184	-1.216	0.988	2.691	2.921	0.912	0.999	0.239	0.079	-4.332	0.001	1.552	0.393	1.736	0.852
year + season + location	Dronten / Twiske	1.580	0.232	3.120	0.078	0.787	0.141	-1.336	0.974	0.694	0.341	-0.743	1.000	2.197	0.826	2.093	0.628	3.137	0.609	5.885	0.000
year + season + location	Dronten / Vaals	1.502	0.233	2.626	0.265	2.629	0.609	4.171	0.002	1.125	0.633	0.210	1.000	3.128	1.426	2.501	0.339	1.900	0.354	3.445	0.028
year + season + location	Dronten / Veldhoven	1.087	0.149	0.612	1.000	1.951	0.345	3.777	0.009	0.569	0.255	-1.258	0.984	0.435	0.117	-3.101	0.082	2.052	0.317	4.652	0.000
year + season + location	Dronten / Wassenaar	1.678	0.224	3.875	0.006	0.168	0.027	-11.303	0.000	0.526	0.233	-1.449	0.954	0.701	0.193	-1.295	0.980	1.260	0.184	1.581	0.916
year + season + location	Ede / Gieten	0.544	0.057	-5.810	0.000	0.313	0.052	-6.940	0.000	1.345	0.445	0.895	0.999	0.753	0.123	-1.741	0.849	1.175	0.167	1.136	0.993
year + season + location	Ede / HoogBaarlo	0.476	0.056	-6.315	0.000	0.867	0.174	-0.709	1.000	0.963	0.323	-0.113	1.000	2.451	0.563	3.905	0.005	3.622	0.743	6.271	0.000
year + season + location	Ede / KwadeHoek	0.197	0.080	-4.000	0.004	0.182	0.054	-5.723	0.000	1.697	1.760	0.510	1.000	0.643	0.245	-1.160	0.992	0.402	0.115	-3.186	0.064
year + season + location	Ede / Montferland	0.746	0.084	-2.614	0.271	0.412	0.074	-4.932	0.000	1.209	0.434	0.529	1.000	1.104	0.209	0.525	1.000	1.001	0.152	0.003	1.000
year + season + location	Ede / Schiermonnikoog	1.421	0.256	1.953	0.725	0.270	0.068	-5.207	0.000	3.779	3.904	1.287	0.981	0.464	0.118	-3.030	0.100	0.832	0.204	-0.752	1.000
year + season + location	Ede / Twiske	1.080	0.123	0.675	1.000	0.288	0.052	-6.868	0.000	0.975	0.350	-0.070	1.000	4.261	1.323	4.668	0.000	1.681	0.307	2.842	0.163
year + season + location	Ede / Vaals	1.027	0.128	0.210	1.000	0.961	0.224	-0.168	1.000	1.580	0.717	1.009	0.998	6.064	2.448	4.466	0.000	1.018	0.178	0.103	1.000
year + season + location	Ede / Veldhoven	0.743	0.075	-2.936	0.128	0.713	0.127	-1.891	0.765	0.798	0.239	-0.751	1.000	0.843	0.139	-1.036	0.997	1.100	0.154	0.682	1.000
year + season + location	Ede / Wassenaar	1.147	0.111	1.416	0.961	0.061	0.010	-17.423	0.000	0.738	0.216	-1.037	0.997	1.358	0.237	1.752	0.844	0.676	0.088	-2.995	0.110
year + season + location	Gieten / HoogBaarlo	0.875	0.098	-1.199	0.989	2.775	0.422	6.709	0.000	0.716	0.227	-1.056	0.996	3.254	0.677	5.667	0.000	3.082	0.601	5.766	0.000
year + season + location	Gieten / KwadeHoek	0.363	0.147	-2.507	0.335	0.582	0.156	-2.014	0.684	1.262	1.302	0.225	1.000	0.853	0.315	-0.430	1.000	0.342	0.096	-3.836	0.007
year + season + location	Gieten / Montferland	1.372	0.146	2.982	0.114	1.317	0.164	2.206	0.545	0.899	0.307	-0.312	1.000	1.466	0.239	2.348	0.442	0.851	0.118	-1.163	0.992
year + season + location	Gieten / Schiermonnikoog	2.613	0.462	5.428	0.000	0.864	0.187	-0.679	1.000	2.810	2.889	1.005	0.998	0.616	0.145	-2.053	0.656	0.708	0.168	-1.454	0.953
year + season + location	Gieten / Twiske	1.985	0.216	6.302	0.000	0.920	0.117	-0.652	1.000	0.725	0.249	-0.936	0.999	5.655	1.674	5.855	0.000	1.431	0.247	2.076	0.640
year + season + location	Gieten / Vaals	1.887	0.226	5.306	0.000	3.076	0.597	5.794	0.000	1.175	0.518	0.366	1.000	8.050	3.158	5.317	0.000	0.866	0.142	-0.878	0.999
year + season + location	Gieten / Veldhoven	1.366	0.128	3.319	0.043	2.283	0.278	6.770	0.000	0.594	0.165	-1.877	0.774	1.119	0.148	0.851	0.999	0.936	0.117	-0.531	1.000
year + season + location	Gieten / Wassenaar	2.108	0.189	8.328	0.000	0.197	0.018	-17.491	0.000	0.549	0.149	-2.217	0.537	1.803	0.262	4.054	0.003	0.575	0.066	-4.835	0.000
year + season + location	HoogBaarlo / KwadeHoek	0.415	0.169	-2.156	0.582	0.210	0.061	-5.380	0.000	1.763	1.823	0.548	1.000	0.262	0.106	-3.317	0.043	0.111	0.035	-6.945	0.000

table continues

Model (~X)	Pairwise comparison terms	*M. mitochondrii*				*R. helvetica*				*B .miyamotoi*				*N. mikurensis*				*B. burgdorferi* s.l.			
		OR	SE	z.ratio	p.value	OR	SE	z.ratio	p.value	OR	SE	z.ratio	p.value	OR	SE	z.ratio	p.value	OR	SE	z.ratio	p.value
year + season + location	HoogBaarlo / Montferland	1.569	0.186	3.790	0.008	0.474	0.079	-4.485	0.000	1.256	0.435	0.657	1.000	0.450	0.104	-3.470	0.026	0.276	0.056	-6.345	0.000
year + season + location	HoogBaarlo / Schiermonnikoog	2.987	0.551	5.931	0.000	0.311	0.075	-4.823	0.000	3.925	4.040	1.328	0.976	0.189	0.054	-5.825	0.000	0.230	0.064	-5.261	0.000
year + season + location	HoogBaarlo / Twiske	2.270	0.275	6.765	0.000	0.332	0.056	-6.561	0.000	1.013	0.353	0.037	1.000	1.738	0.586	1.638	0.895	0.464	0.106	-3.375	0.036
year + season + location	HoogBaarlo / Vaals	2.158	0.283	5.874	0.000	1.108	0.247	0.462	1.000	1.641	0.729	1.116	0.994	2.474	1.050	2.135	0.598	0.281	0.062	-5.760	0.000
year + season + location	HoogBaarlo / Veldhoven	1.562	0.169	4.116	0.002	0.822	0.135	-1.189	0.990	0.829	0.236	-0.658	1.000	0.344	0.072	-5.089	0.000	0.304	0.059	-6.152	0.000
year + season + location	HoogBaarlo / Wassenaar	2.411	0.252	8.425	0.000	0.071	0.010	-18.375	0.000	0.767	0.212	-0.959	0.998	0.554	0.121	-2.708	0.222	0.187	0.035	-8.959	0.000
year + season + location	KwadeHoek / Montferland	3.780	1.534	3.276	0.049	2.262	0.624	2.957	0.122	0.712	0.741	-0.326	1.000	1.718	0.655	1.420	0.960	2.486	0.705	3.209	0.060
year + season + location	KwadeHoek / Schiermonnikoog	7.197	3.093	4.592	0.000	1.483	0.486	1.204	0.989	2.227	3.167	0.563	1.000	0.722	0.301	-0.782	1.000	2.067	0.709	2.116	0.611
year + season + location	KwadeHoek / Twiske	5.469	2.224	4.179	0.002	1.581	0.438	1.652	0.889	0.575	0.598	-0.532	1.000	6.629	3.009	4.167	0.002	4.178	1.261	4.738	0.000
year + season + location	KwadeHoek / Vaals	5.198	2.129	4.024	0.003	5.284	1.655	5.314	0.000	0.931	1.003	-0.066	1.000	9.435	4.923	4.301	0.001	2.530	0.750	3.130	0.075
year + season + location	KwadeHoek / Veldhoven	3.763	1.517	3.287	0.047	3.921	1.079	4.963	0.000	0.471	0.481	-0.737	1.000	1.312	0.485	0.734	1.000	2.733	0.760	3.618	0.016
year + season + location	KwadeHoek / Wassenaar	5.807	2.336	4.374	0.001	0.338	0.089	-4.110	0.002	0.435	0.444	-0.816	1.000	2.113	0.792	1.997	0.696	1.679	0.459	1.893	0.764
year + season + location	Montferland / Schiermonnikoog	1.904	0.345	3.556	0.019	0.656	0.148	-1.867	0.780	3.126	3.243	1.099	0.995	0.420	0.107	-3.406	0.032	0.831	0.202	-0.759	1.000
year + season + location	Montferland / Twiske	1.447	0.167	3.192	0.063	0.699	0.100	-2.494	0.344	0.807	0.299	-0.580	1.000	3.858	1.201	4.339	0.001	1.681	0.304	2.874	0.150
year + season + location	Montferland / Vaals	1.375	0.173	2.532	0.320	2.336	0.478	4.148	0.002	1.307	0.604	0.580	1.000	5.492	2.217	4.219	0.001	1.018	0.175	0.102	1.000
year + season + location	Montferland / Veldhoven	0.996	0.102	-0.042	1.000	1.734	0.241	3.963	0.004	0.660	0.206	-1.331	0.975	0.764	0.125	-1.641	0.894	1.100	0.149	0.698	1.000
year + season + location	Montferland / Wassenaar	1.536	0.151	4.373	0.001	0.149	0.017	-16.542	0.000	0.611	0.186	-1.616	0.904	1.230	0.215	1.183	0.990	0.675	0.086	-3.091	0.084
year + season + location	Schiermonnikoog / Twiske	0.760	0.138	-1.507	0.939	1.066	0.241	0.281	1.000	0.258	0.268	-1.306	0.979	9.185	3.250	6.267	0.000	2.022	0.533	2.671	0.241
year + season + location	Schiermonnikoog / Vaals	0.722	0.137	-1.721	0.859	3.562	0.962	4.705	0.000	0.418	0.449	-0.812	1.000	13.074	5.725	5.870	0.000	1.224	0.316	0.784	1.000
year + season + location	Schiermonnikoog / Veldhoven	0.523	0.091	-3.707	0.011	2.643	0.595	4.320	0.001	0.211	0.215	-1.526	0.934	1.818	0.432	2.514	0.331	1.323	0.313	1.182	0.990

table continues

Model (~X)	Pairwise comparison terms	*M. mitochondrii*				*R. helvetica*				*B .miyamotoi*				*N. mikurensis*				*B. burgdorferi* s.l.			
		OR	SE	z.ratio	p.value	OR	SE	z.ratio	p.value	OR	SE	z.ratio	p.value	OR	SE	z.ratio	p.value	OR	SE	z.ratio	p.value
year + season + location	Schiermonnikoog / Wassenaar	0.807	0.139	-1.244	0.985	0.228	0.048	-7.038	0.000	0.195	0.199	-1.606	0.907	2.928	0.717	4.390	0.001	0.812	0.188	-0.899	0.999
year + season + location	Twiske / Vaals	0.951	0.122	-0.396	1.000	3.343	0.690	5.848	0.000	1.621	0.750	1.044	0.997	1.423	0.674	0.746	1.000	0.606	0.121	-2.508	0.334
year + season + location	Twiske / Veldhoven	0.688	0.072	-3.549	0.020	2.480	0.352	6.405	0.000	0.819	0.257	-0.637	1.000	0.198	0.059	-5.454	0.000	0.654	0.112	-2.484	0.350
year + season + location	Twiske / Wassenaar	1.062	0.107	0.595	1.000	0.214	0.025	-13.202	0.000	0.757	0.232	-0.909	0.999	0.319	0.096	-3.779	0.009	0.402	0.066	-5.588	0.000
year + season + location	Vaals / Veldhoven	0.724	0.084	-2.776	0.190	0.742	0.151	-1.468	0.949	0.505	0.211	-1.634	0.897	0.139	0.055	-5.021	0.000	1.080	0.174	0.480	1.000
year + season + location	Vaals / Wassenaar	1.117	0.126	0.983	0.998	0.064	0.012	-14.643	0.000	0.467	0.193	-1.844	0.794	0.224	0.089	-3.764	0.009	0.664	0.102	-2.667	0.243
year + season + location	Veldhoven / Wassenaar	1.543	0.131	5.100	0.000	0.086	0.010	-21.882	0.000	0.925	0.214	-0.339	1.000	1.611	0.237	3.239	0.055	0.614	0.069	-4.364	0.001
2012																					
year * season + location	autumn / spring	1.693	0.229	3.887	0.000	0.638	0.119	-2.405	0.043	0.330	0.163	-2.244	0.064	1.253	0.285	0.991	0.583	1.250	0.222	1.253	0.422
year * season + location	autumn / summer	1.119	0.176	0.715	0.755	0.714	0.154	-1.565	0.261	0.488	0.270	-1.297	0.397	1.071	0.269	0.271	0.960	0.880	0.169	-0.661	0.786
year * season + location	spring / summer	0.661	0.089	-3.078	0.006	1.120	0.205	0.618	0.810	1.480	0.564	1.030	0.558	0.855	0.199	-0.674	0.779	0.705	0.124	-1.989	0.115
2013																					
year * season + location	autumn / spring	1.027	0.145	0.188	0.981	1.482	0.264	2.206	0.070	0.873	0.353	-0.337	0.939	1.239	0.275	0.969	0.597	1.155	0.206	0.807	0.699
year * season + location	autumn / summer	1.216	0.165	1.441	0.320	0.831	0.140	-1.101	0.513	1.160	0.482	0.357	0.932	1.270	0.272	1.116	0.504	0.963	0.165	-0.220	0.974
year * season + location	spring / summer	1.184	0.150	1.335	0.376	0.561	0.092	-3.529	0.001	1.329	0.513	0.738	0.741	1.025	0.223	0.112	0.993	0.834	0.136	-1.112	0.507
2014																					
year * season + location	autumn / spring	0.839	0.113	-1.307	0.391	1.321	0.239	1.541	0.272	1.542	0.669	0.999	0.577	3.874	0.992	5.289	0.000	1.086	0.236	0.380	0.923
year * season + location	autumn / summer	0.415	0.057	-6.349	0.000	0.907	0.156	-0.564	0.839	0.852	0.331	-0.413	0.910	1.998	0.431	3.208	0.004	0.700	0.145	-1.721	0.197
year * season + location	spring / summer	0.495	0.053	-6.523	0.000	0.687	0.092	-2.808	0.014	0.552	0.183	-1.789	0.173	0.516	0.119	-2.861	0.012	0.644	0.098	-2.889	0.011
2015																					
year * season + location	autumn / spring	1.004	0.109	0.035	0.999	1.117	0.149	0.833	0.683	1.599	0.483	1.552	0.267	0.859	0.158	-0.825	0.688	1.235	0.189	1.381	0.351
year * season + location	autumn / summer	0.735	0.088	-2.562	0.028	0.636	0.088	-3.271	0.003	1.996	0.700	1.969	0.120	1.313	0.278	1.288	0.402	1.059	0.170	0.357	0.932
year * season + location	spring / summer	0.732	0.076	-3.024	0.007	0.569	0.067	-4.771	0.000	1.248	0.422	0.657	0.788	1.529	0.282	2.299	0.056	0.858	0.122	-1.081	0.526

Table S7. Goodness of fit parameters for the climatic models. Deviance and p were obtained in comparison with the null models. Climatic data from the previous season was used in the models in bold (within parenthesis). McFadden, Cox-Snell and Nagelkerke are three different algoritms to calculate pseudo R^2 values (ρ2). EV, evaporation rate; Prec, precipitation; RH, relative humidity; Tmean, mean temperature; logLik, logarithm of the likelihood; AIC, Akaike information criterion; BIC, Bayesian information criterion.

Symbiont (Y)	Model (~X)	Deviance	Pr(>Chi)	logLik	AIC	BIC	McFadden ρ^2	Cox.Snell ρ^2	Nagelkerke ρ^2	Brier. Score
M. mitochondrii	NULL			-598	1197	1200				0.22
M. mitochondrii	EV + Prec + RH + Tmean	50	0.000	-573	1155	1170	0.04	0.30	0.30	0.22
M. mitochondrii	NULL			-598	1197	1200				0.22
M. mitochondrii	**(EV + Prec + RH + Tmean)**	54	0.000	-571	1151	1166	0.04	0.32	0.32	0.22
R. helvetica	NULL			-806	1614	1617				0.13
R. helvetica	EV + Prec + RH + Tmean	108	0.000	-752	1515	1529	0.07	0.54	0.54	0.13
R. helvetica	NULL			-806	1614	1617				0.13
R. helvetica	**(EV + Prec + RH + Tmean)**	70	0.000	-771	1553	1567	0.04	0.40	0.40	0.13
B. miyamotoi	NULL			-177	357	360				0.02
B. miyamotoi	EV + Prec + RH + Tmean	3	0.628	-176	362	377	0.01	0.02	0.02	0.02
B. miyamotoi	NULL			-177	357	360				0.02
B. miyamotoi	**(EV + Prec + RH + Tmean)**	7	0.139	-174	358	373	0.02	0.05	0.05	0.02
N. mikurensis	NULL			-437	876	879				0.07
N. mikurensis	EV + Prec + RH + Tmean	37	0.000	-419	847	862	0.04	0.23	0.23	0.07
N. mikurensis	NULL			-437	876	879				0.07
N. mikurensis	**(EV + Prec + RH + Tmean)**	24	0.000	-425	860	875	0.03	0.16	0.16	0.07
B. burgdorferi s.l.	NULL			-418	837	840				0.11
B. burgdorferi s.l.	EV + Prec + RH + Tmean	8	0.093	-414	837	852	0.01	0.06	0.06	0.11
B. burgdorferi s.l.	NULL			-418	837	840				0.11
B. burgdorferi s.l.	**(EV + Prec + RH + Tmean)**	11	0.032	-412	835	850	0.01	0.07	0.07	0.11

Table S8. Summary results for the climatic models, without and with climatic data season shift. No interactions were introduced. EV, evaporation rate; Prec, precipitation; RH, relative humidity; Tmean, mean temperature; SE, standard error.

Symbiont	term	Climatic Model (no shift)				Climatic Model (shift)			
		estimate	**SE**	**z.value**	**Pr(>|z|)**	**estimate**	**SE**	**z.value**	**Pr(>|z|)**
M. mitochondrii	(Intercept)	0.48	0.56	0.86	0.390	4.27	0.77	5.56	**0.000**
	EV	0.07	0.05	1.24	0.214	-0.14	0.06	-2.25	**0.025**
	Prec	0.15	0.04	4.00	**0.000**	-0.07	0.04	-1.87	0.062
	RH	0.00	0.01	-0.58	0.562	-0.04	0.01	-4.13	**0.000**
	Tmean	0.01	0.01	0.98	0.325	0.01	0.01	0.93	0.355
R. helvetica	(Intercept)	-4.83	0.73	-6.63	**0.000**	-5.53	0.93	-5.96	**0.000**
	EV	0.24	0.07	3.31	**0.001**	0.57	0.09	6.62	**0.000**
	Prec	0.29	0.04	6.90	**0.000**	-0.08	0.05	-1.75	0.079
	RH	0.03	0.01	3.48	**0.000**	0.05	0.01	4.13	**0.000**
	Tmean	-0.03	0.02	-1.59	0.112	-0.06	0.02	-3.49	**0.000**
B. miyamotoi	(Intercept)	-6.14	1.70	-3.60	**0.000**	0.94	2.27	0.41	0.678
	EV	0.20	0.17	1.17	0.242	-0.37	0.17	-2.20	**0.028**
	Prec	-0.01	0.11	-0.07	0.941	0.10	0.12	0.81	0.417
	RH	0.03	0.02	1.45	0.146	-0.06	0.03	-2.05	**0.041**
	Tmean	-0.03	0.04	-0.67	0.501	0.04	0.04	1.02	0.310
N. mikurensis	(Intercept)	-7.91	0.99	-8.03	**0.000**	-7.26	1.32	-5.48	**0.000**
	EV	0.34	0.10	3.30	**0.001**	0.30	0.11	2.67	**0.008**
	Prec	-0.03	0.06	-0.46	0.646	-0.06	0.06	-0.92	0.358
	RH	0.07	0.01	5.79	**0.000**	0.05	0.02	3.35	**0.001**
	Tmean	-0.05	0.03	-2.13	**0.034**	0.00	0.02	0.02	0.985
B. burgdorferi s.l.	(Intercept)	-3.07	0.78	-3.93	**0.000**	-0.88	1.01	-0.87	0.384
	EV	0.00	0.08	-0.05	0.961	0.02	0.08	0.27	0.784
	Prec	-0.03	0.05	-0.60	0.546	-0.04	0.05	-0.70	0.482
	RH	0.01	0.01	1.11	0.268	-0.01	0.01	-0.98	0.328
	Tmean	0.03	0.02	1.65	0.098	0.00	0.02	0.13	0.898

Table S9. Detailed information regarding vegetation descriptions by location transects in this study. Location transects coordinates, the thickness of litter and humus layers (cm), the abundance of moss, berries, and grasses in the understory measured in coverage classes accordingly to Braun-Blanket, adjusted by Van der Maarel, and dominant tree species. GPS Coord, GPS coordinates; Q., Quercus; B., Betulus; P., Pinus; S., Salix; F., Fagus ; P., Popules; Sa., Sambucus; H., Hippophae; R., Robinia

Transect	GPS Coord.	Litter Layer	Humus layer	*Bryophyta*	*Vaccinium*	*Molinia*	*Deschampsia*	*Habitat type*	Dominant Vegetation/Tree species
Bilthoven 1	52°09′22N, 5°13′40E	2.5	4	8	-	-	8	Forest	*Betulo-Quercetum roboris deschampsietosum (Q. robur)*
Bilthoven 2	52°09′27N, 5°13′47E	3	5	8	-	-	9	Forest	*Leuco-Pinetum deschampsietosum (P. sylvestris)*
Dronten 1	52°27′01N, 5°46′25E	4.25	0	6	-	-	-	Forest	*Salicetum cinereae salitosum repentis (Q. robur, S. caprea)*
Dronten 2	52°36′17N, 5°38′20E	3.25	1.25	1	-	-	-	Forest	*Violo odoratae-ulmetum inops (Q. robur, Acer sp., Alnus sp., Fraxinus sp., F. sylvaticus)*
Ede 1	52°01′41N, 5°41′49E	0	0	6	9	-	5	Forest	*Leucobryo-pinetum vaccinietosum (P. sylvestris, B. pendula, Larix sp.)*
Ede 2	52°01′41N, 5°41′49E	0	0	5	7	-	5	Forest	*Betulo - Quercetum roboris vaccinietosum (Q. robur)*
Gieten 1	53°00′54N, 6°45′18E	2	7.25	1	-	-	8	Forest	*Betulo Quercetum roboris deschampsietosum (B. pubescens, P. tremula)*
Gieten 2	53°00′56N, 6°45′12E	2.75	5.75	1	-	-	1	Forest	*Fago Quercetum holcetosum (Q. robur, B. pubescens, Corylus avellane)*
Hoog Baarlo 1	52°06′20N, 5°52′29E	1.5	3.25	5	9	-	-	Forest	*Betulo Quercetum roboris vaccinietosum (Q. robur, Q. petrea, P. sylvestris, B. pendula)*
Hoog Baarlo 2	52°06′20N, 5°52′29E	4.75	3.25	5	9	-	-	Forest	*Betulo Quercetum roboris vaccinietosum (Q. robur, Q. petrea, P. sylvestris, B. pendula)*
Kwade Hoek 1	51°50′25N, 3°58′58E	1.75	2.5	1	-	-	-	Coast/ Dunes	*Hippophao-sambucetum (Sa. nigra, H. rhamnoides)*
Kwade Hoek 2	51°50′25N, 3°58′58E	2.5	4.75	1	-	-	-	Coast/ Dunes	*Rhamno Crataegetum (H. rhamnoides)*
Montferland 1	51°53′29N, 6°13′10E	2.75	5.25	8	8	-	-	Forest	*Leucobryo-pinetum vaccinietosum (B. pendula, Q. robur, Picea abies, Larix sp.)*
Montferland 2	51°55′42N, 6°13′23E	1.75	0	1	-	-	-	Forest	*Quercetea Robori-Petraeae [Robinianum] (R. pseudoacacia, Q. robur)*
Schiermonnikoog 1	53°29′34N, 6°09′46E	4	3.25	3	-	-	-	Coast/ Dunes	*Covallario Quercetum dunense (B. pendula, B. pubescens)*
Schiermonnikoog 2	53°29′44N, 6°09′55E	1.75	4.75	4	-	-	-	Coast/ Dunes	*Carex arenaria-Calamogrostis epigejos-[Dicrano-Pinion] (P. nigra)*
Twiske 1	52°26′52N, 4°53′35E	3.25	0	1	-	-	-	Marsh	*Artemisio-salicetum albae agrostietosum stoloniferae (S. alba, S. caprea)*

table continues

Transect	GPS Coord.	Litter Layer	Humus layer	*Bryophyta*	*Vaccinium*	*Molinia*	*Deschampsia*	*Habitat type*	Dominant Vegetation/Tree species
Twiske 2	52°26′52N, 4°53′35E	0	0	1	-	-	-	Marsh	*Lychnido-Hypericetum Tetrapteri orchietosum morionis (Hardly any trees: S. alba, S.caprea)*
Vaals 1	50°47′38N, 5°57′19E	0.25	0	2	-	-	-	Forest	*Stellario-carpinetum allietosum (Prunus sp., Ulmus sp., Q. robur, Acer sp.)*
Vaals 2	50°45′48N, 5°58′57E	3.5	3	1	1	-	2	Forest	*Luzulo Luzuloides Fagetum (Q. rubus, F. sylvatica)*
Veldhoven 1	51°25′19N, 5°20′02E	3.25	5	5	2	8	8	Forest	*Betulo Quercetum roboris molinietosum (Q. robur, P. sylvestris)*
Veldhoven 2	51°25′19N, 5°20′02E	2	3.25	5	2	8	8	Forest	*Betulo Quercetum roboris molinietosum (Q. robur, P. sylvestris)*
Wassenaar 1	52°09′32N, 4°21′41E	3	2	2	-	-	-	Coast/ Dunes	*Carex arenaria [Dicrano - Pinion] (P. nigra)*
Wassenaar 2	52°09′23N, 4°21′38E	2.25	2.25	5	-	-	-	Coast/ Dunes	*Calamagrostis epigejos [Dicrano - Pinion] (P. nigra)*

Table S10. Goodness of fit parameters and summary results for the spatio-temporal model of Midichloria mitochondrii NIPs including DON as explanatory variable. Pr(>Chi) was obtained in comparison with the null model, and the only significant between all the bacterial symbionts tested in this manuscript. McFadden, Cox-Snell (CS) and Nagelkerke (NG) were the three different algorithms used to calculate pseudo R^2 values (ρ^2). SE, standard error; logLik, logarithm of the likelihood; AIC, Akaike information criterion; BIC, Bayesian information criterion.

Symbiont (Y)	Model (~X)			Pr(>Chi)
M.mitochondrii	year * season + location + DON			0.000
Analysis of variance for individual terms				
term	**estimate**	**SE**	**z.value**	**Pr(>|z|)**
(Intercept)	0.97	0.12	8.334	0.000
year [2013]	0.44	0.12	3.563	0.000
year [2014]	0.00	0.11	0.040	0.968
year [2015]	0.19	0.10	1.908	0.056
season [summer]	0.43	0.13	3.166	0.002
season [autumn]	0.56	0.14	4.099	0.000
location [Bilthoven]	-0.63	0.15	-4.260	0.000
location [Twiske]	-0.80	0.12	-6.588	0.000
location [Gieten]	-0.20	0.11	-1.743	0.081
location [Veldhoven]	-0.48	0.11	-4.319	0.000
location [Vaals]	-0.69	0.13	-5.211	0.000
location [Ede]	-0.70	0.12	-5.910	0.000
location [Montferland]	-0.41	0.12	-3.443	0.001
location [Schiermonnikoog]	-0.99	0.19	-5.298	0.000
location [Wassenaar]	-0.96	0.11	-8.825	0.000
location [Dronten]	-0.32	0.15	-2.122	0.034
location [KwadeHoek]	0.96	0.41	2.354	0.019
DON	0.00	0.00	2.536	0.011
Goodness of fit parameters				
logLik	**AIC**	**BIC**	**McFadden ρ^2**	**CS & NG ρ^2**
-978.992	2006	2102	0.13	0.56

Table S11. Discriminated 21 out of 648 planned collections that had not been performed during this study.

Location	**Date**	**Assumed Nº Ticks**	**Reason**
Bilthoven	Dec-13	0	Occurrence of snow and frost in the winter did not allow the field work to be performed
	Jan-14		
Dronten	Feb-12		
Gieten	Dec-12		
Hoog Baarlo	Feb-15		
Vaals	Dec-14		
Bilthoven	May-13	N/A	Other reasons such as unavailability of volunteers and lack of conditions to be performed
	May-14		
	Jun-14		
	Jul-14		
Ede	Mar-13		
	Jul-13		
Kwade Hoek	Jul-14		
	Aug-14		
Montferland	Sep-12		
	Jun-14		
Twiske	Jul-14		
Vaals	Oct-15		

Table S12. Genes targeted in multiplex qPCR assays for detection of tick microorganisms.

Symbiont	Target	Reference
R. helvetica	gltA	de Bruin et al., 2015
M. mitochondrii	gyrB	Chapter 2
B. miyamotoi	flaB	Hovius et al., 2013
B. burgdorferi s.l	ospA flaB	Heylen et al., 2013b
N. mikurensis	GroEL	Jahfari et al., 2012

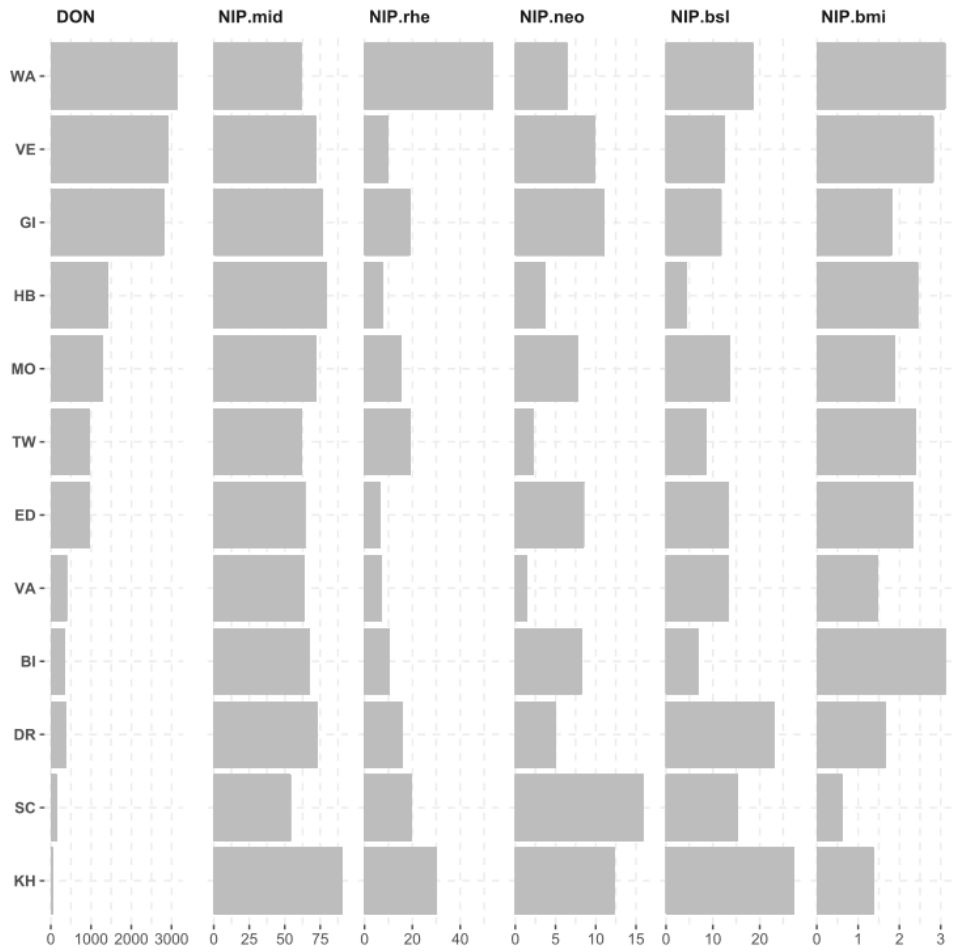


Figure S1. Density of ticks and nymphal infection prevalence (NIP) per site. The densities were calculated using the number of collected ticks per 100m2. The locations are represented in the Y-axis. BI, Bilthoven; DR, Dronten; ED, Ede; GI, Gieten; HB, Hoog Baarlo; KH, Kwade Hoek; MO, Montferland; SC, Schiermonnikoog; TW, Twiske; VA, Vaals; VE, Veldhoven; WA, Wassenaar.

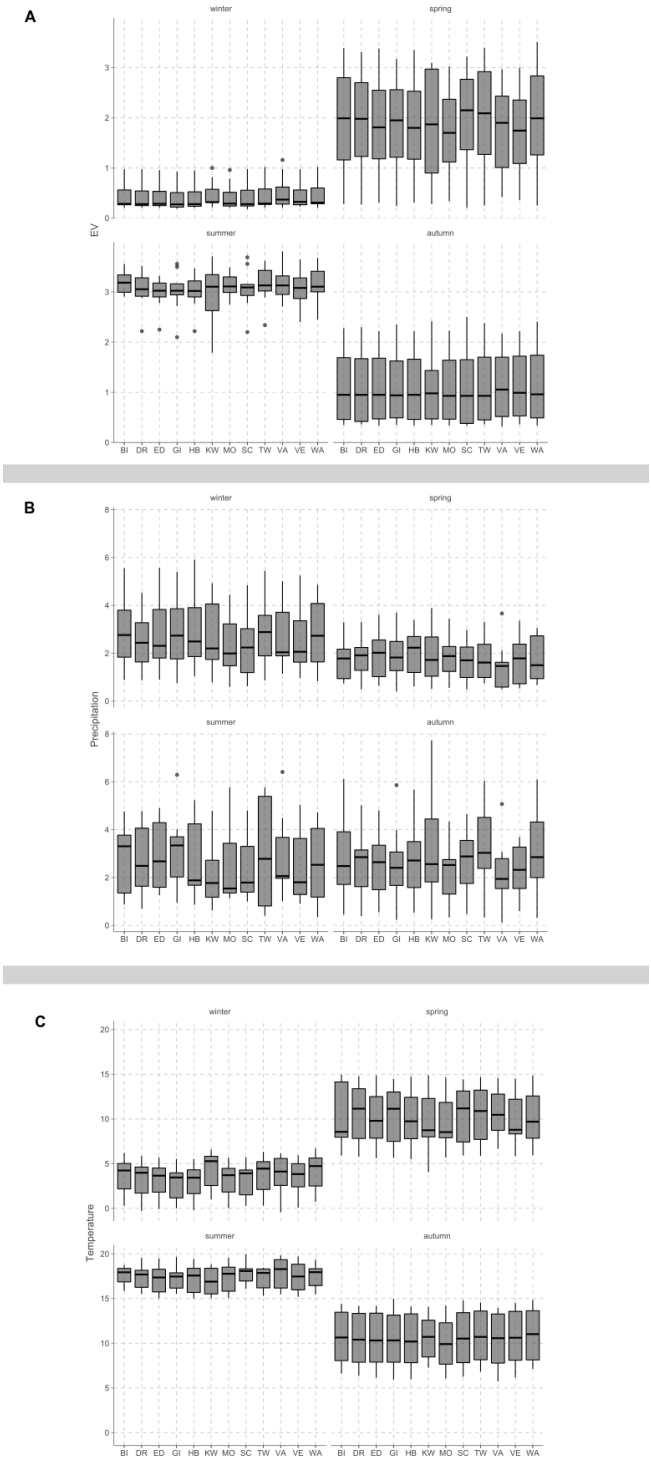


Figure S2. Evaporation rate (A), precipitation (B) and temperature (C) variation per location and season.

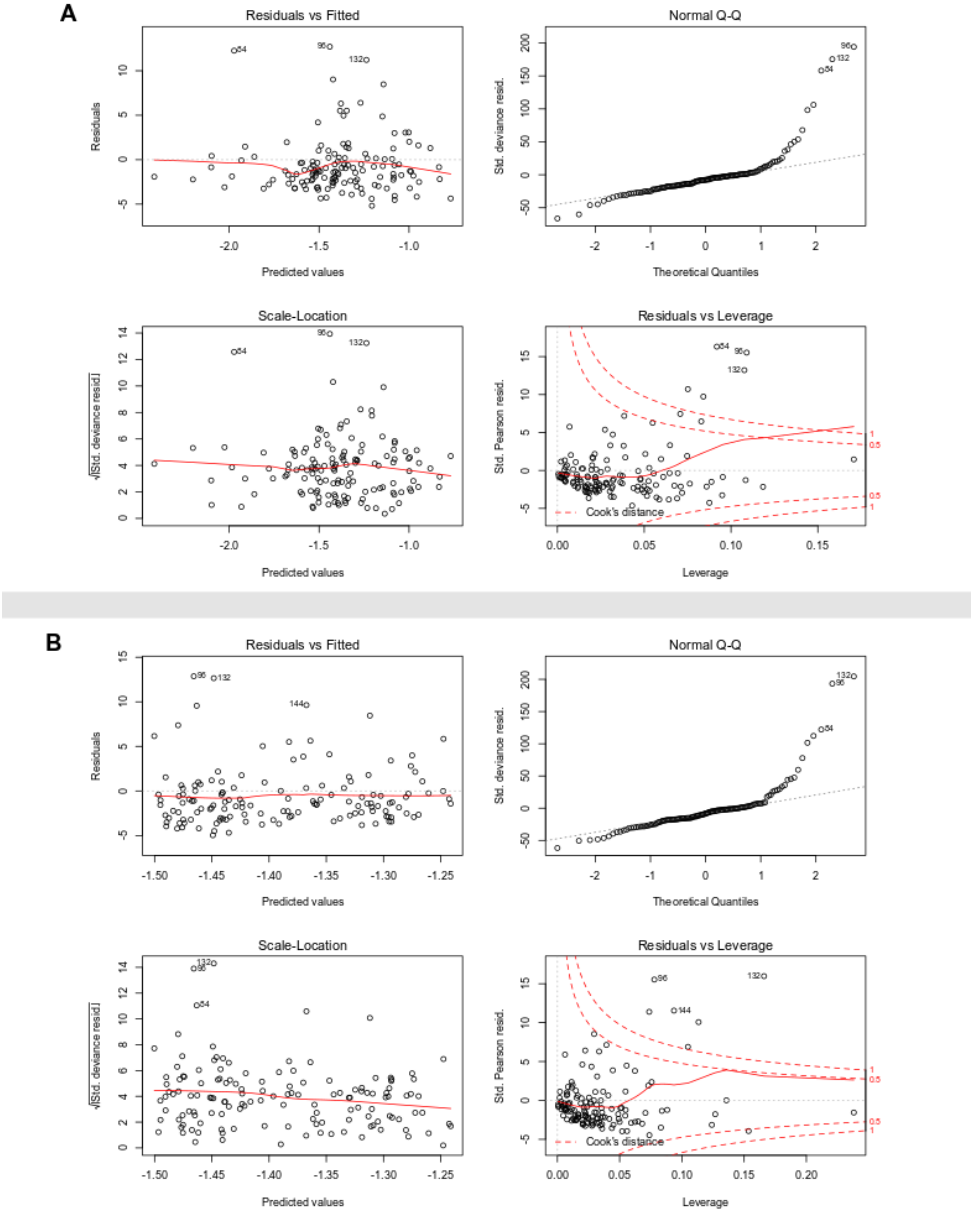
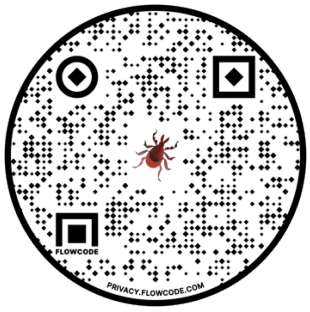


Figure S3. Diagnostic plots of climatic models for *Rickettsia helvetica* applying as the explanatory variables (A) climatic data from the same year and season as the nymphal infection prevalence (no shift) and (B) climatic data from the previous season was used (shift). Outliers corresponding to n = 126 and 123, both from Wassenaar (Summer 2014 and Spring 2015) were removed from further analysis.



Please click here (for the online version) or scan this QR code to listen to an immersive soundscape of a forest in Stameren, the Netherlands recorded by Vitalij Kuzkin. The Stameren forest is one of the study sites where I have collected samples for this research. Listen with headphones for the best experience. The full album is available on: ticksandmicrobes.bandcamp.com

Chapter 6

Tripartite interactions among *Ixodiphagus hookeri*, *Ixodes ricinus,* and deer: differential interference with transmission cycles of tick-borne pathogens

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Abstract

To development of sustainable control of tick-borne diseases, insight is needed into biological factors that affect tick populations. Here, the ecological interactions among *Ixodiphagus hookeri*, *Ixodes ricinus*, and two vertebrate species groups were investigated in relation to their effects on tick-borne disease risk. In 1,129 questing ticks, *I. hookeri* DNA was detected more often in *I. ricinus* nymphs (4.4%) than in larvae (0.5%) and not in adults. Therefore, we determined the infestation rate of *I. hookeri* in nymphs from 19 forest sites, where vertebrate, tick, and tick-borne pathogen communities had been previously quantified. We found higher than expected co-occurrence rates of *I. hookeri* with deer-associated *Anaplasma phagocytophilum*, and lower than expected rates with rodent-associated *Borrelia afzelii*, and *Neoehrlichia mikurensis*. The prevalence of *I. hookeri* in nymphs varied between 0 and 16%, and was positively correlated with the encounter probability of ungulates and the densities of all life stages of *I. ricinus*. Lastly, we investigated the emergence of *I. hookeri* from artificially fed, field-collected nymphs. Adult wasps emerged from seven of the 172 fed nymphs. From these observations, we inferred that *I. hookeri* is parasitizing *I. ricinus* larvae that are feeding on deer, rather than on rodents or in the vegetation. Since *I. hookeri* populations depend on deer abundance, the main propagation host of *I. ricinus*, these wasps have no apparent effect on tick populations. The presence of *I. hookeri* may directly interfere with the transmission cycle of *A. phagocytophilum*, but not with that of *B. afzelii* or *N. mikurensis*.

Keywords: Parasitic Wasp, Biological Control, Tick-Borne Pathogen, Host Preference; Parasitization, Transmission Cycle, Lyme Borreliosis, Human Granulocytic Anaplasmosis, Neoehrlichiosis

Introduction

Lyme borreliosis poses serious health concerns in the northern hemisphere (Eisen & Eisen, 2018; Sprong et al., 2018; Stanek et al., 2012). Increases in incidence have been observed in several countries in Europe. These increases are partially explained by the geographical spread of its vector, *Ixodes ricinus* (Lindgren et al., 2012; Medlock et al., 2013). Other tick-borne diseases (TBDs), such as anaplasmosis and neoehrlichiosis, are also emerging (Azagi et al., 2020; Matei et al., 2019). Understanding which factors drive the population densities of ticks and the transmission cycles of tick-borne pathogens are important steps in assessing disease risk and formulating possible intervention strategies. In north-western Europe, TBDs are generally caused by a bite of an infected *I. ricinus* nymph (Hofhuis et al., 2017). Therefore, the density of infected nymphs (DIN) is an important ecological parameter that contributes to the overall disease risk of TBD (Coipan et al., 2013b; Mannelli et al., 2003; Randolph, 2004).

Ixodes ricinus has a three-host life cycle, and its survival depends on finding vertebrate hosts for feeding and propagation. *Ixodes ricinus* utilizes a multitude of host species, but these species differ in the number of ticks and in the life stages they feed (Jaenson et al., 1994). In a typical north-western European forest, larvae predominantly feed on rodents, nymphs on rodents and birds, and adult ticks on deer, mostly *Capreolus capreolus* (Hofmeester et al., 2017b; Hofmeester et al., 2016; Takumi et al., 2019). The presence of deer generally results in a high abundance of *I. ricinus* (Gilbert et al., 2012; Hofmeester et al., 2017b; Rand et al., 2003). However, fluctuations in rodent density have also been associated with variations in density of (infected) nymphs (Chapter 4; Ostfeld et al., 2001).

Control of *I. ricinus*-borne diseases primarily consists of promoting personal preventive actions, but its effectiveness is questionable (Eisen & Dolan, 2016; Foster et al., 2020; Vazquez et al., 2008). Biological control of ticks is considered to be an environmentally friendly approach. However, so far, this has not been effectively applied in routine settings (Collatz et al., 2011; Hu & Hyland, 1998b; Hu et al., 1998a; Mwangi et al., 1997; Smith & Cole, 1943). Interestingly, the parasitoid wasp, *Ixodiphagus hookeri* (Hymenoptera: *Encyrtidae*), is a natural enemy of *I. ricinus*, and has been a target of interest as a biological control agent (Collatz et al., 2011; Hu et al., 1998b; Larrousse et al., 1928).

Although the total lifecycle of *I. hookeri* generally takes one year, the adult life stage has a lifespan of only two to three days (Hu et al., 1998a; Plantard et al., 2012). It is not well understood how female wasps find *I. ricinus* larvae and nymphs for oviposition (Collatz et al., 2011; Hu et al., 1998a). We hypothesize that female *I. hookeri* randomly infests *I. ricinus* larvae and nymphs in the vegetation. We test this by calculating the infestation rate of the life stages of questing *I. ricinus*. If *I. hookeri* infests ticks in the vegetation, then we expect to find the wasp in nymphs together with horizontally transmitted tick-borne pathogens more or less randomly. The alternative hypothesis is that female *I. hookeri* infest *I. ricinus* larvae while feeding on a vertebrate host. By calculating the co-occurrence rates of the wasp with strictly horizontally transmitted tick-borne pathogens, we might infer a preference for a vertebrate host on which a female wasp would infest feeding ticks with her eggs.

The embryonic development of *I. hookeri* eggs is triggered by the attachment and engorgement of nymphs feeding on a vertebrate host (Collatz et al., 2011; Hu et al., 1998a). *Ixodiphagus hookeri* larvae feed on the internal tissue along with the vertebrate blood ingested by nymphs (Collatz et al., 2011; Hu et al., 1998a). Adult wasps emerge by making a hole in the nymph's body, and

kill the tick before moulting (Wood, 1911). Until now, the emergence of *I. hookeri* has only been observed from fully fed *I. ricinus* nymphs (Collatz et al., 2011; Tijsse-Klasen et al., 2011; Wood, 1911). Although several studies on the interactions between *I. hookeri* and *I. ricinus* have been conducted (Bohacsova et al., 2016; Collatz et al., 2010; Collatz et al., 2011; Plantard et al., 2012; Ramos et al., 2015; Sormunen et al., 2019; Tijsse-Klasen et al., 2011), we lack estimates about the impact of this wasp on *I. ricinus* population dynamics. We hypothesize that the prevalence of *I. hookeri* in nymphs is driven by the abundance of immature stages of *I. ricinus*, and because of their predatory behaviour, an increasing prevalence of wasps is negatively associated with the abundance of *I. ricinus* adults. For this, we determined the infestation rates of *I. hookeri* in questing nymphs and correlated it with the abundances of the different life stages of *I. ricinus* from a cross-sectional study, in which tick density and vertebrate communities were quantified (Hofmeester et al., 2017b; Takumi et al., 2019). The latter allowed us to determine the relationship between *I. hookeri* and tick hosts such as ungulates and rodents.

Lastly, to determine whether the molecular detection of the wasp in ticks reflects the presence of viable wasp eggs, and to determine to what extent these infestations kill nymphs, an artificial tick feeding assay was performed.

Materials and methods

Tick Collection

Ixodes ricinus of all life stages were collected in 2019 by dragging a blanket of 1m^2 from two locations: Buunderkamp and Amsterdamse Waterleidingduinen (Table S4 and Figure S1), the Netherlands. Ticks were identified to species level using morphological keys (Hillyard, 1996). A proportion of nymphs collected in Amsterdamse Waterleidingduinen was used in an artificial blood-feeding assay (see below). The remaining nymphs were tested by PCR-based methods for the presence of tick-borne pathogens and *I. hookeri.*

Cross-sectional study

Extensive field surveys had been carried out previously in 19 sites located in forested areas in the Netherlands in 2013 and 2014 (Hofmeester et al., 2017b; Takumi et al., 2019). Data were collected on the density of questing *I. ricinus* (blanket dragging), vertebrate communities (camera and live trapping), and on the infection rates of tick-borne pathogens (qPCR detection). Only data on *A. phagocytophilum*, *N. mikurensis* and *B. afzelii* were used in this study. Details from these forest sites are provided in Table S4 and Figure S1. All handling procedures of this study were approved by the Animal Experiments Committee of Wageningen University (WUR-2013055 and WUR-2014019) and by the Netherlands Ministry of Economic Affairs (FF/75A/2013/003).

Detection of *I. hookeri* and tick-borne pathogens

DNA extraction from the individual questing ticks was achieved by alkaline lysis in ammonium hydroxide (Wielinga et al., 2006). The lysates were stored at 4 °C. Samples were tested with qPCRs for the presence of *A. phagocytophilum* (Jahfari et al., 2014), *B. burgdorferi* s.l. (Heylen et al., 2013b), and *N. mikurensis* (Jahfari et al., 2012). For the study described here, the presence of *I. hookeri* DNA was detected by qPCR targeting a 104-bp fragment of the Cytochrome Oxidase I gene using primers 5'-AGA TGT TGA TAC TCG AGC TT-3', 5'- AAT TTT ATT CCA TTT ATT

GAA GCT A-3' and a probe 5'-ATTO647- TGC TGT TCC AAC AGG AGT AAA AGT TTT TAG ATG A-BHQ2-3'. This newly developed qPCR was determined as described previously (Heylen et al., 2013b; Tijsse-Klasen et al., 2011). In short, qPCR-positive samples were subjected to conventional PCR targeting a fragment of the 16S rRNA gene (Heylen et al., 2013b; Tijsse-Klasen et al., 2011). Both strands of PCR products were sequenced using Sanger Sequencing (Baseclear, Leiden, Netherlands). The resulting sequences were compared with sequences in Genbank using BLAST. A DNA lysate from an *I. hookeri* specimen, which was both morphologically and genetically identified, was used as a positive control (Heylen et al., 2013b; Tijsse-Klasen et al., 2011). All qPCRs were carried out on a Light Cycler 480 (Roche Diagnostics Nederland B.V, Almere, The Netherlands) in a final volume of 20 µl with iQ multiplex Powermix, 3 µl of sample and 0.2 µM for all primers and different concentrations for probes (30176908). Positive controls and negative water controls were used on every plate tested. The DNA extraction, PCR mix preparation, sample addition, and qPCR analyses were performed in separated air locked dedicated labs to minimize contamination and false-positive samples.

Co-infection analysis

A Fisher's exact test was applied to explore correlations between tick-borne pathogens and *I. hookeri*. For this, the expected co-occurrence was calculated assuming the independent acquisition of tick-borne pathogens and parasitoid wasps by multiplying their prevalence estimates and the observed density of nymphal ticks (Takumi et al., 2019).

Association of *I. hookeri* prevalence in questing nymphs with the density of *I. ricinus*

We performed regression analyses to investigate associations of *I. hookeri* prevalence in questing *I. ricinus* nymphs with densities of *I. ricinus* larvae (DOL), nymphs (DON), and adults (DOA) of *I. ricinus*. Because *I. hookeri* prevalence is represented as proportional data, we chose a binomial generalized linear model taking into account sample size with the logit link transform. A likelihood ratio test was performed to assess the goodness of fit of all models. The ranges of DOL, DON, DOA, as well as *I. hookeri* prevalence in questing nymphs are provided in Table S1. The prevalence of *I. hookeri* was calculated based on a subset of samples tested. Model building was performed in R version 3.6.1 "Action of the Toes" (R Core Team, 2019).

Association of *I. hookeri* prevalence with vertebrate encounter probability

To find out whether *I. hookeri* displays the observed vertebrate host preference as inferred from the co-occurrence data, we considered a scenario in which numbers of *I. hookeri* presence in the local tick collections follow the Beta binomial distribution. Furthermore, the beta mean was considered to relate (via the logit link) to the local ungulate population measured by encounter probability (Takumi et al., 2019). The ungulate population consisted of four species: roe deer (*Capreolus capreolus*), fallow deer (*Dama dama*), red deer (*Cervus elaphus*), and wild boar (*Sus scrofa*). The rodent population consisted of two (major) species, namely wood mouse (*Apodemus sylvaticus*) and bank vole (*Myodes glarelolus*). Other rodent species, which occurred to a negligible extent, were: field vole (*Microtus agrestis*), common shrew (*Sorex araneus*), and pygmy shrew (*Sorex minutus*). The model was fitted by maximizing the beta-binomial likelihood to the numbers of *I. hookeri* presence and absence per forest site. The likelihood-ratio test was applied to test whether *I. hookeri* prevalence is significantly associated with the local ungulate population. In addition to the ungulates, an association of rodents to *I. hookeri* prevalence was tested by following the same procedure. The calculations were performed using R (Team, 2015).

Artificial blood-feeding of *I. ricinus* nymphs and examination of wasp emergence

To determine the wasp emergence rate, *I. ricinus* nymphs were placed on artificial blood-feeding units prepared according to previously described methods (Krull et al., 2017; Oliver et al., 2016). Between 50 and 60 ticks were placed in each of the six blood-feeding units. The nymphs were fed on heparinized bovine blood, which was supplemented with glucose (4 g/L), fungizone (2.5 ug/mL), gentamycin (50 ug/mL), and 5 ul of 100 mM ATP solution per 4 ml of blood. Cow blood was obtained from Carus (Wageningen University, The Netherlands) under animal ethics protocol no. AVD1040020173624 and from Faculty of Veterinary Medicines, Utrecht University.

Blood was replaced every 24 h. Engorged and detached ticks were collected and stored individually in 2 mL Eppendorf tubes with pierced lids, which were kept in a desiccator with approximately 90% relative humidity at room temperature and observed daily for parasitic wasp emergence. After the blood-feeding experiment, all engorged ticks were also tested for the presence of tick-borne pathogens and *I. hookeri* with PCR-based methods.

Results

Prevalence of *I. hookeri* and tick-borne pathogens in different life stages of *I. ricinus*

To determine which life stage of *I. ricinus* was most often infested with *I. hookeri*, larvae (n = 367), nymphs (n = 684), and adults (n = 78) were tested (Table 1). *Ixodiphagus hookeri* DNA was found in two larvae (0.5%), 30 nymphs (4.4%), but not in adult *I. ricinus*. Thus, the highest prevalence of *I. hookeri* was detected in nymphs. The three horizontally transmitted tick-borne pathogens, *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum,* and *Neoehrlichia mikurensis,* were detected in nymphs and adults (Table 1), but not in larvae, except for one larva, which was positive of *B. burgdorferi* s.l. (Table 1).

Table 1. The occurrence of tick symbionts in questing *I. ricinus* ticks. Different life stages of questing *I. ricinus* were collected and tested for the presence of I. hookeri and tick-borne pathogens. Occurrence is presented as n (number of positive ticks), prevalence (%), and the 95% confidence intervals of the prevalence (range), which is calculated according to Armitage et al. (2001).

Symbiont	Larvae (n = 367)			Nymphs (n = 684)			Adults (n = 78)		
	n	**%**	**(range)**	**n**	**%**	**(range)**	**n**	**%**	**(range)**
I. hookeri %	2	0.5%	(0.1 - 2.5)	30	4.4%	(3.0 - 6.2)	0	0%	(0.0 - 4.6)
B. burgdorferi s.l. %	1	0.3%	(0.0 – 1.5)	82	12%	(9.6 - 14.7)	13	16,7%	(9.2 - 26.8)
A. phagocytophilum %	0	0%	(0.0 - 1.0)	19	2.8%	(1.7 - 4.3)	7	9%	(3.7- 17.6)
N. mikurensis %	0	0%	(0.0 - 1.0)	30	4.4%	(3.0 - 6.2)	4	5,1%	(1.4 - 12.6)

Infestations with *I. hookeri* and infection with horizontally transmitted tick-borne pathogens.

The presence of *I. hookeri* DNA was determined in 13,967 nymphs from the cross-sectional study, and which had already been tested for the prevalence of tick-borne pathogens (Hofmeester et al., 2017b; Takumi et al., 2019). *Ixodiphagus hookeri* was detected in nymphs from 18 of the 19 forest sites in the Netherlands, with prevalence varying from 0.1% to 15.9% (Table S1). The infestation with *I. hookeri* and infection with horizontally transmitted pathogens appeared not to be random (Table 2). The presence of *I. hookeri* DNA in nymphs was positively associated with the deer-

associated *A. phagocytophilum* and negatively associated with the rodent-borne *B. afzelii*, and *N. mikurensis* (Table 2 and Table S2). Apparently, the infestation with *I. hookeri* appears somehow to be associated with the first blood meal of the questing nymphs.

Table 2. Observed and expected co-occurrence of *I. hookeri* and tick-borne pathogens in *I. ricinus* nymphs. Questing nymphs (n=13,967) from the 19 forest sites were tested for the presence of *I. hookeri*, *A. phagocytophilum*, *B. afzelii*, and *N. mikurensis* DNA. Odds ratio > 1 and < 1 indicate increased and decreased co-occurrence, respectively. A Fisher's exact test was used to test the statistical significance for an association.

	A. phagocytophilum	*B. afzelii*	*N. mikurensis*
Observed co-occurrence	72	4	9
Expected co-occurrence	26	17	46
Odds ratio	3.3	0.2	0.2
P-value	< 0.001	< 0.001	< 0.001

Association of *I. hookeri* prevalence in questing nymphs with the density of *I. ricinus*

The relationship between prevalence (%) of *I. hookeri* in questing nymphs and the density of all life stages of *I. ricinus* of the 19 forest sites was investigated with generalized linear models. The prevalence (%) of *I. hookeri* was significantly positively associated with the density of questing *I. ricinus* larvae (DOL; $p < 0.0001$), nymphs (DON; $p < 0.0001$), and adults (DOA; $p < 0.0001$; Figure 1). Equations of all models, Akaike information criterion (AIC) values, and likelihood ratio test results are provided in Table S3.

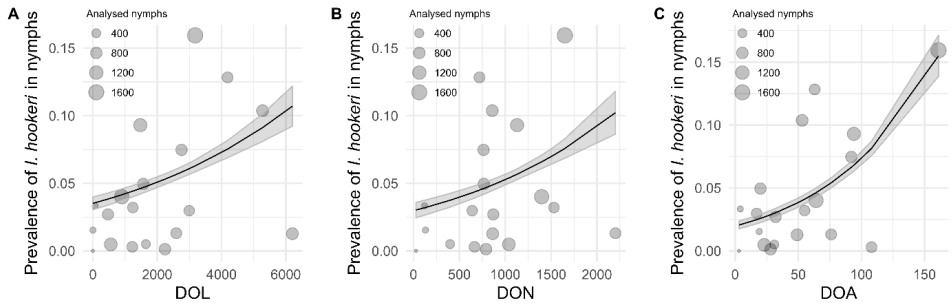

Figure 1. Associations of the prevalence of *I. hookeri* in questing nymphs with (A) the density of *I. ricinus* larvae (DOL), (B) density of nymphs (DON), and (C) density of adults (DOA) in the 19 forest sites (Figure S1 and Table S4). The density is presented per 1200 m^2. Grey shading around the black regression line represents standard errors. All presented associations are significant ($p < 0.0001$). Equations of all models, AIC values, and likelihood ratio test results are provided in Table S3.

Association of *I. hookeri* with densities of ungulates and rodents

Previous analyses of the data from the cross-sectional study showed that the abundance of ungulates was positively associated with the density of the three life stages of *I. ricinus* (Takumi et al., 2019), just like the prevalence of *I. hookeri* in our study (Figure 1). The observed positive association of *I. hookeri* with densities of the different life stages of *I. ricinus* might therefore be caused by the dominant role of deer in the propagation of ticks. Indeed, the occurrence of *I. hookeri* in questing

nymphs was positively correlated with the encounter probability with ungulates (Figure 2, left panel). No significant association between the encounter probability of rodents and *I. hookeri* occurrence was observed (Figure 2, right panel).

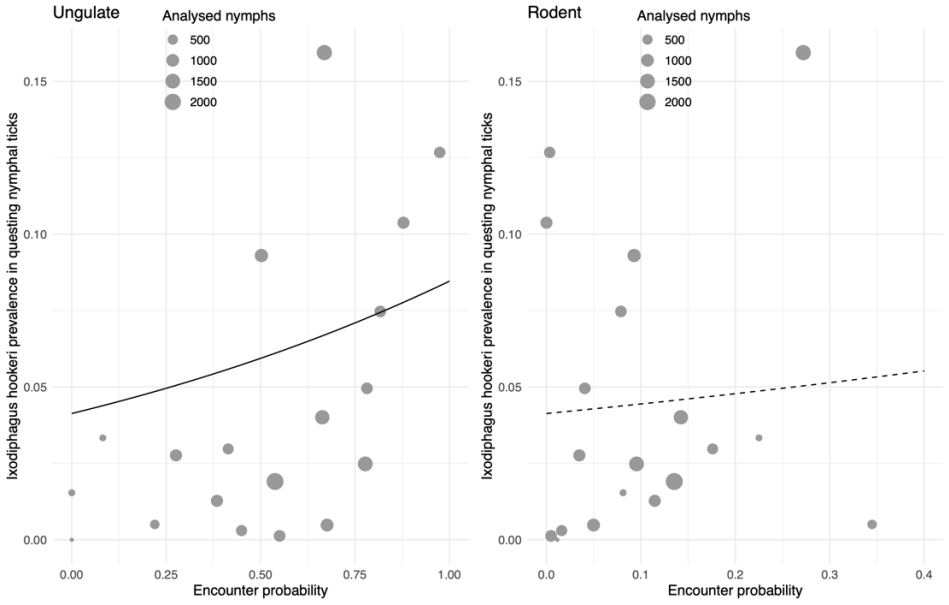

Figure 2. Association of vertebrate species groups (ungulates and rodents) with the occurrence of *I. hookeri* in questing nymphs in the 19 forest sites. The occurrence is presented as the infestation prevalence of *I. hookeri* in questing nymphs (DIN) at each site of 1200 m^2. Horizontal axis: a function of encounter rates (see methods). A circle represents a single forest site. The best-fit beta-binomial model is visualized by a solid line (a significant relationship to the host species, $p = 0.018$) or a dashed line (not significant, $p = 0.7$).

Artificial blood-feeding of *I. ricinus* nymphs

Lastly, to determine whether the detection of *I. hookeri* in ticks reflects the presence of viable wasp eggs and to determine to what extent these infestations kill nymphs, an artificial tick feeding assay was performed. A total of 561 nymphs collected in the Amsterdamse Waterleidingduinen were placed on feeding membranes, of which 172 successfully blood-fed and engorged (Table 3). A total of 151 nymphs (88%) successfully moulted into the adult stage. In total, 21 engorged nymphs did not moult and died (12%); 14 died without, and seven died with the emergence of three to five wasps (Table 3). We detected wasp DNA in seven nymphs the wasps emerged from, as well as in nine of the 14 dead nymphs, and in ten of the 151 ones, which engorged and successfully moulted to adults (Table 3). In total, 15% of the nymphs were infested with *I. hookeri*, which is comparable to the infestation rate found previously in the Amsterdamse Waterleidingduinen (13%; Table S1). Thus, from the nymphs infested with *I. hookeri* (n = 26), sixteen (62%) died, and ten (38%) survived and successfully moulted into an adult tick.

Table 3. The presence of pathogens and *I. hookeri* in nymphs, which successfully blood-fed in the assay. Field collected, questing nymphs were artificially fed in blood feeding units in the laboratory, and the emergence of parasitoid wasps was monitored. After that, all ticks were analysed by molecular methods for the presence of pathogens and I. hookeri. Between brackets are the 95% confidence intervals, as calculated according to Armitage et al. (2001).

Ticks		*I. hookeri* %		*A. phagocytophilum* %		*B. burgdorferi* s.l. %		*N. mikurensis* %	
Female	(n = 64)	7.8	(2.6 - 17.3)	15.6	(7.8 - 26.9)	0.0	(0.0 - 5.6)	0.0	(0.0 - 5.6)
Male	(n = 87)	5.7	(1.9 - 12.9)	19.5	(11.8 - 29.4)	0.0	(0.0 - 4.2)	0.0	(0.0 - 4.2)
Not moulted	(n = 14)	64.3	(35.1 - 87.2)	14.3	(1.8 - 42.8)	0.0	(0.0 - 23.2)	0.0	(0.0 - 23.2)
With wasps	(n = 7)	100	(59.0 - 100.0)	28.6	(3.7 - 71.0)	0.0	(0.0 - 41.0)	0.0	(0.0 - 41.0)
Total	(n = 172)	15.1	(10.1 - 21.4)	18.0	(12.6 - 24.6)	0.0	(0.0 - 2.1)	0.0	(0.0 - 2.1)

Discussion

In this study, we investigated the ecological interactions among the parasitoid wasp *I. hookeri*, the tick *I. ricinus*, and two vertebrate species groups (ungulates and rodents) and their combined effect on tick-borne disease risk. Significant differences in the prevalence of *I. hookeri* between questing *I. ricinus* larvae and nymphs (Table 1) were found as well as different associations of wasps with horizontally transmitted tick-borne pathogens in nymphs (Table 2). From these results, we inferred that female *I. hookeri* wasps infest engorging larvae while feeding on a deer rather than on a rodent or in the vegetation. In addition, the positive association of the *I. hookeri* prevalence with the encounter probability of deer, but not rodents (Figure 2) supports the idea that female wasps are attracted to deer by their odour, and subsequently infest ticks that are feeding on these deer (Demas et al., 2000; Hu & Hyland, 1997b). Since deer act as major propagation hosts for *I. ricinus*, we observed a positive association between *I. hookeri* prevalence and the density of all tick life stages, not only larvae and nymphs (Figure 1). Our findings further indicate that preferential infestation of *I. ricinus* larvae feeding on deer may directly interfere with the transmission cycles of tick-borne pathogens, such as *A. phagocytophilum*, that utilizes deer as amplification hosts (Table 2).

Here, we detected *I. hookeri* in ticks collected in 18 of the 19 locations around the Netherlands with a prevalence in questing *I. ricinus* nymphs ranging from 0.1% to 16% (Figure 1 and Table S1). This wasp has a widespread distribution in Europe (Bohacsova et al., 2016; Collatz et al., 2011; Plantard et al., 2012; Ramos et al., 2015; Sormunen et al., 2019; Tijsse-Klasen et al., 2011) and various factors, including microclimate, tick density, and tick-host abundance, were proposed as possible determinants for the high variation in infestation rates (Hu et al., 1993; Stafford et al., 2003; Tijsse-Klasen et al., 2011). A prospective study of ten years reported a decrease in *I. scapularis* larvae and nymphs together with a decrease in the *I. hookeri* prevalence in questing nymphs when deer populations sharply declined (Stafford et al., 2003). Similar observations were made in our cross-sectional study, where the encounter probability of ungulates, most notably roe deer, was positively associated with the prevalence of all the life stages of *I. ricinus* (Takumi et al., 2019) and with the *I. hookeri* prevalence in questing nymphs (Figure 2, left panel). Interestingly, the prevalence was indifferent to the encounter probability of rodents (Figure 2, right panel). Fluctuating rodent populations have been shown to affect the density of *I. ricinus* nymphs (Chapter 4; Ostfeld et al., 2001); however, the presence of deer is generally responsible for the high density of ticks due to their role as propagation hosts (Gilbert et al., 2012; Hofmeester et al., 2017b; Tagliapietra et al., 2011).

In addition, deer play a role in attracting female *I. hookeri* over some distance (Demas et al., 2000; Hu & Hyland, 1997b). In laboratory tests, it has been demonstrated that *I. hookeri* females preferred mostly unfed *I. ricinus* nymphs for oviposition over unfed larvae, engorged larvae, and fully engorged nymphs (Collatz et al., 2011), however, engorging ticks were not included in the experiment. In addition, it is not known how often wasps and questing nymphs are in spatial proximity in the wild. A strategy for female *I. hookeri* is to locate a nymph with the help of chemical cues from a vertebrate host (Collatz et al., 2010; Takasu et al., 2003). In laboratory experiments, *I. hookeri* females were attracted by carbon dioxide, which is an unspecific vertebrate cue, and by odours from roe deer faeces, roe deer hair, and wild boar hair. In contrast, they were not attracted to odours derived from field mice, cattle, and rabbits (Collatz et al., 2010). Locating and parasitizing feeding ticks on their mammal hosts may be advantageous for *I. hookeri* wasps in several ways. First, the density of ticks feeding on a deer is expected to be (much) larger than the density of ticks in the vegetation (Hofmeester et al., 2016). Second, the probability of an engorging larva to successfully moult to a questing *I. ricinus* nymph is (much) higher than that of a questing larva (Hartemink et al., 2008; Randolph, 2004).

Overall, the interaction of the wasp with the propagation host (deer) of its tick host ensures, to a large extent, the continuation of the wasp lifecycle. This tri-trophic interaction makes it very difficult, if not impossible, to disentangle the effect of deer abundance on the tick and wasp populations as well as the effect of the wasp on the tick population. Indeed, although the parasitic wasp is killing nymphs upon its emergence, hence interfering with the life cycle of ticks, our study was unable to detect any effect of the occurrence of the wasp on the population sizes (densities) of the different life stages of *I. ricinus* (Figure 1).

In this study, the wasp was detected in questing larvae and nymphs but not in adult ticks (Table 1). The wasp DNA has been previously detected in questing nymphs and adults of *I. ricinus* with significantly higher prevalence in nymphs (Ramos et al., 2015). Our results are in line with the idea that nymphs are the main developmental stage affected by the parasitoid wasp. In addition, until now, the emergence of *I. hookeri* has been observed only in fully fed *I. ricinus* nymphs (Collatz et al., 2011; Wood, 1911). Nevertheless, parasitization itself might already occur at the larval stage of questing ticks to some extent. Although we detected the wasp DNA in two larvae (Table 1), we can imagine that they might have been parasitized while briefly feeding on a vertebrate rather than while questing. Some larvae in the vegetation already fed on a host and inadvertently detached due to grooming. The same explanation is used for finding *B. burgdorferi* s.l. infected questing larvae (Table 1; van Duijvendijk et al., 2016). The results on the co-occurrence of both *I. hookeri* and tick-borne pathogens in questing nymphs are contrary to what we expected that *I. hookeri* parasitize ticks in the vegetation. Instead, our results support the idea that parasitization takes place when a larva feeds on a vertebrate. We observed a positive association between infestation with *I. hookeri* and infection with *A. phagocytophilum*, which is a pathogen that an *I. ricinus* larva acquires while feeding on a deer, an *A. phagocytophilum*-competent host (Table 2; (Hamsikova et al., 2019; Jahfari et al., 2014; Stuen et al., 2013)). Thus, both acquisition of a bacterium and parasitization by *I. hookeri* occurs simultaneously. Interestingly, we detected a strong negative association of *I. hookeri* with *B. afzelii* and *N. mikurensis*, rodent-associated pathogens (Table 2). The absence of Lyme spirochetes and *Babesia microti* in *I. scapularis* nymphs infested with parasitic wasps has been observed before (Mather et al., 1987). These results suggest that wasps rarely parasitize larvae feeding on rodents and questing nymphs infected with these pathogens. Our results further imply

that *I. hookeri*, which infests feeding *I. ricinus* larva, can survive its moulting to the nymphal stage (transstadial transmission). The efficiency of the transstadial transmission of the wasp as well as the effects on the survival of the infested tick are unknown and warrants further investigation.

One of the questions of this study was whether *I. hookeri* decreases disease risk by reducing tick and/or associated pathogen communities. Obviously, a fraction of the nymphs will not complete their life cycle because of predation by the wasp, and therefore the abundance of the wasp has a negative impact on the life cycle of *I. ricinus*. Given that survival of wasps depends on their interaction with deer, the propagation hosts of *I. ricinus*, a diminishing or mitigating effect by the wasp on the tick population is rather unlikely (Figure 1). The parasitic wasp may influence tick-borne pathogen cycles to various extent, as it co-occurs in questing nymphal ticks less frequently with *B. afzelii* and *N. mikurensis* and more frequently with *A. phagocytophilum*. Future studies could investigate the potential long-lasting effect of *I. hookeri* on *A. phagocytophilum* and other deer-associated pathogens, such as *Babesia* spp. An Investigation into comprehensive tick-borne disease risk ultimately requires estimating DIN of these and other tick-borne pathogens.

The artificial tick feeding assay results show that molecular detection of *I. hookeri* DNA indicates presence of viable wasps as adult wasps successfully emerged from artificially blood-fed nymphs (Table 3). A total of 7 of the 172 (4.1%) nymphs were killed by emerging wasps (Figure 3), which is in concordance with a previous study (Collatz et al., 2011), but not with another study (Bohacsova et al., 2016). The number of emerged parasitoids from a single *I. ricinus* nymph in our assay ranged from 3 to 5 wasps per tick, which were 1-15 in a German study and 2-20 in a Slovak study (Bohacsova et al., 2016; Collatz et al., 2011). About 8% of the nymphs did not moult, and no wasp emerged (Table 3). From these not moulted nymphs, 64% was infested with *I. hookeri*, which is comparable to a previous study (Collatz et al., 2011). The nymphs of that study were dissected one year later, and dead *I. hookeri* wasps were found inside (Collatz et al., 2011). Adult parasitoids may not be able to emerge if they cannot consume all the material inside the nymphs (Mwangi et al., 1997). Interestingly, *I. hookeri* DNA was also detected in 10 of the 151 (6.6%) engorged ticks that successfully moulted to adults and thus survived infestation with the parasitic wasp. This observation indicates that the development of wasp eggs was suppressed, but the mechanism behind it remains unknown. In other arthropods, it has been shown that some facultative symbionts may enhance the survival of their host infested by a parasitic wasp (Oliver et al., 2003; Xie et al., 2010).

Figure 3. *Ixodiphagus hookeri* (left) and an engorged nymph of *I. ricinus*, which the parasitoid wasp emerged from (right). The field-collected nymph was fed to repletion using an artificial blood-feeding assay. During the moulting process, a wasp emerged in the test tube. Photos were taken with a mobile phone, and three images were processed in photo editing software to one. Original photos are in Figure S2.

The infection rate of the tick-borne pathogens in the ticks from the feeding assay is not in line with field observations (Table 3). The absence of *B. burgdorferi* s.l. and *N. mikurensis* could be explained by antibiotics in the assay or by their incompatibility with the innate immune components in bovine blood (Bhide et al., 2005). Admittedly, little is known about antibiotic susceptibility, immune (in) compatibility, and tissue tropism of *N. mikurensis*. In contrast, *A. phagocytophilum* prevalence in *I. ricinus* nymphs that fed in the feeding assay, was significantly higher (17.6%) than nymphs from the vegetation (Table 1 and Table S1). Perhaps, the intracellular location of *A. phagocytophilum* in ticks might prevent killing by gentamycin (Chen et al., 2012). Further improvements of the artificial feeding system are necessary to enable studies of the microbial interactions with the parasitic wasp in more detail. Results from the artificial feeding assays indicate that the molecular detection of the wasp is indicative for the presence of viable wasps in ticks.

Conclusions

Ungulates, particularly deer, are important drivers of tick populations and facilitate the infestation of *I. ricinus* by *I. hookeri*. This double role of deer diminishes the negative effect of the wasp on the tick abundance by killing *I. ricinus*. As this wasp infests *I. ricinus* larvae feeding on deer rather than on rodents, it has no direct interference with the transmission cycle of *B. afzelii*. Taken together, natural *I. hookeri* populations have a minimal impact on TBD risk. Insights in these ecological interactions might provide new food for thoughts on the biological control of *Ixodes ricinus*-borne diseases.

Acknowledgements

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Supplement

Table S1. Density of larvae (DOL), nymphs (DON) and adults (DOA), and the prevalence of *A. phagoctophilum*, *N. mikurensis*, *B. afzelii* and *I. hookeri* per study site. A column DON tested shows a subset of nymphs tested for prevalence of pathogens and *I. hookeri*. Formula of 95% exact binomial confidence interval from Armitage et al. (2001). The table continues on the following page.

Study site	**DOL/1200 m2**	**DON/1200 m2**	**DOA/1200 m2**	**Nymphs tested**
Amsterdamse Waterleiding Duinen	4186	718	63	678
Bergherbos	468	869	32	742
Buunderkamp	2,591	2,202	76	688
Deelerwoud	3,178	1,650	161	1,650
Duin en Kruidberg	5,274	858	53	858
Halfmijl	1,241	1,531	55	624
Herperduin	72	120	4	120
Kremboong	2,239	791	28	791
Kroondomein	897	1,397	64	1,397
Maashorst	2,994	639	17	639
Pettermerduin	0	130	19	130
Planken Wambuis	1,566	767	20	767
Rheebruggen	6,203	863	49	863
Schoorlse Duinen	1	26	3	26
Stameren	1,474	1,129	94	1,129
Valenberg	2,754	763	92	763
Vijverhof	555	1,039	23	1,039
Vledderhof	1,648	398	31	398
Zwanemeerbos	1,225	665	108	665
Total	38,566	16,555	992	13,967

	A. phagocytophilum			*N. mikurensis*			*B. afzelii*			*I. hookeri*		
Study site	**N positive**	**%**	**CI 95%**	**N positive**	**%**	**CI 95%**	**N positive**	**%**	**CI 95%**	**N positive**	**%**	**CI 95%**
Amsterdamse Waterleiding Duinen	39	5.8	4.1 - 7.8	1	0.1	0.0 - 0.8	5	0.7	0.2 - 1.7	87	12.8	10.4 - 15.6
Bergherbos	6	0.8	0.3 - 1.8	30	4.0	2.7 - 5.7	19	2.6	1.5 - 4.0	20	2.7	1.7 - 4.1
Buunderkamp	0	0.0	0.0 - 0.5	92	13.4	10.9 - 16.1	33	4.8	3.3 - 6.7	9	1.3	0.6 - 2.5
Deelerwoud	68	4.1	3.2 - 5.2	44	2.7	1.9 - 3.6	19	1.2	0.7 - 1.8	263	15.9	14.2 - 17.8
Duin en Kruidberg	31	3.6	2.5 - 5.1	31	3.6	2.5 - 5.1	5	0.6	0.2 - 1.4	89	10.4	8.4 - 12.6
Halfmijl	36	5.8	4.1 - 7.9	50	8.0	6.0 - 10.4	29	4.6	3.1 - 6.6	20	3.2	2.0 - 4.9
Herperduin	1	0.8	0.0 - 4.6	2	1.7	0.2 - 5.9	3	2.5	0.5 - 7.1	4	3.3	0.9 - 8.3
Kremboong	2	0.3	0.0 - 0.9	24	3.0	2.0 - 4.5	2	0.3	0.0 - 0.9	1	0.1	0.0 - 0.7
Kroondomein	62	4.4	3.4 - 5.7	155	11.1	9.5 - 12.9	21	1.5	0.9 - 2.3	56	4.0	3.0 - 5.2
Maashorst	11	1.7	0.9 - 3.1	8	1.3	0.5 - 2.5	7	1.1	0.4 - 2.2	19	3.0	1.8 - 4.6
Pettermerduin	1	0.8	0.0 - 4.2	4	3.1	0.8 - 7.7	15	11.5	6.6 - 18.3	2	1.5	0.2 - 5.4
Planken Wambuis	114	14.9	12.4 - 17.6	69	9.0	7.1 - 11.2	12	1.6	0.8 - 2.7	38	5.0	3.5 - 6.7
Rheebruggen	7	0.8	0.3 - 1.7	30	3.5	2.4 - 4.9	20	2.3	1.4 - 3.6	11	1.3	0.6 - 2.3
Schoorlse Duinen	0	0.0	0.0 - 13.2	0	0.0	0.0 - 13.2	1	3.8	0.1 - 19.6	0	0.0	0.0 - 13.2
Stameren	9	0.8	0.4 - 1.5	70	6.2	4.9 - 7.8	45	4.0	2.9 - 5.3	105	9.3	7.7 - 11.1
Valenberg	42	5.5	4.0 - 7.4	43	5.6	4.1 - 7.5	21	2.8	1.7 - 4.2	57	7.5	5.7 - 9.6
Vijverhof	4	0.4	0.1 - 1.0	121	11.6	9.8 - 13.8	21	2.0	1.3 - 3.1	5	0.5	0.2 - 1.1
Vledderhof	2	0.5	0.1 - 1.8	11	2.8	1.4 - 4.9	2	0.5	0.1 - 1.8	2	0.5	0.1 - 1.8
Zwanemeerbos	21	3.2	2.0 - 4.8	21	3.2	2.0 - 4.8	11	1.7	0.8 - 2.9	2	0.3	0.0 - 1.1
Total	456	3.3	3.0 - 3.6	806	5.8	5.4 - 6.2	291	2.1	1.9 - 2.3	790	5.7	5.3 - 6.1

Table S2. Observed and expected co-occurrence of *I. hookeri* and three tick-borne pathogens. Nymphs (n=13,967) from the 19 forest sites were tested for the presence of I. hookeri DNA, *A. phagocytophilum*, *B. afzelii*, and *N. mikurensis*. Odds ratio > 1 and < 1 indicates increased and decreased occurrence of the event, respectively. A Fisher's exact test was used to test the statistical significance for an association. Prevalences of all pathogens and I. hookeri are provided in Supplementary material S3.

	A. phagocytophilum	*B. afzelii*	*N. mikurensis*
Tick-borne pathogen and *I. hookeri* are absent	12,793	12,890	12,380
Tick-borne pathogen is absent and *I. hookeri* is present	718	786	781
Tick-borne pathogen is present and *I. hookeri* is absent	384	287	797
Tick-borne pathogen and *I. hookeri* are present (observed coinfection)	72	4	9
Expected coinfection	26	17	46
Odds ratio	3.3	0.2	0.2
p value	< 0.001	< 0.001	< 0.001

Table S3. Full equations of all models for prediction of prevalence of *I. hookeri* in questing nymphs, AIC values and results of the likelihood ratio test.

						Likelihood ratio test	
	Model equation	Std. Error	z value	p value	AIC	LogLik	Chiq
Prevalence of I. hookeri in questing nymphs ~ 1	-	-	-	-	809	-403,55	-
Prevalence of I. hookeri in questing nymphs ~ DOL	= -3.312 + 1.919e-04 * DOL	2.031e-05	9.449	<2e-16	726	-361,18	<2.2e-16
Prevalence of I. hookeri in questing nymphs ~ DON	= -3.484 + 5.955e-04 * DON	7.673e-05	7.761	8.44e-15	753	-374,62	2.814e-14
Prevalence of I. hookeri in questing nymphs ~ DON	= -3.9043783 + 0.0137145 * DOA	0.0007385	18.57	<2e-16	481	-238,5	<2.2e-16

Table S4. Summary description of the 19 locations from the cross-sectional study where questing nymphs were collected. In BU and AW, ticks of all developmental stages were collected for symbiont detection. Nymphs from AW were used for the bio-assay in 2019. Detailed information on the cross-sectional study and the study sites have been published before. See Takumi et al., 2019; Hofmeester et al., 2017b.

Study site	Abbreviation	Coordinates	Habitat	Undergrowth vegetation
Amsterdamse Waterleiding Duinen	AW	52°20'36" N 4°33'58"E	Mixed forest	*Calamagrostis epigejos*
Bergherbos	BB	51°55'14"N 6°14'30"E	Mixed forest	*Deschampsia flexuosa*
Buunderkamp	BU	52°00'56" N 5°44'50"E	Scots pine forest	*Vaccinium myrtillus*
Duin en Kruidberg	DK	52°26'16" N 4°36'18"E	Mixed forest	*Calamagrostis epigejos*
Deelerwoud	DW	52°05'51" N 5°56'42"E	Scots pine forest	*Vaccinium myrtillus*
Enkhout	EN	52°16'25" N 5°54'49"E	Scots pine forest	*Vaccinium myrtillus*
Herperduin	HD	51°45'33" N 5°36'53"E	Mixed forest	*Molinia caerulea*
Halfmijl	HM	51°25'23" N 5°19'09"E	Mixed forest	*Molinia caerulea*
Kremboong	KB	52°45'13" N 6°31'16"E	Pedunculate oak forest	*Dryopteris dilatata*
Maashorst	MH	51°42'44" N 5°35'24"E	Mixed forest	*Deschampsia flexuosa*
Pettemerduin	PD	52°46'33" N 4°40'19"E	Pedunculate oak forest	*Polypodium vulgare*
Planken Wambuis	PW	52°01'54" N 5°48'36"E	Scots pine forest	*Vaccinium myrtillus*
Rheebruggen	RB	52°46'60" N 6°17'44"E	Pedunculate oak forest	*Dryopteris dilatata*
Schoorlse Duinen	SD	52°41'47" N 4°40'01"E	Mixed forest	*Molinia caerulea*
Stameren	ST	52°03'38" N 5°21'01"E	Mixed forest	*Deschampsia flexuosa*
Valenberg	VA	52°15'33" N 5°48'47"E	Scots pine forest	*Vaccinium myrtillus*
Vijverhof	VH	52°09'43" N 5°13'43"E	Mixed forest	*Deschampsia flexuosa*
Vledderhof	VL	52°52'46" N 6°14'25"E	Pedunculate oak forest	*Dryopteris dilatata*
Zwanemeerbos	ZM	53°00'46" N 6°45'19"E	Pedunculate oak forest	*Pteridium aquilinum*

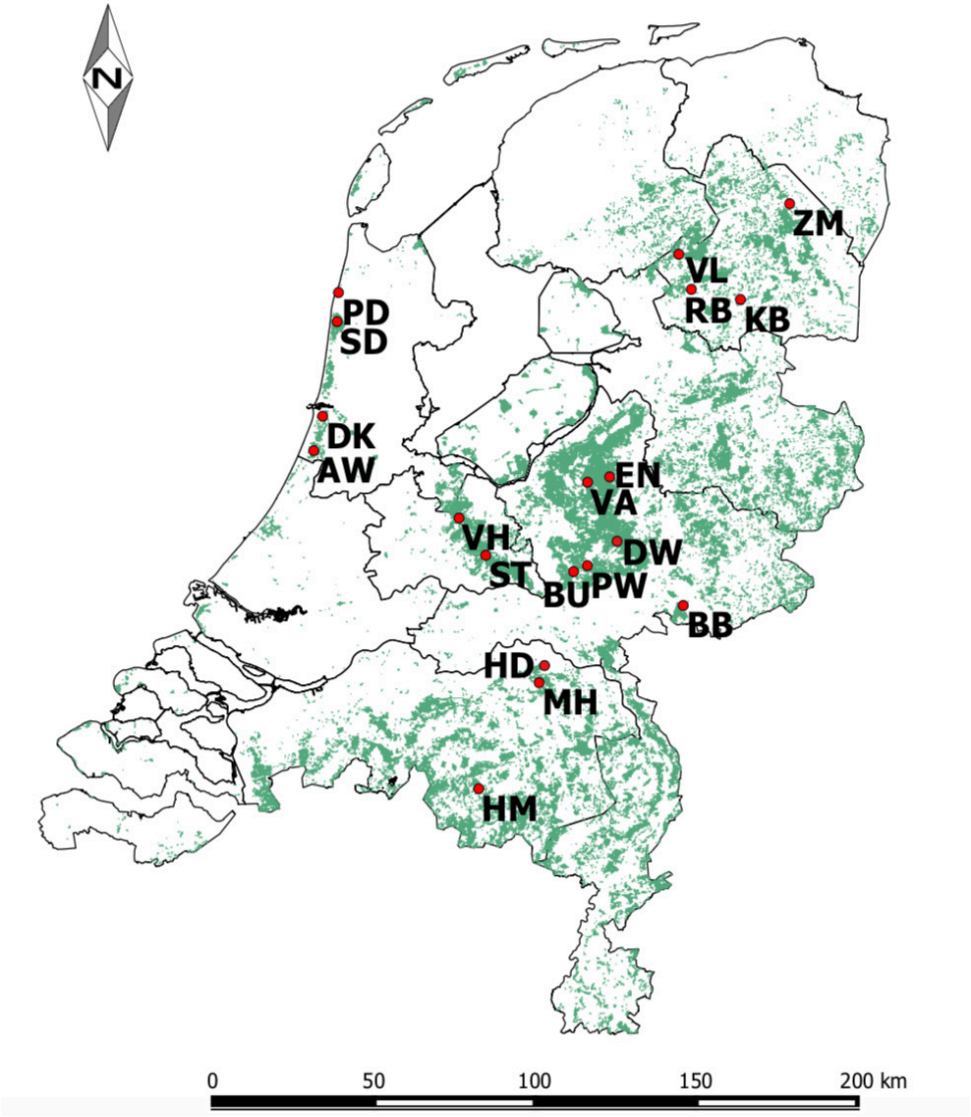


Figure S1. Forest sites in which ticks were sampled. Forest cover in The Netherlands is presented in green.

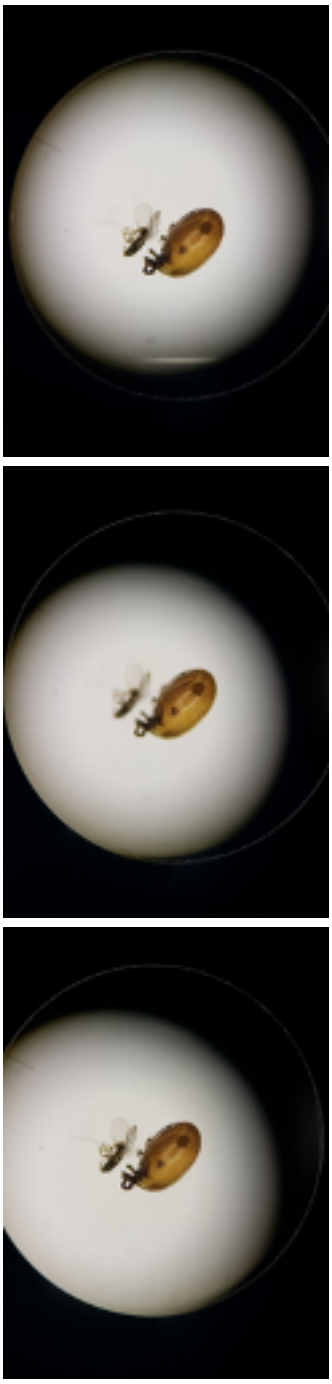
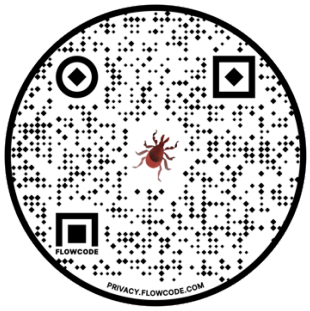


Figure S2. Original photographs used for the creation of Figure 3.



Please click here (for the online version) or scan this QR code to listen to an immersive soundscape of a forest in Zwanemeer, the Netherlands recorded by Vitalij Kuzkin. The Zwanemeer forest is one of the study sites where I have collected samples for this research. Listen with headphones for the best experience. The full album is available on: ticksandmicrobes.bandcamp.com

Chapter 7

General discussion

General discussion

Lyme borreliosis and other *Ixodes ricinus*-borne diseases emerge from interactions of humans with infected ticks in nature. The observed patterns and dynamics of tick-borne diseases result from the multitrophic interactions between pathogenic agents and (a)biotic components of the environment (van Duijvendijk et al., 2015). Since the late 20th century, the approach to study infectious disease ecology departed from a single pathogen and single host and incorporated additional layers of complexity. This approach presumes that the transmission dynamics of most pathogens involve multiple host species and possibly vectors, which can be infected with numerous microorganisms (including pathogens) and influenced by abiotic conditions. In the context of vector-borne pathogens, there is an increasing interest in how microorganisms affect the fitness and physiology and possibly vectorial capacity of the arthropod vectors. Especially the latter, as well as interactions between microorganisms and pathogens, may have consequences for vector-borne disease dynamics.

Nevertheless, to understand the role of microorganisms of *I. ricinus* in disease dynamics, we first have to learn what these microorganisms are and how they are acquired and maintained in this tick species. During my Ph.D. study, I described members of the microbiome of *I. ricinus* and elucidated their distribution in tick populations. Furthermore, I studied the tick microbiome, or its members, concerning tick life stages, geographical location, vertebrates, climatic variables, and the effect of a natural enemy of *I. ricinus*.

Microbiome of *Ixodes ricinus*

The microbiome of *I. ricinus* was studied by sequencing and analysing the V3-V4 region of the 16S rRNA gene using Illumina MiSeq™. The observed microbiome, which differs from the actual microbiome due to technical limitations, consisted of tick-associated bacteria and environmental and laboratory contamination. The top 10 most abundant taxa consisted of *Rickettsia*, two distinguishable *Rickettsiella* operational taxonomic units, *Midichloria*, *Pseudomonas*, *Halomonas*, *Mycobacterium*, *Shewanella*, *Methylobacterium*, and *Williamsia*. *Williamsia* and *Mycobacterium* are opportunistic bacteria often isolated from the environment (Barka et al., 2016; Keikha, 2018), while *Methylobacterium* is a free-living bacterium associated with soil and plants (Sánchez-López et al., 2018). Therefore, it is likely that these bacteria were present on tick exoskeletons. *Pseudomonas, Halomonas*, and *Shewanella* were abundant in our negative controls, indicating that these are laboratory contaminations. *Midichloria*, *Rickettsia*, and *Rickettsiella* are heritable symbionts of *I. ricinus* (Duron et al., 2017). Next to these, we detected another recognized heritable symbiont of ticks, *Spiroplasma* (Duron et al., 2017). Interestingly, reads belonging to tick-borne pathogens such as *Anaplasma, Neoehrlichia*, and *Borrelia* accounted for a small part of the overall tick microbiome (~ 0.4%), even smaller than the supposedly bacterial contaminants part.

Geography

The observed microbiome compositions of *I. ricinus* varied significantly between geographical regions within the Netherlands. Previous studies have given several probable explanations for differences in bacterial communities suggesting that they arose from the distinct habitats with a dissimilar availability of vertebrate hosts and consequently different environmental and animal host-associated bacteria (Carpi et al., 2011; Van Treuren et al., 2015). Interestingly, in our dataset, the strongest determinants of observed microbiome differences were the abundance and prevalence

of the heritable bacteria *Rickettsia* and *Rickettsiella* (Chapter 2 and 3). These results led us to the hypothesis that the prevalences of *R. helvetica* (the most common *Rickettsia* species in *I. ricinus*) and *Rickettsiella* spp. are determined regionally rather than locally. In addition to these microorganisms, we studied other tick-associated bacteria *S. ixodetis* and *M. mitochondrii*, present in our dataset but less responsible for variations in bacterial communities between regions. Lastly, for contrast, we included tick-borne pathogens *B. burgdorferi* s.l., *A. phagocytophilum*, and *N. mikurensis*.

The main difference between the microorganisms mentioned above is the transmission route facilitating their maintenance in tick populations. *Rickettsia helvetica*, *Rickettsiella* spp., *S. ixodetis*, and *M. mitochondrii* are predominantly vertically-transmitted from a female tick to her offspring (Chapter2; Sassera et al., 2006), while *B. burgdorferi* s.l., *A. phagocytophilum*, and *N. mikurensis* are transmitted horizontally from infected to uninfected ticks via a vertebrate host (de la Fuente et al., 2005; Jahfari et al., 2014; Obiegala et al., 2017). Therefore, the prevalences of these bacteria in ticks are affected by different factors, with both quantitative and qualitative epidemiological consequences.

Although all seven studied microorganisms were present in every studied forest site, the prevalences of *R. helvetica*, *Rickettsiella* spp., *M. mitochondrii*, and *N. mikurensis* in questing nymphs differed significantly between three geographical regions. The prevalence of *B. burgdorferi* s.l., *A. phagocytophilum*, and *S. ixodetis* appeared to be determined on a local rather than regional scale. Previously, the prevalences of horizontally-transmitted pathogens have been shown to be primarily determined by the local vertebrate community (Chapter 4; Ostfeld et al., 2006; Takumi et al., 2019). Therefore, my results for *N. mikurensis* which prevalence varied rather regionally than locally, were unexpected.

Rickettsia helvetica, *Rickettsiella* spp., *S. ixodetis*, and *M. mitochondrii* are intracellular heritable symbionts of *I. ricinus* and are suspected of having obligate or facultative relationships with this tick species (Duron et al., 2017). Nevertheless, we do not fully understand which mechanisms caused such regional heterogenicity in the prevalence of *R. helvetica*, *Rickettsiella* spp., and *M. mitochondrii*. It is possible that (meta)populations of ticks from the three studied regions are exposed to different biotic and abiotic stresses; a symbiont is more prevalent in the host population when it provides tolerance to existing stress (strong selective force) and less prevalent when it is less beneficial for mitigating stress (weak selective force; Duron et al., 2017; Gundel et al., 2011).

Variations in abiotic stress may arise from regional differences in soil type, temperature, and humidity related to coastal (the western region) and inland (the central and northern regions) areas of the Netherlands. Interestingly, in another study on the spatio-temporal variation of nymphal infection prevalences (NIP) of symbionts, we observed that the spatial distribution of at least one symbionts, *R. helvetica,* can be explained by spatial variation in climate (Chapter 5). Among the 12 studied locations, the five forest sites with the highest *R. helvetica* NIP had the highest relative humidity in spring and summer (and often the lowest in autumn and winter). All but one of these sites are located near the Dutch coast. The two sites with the lowest average relative humidity in the southernmost in-land part of the Netherlands had one of the lowest *R. helvetica* NIP. In the study mentioned above, we looked at the short-term effects of climatic variables on the prevalence of tick symbionts. We detected a significant but minor association between relative humidity and *R. helvetica* NIP. Although a short-term positive effect of relative humidity was detected, we propose

that the spatial variation of *R. helvetica* NIP in the Netherlands stems from long-term differences in relative humidity between locations. Nevertheless, it remains unclear whether *R. helvetica* confers fitness benefits to *I. ricinus* in certain environmental conditions or whether certain environmental conditions facilitate propagation and transmission of this symbiont in tick populations.

Biotic stresses such as the presence and abundance of tick parasites, including parasitic wasps, fungi, and viruses, may lead to varying prevalences of facultative symbionts between tick populations. Although never documented for ticks, symbionts have been shown to confer defence against parasites in other arthropod orders. For example, in the pea aphid, *Acyrthosiphon pisum*, the bacterium *Hamiltonella defensa* provides resistance to parasitic wasps (Oliver et al., 2008; Oliver et al., 2005) while *Regiella insecticola* resistance to fungal pathogens (Scarborough et al., 2005). However, infections by defensive symbionts come with a cost in the absence of parasitism, which would explain the intermediate prevalences in natural populations (Oliver et al., 2006). As elegantly illustrated in Hopkins et al. (2017). defensive symbiont and parasite densities determine whether net effects on hosts are positive or negative compared to symbiont-free hosts. Therefore, this highly context-dependent nature of defensive symbiont-host interactions makes it challenging to identify and quantify symbiont functions on the ecological level. Laboratory experiments may reveal which symbionts might have a protective effect on, for example, the predation by parasitic wasps (Chapter 6).

Alternatively, the varying prevalences of a symbiont between distinct tick (meta)populations may arise from differences in transmission rate. The transmission rate could be potentially affected by a tick or symbiont genotypes or the compatibility of particular tick – symbiont genotype pairs (Gundel et al., 2011). This evolutionary divergence within *I. ricinus* and/or a symbiont species could be promoted by a geographical barrier and limited exchange of tick individuals between regions. However, this variation has not yet been studied or documented for arthropod – microbe symbiosis. Indeed, investigating the genetic diversity of symbionts and *I. ricinus* originating from different populations may provide more insight.

Life stage

We have observed small but statistically significant differences between microbiome compositions of distinct developmental stages (Chapter 2). The developmental stage on its own did not explain the clustering of tick microbiomes. Contrary to what we had expected, bacterial diversity seems to decrease along with tick development, illustrated with a significant difference between larvae and females. However, an opposite trend was observed from nymphs to adult males. It appears that during the off-host phases of ticks, fewer bacterial species enter a tick than has been speculated before (Narasimhan & Fikrig, 2015). Alternatively, the decreased bacterial diversity that we observed arose from the technical limitations of next-generation sequencing. It has been shown that abundant bacteria recruit more reads and potentially mask less abundant ones making them less likely to be detected (Gofton et al., 2015). In some instances, we were not even able to detect tick-borne pathogens in qPCR positive samples. This would imply that selected bacterial species thrive along with the tick life-span, while others remain at low yields and become undetectable by this technology.

Previous studies have reported significant differences in *Ixodes* microbiome compositions of distinct life stages. However, they reported contradictory results regarding the dynamics of bacterial diversity. Zolnik et al. (2016) documented increasing, while Kwan et al. (2017), Swei &Kwan (2017), and Carpi et al. (2011) showed decreasing diversity along with tick development. Therefore, the role of the life stage in shaping the tick microbiome is still not elucidated, and it is difficult to subtract this information from the data obtained solely with next-generation sequencing. Thus, in future studies, combining this method with other detection techniques is highly advisable. Exploring changes in abundance and prevalence of a specific or several tick symbionts through the ontogeny may help us understand the nature of symbiont-tick relationships (Chapter 2). Tick activity, metabolism, and physiology vary between tick developmental stages with possible consequences for their symbiotic community. Also, given that males and females face distinct challenges related to reproduction, symbionts' functions may be sex-specific. As a consequence, male-associated symbionts would perish in females and vice versa. For instance, in *I. ricinus*, *M. mitochondrii* and *R. helvetica* have been observed at higher infection rates in females suggesting that males lose these symbionts through each subsequent moult (Noda et al., 1997; Stańczak et al., 2009; Thapa et al., 2019; Zhu et al., 1992; Zolnik et al., 2016). Undoubtedly, knowing the sex of larvae and nymphs would be advantageous. However, male and female immature stages are morphologically indistinguishable; no genetic markers are available (Sonenshine & Roe, 2014). In general, within sub-class Acari, little is known about the process of sex determination at the gene level, and at present, there is no perspective for a molecular tool to distinguish sexes.

Vertebrate hosts

We did not detect significant differences between the bacterial microbiomes of ticks with and without pathogens, even when ignoring the geographical effect (Chapter 3). Because the presence of a pathogen indicates a specific blood-feeding history, our findings imply that the vertebrate hosts make little or no contribution to the bacterial community of ticks. Our results are in line with previous findings by Hawlena et al. (2013), but they are in contrast with a study by Swei & Kwan (2017). The latter found differences between the microbiome of *I. pacificus* ticks feeding on lizards and those feeding on mice. We are not aware of any of the nymphs studied here fed on lizards, which are relatively uncommon tick hosts in our study areas (Hofmeester et al., 2016). Therefore, we cannot rule out the potential influence of reptiles on the tick microbiome. Cold-blooded vertebrates, such as reptiles, have less stringent requirements for regulating blood biochemical properties, and their blood may have vastly different osmotic pressure and pH compared to mammals and birds (Dessauer, 1970).

A possible explanation for our finding is that ticks are genetically equipped to resist opportunistic bacteria, such as host skin commensals. Hayes et al. (2020) has discovered that *I. scapularis* horizontally acquired an antimicrobial toxin gene from bacteria. The gene encodes lytic cell wall-degrading enzymes delivered to the host bite site via saliva and responsible for selectively killing vertebrate's skin-associated bacteria. Interestingly, the enzymes had no intrinsic lytic activity against *B. burgdorferi* (Hayes et al., 2020). Therefore, pathogens might still affect the tick gut microbiome to facilitate their colonization, as previously observed in laboratory experiments (Abraham et al., 2017; Narasimhan et al., 2017). Here, we did not investigate the microbiome of engorged ticks, which might have been directly affected by a blood meal. In future work, studying the individual organs deriving from engorged and questing ticks may extend our understanding of microbiome-pathogen interactions.

Limited microbiome of Ixodes ricinus

Interestingly, microbiome analysis showed that many samples (pools of ticks or individual adult ticks) had a very low bacterial load and varying bacterial diversity (Chapter 2). These ticks usually were devoid of vertically-acquired symbionts such as *Midichloria*, *Rickettsia*, *Rickettsiella*, and *Spiroplasma*. Also, in nearly 12% of individual questing nymphs (n = 13 967) tested with qPCR, we did not detect any of the seven microorganisms: the symbionts mentioned above, *B. burgdorferi* s.l., *A. phagocytophilum*, and *N. mikurensis* (Chapter 2). Our results indicate that *I. ricinus* as a species does not possess a stable microbiome and not even an obligate bacterial symbiont present in 100% of the population. These indications are somewhat surprising given that ticks are arthropods feeding exclusively on vertebrate blood, a nutritionally restricted diet. In many hematophagous arthropods, including other tick species, obligate, maternally acquired bacterial symbionts are responsible for providing essential metabolites such as vitamins B that are deficient in vertebrate blood (Duron et al., 2018). These nutrients are required for the maintenance of arthropod metabolic homeostasis, and therefore one would expect to find at least a small set of microorganisms in all tested individual *I. ricinus* ticks. However, our observations are in line with an increasing body of evidence that many animals, including arthropods, are minimally or facultatively dependent on microbes or do not need a microbiome at all (Hammer et al., 2017; Hammer et al., 2019; Husnik, 2018).

A previous study on the microbiome of *I. ricinus* also observes an unstable microbial composition (Aivelo et al., 2019a, 2019b). However, contrary to our findings, Aivelo et al. found *M. mitochondrii* in all sequenced ticks (n = 82). Apart from *M. mitochondrii* being common for all samples, the authors linked the substantial microbiota variation to the presence of the transient (non-residential) bacteria, which could have been acquired during blood-feeding or from the environment through the mouth, the anal pore, or spiracles (Engel & Moran, 2013; Ross et al., 2018; Zolnik et al., 2018; Zolnik et al., 2016).

Other studies explicitly focused on the gut, an organ that plays an essential role in the maintenance and transmission of many vector-borne pathogens. Among arthropod tissues, the gut epithelium is the most exposed to microorganisms. Consequently, it usually harbours a complex bacterial community, which influences arthropod physiology and immunity, and therefore mediates interactions between the host and microbiota (Dong et al., 2009; Oliveira et al., 2011; Ryu et al., 2008). In many hematophagous arthropods, the influx of blood into the digestive system is associated with the growth of the midgut bacterial community as it has been documented in mosquitoes and triatomine bugs (Eichler & Schaub, 2002; Oliveira et al., 2011). Nevertheless, a recent study by Guizzo et al. (2020) showed an extremely low overall bacterial load in the *I. ricinus* and *R. microplus* midguts and did not detect any common bacterial genera. Unexpectedly, the amount of blood consumed was negatively associated with the bacterial load in the midgut of *I. ricinus*. Similarly, authors of the study on the midgut microbiome of *I. scapularis*, a closely related tick species to *I. ricinus*, observed that unfed wild hard ticks possess a limited and unstable internal microbiome (Ross et al., 2018).

One probable explanation for the low bacterial loads in the midgut of ticks lies in the biology of tick blood-feeding. Unlike the majority of hematophagous animals, which feed rapidly and digest blood in an extracellular process in the gut lumen (Billingsley & Rudin, 1992; Briegel & Lea, 1975), ticks feed in a slower process and digest blood intracellularly in the specialized digestive cells of the gut epithelium (Grandjean & Aeschlimann, 1973; Sojka et al., 2013). Thus, along with the blood meal,

some midgut microbiota might be endocytosed and digested by the tick digestive epithelial cells, leading to decreased bacterial load (Guizzo et al., 2020).

Another organ frequently observed as a reservoir of microbes is the ovary. A tropism for the ovaries guarantees the transovarial transmission. Thus, microorganisms residing there are usually involved in an intimate and obligate relationship with their host. For example, *Coxiella* sp. and *Francisella* colonize the ovaries of *R. microplus* and *Ornithodoros moubata*, respectively, and appear to be essential for tick moult and/or survival (Duron et al., 2018; Guizzo et al., 2017). Regarding the overall microbiome of the *I. ricinus* ovaries, Guizzo et al. (2020) described an abundant and stable bacterial community with very low diversity dominated by *Midichloria* sp. Interestingly, the obligate intracellular bacteria situated in tick ovaries seem to play a similar physiological role as the gut microbiota observed in most animals (Guizzo et al., 2020; Hammer et al., 2019).

A bacterial species is often not restricted to a single organ and can establish itself in a variety of organs including salivary glands and Malpighian tubules (Narasimhan et al., 2015). The Malpighian tubules are involved in excretion and osmoregulation, and it has been suggested that microorganisms residing in this tissue have a nutritional role for their hosts (Buysse et al., 2019; Sonenshine et al., 2014). However, in general, little is known about the microbiome of Malpighian tubules in ticks. The salivary glands are also responsible for osmoregulation in ticks during the blood-feeding as well as off-host period (Bowman & Sauer, 2004). During feeding, a tick returns about 70% of the blood-meal fluid to the host via salivation. The salivary glands are the perfect site for microorganisms to develop and are intricately involved in transmitting infectious microbes to vertebrate hosts. There is scarce information on bacterial load in the salivary glands of hard ticks. Nevertheless, the bacterial diversity of the salivary glands (or saliva) in *I. scapularis* and *D. silvarum* has been observed to be higher than in the midgut (Duan et al., 2020; Zolnik et al., 2016). This difference is probably related to the presence of horizontally-transmitted pathogens such as *Borrelia* spp. and *Anaplasma* spp., which can colonize this organ after migrating from the gut (Piesman & Schneider, 2002; Zolnik et al., 2016).

Given the above-mentioned known unstable associations of tick organs with microbiota, some individuals of *I. ricinus* may have no or a limited microbiome. How is it possible that some individuals of this hematophagous arthropod species have no obligate symbionts? Or if they have, how is it possible that we have missed them?

As with *I. ricinus,* there is no strong evidence for an obligatory relationship between *I. scapularis* and bacteria. Previously *R. buchneri* was considered an obligate symbiont as its genome contains all the genes of de novo folate (vitamin B9) biosynthesis (Hunter et al., 2015). However, on many occasions, *I. scapularis* ticks without *R. buchneri* have been reported. In fact, the prevalence of this symbiont in tick populations varied between 46% to 82% depending on the studied location, suggesting a facultative relationship rather than an obligate one (Steiner et al., 2008; Tokarz et al., 2019).

In *I. ricinus*, *M. mitochondrii* has been proposed as an obligate symbiont. This obligate intracellular bacterium exhibits a unique lifestyle and, next to residing in the cytoplasm of tick cells, it survives in host mitochondria (Beninati et al., 2004; Lo et al., 2006; Sacchi et al., 2004). *Midichloria mitochondrii* predominantly colonizes ovaries, but it has also been detected in salivary glands, tracheae, and Malpighian tubules (Epis et al., 2013; Olivieri et al., 2019). An infected female of *I.*

ricinus transmits this symbiont transovarially to both female and male eggs. Nevertheless, only in female eggs, the bacterium continues to thrive in primordial ovarian tissues (Beninati et al., 2004; Lo et al., 2006; Sassera et al., 2008). As a result, its infection rate may reach up to 100% in wild adult females, but it is much lower in males.

Interestingly, in tick colonies maintained in the laboratory for several years, *I. ricinus* females presented a reduced infection rate ranging from 18% to 44% depending on a laboratory population (Lo et al., 2006). Our results also suggest that some of the questing wild females are free of *M. mitochondrii*. Indeed, in the microbiome study, the majority of the adult female individuals presented a high abundance of *Midichloria.* However some had only singular reads (Chapter 2). We also did not detect *M. mitochondrii* in two out of 40 questing females tested with qPCR (Chapter 2). Lastly, the prevalence of this symbiont in nymphal populations from 19 forest sites across the Netherlands ranged from 47% to 77%, indicating that either the sex ratio differs largely between the locations or that under certain conditions, males preserve *M. mitochondrii.* Alternatively, in some locations, this bacterium is redundant in female nymphs. Nevertheless, with the methodology used in this study, it is impossible to assess whether uninfected female ticks are more sensitive to changing conditions and whether they are physiologically able to oviposit and complete their cycle.

The study by Olivieri et al. (2019) on the functional roles of *M. mitochondrii* in *I. ricinus* concluded that this symbiont interacts with its host in various ways depending on the tissue it colonizes. These would include enhancing the host fitness by contributing to nutrition supplementation, reproduction, energy production, coping with oxidative stress, and maintaining homeostasis and water balance (Sassera et al., 2008; Stavru et al., 2020). However, because *M. mitochondrii* is not essential for the survival of each individual tick, scientists are hesitant to call it an obligate symbiont. According to the strict definition, a symbiosis is considered to be obligatory when the relationship between a symbiont and its host has a mutualistic character and when the symbiont is present in most individuals of given species (Duron et al., 2017). Nevertheless, *M. mitochondrii* appears to be obligate for *I. ricinus* to thrive under natural conditions successfully.

Overall, in our study, all maternally-inherited symbionts such as *M. mitochondrii*, *R. helvetica*, *S. ixodetis*, and *Rickettsiella* were present in all 19 forest sites studied (Chapter 2, Chapter 3). However, they presented significant distinct infection rates in questing nymphs between locations or regions, and they appeared in different proportions depending on the population (Chapter 2, Chapter 3). This result is in accordance with a study by Duron et al. (2017), who investigated a community of 10 maternally inheritable symbionts in 81 tick species. They showed that next to being unstable across the tick species and phylogeny, the symbiotic community structure varied among tick individuals and populations within species. The authors suggested that the flow of bacterial symbionts between tick species results from occasional horizontal transmission (Duron et al., 2017). On a species level, the horizontal transmission can probably complement the imperfect maternal inheritance (vertical transmission) as one of the mechanisms for maintaining stable infection rates in a population. In fact, on many occasions, symbionts such as *M. mitochondrii*, *R. helvetica*, and *S. ixodetis* were suspected to be transmitted via a vertebrate host either by surviving in vertebrate tissues or via co-feeding of infected and uninfected ticks (Binetruy et al., 2019; Cafiso et al., 2019; Heylen et al., 2016; Hornok et al., 2014).

Nevertheless, the striking difference in their infection rates between the populations could be derived from the facultative relationship between these symbionts and *I. ricinus.* It appears that under certain environmental conditions, specific symbionts confer fitness benefits (as they do in many insects), and therefore their infection rate in ticks is fixed on a higher level (Gavotte et al., 2010; Weiss & Aksoy, 2011). Probably, under other circumstances, the same symbionts can be a burden, and therefore its infection rate is compromised.

There is a probability that *I. ricinus* populations from distant geographical regions are engaged in relationships with different bacterial symbionts. Thus, it is of interest to study communities of symbionts of *I. ricinus* originating from varying biotic and abiotic conditions. Correlative studies could help to identify possible tick traits mediated by bacterial symbionts. To date, there are several indications that *I. ricinus* may harbour other symbiotic bacteria than the ones described in this thesis. In studies from Slovakia, Poland, and Switzerland, *I. ricinus* has been shown to carry *Coxiella* spp. and *Francisella* spp., and *Lariskella* spp. (Aivelo et al., 2019a, 2019b; Spitalska et al., 2018; Wojcik-Fatla et al., 2015). However, it is unknown what their functional role is and whether they are genuinely associated with this tick species. Nevertheless, our results, obtained solely in the Netherlands, already show that *I. ricinus,* as a species, has a flexible relationship with a set of maternally heritable symbionts.

A flexible relationship with a set of facultative instead of strictly obligate symbionts could benefit *I. ricinus* as a species. Although obligate symbionts are undoubtedly beneficial, in some cases, they may impose limitations on their hosts as they are often more sensitive to, for example, changes in temperature than the hosts themselves. This sensitivity is usually a consequence of the degenerative evolution of the bacterial genome, driven by intimate symbioses (Kikuchi et al., 2016; Moran, 2016; Musolin et al., 2010). As a result, even small increases in average ambient temperature may kill symbionts and, with them, their hosts. Therefore, the lack of obligate symbiosis and a partial dependence on a set of facultative heritable symbionts, which can be occasionally transmitted horizontally, can facilitate *I. ricinus*' geographical expansion.

Currently, *I. ricinus* can be found further north and at higher altitudes than described in historical records (Garcia-Vozmediano et al., 2020; Jore et al., 2011). Although the main factors driving the observed expansion of *I. ricinus* are changes in the distribution of tick hosts and vegetation (mainly caused by land management and climate change; Medlock et al., 2013), various facultative bacteria can provide adaptative traits under new environmental conditions. For example, such flexibility in symbiotic association enables *Atta texana*, a leafcutter ant, to have a broad geographic range (Mueller et al., 2011). Populations of *A. texana* experiencing different climatic conditions have been shown to engage in relationships with fungal symbionts of varying temperature tolerance (Mueller et al., 2011).

Apart from adaptation to the abiotic environment, facultative symbionts may confer a range of ecologically important traits, occasionally exchanged via horizontal transmission within and between tick species (Feldhaar, 2011). Due to the impact of symbioses on various aspects of their arthropod host biology, the hosts are often studied more holistically; this is as holobionts (Rosenberg et al., 2010; Zilber-Rosenberg & Rosenberg, 2008). The concept of the holobiont presumes that the organism's phenotype is determined by both host's genome and genome(s) of all symbionts inhabiting the host. Using this approach in studying tick ecology may increase our

understanding of the vectorial capacity of given tick populations.

Technical limitations

It is possible that in some of the ticks collected in our study, we have not detected symbionts due to technical limitations of both qPCR and high-throughput sequencing technology. For example, our choice for the 16S rRNA region might have significantly influenced the estimated taxonomic diversity (Bukin et al., 2019; Yang et al., 2016). In particular, targeting distinct regions may lead to different estimated proportions of taxa in the same sample. One possible solution is to use two or more 16S rRNA regions to study the same communities.

In addition, both detection methods may fail when the abundance of bacteria is too low. A load of bacteria may potentially fluctuate depending on a tick physiological state, however, not much is known about it. Bacterial communities may change as ticks proceed through feeding, digestion, and moulting, suggesting that some microorganisms perish and others are lost during these life events (Zolnik et al., 2018). Given the shortcomings of high-throughput sequencing technology, we advocate that this method is suitable for generating hypotheses but not testing them.

Assessment of disease risk

In the Netherlands, several pathogens of humans and domestic animals circulate between ticks and wildlife. These pathogens include *B. burgdorferi* sensu lato, *B. miyamotoi*, *A. phagocytophilum*, and *N. mikurensis*, which are acquired predominantly horizontally by ticks from various animal species (Hofmeester et al., 2016; Hoornstra et al., 2018; Jahfari et al., 2014; Silaghi et al., 2016). Their prevalences in questing nymphs have been, therefore, associated with the diversity and abundance of vertebrates (Takumi et al., 2019). My study contributed to these observations by showing that the prevalences of rodent-associated *B. afzelii* and *N. mikurensis* in questing nymphs are positively associated with rodent density (Chapter 4). Because rodents are the primary hosts of larval ticks and consistently contribute to a new generation of nymphs (Chapter 4; Hofmeester et al., 2016; Ostfeld et al., 2006), rodent density has a strong synergistic effect on the density of nymphs infected with these pathogens.

Knowing what determines the density of infected nymphs allows for a more accurate assessment of tick-borne disease risk. Therefore, it is crucial to understand what drives the density of nymphs infected with other tick microorganisms such as *I. ricinus* heritable symbionts, potentially opportunistic human and animal pathogens. For instance, both *R. helvetica* and *S. ixodetis* are suspected of persisting in vertebrate tissues (Binetruy et al., 2019; Hornok et al., 2014) and are associated with cases of human disease (Fournier et al., 2000; Matet et al., 2020; Aquilino et al., 2015). In Chapter 4, we showed that the association between the fluctuations in rodents and *R helvetica* NIP differed between years, and the association between the fluctuations in rodents and *S. ixodetis* NIP was negative. This difference probably arose from distinct primary transmission modes, and that amplification of these microorganisms rely to a different extent on rodent hosts. Interestingly, from the two rodent species investigated in the study, we detected *S. ixodetis* almost exclusively in wood mice, which indicates that the symbiont is occasionally horizontally-transmitted via this rodent species. Therefore, a possible explanation for the observed negative association is that the increasing bank vole population diluted the density of ticks infected with *S. ixodetis* in our experiment. Nevertheless, increased rodent density was positively associated with the density of nymphs infected with *R. helvetica* and *S. ixodetis*. These findings show that changes

in vertebrate abundance, directly and indirectly, affect the risk of infection with *S. ixodetis* and *R. helvetica*, respectively.

In another study (Chapter 2), *S. ixodetis* was the only heritable symbiont with a local difference in prevalence possibly driven by differences in rodent community, particularly a ratio between wood mice and bank voles. Therefore, similarly to horizontally-transmitted pathogens, the local vertebrate community should be considered when performing an accurate *S. ixodetis* risk assessment.

Contrary, to assess the risk of *R. helvetica* and *M. mitochondrii* infections, other factors should be considered as their distribution appears to be independent of the local vertebrate community (Chapter 2). Although *M. mitochondrii* has never been associated with human disease, it is potentially pathogenic as it colonizes salivary glands of ticks, and its DNA has been detected in several mammalian species (Bazzocchi et al., 2013). Regarding *R. helvetica*, several studies performed in the Netherlands showed that this microorganism is significantly more prevalent and reaching up to 60% in tick populations in the western part of the country (Chapter 2 and Chapter 5; Sprong et al., 2009). Despite this, rickettsiosis caused by *R. helvetica* has never been documented in the Netherlands, probably because the early signs and symptoms of rickettsial infections are non-specific, mimicking flu-like illnesses. Therefore, studies aimed at identifying disease cases are best conducted in close collaboration with the Western region medical community.

Parasitic wasp and symbionts as potential biological control agents

Parasitic wasp

Biological control of ticks is considered an environmentally friendly approach (Collatz et al., 2011; Hu et al., 1998a; Hu & Hyland, 1998b; Mwangi et al., 1997) but has not been effectively applied in routine settings for *Ixodes ricinus*. In Chapter 6, we studied the parasitoid wasp, *Ixodiphagus hookeri* (Hymenoptera: Encyrtidae), which is a natural enemy of *I. ricinus*, and a target of interest as a biological control agent (Collatz et al., 2011).

We investigated the associations of *hookeri* wasps with the propagation hosts of *I. ricinus*. We observed a positive association between *I. hookeri* prevalence and encounter probability of deer, but not rodents. This observation supports the idea that female wasps are attracted to deer, and subsequently infest ticks that feed on these deer (Hu & Hyland, 1997b). The interaction of the wasp with the propagation host (deer) of its tick host ensures, to a large extent, the continuation of its lifecycle. This tri-trophic interaction makes it very difficult, if not impossible, to disentangle the effect of deer abundance on the tick and wasp populations as well as the effect of the wasp on the tick population. Indeed, our study shows that the high prevalence of the wasp, which kills nymphs upon its emergence, had no apparent negative impact on the density of adult ticks (Chapter 6). Furthermore, it is costly and labour-intensive to rear these parasitic wasps in the laboratory for application in field settings, making this approach less environmentally friendly.

Further, we showed that the parasitic wasp and *A. phagocytophilum* frequently co-occur in questing nymphs, negatively influencing the pathogen's cycle. Future studies could investigate the potential long-lasting effect of *I. hookeri* on *A. phagocytophilum* and other deer-associated pathogens, such as *Babesia* spp.

Symbionts

In a study investigating co-infections of microorganisms in *I. ricinus* ticks (Chapter 3), we observed positive relationships of *M. mitochondrii* and *Rickettsiella* spp. with *B. burgdorferi* s.l. indicating that these two symbionts have facilitative properties for pathogen acquisition. Therefore, symbiotic control strategies aiming at decreasing disease risk could focus on diminishing prevalences of *M. mitochondrii* and *Rickettsiella* spp. in *I. ricinus*. This could be achieved by developing a symbiont-targeted vaccine and administrating it to tick propagation hosts such as deer. Interestingly, a recent study investigated the suitability of anti-tick microbiota vaccines to control ticks and tick-borne pathogens. In the experiment, the anti-tick vaccine targeting *Escherichia coli* impacted *I. ricinus* performance during feeding (Mateos-Hernández et al., 2020).

Ixodes ricinus symbionts could be considered for paratransgenesis, although we did not observe natural interference between symbionts and pathogens. Paratransgenesis relies on genetically-modified bacterial symbionts interfering with pathogens transmission via, for instance, delivering anti-pathogen effector molecules (Durvasula et al., 1997; Wilke & Marrelli, 2015). This strategy successfully reduces the mosquito vectorial capacity and involves genetically-modified mosquito symbionts interfering with malaria parasite development and transmission (Mancini et al., 2016; Ricci et al., 2011).

Conclusions and implications

Individual *I. ricinus* ticks can survive with a limited microbiome, but a bacterial community is required for a tick population to thrive. This community differs on a spatial scale, likely driven by habitat characteristics, vertebrate communities, and (micro)climate. The relationship between ticks and microorganisms can probably change throughout tick ontogeny as some symbionts may confer specific functions in a particular tick stage. Therefore, the transmission dynamics of tick-borne microorganisms is dictated by various (a)biotic factors, which could be elucidated by thoroughly studying microbiomes of distinct tick life stages across Europe's geographical scale.

To what extent (a)biotic factors can influence microorganisms depends vastly on the transmission mode the microorganisms utilize for their maintenance in tick populations. The prevalence of horizontally-transmitted bacteria appears to be determined primarily by local factors such as the vertebrate community. Contrary, the prevalence of vertically-transmitted bacteria is probably mirroring the character of symbioses with *I. ricinus*, which can be context-dependent. Therefore, to improve our ability to predict the distribution of tick-borne microorganisms, including human pathogens, we need to understand whether they are transmitted horizontally or vertically or both. Under the latter condition, the contribution of each mode should be quantified.

Lastly, the *I. ricinus* symbionts facilitate rather than impede the transmission dynamics of tick-borne pathogens, probably indirectly via enhancing tick fitness. Thus, strategies aiming at decreasing disease risk could focus on reducing prevalences of *I. ricinus* symbionts via symbiont-targeted vaccines administered to tick hosts. Alternatively, tick symbionts could be assessed for their potential to be used in paratransgenic strategies.

References

Abraham, N. M., Liu, L., Jutras, B. L., Yadav, A. K., Narasimhan, S., Gopalakrishnan, V., et al. (2017). Pathogen-mediated manipulation of arthropod microbiota to promote infection. *Proc Natl Acad Sci U S A, 114*(5), E781-E790.

Aivelo, T., Norberg, A., & Tschirren, B. (2019a). Bacterial microbiota composition of Ixodes ricinus ticks: the role of environmental variation, tick characteristics and microbial interactions. *PeerJ, 7*, e8217.

Aivelo, T., Norberg, A., & Tschirren, B. (2019b). Human pathogen co-occurrence in Ixodes ricinus ticks: effects of landscape topography, climatic factors and microbiota interactions. *bioRxiv*, 559245.

Akman, L., Yamashita, A., Watanabe, H., Oshima, K., Shiba, T., Hattori, M., et al. (2002). Genome sequence of the endocellular obligate symbiont of tsetse flies, Wigglesworthia glossinidia. *Nat Genet, 32*(3), 402-407.

Anderson, M. J. (2014). Permutational multivariate analysis of variance (PERMANOVA). *Wiley statsref: statistics reference online*, 1-15.

Andersson, M., Bartkova, S., Lindestad, O., & Raberg, L. (2013). Co-infection with 'Candidatus Neoehrlichia Mikurensis' and Borrelia afzelii in Ixodes ricinus ticks in southern Sweden. *Vector Borne Zoonotic Dis, 13*(7), 438-442.

Andersson, M., Scherman, K., & Raberg, L. (2014). Infection dynamics of the tick-borne pathogen "Candidatus Neoehrlichia mikurensis" and coinfections with Borrelia afzelii in bank voles in Southern Sweden. *Appl Environ Microbiol, 80*(5), 1645-1649.

Andreotti, R., Perez de Leon, A. A., Dowd, S. E., Guerrero, F. D., Bendele, K. G., & Scoles, G. A. (2011). Assessment of bacterial diversity in the cattle tick Rhipicephalus (Boophilus) microplus through tag-encoded pyrosequencing. *BMC Microbiol, 11*(1), 6.

Aquilino, A., Masiá, M., López, P., Galiana, A. J., Tovar, J., Andrés, M., et al. (2015). First human systemic infection caused by Spiroplasma. *J Clin Microbiol, 53*(2), 719-721.

Arevalo, P., VanInsberghe, D., Elsherbini, J., Gore, J., & Polz, M. F. (2019). A reverse ecology approach based on a biological definition of microbial populations. *Cell, 178*(4), 820-834.

Armitage, P., Berry, G., & Matthews, J. N. S. (2001). *Statistical methods in medical research* (4 ed.): Blackwell Publishers. Malden, MA.

Azad, A. F., & Beard, C. B. (1998). Rickettsial pathogens and their arthropod vectors. *Emerging infectious diseases, 4*(2), 179.

Azagi, T., Hoornstra, D., Kremer, K., Hovius, J. W., & Sprong, H. (2020). Evaluation of disease causality of rare Ixodes ricinus-borne infections in Europe. *Pathogens, 9*(2), 150.

Azagi, T., Klement, E., Perlman, G., Lustig, Y., Mumcuoglu, K. Y., Apanaskevich, D. A., et al. (2017). Francisella-like endosymbionts and Rickettsia species in local and imported Hyalomma ticks. *Appl Environ Microbiol, 83*(18).

Balashov, Y. S. (1972). Bloodsucking ticks (Ixodoidea)-vectors of disease in man and animals. *Misc publ Entomol Soc Am, 8*(5).

Barka, E. A., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Klenk, H.-P., et al. (2016). Taxonomy, physiology, and natural products of Actinobacteria. *Microbiol Mol Biol Rev, 80*(1), 1-43.

Bazzocchi, C., Mariconti, M., Sassera, D., Rinaldi, L., Martin, E., Cringoli, G., et al. (2013). Molecular and serological evidence for the circulation of the tick symbiont Midichloria (Rickettsiales: Midichloriaceae) in different mammalian species. *Parasit Vectors, 6*(1), 350.

Bell-Sakyi, L., Palomar, A. M., & Kazimirova, M. (2015). Isolation and propagation of a Spiroplasma sp. from Slovakian Ixodes ricinus ticks in Ixodes spp. cell lines. *Ticks Tick Borne Dis, 6*(5), 601-606.

Belozerov, V. (1982). Diapause and biological rhythms in ticks. In *Physiology of ticks* (pp. 469-500): Pergamon.

Beninati, T., Lo, N., Sacchi, L., Genchi, C., Noda, H., & Bandi, C. (2004). A novel alpha-Proteobacterium resides in the mitochondria of ovarian cells of the tick Ixodes ricinus. *Appl Environ Microbiol, 70*(5), 2596-2602.

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Series B Stat Methodol, 57*(1), 289-300.

Berg, G., Rybakova, D., Fischer, D., Cernava, T., Vergès, M.-C. C., Charles, T., et al. (2020). Microbiome definition re-visited: old concepts and new challenges. *Microbiome, 8*(1), 1-22.

Beugnet, F., & Marie, J. L. (2009). Emerging arthropod-borne diseases of companion animals in Europe. *Vet Parasitol, 163*(4), 298-305.

Bhide, M. R., Travnicek, M., Levkutova, M., Curlik, J., Revajova, V., & Levkut, M. (2005). Sensitivity of Borrelia genospecies to serum complement from different animals and human: a host—pathogen relationship. *FEMS Immunology & Medical Microbiology, 43*(2), 165-172.

Billingsley, P. F., & Rudin, W. (1992). The role of the mosquito peritrophic membrane in bloodmeal digestion and infectivity of Plasmodium species. *J Parasitol, 78*(3), 430-440.

Binetruy, F., Bailly, X., Chevillon, C., Martin, O. Y., Bernasconi, M. V., & Duron, O. (2019). Phylogenetics of the Spiroplasma ixodetis endosymbiont reveals past transfers between ticks and other arthropods. *Ticks Tick Borne Dis, 10*(3), 575-584.

Bogaert, D., Keijser, B., Huse, S., Rossen, J., Veenhoven, R., Van Gils, E., et al. (2011). Variability and diversity of nasopharyngeal microbiota in children: a metagenomic analysis. *PLoS One, 6*(2), e17035.

Bogdziewicz, M., Zwolak, R., & Crone, E. E. (2016). How do vertebrates respond to mast seeding? *Oikos, 125*(3), 300-307.

Bohacsova, M., Mediannikov, O., Kazimirova, M., Raoult, D., & Sekeyova, Z. (2016). Arsenophonus nasoniae and Rickettsiae Infection of Ixodes ricinus Due to Parasitic Wasp Ixodiphagus hookeri. *PLoS One, 11*(2), e0149950.

Bonnet, S. I., Binetruy, F., Hernández-Jarguín, A. M., & Duron, O. (2017). The tick microbiome: why non-pathogenic microorganisms matter in tick biology and pathogen transmission. *Front Cell Infect Microbiol, 7*, 236.

Boretti, F. S., Perreten, A., Meli, M. L., Cattori, V., Willi, B., Wengi, N., et al. (2009). Molecular investigations of Rickettsia helvetica infection in dogs, foxes, humans, and Ixodes ticks. *Appl Environ Microbiol, 75*(10), 3230--3237.

Bouchard, C., Dibernardo, A., Koffi, J., Wood, H., Leighton, P. A., & Lindsay, L. R. (2019). Increased risk of tick-borne diseases with climate and environmental changes. *Can Commun Dis Rep, 45*(4), 83-89.

Bowman, A. S., & Sauer, J. (2004). Tick salivary glands: function, physiology and future. *Parasitology, 129*(S1), S67.

Bown, K. J., Lambin, X., Telford, G. R., Ogden, N. H., Telfer, S., Woldehiwet, Z., et al. (2008). Relative importance of Ixodes ricinus and Ixodes trianguliceps as vectors for Anaplasma phagocytophilum and Babesia microti in field vole (Microtus agrestis) populations. *Appl Environ Microbiol, 74*(23), 7118-7125.

Breuner, N. E., Hojgaard, A., & Eisen, L. (2018). Lack of evidence for transovarial transmission of

the Lyme disease spirochete Borrelia mayonii by infected female Ixodes scapularis (Acari: Ixodidae) ticks. *J Med Entomol, 55*(3), 739-741.

Briegel, H., & Lea, A. O. (1975). Relationship between protein and proteolytic activity in the midgut of mosquitoes. *J Insect Physiol, 21*(9), 1597-1604.

Bruinderink, G. G., & Broekhuizen, S. (2016). Edelhert: Cervus elaphus. In *Atlas van de Nederlandse zoogdieren: Natuur van Nederland 12* (pp. 296-299): Naturalis Biodiversity Center.

Budachetri, K., Kumar, D., Crispell, G., Beck, C., Dasch, G., & Karim, S. (2018). The tick endosymbiont Candidatus Midichloria mitochondrii and selenoproteins are essential for the growth of Rickettsia parkeri in the Gulf Coast tick vector. *Microbiome, 6*(1), 141.

Bukin, Y. S., Galachyants, Y. P., Morozov, I., Bukin, S., Zakharenko, A., & Zemskaya, T. (2019). The effect of 16S rRNA region choice on bacterial community metabarcoding results. *Sci Data, 6*, 190007.

Burgdorfer, W., Aeschlimann, A., Peter, O., Hayes, S. F., & Philip, R. N. (1979). Ixodes ricinus: vector of a hitherto undescribed spotted fever group agent in Switzerland. *Acta Trop, 36*(4), 357-367.

Burgdorfer, W., Hayes, S., Thomas, L., & Lancaster Jr, J. (1981). New spotted fever group Rickettsia from the lone star tick, Amblyomma americanum. *Rickettsiae and rickettsial diseases/edited by W. Burgdorfer; RL Anacker*.

Burri, C., Dupasquier, C., Bastic, V., & Gern, L. (2011). Pathogens of emerging tick-borne diseases, Anaplasma phagocytophilum, Rickettsia spp., and Babesia spp., in ixodes ticks collected from rodents at four sites in Switzerland (Canton of Bern). *Vector Borne Zoonotic Dis, 11*(7), 939-944.

Burri, C., Schumann, O., Schumann, C., & Gern, L. (2014). Are Apodemus spp. mice and Myodes glareolus reservoirs for Borrelia miyamotoi, Candidatus Neoehrlichia mikurensis, Rickettsia helvetica, R. monacensis and Anaplasma phagocytophilum? *Ticks Tick Borne Dis, 5*(3), 245-251.

Buysse, M., Plantard, O., McCoy, K. D., Duron, O., & Menard, C. (2019). Tissue localization of Coxiella-like endosymbionts in three European tick species through fluorescence in situ hybridization. *Ticks Tick Borne Dis, 10*(4), 798-804.

Cafiso, A., Sassera, D., Romeo, C., Serra, V., Hervet, C., Bandi, C., et al. (2019). Midichloria mitochondrii, endosymbiont of Ixodes ricinus: evidence for the transmission to the vertebrate host during the tick blood meal. *Ticks Tick Borne Dis, 10*(1), 5-12.

Carpi, G., Cagnacci, F., Wittekindt, N. E., Zhao, F., Qi, J., Tomsho, L. P., et al. (2011). Metagenomic profile of the bacterial communities associated with Ixodes ricinus ticks. *PLoS One, 6*(10), e25604.

Cayol, C., Jaaskelainen, A., Koskela, E., Kyrolainen, S., Mappes, T., Siukkola, A., et al. (2018). Sympatric Ixodes-tick species: pattern of distribution and pathogen transmission within wild rodent populations. *Sci Rep, 8*(1), 16660.

Chen, G., Severo, M. S., Sohail, M., Sakhon, O. S., Wikel, S. K., Kotsyfakis, M., et al. (2012). Ixodes scapularis saliva mitigates inflammatory cytokine secretion during Anaplasma phagocytophilum stimulation of immune cells. *Parasit Vectors, 5*, 229.

Cirimotich, C. M., Ramirez, J. L., & Dimopoulos, G. (2011). Native microbiota shape insect vector competence for human pathogens. *Cell Host Microbe, 10*(4), 307-310.

Cisak, E., Wójcik-Fatla, A., Zajac, V., Sawczyn, A., Sroka, J., & Dutkiewicz, J. (2015). Spiroplasma-an emerging arthropod-borne pathogen? *Ann Agric Environ Med, 22*(4).

Clay, K., Klyachko, O., Grindle, N., Civitello, D., Oleske, D., & Fuqua, C. (2008). Microbial

communities and interactions in the lone star tick, Amblyomma americanum. *Mol Ecol, 17*(19), 4371-4381.

Clotfelter, E. D., Pedersen, A. B., Cranford, J. A., Ram, N., Snajdr, E. A., Nolan, V., Jr., et al. (2007). Acorn mast drives long-term dynamics of rodent and songbird populations. *Oecologia, 154*(3), 493-503.

Coipan, C. E., van Duijvendijk, G. L. A., Hofmeester, T. R., Takumi, K., & Sprong, H. (2018). The genetic diversity of Borrelia afzelii is not maintained by the diversity of the rodent hosts. *Parasit Vectors, 11*(1), 454.

Coipan, E. C., Fonville, M., Tijsse-Klasen, E., van der Giessen, J. W., Takken, W., Sprong, H., et al. (2013a). Geodemographic analysis of Borrelia burgdorferi sensu lato using the 5S-23S rDNA spacer region. *Infect Genet Evol, 17*, 216-222.

Coipan, E. C., Jahfari, S., Fonville, M., Maassen, C. B., van der Giessen, J., Takken, W., et al. (2013b). Spatiotemporal dynamics of emerging pathogens in questing Ixodes ricinus. *Front Cell Infect Microbiol, 3*, 36.

Collatz, J., Fuhrmann, A., Selzer, P., Oehme, R. M., Hartelt, K., Kimmig, P., et al. (2010). Being a parasitoid of parasites: host finding in the tick wasp Ixodiphagus hookeri by odours from mammals. *Entomologia experimentalis et applicata, 134*(2), 131-137.

Collatz, J., Selzer, P., Fuhrmann, A., Oehme, R., Mackenstedt, U., Kahl, O., et al. (2011). A hidden beneficial: biology of the tick-wasp Ixodiphagus hookeri in Germany. *J Appl Entomol, 135*(5), 351-358.

Cook, P. E., & McGraw, E. A. (2010). Wolbachia pipientis: an expanding bag of tricks to explore for disease control. *Trends Parasitol, 26*(8), 373-375.

Courtney, J. W., Kostelnik, L. M., Zeidner, N. S., & Massung, R. F. (2004). Multiplex real-time PCR for detection of anaplasma phagocytophilum and Borrelia burgdorferi. *J Clin Microbiol, 42*(7), 3164-3168.

De Boer, R., Hovius, K., Nohlmans, M., & Gray, J. (1993). The woodmouse (Apodemus sylvaticus) as a reservoir of tick-transmitted spirochetes (Borrelia burgdorferi) in The Netherlands. *Zentralbl Bakteriol, 279*(3), 404-416.

de Bruin, A., van Leeuwen, A. D., Jahfari, S., Takken, W., Foldvari, M., Dremmel, L., et al. (2015). Vertical transmission of Bartonella schoenbuchensis in Lipoptena cervi. *Parasit Vectors, 8*(1), 176.

de la Fuente, J., Antunes, S., Bonnet, S., Cabezas-Cruz, A., Domingos, A. G., Estrada-Pena, A., et al. (2017). Tick-pathogen interactions and vector competence: identification of molecular drivers for tick-borne diseases. *Front Cell Infect Microbiol, 7*, 114.

de la Fuente, J., Estrada-Pena, A., Venzal, J. M., Kocan, K. M., & Sonenshine, D. E. (2008). Overview: Ticks as vectors of pathogens that cause disease in humans and animals. *Front Biosci, 13*(13), 6938-6946.

de la Fuente, J., Torina, A., Caracappa, S., Tumino, G., Furla, R., Almazan, C., et al. (2005). Serologic and molecular characterization of Anaplasma species infection in farm animals and ticks from Sicily. *Vet Parasitol, 133*(4), 357-362.

Degnan, P. H., Yu, Y., Sisneros, N., Wing, R. A., & Moran, N. A. (2009). Hamiltonella defensa, genome evolution of protective bacterial endosymbiont from pathogenic ancestors. *Proc Natl Acad Sci U S A, 106*(22), 9063-9068.

Demas, F. A., Hassanali, A., Mwangi, E. N., Kunjeku, E. C., & Mabveni, A. R. (2000). Cattle and Amblyomma variegatum odors used in host habitat and host finding by the tick parasitoid, Ixodiphagus hookeri. *Journal of chemical ecology, 26*(4), 1079-1093.

Dessauer, H. C. (1970). Blood chemistry of reptiles: physiological and evolutionary aspects. *Biol Reptil, 3*(1), 1-72.

Dizij, A., & Kurtenbach, K. (1995). Clethrionomys glareolus, but not Apodemus flavicollis, acquires resistance to Ixodes ricinus L., the main European vector of Borrelia burgdorferi. *Parasite Immunol, 17*(4), 177-183.

Dong, Y., Manfredini, F., & Dimopoulos, G. (2009). Implication of the mosquito midgut microbiota in the defense against malaria parasites. *PLoS Pathog, 5*(5), e1000423.

Duan, D.-Y., Liu, G.-H., & Cheng, T.-Y. (2020). Microbiome analysis of the saliva and midgut from partially or fully engorged female adult Dermacentor silvarum ticks in China. *Exp Appl Acarol, 80*(4), 543-558.

Duron, O., Binetruy, F., Noel, V., Cremaschi, J., McCoy, K. D., Arnathau, C., et al. (2017). Evolutionary changes in symbiont community structure in ticks. *Mol Ecol, 26*(11), 2905-2921.

Duron, O., Morel, O., Noel, V., Buysse, M., Binetruy, F., Lancelot, R., et al. (2018). Tick-bacteria mutualism depends on B vitamin synthesis pathways. *Curr Biol, 28*(12), 1896-1902.

Durvasula, R. V., Gumbs, A., Panackal, A., Kruglov, O., Aksoy, S., Merrifield, R. B., et al. (1997). Prevention of insect-borne disease: an approach using transgenic symbiotic bacteria. *Proc Natl Acad Sci U S A, 94*(7), 3274-3278.

Dusbabek, F. (1996). Nymphal sexual dimorphism in the sheep tick Ixodes ricinus (Acari: Ixodidae). *Folia Parasitol (Praha), 43*(1), 75-79.

Ehrmann, S., Ruyts, S. C., Scherer-Lorenzen, M., Bauhus, J., Brunet, J., Cousins, S. A. O., et al. (2018). Habitat properties are key drivers of Borrelia burgdorferi (s.l.) prevalence in Ixodes ricinus populations of deciduous forest fragments. *Parasit Vectors, 11*(1), 23.

Eichler, S., & Schaub, G. (2002). Development of symbionts in triatomine bugs and the effects of infections with trypanosomatids. *Exp Parasitol, 100*(1), 17-27.

Eisen, L., Breuner, N. E., Hojgaard, A., Hoxmeier, J. C., Pilgard, M. A., Replogle, A. J., et al. (2017). Comparison of Vector Efficiency of Ixodes scapularis (Acari: Ixodidae) From the Northeast and Upper Midwest of the United States for the Lyme Disease Spirochete Borrelia mayonii. *J Med Entomol, 54*(1), 239-242.

Eisen, L., & Dolan, M. C. (2016). Evidence for Personal Protective Measures to Reduce Human Contact With Blacklegged Ticks and for Environmentally Based Control Methods to Suppress Host-Seeking Blacklegged Ticks and Reduce Infection with Lyme Disease Spirochetes in Tick Vectors and Rodent Reservoirs. *J Med Entomol*.

Eisen, R. J., & Eisen, L. (2018). The Blacklegged Tick, Ixodes scapularis: An Increasing Public Health Concern. *Trends Parasitol, 34*(4), 295-309.

Eisenhofer, R., Minich, J. J., Marotz, C., Cooper, A., Knight, R., & Weyrich, L. S. (2019). Contamination in low microbial biomass microbiome studies: issues and recommendations. *Trends Microbiol, 27*(2), 105-117.

Engel, P., & Moran, N. A. (2013). The gut microbiota of insects–diversity in structure and function. *FEMS Microbiol Ecol, 37*(5), 699-735.

Engelstdter, J., & Hurst, G. D. D. (2009). The ecology and evolution of microbes that manipulate host reproduction. *Annu Rev Ecol Evol Syst, 40*, 127-149.

Epis, S., Mandrioli, M., Genchi, M., Montagna, M., Sacchi, L., Pistone, D., et al. (2013). Localization of the bacterial symbiont Candidatus Midichloria mitochondrii within the hard tick Ixodes ricinus by whole-mount FISH staining. *Ticks Tick Borne Dis, 4*(1-2), 39-45.

Excoffier, L., & Lischer, H. E. (2010). Arlequin suite ver 3.5: a new series of programs to perform

population genetics analyses under Linux and Windows. *Mol Ecol Resour, 10*(3), 564-567.
Feldhaar, H. (2011). Bacterial symbionts as mediators of ecologically important traits of insect hosts. *Ecol Entomol, 36*(5), 533-543.
Foster, E., Fleshman, A. C., Ford, S. L., Levin, M. L., Delorey, M. J., Eisen, R. J., et al. (2020). Preliminary evaluation of human personal protective measures against the nymphal stage of the Asian longhorned tick (Acari: Ixodidae). *Journal of medical entomology*.
Fournier, P. E., Grunnenberger, F., Jaulhac, B., Gastinger, G., & Raoult, D. (2000). Evidence of Rickettsia helvetica infection in humans, eastern France. *Emerg Infect Dis, 6*(4), 389-392.
Fox, J., & Weisberg, S. (2019). *An R Companion to Applied Regression, Third Edition*: Sage.
Fukatsu, T., Tsuchida, T., Nikoh, N., & Koga, R. (2001). Spiroplasma symbiont of the pea aphid, Acyrthosiphon pisum (Insecta: Homoptera). *Appl Environ Microbiol, 67*(3), 1284-1291.
Gall, C. A., Reif, K. E., Scoles, G. A., Mason, K. L., Mousel, M., Noh, S. M., et al. (2016). The bacterial microbiome of Dermacentor andersoni ticks influences pathogen susceptibility. *ISME J, 10*(8), 1846-1855.
Garcia-Marti, I., Zurita-Milla, R., Harms, M. G., & Swart, A. (2018). Using volunteered observations to map human exposure to ticks. *Sci Rep, 8*(1), 15435.
Garcia-Vozmediano, A., Krawczyk, A. I., Sprong, H., Rossi, L., Ramassa, E., & Tomassone, L. (2020). Ticks climb the mountains: ixodid tick infestation and infection by tick-borne pathogens in the Western Alps. *Ticks Tick Borne Dis, 11*(5), 101489.
Gassner, F., Takken, W., Lombaers-van der Plas, C., Kastelein, P., Hoetmer, A. J., Holdinga, M., et al. (2013). Rodent species as natural reservoirs of Borrelia burgdorferi sensu lato in different habitats of Ixodes ricinus in The Netherlands. *Ticks Tick Borne Dis, 4*(5), 452-458.
Gassner, F., van Vliet, A. J., Burgers, S. L., Jacobs, F., Verbaarschot, P., Hovius, E. K., et al. (2011). Geographic and temporal variations in population dynamics of Ixodes ricinus and associated Borrelia infections in The Netherlands. *Vector Borne Zoonotic Dis, 11*(5), 523-532.
Gavotte, L., Mercer, D. R., Stoeckle, J. J., & Dobson, S. L. (2010). Costs and benefits of Wolbachia infection in immature Aedes albopictus depend upon sex and competition level. *J Invertebr Pathol, 105*(3), 341-346.
Gern, L. (2008). Borrelia burgdorferi sensu lato, the agent of lyme borreliosis: life in the wilds. *Parasite, 15*(3), 244-247.
Gern, L., Estrada-Pena, A., Frandsen, F., Gray, J. S., Jaenson, T. G., Jongejan, F., et al. (1998). European reservoir hosts of Borrelia burgdorferi sensu lato. *Zentralbl Bakteriol, 287*(3), 196-204.
Gern, L., & Rais, O. (1996). Efficient transmission of Borrelia burgdorferi between cofeeding Ixodes ricinus ticks (Acari: Ixodidae). *J Med Entomol, 33*(1), 189-192.
Gilbert, L., Aungier, J., & Tomkins, J. L. (2014). Climate of origin affects tick (Ixodes ricinus) host-seeking behavior in response to temperature: implications for resilience to climate change? *Ecol Evol, 4*(7), 1186-1198.
Gilbert, L., Maffey, G. L., Ramsay, S. L., & Hester, A. J. (2012). The effect of deer management on the abundance of Ixodes ricinus in Scotland. *Ecol Appl, 22*(2), 658-667.
Glass, G. E., Amerasinghe, F. P., Morgan, J. M., 3rd, & Scott, T. W. (1994). Predicting Ixodes scapularis abundance on white-tailed deer using geographic information systems. *Am J Trop Med Hyg, 51*(5), 538-544.
Glass, G. E., Schwartz, B. S., Morgan, J. M., 3rd, Johnson, D. T., Noy, P. M., & Israel, E. (1995). Environmental risk factors for Lyme disease identified with geographic information systems. *Am J Public Health, 85*(7), 944-948.

Gofton, A. W., Oskam, C. L., Lo, N., Beninati, T., Wei, H., McCarl, V., et al. (2015). Inhibition of the endosymbiont "Candidatus Midichloria mitochondrii" during 16S rRNA gene profiling reveals potential pathogens in Ixodes ticks from Australia. *Parasit Vectors, 8*(1), 345.

Golub, G. H., & Van Loan, C. F. (1996). The singular value decomposition and unitary matrices. *Matrix Comp*, 70-71.

Gommers, L. M., Ederveen, T. H., van der Wijst, J., Overmars-Bos, C., Kortman, G. A., Boekhorst, J., et al. (2019). Low gut microbiota diversity and dietary magnesium intake are associated with the development of PPI-induced hypomagnesemia. *The FASEB Journal, 33*(10), 11235-11246.

Goto, S., Anbutsu, H., & Fukatsu, T. (2006). Asymmetrical interactions between Wolbachia and Spiroplasma endosymbionts coexisting in the same insect host. *Appl Environ Microbiol, 72*(7), 4805-4810.

Gottlieb, Y., Lalzar, I., & Klasson, L. (2015). Distinctive genome reduction rates revealed by genomic analyses of two Coxiella-like endosymbionts in ticks. *Genome Biol Evol, 7*(6), 1779-1796.

Grandjean, O., & Aeschlimann, A. (1973). Contribution to the study of digestion in ticks: histology and fine structure of the midgut ephithelium of Ornithodorus moubata, Murray (Ixodoidea, Argasidae). *Acta Trop, 30*(4), 193-212.

Gray, J. (1998a). Review the ecology of ticks transmitting Lyme borreliosis. *Exp Appl Acarol, 22*(5), 249-258.

Gray, J. S. (1987). Mating and behavioural diapause in Ixodes ricinus L. *Exp Appl Acarol, 3*(1), 61-71.

Gray, J. S., Kahl, O., Robertson, J. N., Daniel, M., Estrada-Pena, A., Gettinby, G., et al. (1998b). Lyme borreliosis habitat assessment. *Zentralbl Bakteriol, 287*(3), 211-228.

Greay, T. L., Gofton, A. W., Paparini, A., Ryan, U. M., Oskam, C. L., & Irwin, P. J. (2018). Recent insights into the tick microbiome gained through next-generation sequencing. *Parasit Vectors, 11*(1), 12.

Guizzo, M. G., Neupane, S., Kucera, M., Perner, J., Frantová, H., da Silva Vaz, I. J., et al. (2020). Poor unstable midgut microbiome of hard ticks contrasts with abundant and stable monospecific microbiome in ovaries. *Front Cell Infect Microbiol, 10*, 211.

Guizzo, M. G., Parizi, L. F., Nunes, R. D., Schama, R., Albano, R. M., Tirloni, L., et al. (2017). A Coxiella mutualist symbiont is essential to the development of Rhipicephalus microplus. *Sci Rep, 7*(1), 1-10.

Gundel, P. E., Rudgers, J., & Ghersa, C. M. (2011). Incorporating the process of vertical transmission into understanding of host–symbiont dynamics. *Oikos, 120*(8), 1121-1128.

Hammer, T. J., Janzen, D. H., Hallwachs, W., Jaffe, S. P., & Fierer, N. (2017). Caterpillars lack a resident gut microbiome. *Proc Natl Acad Sci U S A, 114*(36), 9641-9646.

Hammer, T. J., Sanders, J. G., & Fierer, N. (2019). Not all animals need a microbiome. *FEMS Microbiol Ecol, 366*(10), fnz117.

Hamšíková, Z., Silaghi, C., Takumi, K., Rudolf, I., Gunár, K., Sprong, H., et al. (2019). Presence of roe deer affects the occurrence of Anaplasma phagocytophilum ecotypes in questing Ixodes ricinus in different habitat types of Central Europe. *International journal of environmental research and public health, 16*(23), 4725.

Hanincova, K., Schafer, S. M., Etti, S., Sewell, H. S., Taragelova, V., Ziak, D., et al. (2003a). Association of Borrelia afzelii with rodents in Europe. *Parasitology, 126*(Pt 1), 11-20.

Hanincova, K., Taragelova, V., Koci, J., Schafer, S. M., Hails, R., Ullmann, A. J., et al. (2003b).

Association of Borrelia garinii and B. valaisiana with songbirds in Slovakia. *Appl Environ Microbiol, 69*(5), 2825-2830.

Hartemink, N., van Vliet, A., Sprong, H., Jacobs, F., Garcia-Martí, I., Zurita-Milla, R., et al. (2019). Temporal-spatial variation in questing tick activity in the Netherlands: The effect of climatic and habitat factors. *Vector Borne Zoonotic Dis, 19*(7), 494-505.

Hartemink, N. A., Randolph, S. E., Davis, S. A., & Heesterbeek, J. A. (2008). The basic reproduction number for complex disease systems: defining R(0) for tick-borne infections. *Am Nat, 171*(6), 743-754.

Hawlena, H., Rynkiewicz, E., Toh, E., Alfred, A., Durden, L. A., Hastriter, M. W., et al. (2013). The arthropod, but not the vertebrate host or its environment, dictates bacterial community composition of fleas and ticks. *ISME J, 7*(1), 221.

Hayes, B. M., Radkov, A. D., Yarza, F., Flores, S., Kim, J. R., Zhao, Z., et al. (2020). Immune factor of bacterial origin protects ticks against host microbial commensals. *bioRxiv*.

Henning, K., Greiner-Fischer, S., Hotzel, H., Ebsen, M., & Theegarten, D. (2006). Isolation of Spiroplasma sp. from an Ixodes tick. *Int J Med Microbiol, 296*, 157-161.

Henry, L., & Wickham, H. (2019). purrr: Functional Programming Tools (2019). *R package version 0.2, 3*.

Herren, J. K., Mbaisi, L., Mararo, E., Makhulu, E. E., Mobegi, V. A., Butungi, H., et al. (2020). A microsporidian impairs Plasmodium falciparum transmission in Anopheles arabiensis mosquitoes. *Nat Commun, 11*(1), 1-10.

Hersh, M. H., Tibbetts, M., Strauss, M., Ostfeld, R. S., & Keesing, F. (2012). Reservoir competence of wildlife host species for Babesia microti. *Emerg Infect Dis, 18*(12), 1951-1957.

Heylen, D., Adriaensen, F., Van Dongen, S., Sprong, H., & Matthysen, E. (2013a). Ecological factors that determine Ixodes ricinus tick burdens in the great tit (Parus major), an avian reservoir of Borrelia burgdorferi s.l. *Int J Parasitol, 43*(8), 603-611.

Heylen, D., Fonville, M., van Leeuwen, A. D., & Sprong, H. (2016). Co-infections and transmission dynamics in a tick-borne bacterium community exposed to songbirds. *Environ Microbiol, 18*(3), 988-996.

Heylen, D., Tijsse, E., Fonville, M., Matthysen, E., & Sprong, H. (2013b). Transmission dynamics of Borrelia burgdorferi s.l. in a bird tick community. *Environ Microbiol, 15*(2), 663-673.

Heyman, P., Cochez, C., Hofhuis, A., van der Giessen, J., Sprong, H., Porter, S. R., et al. (2010). A clear and present danger: tick-borne diseases in Europe. *Expert Rev Anti Infect Ther, 8*(1), 33-50.

Hillyard, P. D. (1996). *Ticks of north-west Europe*: Field Studies Council.

Hofhuis, A., van de Kassteele, J., Sprong, H., van den Wijngaard, C. C., Harms, M. G., Fonville, M., et al. (2017). Predicting the risk of Lyme borreliosis after a tick bite, using a structural equation model. *PLoS One, 12*(7), e0181807.

Hofmeester, T., Coipan, E., Van Wieren, S., Prins, H., Takken, W., & Sprong, H. (2016a). Few vertebrate species dominate the Borrelia burgdorferi sl life cycle. *Environ Res Lett, 11*(4), 043001.

Hofmeester, T. R., Jansen, P. A., Wijnen, H. J., Coipan, E. C., Fonville, M., Prins, H. H. T., et al. (2017a). Cascading effects of predator activity on tick-borne disease risk. *Proc Biol Sci, 284*(1859), 20170453.

Hofmeester, T. R., Sprong, H., Jansen, P. A., Prins, H. H. T., & van Wieren, S. E. (2017b). Deer presence rather than abundance determines the population density of the sheep tick, Ixodes ricinus, in Dutch forests. *Parasit Vectors, 10*(1), 433.

Hofmeester, T. R., van der Lei, P. B., Docters van Leeuwen, A., Sprong, H., & van Wieren, S. E. (2016b). New foci of Haemaphysalis punctata and Dermacentor reticulatus in the Netherlands. *Ticks Tick Borne Dis, 7*(2), 367-370.

Hoornstra, D., Koetsveld, J., Sprong, H., Platonov, A. E., & Hovius, J. W. (2018). Borrelia miyamotoi disease in an immunocompetent patient, Western Europe. *Emerg Infect Dis, 24*(9), 1770.

Hopkins, S. R., Wojdak, J. M., & Belden, L. K. (2017). Defensive symbionts mediate host–parasite interactions at multiple scales. *Trends Parasitol, 33*(1), 53-64.

Hornok, S., Kovats, D., Csorgo, T., Meli, M. L., Gonczi, E., Hadnagy, Z., et al. (2014). Birds as potential reservoirs of tick-borne pathogens: first evidence of bacteraemia with Rickettsia helvetica. *Parasit Vectors, 7*(1), 128.

Hovius, J. W., de Wever, B., Sohne, M., Brouwer, M. C., Coumou, J., Wagemakers, A., et al. (2013). A case of meningoencephalitis by the relapsing fever spirochaete Borrelia miyamotoi in Europe. *Lancet, 382*(9892), 658.

Hu, C. M., Humair, P. F., Wallich, R., & Gern, L. (1997a). Apodemus sp. rodents, reservoir hosts for Borrelia afzelii in an endemic area in Switzerland. *Zentralbl Bakteriol, 285*(4), 558-564.

Hu, R., Hyland, K., & Oliver, J. (1998a). A review on the use of Ixodiphagus wasps (Hymenoptera: Encyrtidae) as natural enemies for the control of ticks (Acari: Ixodidae). *Syst Appl Microbiol, 3*(1), 19-28.

Hu, R., & Hyland, K. E. (1997b). Prevalence and seasonal activity of the wasp parasitoid, Ixodiphagus hookeri (Hymenoptera: Encyrtidae) in its tick host, Ixodes scapularis (Acari: Ixodidae). *Syst Appl Acarol, 2*(1), 95-100.

Hu, R., & Hyland, K. E. (1998b). Effects of the feeding process of Ixodes scapularis (Acari: Ixodidae) on embryonic development of its parasitoid, Ixodiphagus hookeri (Hymenoptera: Encyrtidae). *J Med Entomol, 35*(6), 1050-1053.

Hu, R., Hyland, K. E., & Mather, T. N. (1993). Occurrence and distribution in Rhode Island of Hunterellus hookeri (Hymenoptera: Encyrtidae), a wasp parasitoid of Ixodes dammini. *J Med Entomol, 30*(1), 277-280.

Humair, P., Rais, O., & Gern, L. (1999). Transmission of Borrelia afzelii from Apodemus mice and Clethrionomys voles to Ixodes ricinus ticks: differential transmission pattern and overwintering maintenance. *Parasitology, 118*(1), 33-42.

Humair, P. F., Postic, D., Wallich, R., & Gern, L. (1998). An avian reservoir (Turdus merula) of the Lyme borreliosis spirochetes. *Zentralbl Bakteriol, 287*(4), 521-538.

Humair, P. F., Turrian, N., Aeschilimann, A., & Gern, L. (1993). Borrelia burgdorferi in a focus of Lyme borreliosis: epizootiologic contribution of small mammals. *Folia Parasitol (Praha), 40*(1), 65-70.

Hunter, D. J., Torkelson, J. L., Bodnar, J., Mortazavi, B., Laurent, T., Deason, J., et al. (2015). The Rickettsia Endosymbiont of Ixodes pacificus Contains All the Genes of De Novo Folate Biosynthesis. *PLoS One, 10*(12), e0144552.

Hurst, G. D., Anbutsu, H., Kutsukake, M., & Fukatsu, T. (2003). Hidden from the host: Spiroplasma bacteria infecting Drosophila do not cause an immune response, but are suppressed by ectopic immune activation: SHORT NOTE. *Insect Mol Biol, 12*(1), 93-97.

Husnik, F. (2018). Host–symbiont–pathogen interactions in blood-feeding parasites: nutrition, immune cross-talk and gene exchange. *Parasitology, 145*(10), 1294-1303.

Jaenson, T. G., Jaenson, D. G., Eisen, L., Petersson, E., & Lindgren, E. (2012). Changes in the geographical distribution and abundance of the tick Ixodes ricinus during the past 30 years in Sweden. *Parasit Vectors, 5*(1), 8.

Jaenson, T. G., Talleklint, L., Lundqvist, L., Olsen, B., Chirico, J., & Mejlon, H. (1994). Geographical distribution, host associations, and vector roles of ticks (Acari: Ixodidae, Argasidae) in Sweden. *J Med Entomol, 31*(2), 240-256.

Jahfari, S., Coipan, E. C., Fonville, M., van Leeuwen, A. D., Hengeveld, P., Heylen, D., et al. (2014). Circulation of four Anaplasma phagocytophilum ecotypes in Europe. *Parasit Vectors, 7*(1), 365.

Jahfari, S., Fonville, M., Hengeveld, P., Reusken, C., Scholte, E. J., Takken, W., et al. (2012). Prevalence of Neoehrlichia mikurensis in ticks and rodents from North-west Europe. *Parasit Vectors, 5*(1), 74.

Jensen, T. S. (1982). Seed production and outbreaks of non-cyclic rodent populations in deciduous forests. *Oecologia, 54*(2), 184-192.

Jones, C. G., Ostfeld, R. S., Richard, M. P., Schauber, E. M., & Wolff, J. O. (1998). Chain reactions linking acorns to gypsy moth outbreaks and Lyme disease risk. *Science, 279*(5353), 1023-1026.

Jore, S., Vanwambeke, S. O., Viljugrein, H., Isaksen, K., Kristoffersen, A. B., Woldehiwet, Z., et al. (2014). Climate and environmental change drives Ixodes ricinus geographical expansion at the northern range margin. *Parasit Vectors, 7*(1), 11.

Jore, S., Viljugrein, H., Hofshagen, M., Brun-Hansen, H., Kristoffersen, A. B., Nygard, K., et al. (2011). Multi-source analysis reveals latitudinal and altitudinal shifts in range of Ixodes ricinus at its northern distribution limit. *Parasit Vectors, 4*(1), 84.

Kallio, E. R., Voutilainen, L., Vapalahti, O., Vaheri, A., Henttonen, H., Koskela, E., et al. (2007). Endemic hantavirus infection impairs the winter survival of its rodent host. *88*(8), 1911-1916.

Karbowiak, G. (2004). Zoonotic reservoir of Babesia microti in Poland. *Pol J Microbiol, 53 Suppl*, 61-65.

Kassambara, A. (2019). ggpubr: ggplot2 Based Publication Ready Plots. In.

Keikha, M. (2018). Williamsia spp. are emerging opportunistic bacteria. *New Microbes New Infect, 21*, 88.

Kikuchi, Y., Tada, A., Musolin, D. L., Hari, N., Hosokawa, T., Fujisaki, K., et al. (2016). Collapse of insect gut symbiosis under simulated climate change. *MBio, 7*(5).

Korn, H. (1986). Changes in home range size during growth and maturation of the wood mouse (Apodemus sylvaticus) and the bank vole (Clethrionomys glareolus). *Oecologia, 68*(4), 623-628.

Krull, C., Böhme, B., Clausen, P.-H., & Nijhof, A. M. (2017). Optimization of an artificial tick feeding assay for Dermacentor reticulatus. *Parasites & Vectors, 10*(1), 60.

Kurtenbach, K., Carey, D., Hoodless, A. N., Nuttall, P. A., & Randolph, S. E. (1998a). Competence of pheasants as reservoirs for Lyme disease spirochetes. *J Med Entomol, 35*(1), 77-81.

Kurtenbach, K., Kampen, H., Dizij, A., Arndt, S., Seitz, H. M., Schaible, U. E., et al. (1995). Infestation of rodents with larval Ixodes ricinus (Acari: Ixodidae) is an important factor in the transmission cycle of Borrelia burgdorferi s.l. in German woodlands. *J Med Entomol, 32*(6), 807-817.

Kurtenbach, K., Peacey, M., Rijpkema, S. G., Hoodless, A. N., Nuttall, P. A., & Randolph, S. E. (1998b). Differential transmission of the genospecies of Borrelia burgdorferi sensu lato by game birds and small rodents in England. *Appl Environ Microbiol, 64*(4), 1169-1174.

Kwan, J. Y., Griggs, R., Chicana, B., Miller, C., & Swei, A. (2017). Vertical vs. horizontal transmission of the microbiome in a key disease vector, Ixodes pacificus. *Mol Ecol, 26*(23), 6578-6589.

Kybicova, K., Kurzova, Z., & Hulinska, D. (2008). Molecular and serological evidence of Borrelia burgdorferi sensu lato in wild rodents in the Czech Republic. *Vector Borne Zoonotic Dis, 8*(5), 645-652.

Lalzar, I., Harrus, S., Mumcuoglu, K. Y., & Gottlieb, Y. J. A. E. M. (2012). Composition and seasonal variation of Rhipicephalus turanicus and Rhipicephalus sanguineus bacterial communities. *78*(12), 4110-4116.

Larrousse, F., King, A. G., & Wolbach, S. (1928). The overwintering in Massachusetts of Ixodiphagus caucurtei. *Science, 67*(1735), 351-353.

Lederberg, J., & McCray, A. T. (2001). Ome SweetOmics--A genealogical treasury of words. *The scientist, 15*(7), 8-8.

Lees, A. D. (1946). The water balance in Ixodes ricinus L. and certain other species of ticks. *Parasitology, 37*(1-2), 1-20.

Leirs, H., Stenseth, N. C., Nichols, J. D., Hines, J. E., Verhagen, R., & Verheyen, W. (1997). Stochastic seasonality and nonlinear density-dependent factors regulate population size in an African rodent. *Nature, 389*(6647), 176.

Lenth, R., Singmann, H., Love, J., Buerkner, P., & Herve, M. (2020). emmeans: estimated marginal means. R package version 1.4. 4. In.

Levi, T., Keesing, F., Oggenfuss, K., & Ostfeld, R. S. (2015). Accelerated phenology of blacklegged ticks under climate warming. *Philos Trans R Soc Lond B Biol Sci, 370*(1665).

Li, S., Gilbert, L., Harrison, P. A., & Rounsevell, M. D. (2016). Modelling the seasonality of Lyme disease risk and the potential impacts of a warming climate within the heterogeneous landscapes of Scotland. *J R Soc Interface, 13*(116).

Lindgren, E., Andersson, Y., Suk, J. E., Sudre, B., & Semenza, J. C. (2012). Monitoring EU emerging infectious disease risk due to climate change. *Science, 336*(6080), 418-419.

Lo, N., Beninati, T., Sassera, D., Bouman, E. A., Santagati, S., Gern, L., et al. (2006). Widespread distribution and high prevalence of an alpha-proteobacterial symbiont in the tick Ixodes ricinus. *Environ Microbiol, 8*(7), 1280-1287.

Lorenz, B., Schroeder, J., & Reischl, U. (2002). First evidence of an endogenous Spiroplasma sp. infection in humans manifesting as unilateral cataract associated with anterior uveitis in a premature baby. *Graefes Arch Clin Exp Ophthalmol, 240*(5), 348-353.

Louviere, J. J., Hensher, D. A., & Swait, J. D. (2000). *Stated choice methods: analysis and applications*: Cambridge university press.

Lüdecke, D. (2019). sjPlot: Data Visualization for Statistics in Social Science. 2.7. 2. In.

Łukasik, P., van Asch, M., Guo, H., Ferrari, J., & Charles J. Godfray, H. (2013). Unrelated facultative endosymbionts protect aphids against a fungal pathogen. *Ecol Lett, 16*(2), 214-218.

Macaluso, K. R., Sonenshine, D. E., Ceraul, S. M., & Azad, A. F. (2002). Rickettsial infection in Dermacentor variabilis (Acari: Ixodidae) inhibits transovarial transmission of a second Rickettsia. *J Med Entomol, 39*(6), 809-813.

Macleod, J. (1936). Studies in Tick-borne Fever of Sheep: II. Experiments on Transmission and Distribution of the Disease. *Parasitology, 28*(3), 320-329.

Mancini, M. V., Spaccapelo, R., Damiani, C., Accoti, A., Tallarita, M., Petraglia, E., et al. (2016). Paratransgenesis to control malaria vectors: a semi-field pilot study. *Parasit Vectors, 9*(1), 1-9.

Mangiafico, S. (2020). rcompanion: Functions to Support Extension Education Program Evaluation. In.

Mangiafico, S., & Mangiafico, M. S. (2017). Package 'rcompanion'. *Cran Repos*, 1-71.

Mannelli, A., Bertolotti, L., Gern, L., & Gray, J. (2012). Ecology of Borrelia burgdorferi sensu lato

in Europe: transmission dynamics in multi-host systems, influence of molecular processes and effects of climate change. *FEMS Microbiol Rev, 36*(4), 837-861.

Mannelli, A., Boggiatto, G., Grego, E., Cinco, M., Murgia, R., Stefanelli, S., et al. (2003). Acarological risk of exposure to agents of tick-borne zoonoses in the first recognized Italian focus of Lyme borreliosis. *Epidemiol Infect, 131*(3), 1139-1147.

Manzano-Marín, A., Oceguera-Figueroa, A., Latorre, A., Jiménez-García, L. F., & Moya, A. (2015). Solving a bloody mess: B-vitamin independent metabolic convergence among gammaproteobacterial obligate endosymbionts from blood-feeding arthropods and the leech Haementeria officinalis. *Genome Biol Evol, 7*(10), 2871-2884.

Marchesi, J. R., & Ravel, J. (2015). The vocabulary of microbiome research: a proposal. In: Springer.

Matei, I. A., Estrada-Peña, A., Cutler, S. J., Vayssier-Taussat, M., Varela-Castro, L., Potkonjak, A., et al. (2019). A review on the eco-epidemiology and clinical management of human granulocytic anaplasmosis and its agent in Europe. *Parasites & Vectors, 12*(1), 1-19.

Mateos-Hernández, L., Obregón, D., Maye, J., Versille, N., de la Fuente, J., Estrada-Peña, A., et al. (2020). Anti-tick microbiota vaccine impacts Ixodes ricinus performance during feeding. *Vaccines, 8*(4), 702.

Matet, A., Le Flèche-Matéos, A., Doz, F., Dureau, P., & Cassoux, N. (2020). Ocular Spiroplasma ixodetis in Newborns, France. *Emerg Infect Dis, 26*(2), 340.

Mather, T. N., Piesman, J., & Spielman, A. (1987). Absence of spirochaetes (Borrelia burgdorferi) and piroplasms (Babesia microti) in deer ticks (Ixodes dammini) parasitized by chalcid wasps (Hunterellus hookeri). *Med Vet Entomol, 1*(1), 3-8.

McShea, W. (2000). The influence of acorn crops on annual variation in rodent and bird populations. *Ecology, 81*(1), 228-238.

Medlock, J. M., Hansford, K. M., Bormane, A., Derdakova, M., Estrada-Pena, A., George, J. C., et al. (2013). Driving forces for changes in geographical distribution of Ixodes ricinus ticks in Europe. *Parasit Vectors, 6*(1), 1.

Mejlon, H., & Jaenson, T. G. (1997). Questing behaviour of Ixodes ricinus ticks (Acari: Ixodidae). *Exp Appl Acarol, 21*(12), 747-754.

Miťková, K., Berthová, L., Kalúz, S., Kazimírová, M., Burdová, L., & Kocianová, E. (2015). First detections of Rickettsia helvetica and R. monacensis in ectoparasitic mites (Laelapidae and Trombiculidae) infesting rodents in south-western Slovakia. *Parasitol Res, 114*(7), 2465-2472.

Montgomery, W. J. T. J. o. A. E. (1989). Population regulation in the wood mouse, Apodemus sylvaticus. II. Density dependence in spatial distribution and reproduction. *J Anim Ecol, 58*(2), 477-494.

Moran, N. A. (2016). When obligate partners melt down. *MBio, 7*(6).

Moutailler, S., Valiente Moro, C., Vaumourin, E., Michelet, L., Tran, F. H., Devillers, E., et al. (2016). Co-infection of ticks: The rule rather than the exception. *PLoS Negl Trop Dis, 10*(3), e0004539.

Mueller, U. G., Mikheyev, A. S., Hong, E., Sen, R., Warren, D. L., Solomon, S. E., et al. (2011). Evolution of cold-tolerant fungal symbionts permits winter fungiculture by leafcutter ants at the northern frontier of a tropical ant–fungus symbiosis. *Proc Natl Acad Sci U S A, 108*(10), 4053-4056.

Muller, K., & Wickham, H. (2019). tibble: Simple Data Frames (R package version 2.1. 3). In.

Musolin, D. L., Tougou, D., & Fujisaki, K. (2010). Too hot to handle? Phenological and life-history responses to simulated climate change of the southern green stink bug Nezara viridula

(Heteroptera: Pentatomidae). *Glob Chang Biol, 16*(1), 73-87.
Mwangi, E. N., Hassan, S. M., Kaaya, G. P., & Essuman, S. (1997). The impact of Ixodiphagus hookeri, a tick parasitoid, on Ambl yomma variegatum (Acari: Ixodidae) in a field trial in Kenya. *Exp Appl Acarol, 21*(2), 117-126.
Mysterud, A., Easterday, W. R., Qviller, L., Viljugrein, H., & Ytrehus, B. (2013). Spatial and seasonal variation in the prevalence of Anaplasma phagocytophilum and Borrelia burgdorferi sensu lato in questing Ixodes ricinus ticks in Norway. *Parasit Vectors, 6*(1), 187.
Mysterud, A., Easterday, W. R., Stigum, V. M., Aas, A. B., Meisingset, E. L., & Viljugrein, H. (2016). Contrasting emergence of Lyme disease across ecosystems. *Nat Commun, 7*(1), 1-11.
Mysterud, A., Stigum, V. M., Jaarsma, R. I., & Sprong, H. (2019). Genospecies of Borrelia burgdorferi sensu lato detected in 16 mammal species and questing ticks from northern Europe. *Sci Rep, 9*(1), 5088.
Narasimhan, S., & Fikrig, E. (2015). Tick microbiome: the force within. *Trends Parasitol, 31*(7), 315-323.
Narasimhan, S., Rajeevan, N., Liu, L., Zhao, Y. O., Heisig, J., Pan, J., et al. (2014). Gut microbiota of the tick vector Ixodes scapularis modulate colonization of the Lyme disease spirochete. *Cell Host Microbe, 15*(1), 58-71.
Narasimhan, S., Schuijt, T. J., Abraham, N. M., Rajeevan, N., Coumou, J., Graham, M., et al. (2017). Modulation of the tick gut milieu by a secreted tick protein favors Borrelia burgdorferi colonization. *Nat Commun, 8*(1), 184.
Needham, G. R., & Teel, P. D. (1991). Off-host physiological ecology of ixodid ticks. *Annu Rev Entomol, 36*(1), 659-681.
Nemirov, K., Vaheri, A., & Pljusnin, A. (2004). Hantaviruses: co-evolution with natural hosts. *Recent Res Devel Viro, 6*, 201-228.
Nijhof, A. M., Bodaan, C., Postigo, M., Nieuwenhuijs, H., Opsteegh, M., Franssen, L., et al. (2007). Ticks and associated pathogens collected from domestic animals in the Netherlands. *Vector Borne Zoonotic Dis, 7*(4), 585-595.
Nilsson, A., & Lundqvist, L. (1978). Host selection and movements of Ixodes ricinus (Acari) larvae on small mammals. *Oikos, 31*(3), 313-322.
Nilsson, K., Elfving, K., & Påhlson, C. (2010). Rickettsia helvetica in patient with meningitis, Sweden, 2006. *Emerg Infect Dis, 16*(3), 490.
Noda, H., Munderloh, U. G., & Kurtti, T. J. (1997). Endosymbionts of ticks and their relationship to Wolbachia spp. and tick-borne pathogens of humans and animals. *Appl Environ Microbiol, 63*(10), 3926-3932.
Noden, B. H., Roselli, M. A., & Loss, S. R. (2020). Rickettsia parkeri and Candidatus Rickettsia andeanae in Amblyomma maculatum Group Ticks. *Emerg Infect Dis, 26*(2), 371.
Obiegala, A., Krol, N., Oltersdorf, C., Nader, J., & Pfeffer, M. (2017). The enzootic life-cycle of Borrelia burgdorferi (sensu lato) and tick-borne rickettsiae: an epidemiological study on wild-living small mammals and their ticks from Saxony, Germany. *Parasit Vectors, 10*(1), 115.
Obiegala, A., Oltersdorf, C., Silaghi, C., Kiefer, D., Kiefer, M., Woll, D., et al. (2016). Rickettsia spp. in small mammals and their parasitizing ectoparasites from Saxony, Germany. *Vet Parasitol Reg, 5*, 19-24.
Øines, Ø., Radzijevskaja, J., Paulauskas, A., & Rosef, O. (2012). Prevalence and diversity of Babesia spp. in questing Ixodes ricinus ticks from Norway. *Parasit Vectors, 5*(1), 156.
Okeyo, M., Hepner, S., Rollins, R. E., Hartberger, C., Straubinger, R. K., Marosevic, D., et al. (2020). Longitudinal study of prevalence and spatio-temporal distribution of Borrelia burgdorferi

sensu lato in ticks from three defined habitats in Latvia, 1999–2010. *Environ Microbiol, 00*, 1999-2010.

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M. H. H., Oksanen, M. J., et al. (2007). The vegan package. *Community ecology package, 10*, 631-637.

Oliveira, J. H. M., Gonçalves, R. L., Lara, F. A., Dias, F. A., Gandara, A. C. P., Menna-Barreto, R. F., et al. (2011). Blood meal-derived heme decreases ROS levels in the midgut of Aedes aegypti and allows proliferation of intestinal microbiota. *PLoS Pathog, 7*(3), e1001320.

Oliver, J. D., Lynn, G. E., Burkhardt, N. Y., Price, L. D., Nelson, C. M., Kurtti, T. J., et al. (2016). Infection of Immature Ixodes scapularis (Acari: Ixodidae) by Membrane Feeding. *J Med Entomol, 53*(2), 409-415.

Oliver, K. M., Campos, J., Moran, N. A., & Hunter, M. S. (2008). Population dynamics of defensive symbionts in aphids. *Proc Royal Soc B, 275*(1632), 293-299.

Oliver, K. M., Degnan, P. H., Burke, G. R., & Moran, N. A. (2010). Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. *Annu Rev Entomol, 55*(1), 247-266.

Oliver, K. M., Moran, N. A., & Hunter, M. S. (2005). Variation in resistance to parasitism in aphids is due to symbionts not host genotype. *Proc Natl Acad Sci U S A, 102*(36), 12795-12800.

Oliver, K. M., Moran, N. A., & Hunter, M. S. (2006). Costs and benefits of a superinfection of facultative symbionts in aphids. *Proc Royal Soc B, 273*(1591), 1273-1280.

Oliver, K. M., Russell, J. A., Moran, N. A., & Hunter, M. S. (2003). Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proceedings of the National Academy of Sciences, 100*(4), 1803-1807.

Olivieri, E., Epis, S., Castelli, M., Boccazzi, I. V., Romeo, C., Desirò, A., et al. (2019). Tissue tropism and metabolic pathways of Midichloria mitochondrii suggest tissue-specific functions in the symbiosis with Ixodes ricinus. *Ticks Tick Borne Dis, 10*(5), 1070-1077.

Ostfeld, R. S., Brisson, D., Oggenfuss, K., Devine, J., Levy, M. Z., & Keesing, F. (2018). Effects of a zoonotic pathogen, Borrelia burgdorferi, on the behavior of a key reservoir host. *Ecol Evol, 8*(8), 4074-4083.

Ostfeld, R. S., Canham, C. D., Oggenfuss, K., Winchcombe, R. J., & Keesing, F. (2006). Climate, deer, rodents, and acorns as determinants of variation in lyme-disease risk. *PLoS Biol, 4*(6), e145.

Ostfeld, R. S., Jones, C. G., & Wolff, J. O. (1996). Of mice and mast. *BioScience, 46*(5), 323-330.

Ostfeld, R. S., Keesing, F., & Evolution. (2000). Pulsed resources and community dynamics of consumers in terrestrial ecosystems. *Trends Ecol Evol, 15*(6), 232-237.

Ostfeld, R. S., Schauber, E. M., Canham, C. D., Keesing, F., Jones, C. G., & Wolff, J. O. (2001). Effects of acorn production and mouse abundance on abundance and Borrelia burgdorferi infection prevalence of nymphal Ixodes scapularis ticks. *Vector Borne Zoonotic Dis, 1*(1), 55-63.

Paddock, C. D., Denison, A. M., Dryden, M. W., Noden, B. H., Lash, R. R., Abdelghani, S. S., et al. (2015). High prevalence of "Candidatus Rickettsia andeanae" and apparent exclusion of Rickettsia parkeri in adult Amblyomma maculatum (Acari: Ixodidae) from Kansas and Oklahoma. *Ticks Tick Borne Dis, 6*(3), 297-302.

Pais, R., Lohs, C., Wu, Y., Wang, J., & Aksoy, S. (2008). The obligate mutualist Wigglesworthia glossinidia influences reproduction, digestion, and immunity processes of its host, the tsetse fly. *Appl Environ Microbiol, 74*(19), 5965-5974.

Panzacchi, M., Linnell, J. D., Melis, C., Odden, M., Odden, J., Gorini, L., et al. (2010). Effect of

land-use on small mammal abundance and diversity in a forest–farmland mosaic landscape in south-eastern Norway. *For Ecol Manag, 259*(8), 1536-1545.
Parola, P. (2004). Tick-borne rickettsial diseases: emerging risks in Europe. *Comp Immunol Microbiol Infect Dis, 27*(5), 297-304.
Parola, P., Beati, L., Cambon, M., & Raoult, D. (1998). First isolation of Rickettsia helvetica from Ixodes ricinus ticks in France. *Eur J Clin Microbiol Infect Dis, 17*(2), 95-100.
Parola, P., & Raoult, D. (2001). Tick-borne bacterial diseases emerging in Europe. *Clin Microbiol Infect, 7*(2), 80-83.
Perlman, S. J., Hunter, M. S., & Zchori-Fein, E. (2006). The emerging diversity of Rickettsia. *Proceedings of the Royal Society B: Biological Sciences, 273*(1598), 2097-2106.
Perret, J. L., Guigoz, E., Rais, O., & Gern, L. (2000). Influence of saturation deficit and temperature on Ixodes ricinus tick questing activity in a Lyme borreliosis-endemic area (Switzerland). *Parasitol Res, 86*(7), 554-557.
Pickard, J. M., Zeng, M. Y., Caruso, R., & Núñez, G. (2017). Gut microbiota: Role in pathogen colonization, immune responses, and inflammatory disease. *Immunol Rev, 279*(1), 70-89.
Piesman, J., & Schneider, B. S. (2002). Dynamic changes in Lyme disease spirochetes during transmission by nymphal ticks. *Exp Appl Acarol, 28*(1-4), 141-145.
Plantard, O., Bouju-Albert, A., Malard, M. A., Hermouet, A., Capron, G., & Verheyden, H. (2012). Detection of Wolbachia in the tick Ixodes ricinus is due to the presence of the hymenoptera endoparasitoid Ixodiphagus hookeri. *PLoS One, 7*(1), e30692.
Pucek, Z., Jędrzejewski, W., Jędrzejewska, B., & Pucek, M. J. A. T. (1993). Rodent population dynamics in a primeval deciduous forest (Białowieża National Park) in relation to weather, seed crop, and predation. *Acta Theriol, 38*(2), 199-232.
R Core Team (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
Ramos, R. A., Campbell, B. E., Whittle, A., Lia, R. P., Montarsi, F., Parisi, A., et al. (2015). Occurrence of Ixodiphagus hookeri (Hymenoptera: Encyrtidae) in Ixodes ricinus (Acari: Ixodidae) in southern Italy. *Ticks Tick Borne Dis, 6*(3), 234-236.
Rand, P. W., Lubelczyk, C., Lavigne, G. R., Elias, S., Holman, M. S., Lacombe, E. H., et al. (2003). Deer density and the abundance of Ixodes scapularis (Acari: Ixodidae). *J Med Entomol, 40*(2), 179-184.
Randolph, S. (1995). Quantifying parameters in the transmission of Babesia microti by the tick Ixodes trianguliceps amongst voles (Clethrionomys glareolus). *Parasitology, 110*(3), 287-295.
Randolph, S. (2002). Quantitative ecology of ticks as a basis for transmission models of tick-borne pathogens. *Vector Borne Zoonotic Dis, 2*(4), 209-215.
Randolph, S., & Šumilo, D. (2007). Tick-borne encephalitis in Europe: dynamics of changing risk. In:Takken Wand Knols BGJ (eds.) Emerging pests and vector-borne diseases in Europe. In (Vol. 1, pp. 187-206). Wageningen Academic Publishers.
Randolph, S. E. (2004). Tick ecology: processes and patterns behind the epidemiological risk posed by ixodid ticks as vectors. *Parasitology, 129 Suppl*, S37-65.
Raulf, M. K., Jordan, D., Fingerle, V., & Strube, C. (2018). Association of Borrelia and Rickettsia spp. and bacterial loads in Ixodes ricinus ticks. *Ticks Tick Borne Dis, 9*(1), 18-24.

Ricci, I., Damiani, C., Rossi, P., Capone, A., Scuppa, P., Cappelli, A., et al. (2011). Mosquito symbioses: from basic research to the paratransgenic control of mosquito-borne diseases. *J Appl Entomol, 135*(7), 487-493.

Rizzoli, A., Hauffe, H., Carpi, G., Vourc, H. G., Neteler, M., & Rosa, R. (2011). Lyme borreliosis in Europe. *Euro Surveill, 16*(27), 19906.

Robertson, J. N., Gray, J. S., & Stewart, P. (2000). Tick bite and Lyme borreliosis risk at a recreational site in England. *Eur J Epidemiol, 16*(7), 647-652.

Robinson, D., & Hayes, A. (2020). broom: Convert Statistical Analysis Objects into Tidy Tibbles. In.

Rochlin, I., & Toledo, A. (2020). Emerging tick-borne pathogens of public health importance: a mini-review. *J Med Microbiol, 69*(6), 781-791.

Rosef, O., Paulauskas, A., & Radzijevskaja, J. (2009). Prevalence of Borrelia burgdorferi sensu lato and Anaplasma phagocytophilum in questing Ixodes ricinus ticks in relation to the density of wild cervids. *Acta Vet Scand, 51*, 47.

Rosenberg, E., Sharon, G., Atad, I., & Zilber-Rosenberg, I. (2010). The evolution of animals and plants via symbiosis with microorganisms. *Environ Microbiol Rep, 2*(4), 500-506.

Ross, B. D., Hayes, B., Radey, M. C., Lee, X., Josek, T., Bjork, J., et al. (2018). Ixodes scapularis does not harbor a stable midgut microbiome. *ISME J, 12*(11), 2596-2607.

RStudio Team (2015). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA.

RStudio Team (2019). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA.

RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA.

Rudzinska, M. A., Spielman, A., Riek, R. F., Lewengrub, S. J., & Plesman, J. (1979). Intraerythrocytic'gametocytes' of Babesia microti and their maturation in ticks. *Can J Zool, 57*(2), 424-434.

Ryu, J.-H., Kim, S.-H., Lee, H.-Y., Bai, J. Y., Nam, Y.-D., Bae, J.-W., et al. (2008). Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in Drosophila. *Science, 319*(5864), 777-782.

Sacchi, L., Bigliardi, E., Corona, S., Beninati, T., Lo, N., & Franceschi, A. (2004). A symbiont of the tick Ixodes ricinus invades and consumes mitochondria in a mode similar to that of the parasitic bacterium Bdellovibrio bacteriovorus. *Tissue Cell, 36*(1), 43-53.

Sakai, R. K., Costa, F. B., Ueno, T. E., Ramirez, D. G., Soares, J. F., Fonseca, A. H., et al. (2014). Experimental infection with Rickettsia rickettsii in an Amblyomma dubitatum tick colony, naturally infected by Rickettsia bellii. *Ticks Tick Borne Dis, 5*(6), 917-923.

Sanada-Morimura, S., Matsumura, M., & Noda, H. (2013). Male killing caused by a Spiroplasma symbiont in the small brown planthopper, Laodelphax striatellus. *J Hered, 104*(6), 821-829.

Sassera, D., Beninati, T., Bandi, C., Bouman, E. A., Sacchi, L., Fabbi, M., et al. (2006). 'Candidatus Midichloria mitochondrii', an endosymbiont of the tick Ixodes ricinus with a unique intramitochondrial lifestyle. *Int J Syst Evol Microbiol, 56*(Pt 11), 2535-2540.

Sassera, D., Lo, N., Bouman, E. A., Epis, S., Mortarino, M., & Bandi, C. (2008). "Candidatus Midichloria" endosymbionts bloom after the blood meal of the host, the hard tick Ixodes ricinus. *Appl Environ Microbiol, 74*(19), 6138-6140.

Scarborough, C. L., Ferrari, J., & Godfray, H. (2005). Aphid protected from pathogen by endosymbiont. *Science, 310*(5755), 1781-1781.

Schauber, E. M., Ostfeld, R. S., & Evans, J., Andrew S. (2005). What is the best predictor of annual Lyme disease incidence: weather, mice, or acorns? *Ecol Appl, 15*(2), 575-586.

Schnabel, Z. E. (1938). The estimation of the total fish population of a lake. *Am Math Mon, 45*(6),

348-352.
Schwartz, A. M., Hinckley, A. F., Mead, P. S., Hook, S. A., & Kugeler, K. J. (2017). Surveillance for Lyme Disease - United States, 2008-2015. *MMWR Surveill Summ, 66*(22), 1-12.
Scoles, G. A. (2004). Phylogenetic analysis of the Francisella-like endosymbionts of Dermacentor ticks. *J Med Entomol, 41*(3), 277-286.
Scoles, G. A., Papero, M., Beati, L., & Fish, D. (2001). A relapsing fever group spirochete transmitted by Ixodes scapularis ticks. *Vector Borne Zoonotic Dis, 1*(1), 21-34.
Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., et al. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res, 13*(11), 2498-2504.
Sheldon, A. L. (1969). Equitability indices: dependence on the species count. *Ecology, 50*(3), 466-467.
Silaghi, C., Beck, R., Oteo, J. A., Pfeffer, M., & Sprong, H. (2016). Neoehrlichiosis: an emerging tick-borne zoonosis caused by Candidatus Neoehrlichia mikurensis. *Exp Appl Acarol, 68*(3), 279-297.
Smith, C. N., & Cole, M. M. (1943). Studies of parasites of the American dog tick. *Journal of Economic Entomology, 36*(4).
Smith, T. A., Driscoll, T., Gillespie, J. J., & Raghavan, R. (2015). A Coxiella-like endosymbiont is a potential vitamin source for the Lone Star tick. *Genome Biol, 7*(3), 831-838.
Sojka, D., Franta, Z., Horn, M., Caffrey, C. R., Mareš, M., & Kopáček, P. (2013). New insights into the machinery of blood digestion by ticks. *Trends Parasitol, 29*(6), 276-285.
Sonenshine, D. E., & Roe, R. M. (2014). External and Internal Anatomy of Ticks in. *Biology of Ticks Volume 1*, 74-98.
Sormunen, J. J., Sippola, E., Kaunisto, K. M., Vesterinen, E. J., & Sääksjärvi, I. E. (2019). First evidence of Ixodiphagus hookeri (Hymenoptera: Encyrtidae) parasitization in Finnish castor bean ticks (Ixodes ricinus). *Experimental and applied acarology, 79*(3), 395-404.
Spitalska, E., Sparagano, O., Stanko, M., Schwarzova, K., Spitalsky, Z., Skultety, L., et al. (2018). Diversity of Coxiella-like and Francisella-like endosymbionts, and Rickettsia spp., Coxiella burnetii as pathogens in the tick populations of Slovakia, Central Europe. *Ticks Tick Borne Dis, 9*(5), 1207-1211.
Sprong, H., Azagi, T., Hoornstra, D., Nijhof, A. M., Knorr, S., Baarsma, M. E., et al. (2018). Control of Lyme borreliosis and other Ixodes ricinus-borne diseases. *Parasit Vectors, 11*(1), 145.
Sprong, H., Hofhuis, A., Gassner, F., Takken, W., Jacobs, F., van Vliet, A. J., et al. (2012). Circumstantial evidence for an increase in the total number and activity of Borrelia-infected Ixodes ricinus in the Netherlands. *Parasit Vectors, 5*(1), 294.
Sprong, H., Wielinga, P. R., Fonville, M., Reusken, C., Brandenburg, A. H., Borgsteede, F., et al. (2009). Ixodes ricinus ticks are reservoir hosts for Rickettsia helvetica and potentially carry flea-borne Rickettsia species. *Parasit Vectors, 2*(1), 41.
Stafford, K. C., 3rd, Denicola, A. J., & Kilpatrick, H. J. (2003). Reduced abundance of Ixodes scapularis (Acari: Ixodidae) and the tick parasitoid Ixodiphagus hookeri (Hymenoptera: Encyrtidae) with reduction of white-tailed deer. *J Med Entomol, 40*(5), 642-652.
Stańczak, J., Racewicz, M., Michalik, J., Cieniuch, S., Sikora, B., & Skoracki, M. (2009). Prevalence of infection with Rickettsia helvetica in feeding ticks and their hosts in western Poland. *Clin Microbiol Infect, 15*(s2), 328-329.
Stanek, G. (2009). Pandora's Box: pathogens in Ixodes ricinus ticks in Central Europe. *Wien Klin Wochenschr, 121*(21-22), 673-683.

Stanek, G., Wormser, G. P., Gray, J., & Strle, F. (2012). Lyme borreliosis. *Lancet, 379*(9814), 461-473.

Stavru, F., Riemer, J., Jex, A., & Sassera, D. (2020). When bacteria meet mitochondria: The strange case of the tick symbiont Midichloria mitochondrii. *Cell Microbiol, 22*(4), e13189.

Steiner, F. E., Pinger, R. R., Vann, C. N., Grindle, N., Civitello, D., Clay, K., et al. (2008). Infection and co-infection rates of Anaplasma phagocytophilum variants, Babesia spp., Borrelia burgdorferi, and the rickettsial endosymbiont in Ixodes scapularis (Acari: Ixodidae) from sites in Indiana, Maine, Pennsylvania, and Wisconsin. *J Med Entomol, 45*(2), 289-297.

Steiner, F. E., Pinger, R. R., Vann, C. N., Grindle, N., Civitello, D., Clay, K., et al. (2014). Infection and co-infection rates of Anaplasma phagocytophilum variants, Babesia spp., Borrelia burgdorferi, and the rickettsial endosymbiont in Ixodes scapularis (Acari: Ixodidae) from sites in Indiana, Maine, Pennsylvania, and Wisconsin. *J Med Entomol, 45*(2), 289-297.

Stenos, J., Graves, S. R., & Unsworth, N. B. (2005). A highly sensitive and specific real-time PCR assay for the detection of spotted fever and typhus group Rickettsiae. *Am J Trop Med Hyg, 73*(6), 1083-1085.

Strnad, M., & Rego, R. O. M. (2020). The need to unravel the twisted nature of the Borrelia burgdorferi sensu lato complex across europe. *Microbiology (United Kingdom), 166*(5), 428--435.

Stuen, S., Granquist, E. G., & Silaghi, C. (2013). Anaplasma phagocytophilum--a widespread multi-host pathogen with highly adaptive strategies. *Front Cell Infect Microbiol, 3*, 31.

Sunyer, P., Muñoz, A., Mazerolle, M. J., Bonal, R., & Espelta, J. M. (2016). Wood mouse population dynamics: Interplay among seed abundance seasonality, shrub cover and wild boar interference. *Mamm Biol, 81*(4), 372-379.

Swart, A., Bekker, D. L., Maas, M., de Vries, A., Pijnacker, R., Reusken, C. B., et al. (2017). Modelling human Puumala hantavirus infection in relation to bank vole abundance and masting intensity in the Netherlands. *Infect Ecol Epidemiol, 7*(1), 1287986.

Swei, A., & Kwan, J. Y. (2017). Tick microbiome and pathogen acquisition altered by host blood meal. *ISME J, 11*(3), 813-816.

Sykes, R. A., & Makiello, P. (2017). An estimate of Lyme borreliosis incidence in Western Europe. *J Public Health (Oxf), 39*(1), 74-81.

Tagliapietra, V., Rosa, R., Arnoldi, D., Cagnacci, F., Capelli, G., Montarsi, F., et al. (2011). Saturation deficit and deer density affect questing activity and local abundance of Ixodes ricinus (Acari, Ixodidae) in Italy. *Vet Parasitol, 183*(1-2), 114-124.

Takasu, K., Takano, S.-I., Sasaki, M., Yagi, S., & Nakamura, S. (2003). Host recognition by the tick parasitoid Ixodiphagus hookeri (Hymenoptera: Encyrtidae). *Environmental entomology, 32*(3), 614-617.

Takken, W., van Vliet, A. J., Verhulst, N. O., Jacobs, F. H., Gassner, F., Hartemink, N., et al. (2017). Acarological Risk of Borrelia burgdorferi Sensu Lato Infections Across Space and Time in The Netherlands. *Vector Borne Zoonotic Dis, 17*(2), 99-107.

Takumi, K., Sprong, H., & Hofmeester, T. R. (2019). Impact of vertebrate communities on Ixodes ricinus-borne disease risk in forest areas. *Parasit Vectors, 12*(1), 1-12.

Talleklint, L., & Jaenson, T. G. (1994). Transmission of Borrelia burgdorferi s.l. from mammal reservoirs to the primary vector of Lyme borreliosis, Ixodes ricinus (Acari: Ixodidae), in Sweden. *J Med Entomol, 31*(6), 880-886.

Talleklint, L., & Jaenson, T. G. (1995). Is the small mammal (Clethrionomys glareolus) or the tick vector (Ixodes ricinus) the primary overwintering reservoir for the Lyme borreliosis

spirochete in Sweden? *J Wildl Dis, 31*(4), 537-540.
Taragel'ova, V., Koci, J., Hanincova, K., Kurtenbach, K., Derdakova, M., Ogden, N. H., et al. (2008). Blackbirds and song thrushes constitute a key reservoir of Borrelia garinii, the causative agent of borreliosis in Central Europe. *Appl Environ Microbiol, 74*(4), 1289-1293.
Taylor, K. R., Takano, A., Konnai, S., Shimozuru, M., Kawabata, H., & Tsubota, T. (2013). Borrelia miyamotoi infections among wild rodents show age and month independence and correlation with Ixodes persulcatus larval attachment in Hokkaido, Japan. *Vector Borne Zoonotic Dis, 13*(2), 92-97.
Telford, S. R. (2009). Status of the east side hypothesis (Transovarial Interference) 25 years later. *Ann N Y Acad Sci, 1166*, 144-150.
Tersago, K., Verhagen, R., Servais, A., Heyman, P., Ducoffre, G., Leirs, H., et al. (2009). Hantavirus disease (nephropathia epidemica) in Belgium: effects of tree seed production and climate. *Epidemiol Infect, 137*(2), 250-256.
Thapa, S., Zhang, Y., & Allen, M. S. (2019). Bacterial microbiomes of Ixodes scapularis ticks collected from Massachusetts and Texas, USA. *BMC Microbiol, 19*(1), 138.
Thu, M. J., Qiu, Y., Kataoka-Nakamura, C., Sugimoto, C., Katakura, K., Isoda, N., et al. (2019). Isolation of Rickettsia, Rickettsiella, and Spiroplasma from questing ticks in Japan using arthropod cells. *Vector Borne Zoonotic Dis, 19*(7), 474-485.
Tijsse-Klasen, E., Braks, M., Scholte, E. J., & Sprong, H. (2011). Parasites of vectors--Ixodiphagus hookeri and its Wolbachia symbionts in ticks in The Netherlands. *Parasit Vectors, 4*(1), 228.
Tinsley, M., & Majerus, M. (2006). A new male-killing parasitism: Spiroplasma bacteria infect the ladybird beetle Anisosticta novemdecimpunctata (Coleoptera: Coccinellidae). *Parasitology, 132*(6), 757.
Tokarz, R., Tagliafierro, T., Sameroff, S., Cucura, D. M., Oleynik, A., Che, X., et al. (2019). Microbiome analysis of Ixodes scapularis ticks from New York and Connecticut. *Ticks Tick Borne Dis, 10*(4), 894-900.
Tomassone, L., Ceballos, L. A., Ragagli, C., Martello, E., De Sousa, R., Stella, M. C., et al. (2017). Importance of common wall lizards in the transmission dynamics of tick-borne pathogens in the northern Apennine mountains, Italy. *Microb Ecol, 74*(4), 961-968.
Tully, J. G., Rose, D. L., Yunker, C. E., Carle, P., BOV, J. M., Williamson, D. L., et al. (1995). Spiroplasma ixodetis sp. nov., a new species from Ixodes pacificus ticks collected in Oregon. *Int J Syst Evol Microbiol, 45*(1), 23-28.
Van Buskirk, J., & Ostfeld, R. S. (1995). Controlling Lyme disease by modifying the density and species composition of tick hosts. *Ecol Appl, 5*(4), 1133-1140.
van Duijvendijk, G., Coipan, C., Wagemakers, A., Fonville, M., Ersoz, J., Oei, A., et al. (2016). Larvae of Ixodes ricinus transmit Borrelia afzelii and B. miyamotoi to vertebrate hosts. *Parasit Vectors, 9*(1), 97.
van Duijvendijk, G., Sprong, H., & Takken, W. (2015). Multi-trophic interactions driving the transmission cycle of Borrelia afzelii between Ixodes ricinus and rodents: a review. *Parasit Vectors, 8*(1), 643.
Van Oosten, A. R., Duron, O., & Heylen, D. J. A. (2018). Sex ratios of the tick Ixodes arboricola are strongly female-biased, but there are no indications of sex-distorting bacteria. *Ticks Tick Borne Dis, 9*(2), 307-313.
Van Treuren, W., Ponnusamy, L., Brinkerhoff, R. J., Gonzalez, A., Parobek, C. M., Juliano, J. J., et al. (2015). Variation in the microbiota of Ixodes ticks with regard to geography, species, and sex. *Appl Environ Microbiol, 81*(18), 6200-6209.

Vazquez, M., Muehlenbein, C., Cartter, M., Hayes, E. B., Ertel, S., & Shapiro, E. D. (2008). Effectiveness of personal protective measures to prevent Lyme disease. *Emerg Infect Dis, 14*(2), 210-216.

Voordouw, M. J. (2015). Co-feeding transmission in Lyme disease pathogens. *Parasitology, 142*(2), 290-302.

Wagemakers, A., Koetsveld, J., Narasimhan, S., Wickel, M., Deponte, K., Bleijlevens, B., et al. (2016b). Variable Major Proteins as Targets for Specific Antibodies against Borrelia miyamotoi. *J Immunol, 196*(10), 4185-4195.

Walter, M., Vogelgesang, J. R., Rubel, F., & Brugger, K. (2020). Tick-borne encephalitis virus and its European distribution in ticks and endothermic mammals. *Microorganisms, 8*(7), 1065.

Weiss, B., & Aksoy, S. (2011). Microbiome influences on insect host vector competence. *Trends Parasitol, 27*(11), 514-522.

Whipps, J., Lewis, K., & Cooke, R. (1988). Mycoparasitism and plant disease control. *Fungi in biological control systems*, 161-187.

Wickham, H. (2016). ggplot2: Elegant Graphics for Data Analysis. In.

Wickham, H., Chang, W., Henry, L., Pedersen, T., Takahashi, K., Wilke, C., et al. (2020). ggplot2: Create Elegant Data Visualisations Using the Grammar of Graphics; R package version 3.3.0. In.

Wickham, H., Francois, R., Henry, L., & Mller, K. (2019a). dplyr: A Grammar of Data Manipulation. In.

Wickham, H., & Henry, L. (2019b). tidyr: Easily Tidy Data with 'spread()' and 'gather()' Functions. In.

Wielinga, P. R., Gaasenbeek, C., Fonville, M., de Boer, A., de Vries, A., Dimmers, W., et al. (2006). Longitudinal analysis of tick densities and Borrelia, Anaplasma, and Ehrlichia infections of Ixodes ricinus ticks in different habitat areas in The Netherlands. *Appl Environ Microbiol, 72*(12), 7594-7601.

Wilke, A. B. B., & Marrelli, M. T. (2015). Paratransgenesis: a promising new strategy for mosquito vector control. *Parasit Vectors, 8*(1), 1-9.

Wojcik-Fatla, A., Zajac, V., Sawczyn, A., Cisak, E., Sroka, J., & Dutkiewicz, J. (2015). Occurrence of Francisella spp. in Dermacentor reticulatus and Ixodes ricinus ticks collected in eastern Poland. *Ticks Tick Borne Dis, 6*(3), 253-257.

Wolff, J. O. (1996). Coexistence of white-footed mice and deer mice may be mediated by fluctuating environmental conditions. *Oecologia, 108*(3), 529-533.

Wood, H. (1911). Notes on the life history of the tick parasite. *J Econ Entomol, 4*(5), 425-431.

Xie, J., Vilchez, I., & Mateos, M. (2010). Spiroplasma bacteria enhance survival of Drosophila hydei attacked by the parasitic wasp Leptopilina heterotoma. *PLoS One, 5*(8), e12149.

Yang, B., Wang, Y., & Qian, P.-Y. (2016). Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analysis. *BMC Bioinform, 17*(1), 1-8.

Yordanova, I. A., Zakovic, S., Rausch, S., Costa, G., Levashina, E., & Hartmann, S. (2018). Micromanaging immunity in the murine host vs. the mosquito vector: microbiota-dependent immune responses to intestinal parasites. *Front Cell Infect Microbiol, 8*, 308.

Zemtsova, G., Killmaster, L., Mumcuoglu, K., & Levin, M. (2010). Co-feeding as a route for transmission of Rickettsia conorii israelensis between Rhipicephalus sanguineus ticks. *Exp Appl Acarol, 52*(4), 383-392.

Zhang, X. C., Yang, Z. N., Lu, B., Ma, X. F., Zhang, C. X., & Xu, H. J. (2014). The composition and transmission of microbiome in hard tick, Ixodes persulcatus, during blood meal. *Ticks Tick*

Borne Dis, 5(6), 864-870.
Zhu, Z., Aeschlimann, A., & Gern, L. (1992). Rickettsia-like microorganisms in the ovarian primordial of molting Ixodes ricinus (acari: ixodidae) larvae and nymphs. *Ann Parasitol Hum Comp, 67*(4), 99-110.
Zilber-Rosenberg, I., & Rosenberg, E. (2008). Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *FEMS Microbiol Ecol, 32*(5), 723-735.
Zolnik, C. P., Falco, R. C., Daniels, T. J., & Kolokotronis, S. O. (2018). Transient influence of blood meal and natural environment on blacklegged tick bacterial communities. *Ticks Tick Borne Dis, 9*(3), 563-572.
Zolnik, C. P., Prill, R. J., Falco, R. C., Daniels, T. J., & Kolokotronis, S. O. (2016). Microbiome changes through ontogeny of a tick pathogen vector. *Mol Ecol, 25*(19), 4963-4977.

Summary

In the northern hemisphere, the sheep tick, *Ixodes ricinus,* transmits many pathogens to humans and animals, posing severe health concerns and economic losses. The observed patterns and dynamics of tick-borne diseases result from the multitrophic interactions between the pathogenic agents, ticks, and (a)biotic components of the environment. In the context of vector-borne pathogens, there is an increasing interest in the question of if and how microorganisms affect the fitness and physiology, and possibly, the vectorial capacity of the arthropod vectors. The latter, as well as interactions between microorganisms and pathogens, may have consequences for vector-borne disease dynamics.

Nevertheless, little is known about how microorganisms, other than pathogenic agents, are acquired and propagated in *I. ricinus* ticks. The research presented in this thesis focused on describing the microbiome of *I. ricinus* and aiming to elucidate how it is generated. As *I. ricinus* is responsible for transmitting pathogenic agents to humans and animals, questions and hypotheses of my studies were framed in the context of tick-borne disease risk.

Chapter 2 describes the microbiome of *I. ricinus* in the Netherlands and brings understanding towards the distribution of tick symbionts across tick populations. This study explored bacterial communities of *I. ricinus* across developmental stages and six geographic locations by 16S rRNA amplicon sequencing combined with accurate quantification of the bacterial load. The bacterial communities of ticks were strongly associated with geographical region rather than the tick life stage. Strictly speaking, the microbiome of ticks originating from locations situated in the same geographical region (western, northern, and central) clustered together, and the clusters were mostly determined by differences in *Rickettsia* abundance. The most prominent vertically-transmitted tick symbionts included *Rickettsia*, *Rickettsiella* spp., *Midichloria* and *Spiroplasma*. Overall, there was a surprisingly wide range of total bacterial loads across pools of ticks analysed in our experiment. Accurate quantification of the bacterial load permitted comparisons of low and high microbial biomass samples, despite the significant influence of contaminating DNA. Many tick samples expressed a limited microbiome, which was a surprising result given that ticks are haematophagous arthropods.

Secondly, we screened almost 17,000 questing nymphs from 19 locations around the Netherlands for the presence of the vertically-transmitted tick symbionts *R. helvetica*, *Rickettsiella* spp., *M. mitochondrii*, and *S. ixodetis*, and the horizontally-transmitted pathogens *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum*, and *Neoehrlichia mikurensis*. All microorganisms were present in all studied forest sites. However, around 12% of tested nymphs were free of any infection, indicating that *I. ricinus* does not contain an obligate symbiont. The prevalences of *R. helvetica*, *Rickettsiella* spp., and *M. mitochondrii* differed significantly between the regions in which the symbionts occurred in different proportions. Therefore, we speculate that selective pressures on a regional scale determine infection rates of some vertically-transmitted symbionts in *I. ricinus*. These findings can explain regional differences in the risk of disease caused by *R. helvetica*, a documented human pathogen. By contrast, our analyses showed that the community of horizontally-transmitted pathogens is determined locally rather than regionally. This result is in line with previous studies showing that the local vertebrate community determined pathogen infection rates in ticks.

Furthermore, it is poorly understood how the underlying microbiome of a tick affects the acquisition of zoonotic pathogens and how a blood meal and pathogens contribute to the overall change in the bacterial community. In **Chapter 3**, we addressed these questions and, firstly, investigated the microbiome of nymphs infected with distinct pathogens. Ticks from eight forest sites were tested for the presence of *B. burgdorferi* s.l., *Babesia* spp., *A. phagocytophilum*, and *N. mikurensis* by qPCR. Subsequently, ticks were pooled by a pathogen, and their microbiomes were determined with the 16S rRNA amplicon sequencing. Tick bacterial communities clustered poorly by pathogen infection status but better by geographic regions. As the presence of a pathogen in nymphs indicates a specific vertebrate host on which it fed as a larva, our findings imply that the vertebrate host does not, or hardly, contributes to the bacterial community of ticks. Secondly, we studied associations within tick microbiota to unravel possible facilitative or adverse effects of vertically-transmitted symbionts on acquiring zoonotic pathogens. Using the database of Chapter 2, we compared expected and observed co-infections of tick microorganisms such as horizontally-transmitted pathogens *B. burgdorferi* s.l., *A. phagocytophilum*, and *N. mikurensis*, and the vertically-transmitted tick symbionts *R. helvetica*, *Rickettsiella* spp., *S. ixodetis*, and *M. mitochondrii.* Some associations were observed only on a country level and lost while investigated on a regional scale, while others were observed on both levels. Among the most striking associations were the ones of *M. mitochondrii* and *Rickettsiella* spp. with *B. burgdorferi* s.l. Therefore, we propose that some vertically-transmitted symbionts increase tick fitness and alter the propensity of ticks to acquire or maintain horizontally-acquired pathogens. We also detected a significant negative association of *R. helvetica* with *S. ixodetis*, which implies that ticks cannot simultaneously maintain these two symbiont species by vertical transmission. In this chapter, the possible underlying mechanisms for some of these remarkable interactions are discussed and merit further investigation.

Although we did not observe any effect of vertebrates on the overall tick microbiome, vertebrates are the driving force of the distribution and abundance of ticks and the prevalence of pathogens in questing ticks. For instance, rodents are considered to contribute strongly to the risk of tick-borne diseases by feeding *I. ricinus* larvae and by acting as amplifying hosts for pathogens. In **Chapter 4**, we tested to what extent these two processes depend on rodent density and for which pathogen species rodents synergistically contribute to the local disease risk, i.e., the density of infected nymphs. In a natural woodland, the rodent population was experimentally manipulated either by removing rodents from plots to reduce or adding rodent food (acorns) to increase the density. Rodent densities were monitored for 2.5 years, and tick densities and infection prevalences in ticks were measured for 3.5 years. Collected questing nymphs and rodent ear biopsies were tested for seven tick-borne microorganisms, including *B. afzelii*, *B. garinii*, *B. miyamotoi*, *N. mikurensis*, *R. helvetica*, *S. ixodetis*, and *B. microti*. The strongest associations were found in rodent-associated pathogens *B. afzelii* and *N. mikurensis* that most heavily rely on horizontal transmission. In fact, given that rodents are the primary hosts of larval ticks and consistently contribute to a new generation of nymphs, rodent density had a strong synergistic effect on the density of nymphs infected with these pathogens. The prevalence, but not the density of infected nymphs of the bird-associated *B. garinii* decreased with increasing rodent density. The prevalences of *B. miyamotoi* and *R. helvetica* were independent of rodent density, and rising rodent density moderately increased the densities of infected nymphs. Lastly, prevalences of *B. microti* and *S. ixodetis* decreased with increasing rodent density, which had a non-linear association with densities of infected nymphs. The associations of rodent density with tick microorganisms varied, more than likely due to contrasts in microorganisms' biology, including differences in the transmission mode and efficiency and host specificity. Nevertheless,

even for rodent-associated pathogens, it seems impossible to predict disease risk solely based on rodent density since we have shown that other factors, independent from our experiment, strongly affected tick density. Our results draw attention to the importance of considering the transmission mode of a pathogen as well as other (spatial and temporal) factors while developing models to predict the tick-borne disease risk.

Although the environmental requirements for tick survival are well established, it is not known if and how fluctuating climatic conditions influence transmission dynamics of tick symbionts and pathogens. **Chapter 5** describes a longitudinal study, where we investigated the dynamics behind the little-understood variation in the infection prevalence of the vertically-transmitted symbionts, *M. mitochondrii* and *R. helvetica*, as well as the pathogens *B. burgdorferi* s.l., *B. miyamotoi* and *N. mikurensis* in relation to climatic conditions. In this study, questing nymphs of *I. ricinus* from 12 locations in the Netherlands were collected monthly for four years, and the infection prevalences of the microorganisms mentioned above were determined. Subsequently, we investigated the spatio-temporal variations in observed prevalences and their associations with precipitation, relative humidity, temperature, and evaporation rate measured in the same or previous season. All but *B. miyamotoi* presented spatio-temporal variation. The prevalence of *R. helvetica* was the most variable factor and positively associated with all climatic factors, except for temperature. Interestingly, among the 12 studied locations, the five forest sites with the highest *R. helvetica* prevalence in questing nymphs expressed the highest relative humidity in spring and summer (and often the lowest in autumn and winter). Although a short-term positive effect of relative humidity was detected, we propose that the spatial distribution of *R. helvetica* prevalence in Dutch tick populations derives from long-term differences in relative humidity between locations. *Midichloria mitochondrii* and, surprisingly, *N. mikurensis* were mainly associated with evaporation rate and relative humidity; the former negatively and the latter positively. Concerning *B. miyamotoi* and *B. burgdorferi* s.l., a weak and no association were detected, respectively. We propose that the effect of climatic factors on the infection prevalences of tick bacteria depends on bacterial transmission route and type of symbiosis with *I. ricinus*. Long-term studies using modelling approaches should be applied to a broader area of Europe with distinct habitats and environmental conditions and varying symbionts communities to further investigate these associations. This approach could improve our understanding of the roles of symbionts in tick physiology in the context of tick-borne disease transmission dynamics.

Another possible biotic factor shaping the tick microbiota is the presence and abundance of a natural tick enemy, the parasitoid wasps *Ixodiphagus hookeri*. This parasitic wasp infests both unfed and feeding larvae and nymphs, but the egg development only occurs in fully-engorged nymphs. *Ixodiphagus hookeri* larvae feed on the internal tissue along with the vertebrate blood ingested by their tick host. Subsequently, wasps moult into adults and emerge by making a hole in the tick's body. Since the emergence of wasps kills its host, a tick cycle is disrupted. Therefore, in **Chapter 6**, we examined whether this phenomenon affects the transmission dynamics of zoonotic pathogens. The ecological interactions among *I. hookeri*, *I. ricinus*, and two vertebrate species groups (ungulates and rodents) were explored. *Ixodes hookeri* DNA was detected significantly more often in *I. ricinus* nymphs (4.4%) than in larvae (0.5%) and not in adults. Therefore, we determined the infestation rate of *I. hookeri* in questing nymphs from 19 forest sites, where vertebrate, tick, and tick-borne pathogen communities had been previously quantified. We found higher than expected co-occurrence rates of *I. hookeri* with deer-associated *A. phagocytophilum*, and lower than expected rates with rodent-associated *B. afzelii* and *N. mikurensis*. We detected *I. hookeri* in ticks collected

in 18 of the 19 locations around the Netherlands with a prevalence in questing *I. ricinus* nymphs ranging from 0.1% to 16%. The prevalence of *I. hookeri* was positively correlated with the encounter probability of ungulates and the densities of all life stages of *I. ricinus*. Our observation supported the idea that female wasps are attracted to deer by their odour, and subsequently infest ticks that feed on these animals, which increases chances for a wasp and *A. phagocytophilum* to co-occur. The interaction of *I. hookeri* with the propagation host (deer) of its tick host ensures, to a large extent, the continuation of its lifecycle. Interestingly, this study showed that the high prevalence of the wasp, which kills nymphs upon its emergence, had no apparent negative impact on the density of adult ticks, indicating a diminishing or mitigating effect of wasps on the tick population is rather unlikely. Nevertheless, the presence of *I. hookeri* may directly interfere with the transmission cycle of *A. phagocytophilum*, and probably other deer-associated pathogens. Lastly, we investigated the emergence of *I. hookeri* from artificially fed, field-collected nymphs. Adult wasps emerged from seven of the 172 fed nymphs, implying that molecular detection of *I. hookeri* DNA indicates the presence of viable wasps. Further improvements of the artificial feeding system are necessary to enable studies of the microbial interactions with the parasitic wasp in more detail.

Chapter 7, the general discussion, discusses the most salient findings of my studies in the light of current scientific developments in arthropod microbiome research.

Based on the results from my research, as well as the literature published previously, I interpret that *I. ricinus* has a limited microbiome and lacks an obligate symbiont. Nevertheless, given the relatively high prevalence of symbionts in questing ticks, symbiotic bacteria appear to be essential for this tick species to thrive under different (a)biotic conditions.

It is also concluded that to improve our ability to assess the risk of tick-borne diseases, we should describe the transmission route of disease agents and their symbiosis with ticks. Tick-associated microorganisms, including human pathogens, may utilize different transmission routes and play various roles in tick fitness and physiology, which defines to what extent (a)biotic factors influence microorganisms' dynamics.

Lastly, the chapter discusses *I. hookeri* and tick symbionts as potential biological control agents for ticks and tick-borne diseases. Based on the results obtained in this research, it is advocated that wasps have an unlikely diminishing or mitigating effect on the tick population. Nevertheless, strategies aiming at decreasing disease risk could focus on reducing the prevalences of *I. ricinus* symbionts.

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I have never envisioned that the end of my Ph.D. would fall in the midst of a pandemic. In the last months of my project, I worked from home and could not say a proper goodbye to my colleagues. In fact, I had realised how many of them I will miss before my project even ended. I hope that sooner than later, we will meet in person to tell you how grateful I am for your contribution to this booklet. By the way, it is incredible how many people are needed for one person to write a thesis. Meanwhile, here, before our humanity successfully fights the virus, I would like to – at least partially – express my gratitude.

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Vitalij and Jeremi, my men, you are my home, and I love you so much. Vitalij, you are the reason I have moved to the Netherlands in the first place, and you are the one who has always believed that I could continue my education here. Thank you for your love and support! Jeremi, you know you made it even more of a challenge, right? But you also made it more meaningful.

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Last but not least – Vitalij, Hania, Michał – thank you for breathing art into my thesis and making it extraordinary. Michał, the piece you have composed is very touching, and I can really hear in it the intertwined worlds of ticks and microbes. Hania, your illustrations made ticks much, much cuter to look at and think about. Vitalij, I am thrilled we could combine our passions in one book: biology and field recordings. Your recordings bring me back to one of the nicest things about my job: the fieldwork.

Curriculum vitae

Aleksandra was born in Wroclaw, Poland, on 24 August 1988. She completed her bachelor's degree in General Biology with a specialisation in Zoology at the University of Wroclaw, and she subsequently moved to Amsterdam to pursue her master's degree. Here, she studied General Biology at the University of Amsterdam, conducting a number of short studies from various fields, including developmental psychology, animal behaviour, ecology, and parasitology. The latter project, carried out at the Wageningen University, particularly got her interest, and she brought on the project to the National Institute for Public Health and the Environment (RIVM) in Bilthoven, where she obtained her first position as a research assistant. At this institute, she gained experience in molecular biology, and she worked - among others - on *Bartonella* communities in ectoparasites of bats. This project involved a large amount of exciting fieldwork and allowed her to expand her network and reach out to bat ecologists and enthusiasts from all over the Netherlands and beyond. This cooperation resulted in a joint publication by researchers from the Netherlands, Belgium, Hungary, and the US (McKee et al., 2019).

Following a year of work as a research assistant, Aleksandra continued her career as a Ph.D. student and focused on the ecology of tick-borne diseases exploring the interactions between microbes, ticks, vertebrates, and the environment. The results of her research can be found in this thesis. During her Ph.D., she has developed a passion for host-symbiont interactions and a fascination with the impact of symbiotic microbial communities on the health and well-being of their hosts. As her Ph.D. has now come to an end, she intends to pursue an academic career.

Publications

Garcia-Vozmediano, A., **Krawczyk, A. I.**, Sprong, H., Rossi, L., Ramassa, E., & Tomassone, L. (2020). Ticks climb the mountains: ixodid tick infestation and infection by tick-borne pathogens in the Western Alps. *Ticks Tick Borne Dis, 11*(5), 101489.

Hansford, K. M., Fonville, M., Gillingham, E. L., Coipan, E. C., Pietzsch, M. E., **Krawczyk, A. I.**, et al. (2017). Ticks and Borrelia in urban and peri-urban green space habitats in a city in southern England. *Ticks Tick Borne Dis, 8*(3), 353-361.

Heylen, D., **Krawczyk, A. I.**, Lopes de Carvalho, I., Nuncio, M. S., Sprong, H., & Norte, A. C. (2017a). Bridging of cryptic Borrelia cycles in European songbirds. *Environ Microbiol, 19*(5), 1857-1867.

Heylen, D. J., Sprong, H., **Krawczyk, A. I.**, Van Houtte, N., Genne, D., Gomez-Chamorro, A., et al. (2017b). Inefficient co-feeding transmission of Borrelia afzelii in two common European songbirds. *Sci Rep, 7*, 39596.

Hofmeester, T. R., **Krawczyk, A. I.**, Van Leeuwen, A. D., Fonville, M., Montizaan, M. G., van den Berge, K., et al. (2018). Role of mustelids in the life-cycle of ixodid ticks and transmission cycles of four tick-borne pathogens. *Parasit Vectors, 11*(1), 600.

Hornok, S., Estók, P., Kováts, D., Flaisz, B., Takács, N., Szőke, K., **Krawczyk, A. I.** et al. (2015). Screening of bat faeces for arthropod-borne apicomplexan protozoa: Babesia canis and Besnoitia besnoiti-like sequences from Chiroptera. *Parasit Vectors, 8*(1), 1-6.

Hornok, S., & **Krawczyk, A. I.** (2016). First record of Ixodes ariadnae in western Europe, Belgium. *Acta Vet Hung, 64*(4), 467-471.

Hornok, S., Szőke, K., Estók, P., **Krawczyk, A. I.**, Haarsma, A.-J., Kováts, D., et al. (2018). Assessing bat droppings and predatory bird pellets for vector-borne bacteria: molecular evidence of bat-associated Neorickettsia sp. in Europe. *Anton Leeuw Int J G, 111*(9), 1707-1717.

Jahfari, S., **Krawczyk, A. I.**, Coipan, E. C., Fonville, M., Hovius, J. W., Sprong, H., et al. (2017). Enzootic origins for clinical manifestations of Lyme borreliosis. *Infect Genet Evol, 49*, 48-54.

Krawczyk, A. I., Bakker, J. W., Koenraadt, C. J., Fonville, M., Takumi, K., Sprong, H., et al. (2020a). Tripartite interactions among Ixodiphagus hookeri, ixodes ricinus and deer: Differential interference with transmission cycles of tick-borne pathogens. *Pathogens, 9*(5), 339.

Krawczyk, A. I., Van Duijvendijk, G. L., Swart, A., Heylen, D., Jaarsma, R. I., Jacobs, F. H., et al. (2020b). Effect of rodent density on tick and tick-borne pathogen populations: consequences for infectious disease risk. *Parasit Vectors, 13*(1), 1-17.

Krawczyk, A. I., van Leeuwen, A. D., Jacobs-Reitsma, W., Wijnands, L. M., Bouw, E., Jahfari, S., et al. (2015). Presence of zoonotic agents in engorged ticks and hedgehog faeces from Erinaceus

europaeus in (sub) urban areas. *Parasit Vectors, 8*(1), 210.

Krawczyk, A. I., McKee, C. D., Sándor, A. D., Görföl, T., Földvári, M., Földvári, G., et al. (2019). Host phylogeny, geographic overlap, and roost sharing shape parasite communities in European bats. *Front Ecol Evol, 7*, 69.

Pollet, T., Sprong, H., Lejal, E., **Krawczyk, A. I.**, Moutailler, S., Cosson, J.-F., et al. (2020). The scale affects our view on the identification and distribution of microbial communities in ticks. *Parasit Vectors, 13*(1), 36.

Ravagnan, S., Tomassone, L., Montarsi, F., **Krawczyk, A. I.**, Mastrorilli, E., Sprong, H., et al. (2018). First detection of Borrelia miyamotoi in Ixodes ricinus ticks from northern Italy. *Parasit Vectors, 11*(1), 130.

Sándor, A. D., Földvári, M., **Krawczyk, A. I.**, Sprong, H., Corduneanu, A., Barti, L., et al. (2018). Eco-epidemiology of novel Bartonella genotypes from parasitic flies of insectivorous bats. *Microb Ecol, 76*(4), 1076-1088.

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the 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 (4.5 ECTS)
- Interactions of microbes, ticks, vertebrates and the environment

Writing of project proposal (4.5 ECTS)
- Tick pathobiome dynamics and disease risk

Post-graduate courses (4.7 ECTS)
- Introduction to next generation sequencing; EMBL-EBI (2016)
- Grasping sustainability; SENSE (2020)
- MGC Next generation sequencing data analysis; LUMC (2016)
- Data carpentry genomics workshop; RIVM (2018)

Laboratory training and working visits (4.5 ECTS)
- Statistical analysis of HTS data and identification of bacterial endosymbionts in *Ixodes ricinus*; The Hebrew University of Jerusalem, Israel (2017)

Invited review of (unpublished) journal manuscripts (1 ECTS)
- Frontiers Cellular and Infection Microbiology: sharing the ride: *Ixodes scapularis* symbionts and their interactions (2017)

Competence strengthening / skills courses (1 ECTS)
- Endnote course; RIVM (2018)
- Brain friendly working and writing; WUR (2018)
- Writing in English; RIVM (2018)

Scientific integrity / ethics in science activity (1.5 ECTS)
- Scientific integrity & ethics and animal science; WUR (2019)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.2 ECTS)
- PE&RC Day (2018, 2019)
- PE&RC Weekend (2020)

Discussion groups / local seminars / other scientific meetings (4.6 ECTS)
- Tick tactics; Amsterdam (2014)
- 3rd Conference on Neglected Vectors and Vector-Borne Diseases (EurNegVec) (2016)
- NEV Entomologendag; Wageningen (2016)

- The community ecology of pathogens: coinfection, coexistence and community composition; NIOO (2016)
- Science with impact; Wageningen PhD symposium (2019)
- Biodiversity in Crisis (2019)
- Interactions among infectious agents: why they're important and how to detect them (2019)
- Lyme & Co discussion group (2019, 2020)
- British ecological society (2020)

International symposia, workshops and conferences (7 ECTS)

- Summer workshop ecology and evolution of parasites and infections; oral presentation; Antwerp (2015)
- 4th And final conference on Neglected Vectors and Vector-Borne Diseases (EurNegVec); oral presentation; Chania (2017)
- NEV Entomologendag; oral presentation; Wageningen (2018)
- 17th Ecology and evolution of infectious diseases; poster presentation; Princeton (2019)

BSc / MSc thesis supervision (3 ECTS)

- Ecology of the tick microbiome: role on the tick-borne pathogen dynamic
- Ticks climb the mountains: Ixodid tick infestation and infection by tick-borne pathogens in the Western Alps
- Spatiotemporal variations of heritable *Ixodes ricinus* symbionts is associated with climatic variables

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