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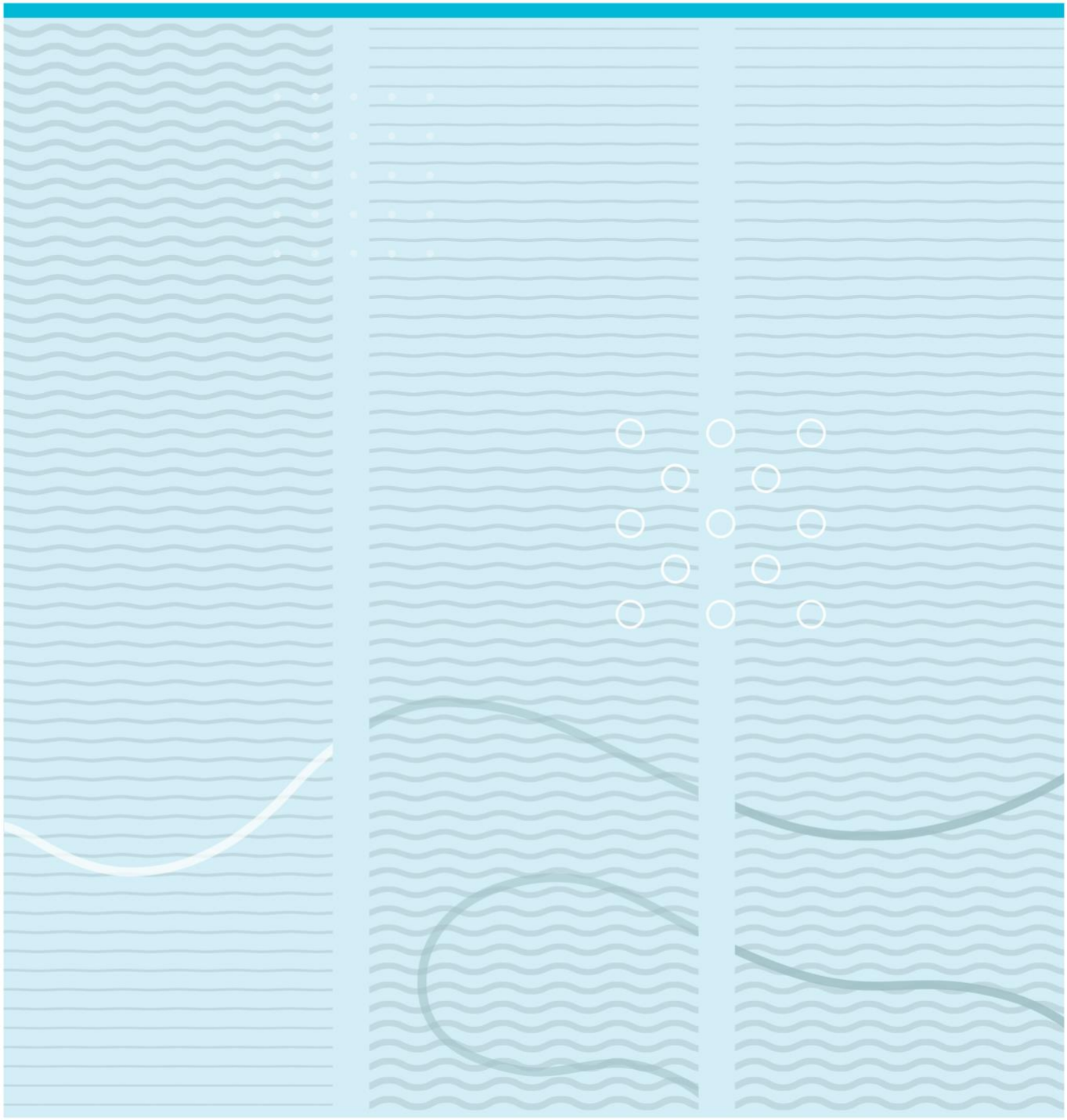
Jamila Synnøve Saber

Co-occurrence of *Borrelia afzelii* and *Neoehrlichia mikurensis* in *Ixodes ricinus* ticks along the coast of Norway



University of South-Eastern Norway
Faculty of Technology, Natural Sciences and Maritime Sciences
Department of Natural Sciences and Environmental Health
PO Box 235
NO-3603 Kongsberg, Norway

http://www.usn.no

This thesis is worth 60 study points

Summary

Borrelia afzelii (*B. afzelii*) and *Neoehrlichia mikurensis* (*N. mikurensis*) are the two most common tick-borne pathogens found in *Ixodes ricinus* ticks (*I. ricinus*) in Norway. A previous study by Pedersen et al. (2019) found a high prevalence of *N. mikurensis* in *I. ricinus* ticks collected in Norway's southern and northwestern coastal parts. However, the pathogen was almost absent along the southwest coast. Also, co-infections with *B. afzelii* and *N. mikurensis* in *I. ricinus* ticks have been higher than expected by random chance. This master thesis raises whether the low prevalence of *N. mikurensis* along the southwest coast is paralleled with a low prevalence of *B. afzelii*. The study's first aim was to investigate the prevalence of *B. afzelii* in *I. ricinus* ticks along the Norwegian coast. The second aim was to compare the prevalence of *B. afzelii* with that of *N. mikurensis* and investigate whether they co-occur.

The ticks of this study had previously been collected from 10 locations along the coast of Norway, from Spjærøy (59.1°N 10.9°E) in the southeast to Brønnøysund (65.4°N 12.1°E) in the northwest. Samples of 185 individual adult ticks and 671 nymph pools were analyzed for *B. afzelii* with real-time PCR. The total prevalence of *B. afzelii* in adult ticks was 15%, and the total estimated pooled prevalence in nymphs was 11.4%. The prevalence was the highest in the most southern and northern locations. The locations in-between were described as a low-prevalence area. This study suggested that the difference in the prevalence of *B. afzelii* was due to climate, seasonal variation, the availability, and compatibility of vertebrate hosts.

B. afzelii in *N. mikurensis* in *I. ricinus* ticks co-occurred at seven out of 10 locations. The prevalence of both pathogens was the highest in the southernmost and northernmost locations and lower at the locations in-between, along the southwest coast. This study suggested that their co-occurrence was due to the availability of common reservoir hosts such as bank voles and field mice. The lower prevalence along the southwest coast might have been due to absence, less availability of reservoir hosts or climate. However, *B. afzelii* in ticks was present at locations in the southwest where *N. mikurensis* was absent. *B. afzelii* may therefore have a broader spectrum of reservoir hosts than *N. mikurensis*, which was suggested to be the common shrew. This is the first study to investigate the co-occurrence of *B. afzelii* and *N. mikurensis* in *I. ricinus* ticks at such geographical scale.

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Preface

This master thesis is part of the master's program in Environmental Science at the University of South-Eastern Norway, department Bø in Vestfold and Telemark County. The laboratory work was conducted at the university's laboratories.

I especially want to thank my supervisor Andrew Jenkins (USN), for sharing his knowledge about tick-borne pathogens and real-time PCR. Thank you for teaching me how to work at the laboratories, answering all my questions, giving feedback on the writing, and helping with all the statistics. I am grateful for your patience and the interesting and fun conversations about ticks and pathogens. It has been truly inspiring. Many thanks to Åshild Andreassen (NIPH), Benedikte Pedersen (NIPH), and colleagues at the Norwegian Institute of Public Health (NIPH) for letting me use the samples analyzed in this study and sending me the *N. mikurensis* data. A thank you to Frode Bergan (USN) for ordering supplies necessary for the laboratory work and handling technical matters. And to Øyvind Steifetten (USN), thank you for the enlightening conversation about rodents in Norway.

I would also like to thank my two good friends, Melanie Schreier (USN) and Kamilla Häusler (NIPH), for helpful tips and kicking me in the right direction in terms of thinking and writing. You are two hardworking people that I look up to.

Finally, my deepest thanks to my family, who cheered me on and took their time to listen to me continuously talk about ticks, pathogens, and rodents for a year. I truly appreciate your patience and interest. And to my partner, Andreas, thank you for supporting me every day.

Abbreviations and definitions

***A. phagocytophilum* (*Anaplasma phagocytophilum*)** – Tick-borne pathogen that can cause anaplasmosis in cattle and granulocytic anaplasmosis in humans.

Annealing temperature – The temperature at which a primer binds to the complementary DNA strand.

Bartonella – Genus of bacteria which can cause various diseases in humans, such as bartonellosis.

***B. burgdorferi* s.l** – *Borrelia burgdorferi* sensu lato is the collective term for the various *Borrelia* genospecies.

cDNA – Complementary deoxyribonucleic acid (cDNA) is a single stranded DNA molecule made from reverse transcription of RNA.

Ct value – Cycle threshold value.

Cycling stage – The amplification step in real-time PCR which consists of repeated denaturing, primer annealing and polymerization. It is a two-step PCR which means that annealing and extension take place at the same temperature.

DNA – Deoxyribonucleic acid (DNA) is a double helix molecule that encodes genetic information of living organisms.

DNA polymerase – Enzyme involved in the synthesis of DNA molecules from nucleoside triphosphates (dNTPs).

Holding stage – First step in real-time PCR which includes denaturing of genomic DNA and hot-start inhibitors.

Melt curve stage – Last step in real-time PCR. Data is collected and melt curves are formed.

MSIS – Norwegian Surveillance System of Communicable Diseases.

NBIC – Norwegian Biodiversity Information Center.

NIPH – Norwegian Institute of Public Health.

OspA – Outer surface protein A (OspA) is a gene found on the outer membrane of the *Borrelia* plasmid. The OspA gene ensures migration of the pathogen from the tick's midgut to the salivary gland, which is an essential step in the infection process.

Pathogen – An organism (bacteria, virus, protozoan etc.) that can cause disease.

Pool – Analyzing several organisms together is a pooled analysis. Pooled analysis is helpful when the pathogenic load is low. It is also time and cost efficient.

Primer – Short single stranded DNA molecule used in amplification of a specific target during PCR. A primer initiates DNA synthesis with the help of DNA polymerase.

Primer-dimer – Primer-dimers can be formed during PCR when primers bind to random locations on the complementary DNA strand or when primers bind to themselves.

RNA – Ribonucleic acid (RNA).

SYBR Green – Small molecules used in real-time PCR that fluoresce when binding to double stranded DNA.

TBE-virus – Tick-borne encephalitis virus; virus transmitted by ticks and infects the nervous system.

Tick-borne pathogen – Pathogen transmitted by ticks to humans and animals.

Tm – Melting temperature.

WHO – World Health Organisation.

Western Palearctic – Region that consists of Europe, North Africa, parts of the Middle East and Asia.

1 Introduction

1.1 *Ixodes ricinus*; vector of tick-borne pathogens

The tick-species *Ixodes ricinus* (*I. ricinus*) is an eight-legged parasitic arachnid (Kahl et al., 2002; Apanaskevich & Oliver, 2014). It belongs to the family Ixodidae, also known as the family of hard ticks, consisting of almost 700 species (Sonenshine & Roe, 2014). *I. ricinus* is a generalist tick (Herrmann & Gern, 2015) that feeds on more than 300 vertebrate hosts, from mammals to reptiles and birds (Anderson, 1991; Medlock et al., 2013). It is a blood-sucking animal can acquire disease-causing pathogens when feeding on its host and transmit them to humans and other animals (Burri et al., 2014; Randolph, 2011). Because of its versatility, *I. ricinus* is marked as a problematic species, especially in Europe, representing the most important vector of human zoonotic diseases (Gyllemark et al., 2021).

The pathogens transmitted by the *I. ricinus* tick include viruses, such as the tick-borne encephalitis virus (TBEV), protozoa, such as *Babesia* spp., and multiple pathogenic bacteria, such as *A. phagocytophilum*, the new and emerging pathogen *N. mikurensis* and the bacterial species complex *B. burgdorferi* s.l (hereafter *Borrelia*) (Andersson et al., 2013; Lommano et al., 2012). The species complex consists of 20 *Borrelia* genospecies (Casjens et al., 2011; Ivanova et al., 2014; Stanek & Reiter, 2011) ; 4 are common in northwestern Europe and are *B. burgdorferi* sensu stricto, *B. garinii*, *B. valaisiana,* and *B. afzelii* (Stanek & Reiter, 2011).

1.1.1 Distribution

I. ricinus is found in the western Palearctic (Estrada-Peña et al., 2013). Its distribution extends from North Africa to Scandinavia in latitude and from Ireland to Russia in longitude (Černý et al., 2020; Estrada-Peña et al. 2017). In Norway, the tick is found along the southern and western coastlines, starting from Østfold county in the southeast until reaching its northern distribution limit in Nordland County at 66°N (Hvidsten et al., 2020; Mehl, 1983; Soleng et al., 2018; Tambs-Lyche 1943; Vikse et al., 2020). According to Mehl (1983), *I. ricinus* was mainly found along the coastline but also distant from the coast at 800 m above sea level. Moreover,

a very recent study by De Pelsmaeker et al., (2021) found *I. ricinus* larvae on bank voles (*Myodes glareolus*) caught at 1000 m above sea level in southeastern Norway.

I. ricinus thrives in mixed and deciduous forests with dense ground vegetation that provides mild habitats with high humidity at the ground surface (Ribeiro et al., 2019). The distribution of *I. ricinus* in Europe continues to move upwards in altitude and latitude (Jore et al., 2011; Medlock et al., 2013). Some of the factors that are believed to affect the distribution and abundance of the tick are microhabitat, climate trends, seasonal variation, the density of key hosts, and continuous migration of tick-infested birds (Medlock et al., 2013; Mysterud et al., 2016; Qviller et al., 2016; Randolph & Storey, 1999; Ribeiro et al., 2019; Soleng et al., 2018).

1.1.2 Development stages and hosts

The *I. ricinus* tick has a four-stage life cycle, starting with an egg, then developing into three remaining active stages: larva, nymph, and adult (Figure 1) (Apanaskevich & Oliver, 2014). It is a three-host tick that detaches itself from its host in-between stages after feeding (Leal et al., 2020); larvae usually feed on small mammals such as rodents, nymphs on rodents and birds, while adult ticks feed on larger mammals such as deer (Hofmeester et al., 2016). However, larvae and nymphal ticks have also been found feeding on deer (Tälleklint & Jaenson, 1997). The tick finds its host by "questing," which means it seeks its host by climbing up the vegetation or rocks. The tick then extends its front legs and waits for a passing animal to attach itself to (Leal et al., 2020). Larvae are especially exposed to dehydration during questing because they leave the moist ground unnourished (Randolph, 2004).

After feeding for multiple days, the tick detaches from its host and drops to the ground to molt into the next stage (Apanaskevich & Oliver, 2014). In the case of a female adult tick, she lays her eggs when fully fed and dies shortly after. The male adult tick's only purpose is to mate with a female. It may or may not have a blood meal (Apanaskevich & Oliver, 2014). Based on climatic conditions and host availability, the life cycle of an *I. ricinus* tick varies from 2 to 6 years (Piesman & Gern, 2004) and is usually active from April to November in Norway, depending on weather and temperature (Soleng, 2019).

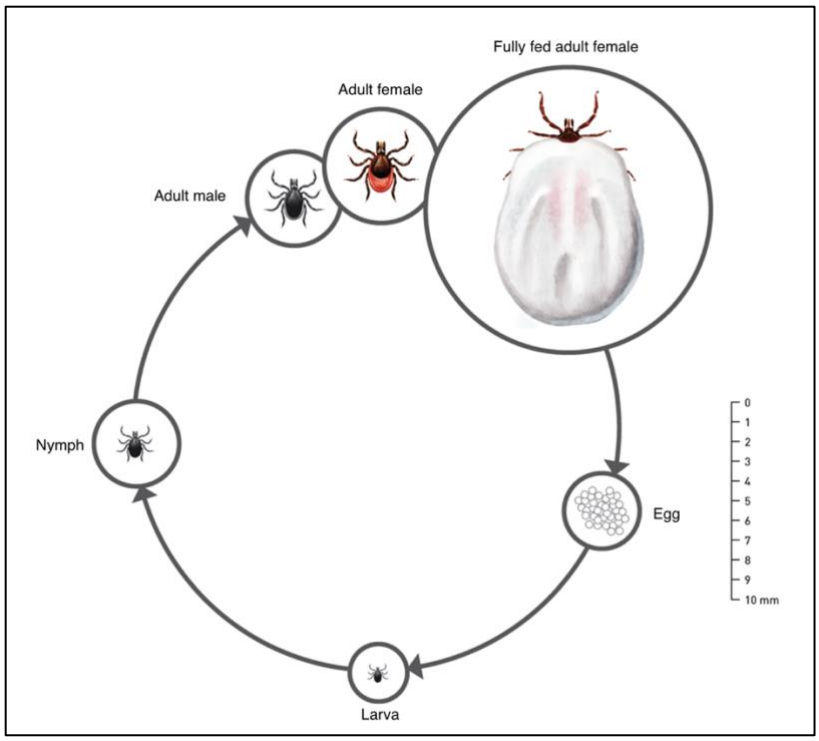

Figure 1: Different stages of an I. ricinus tick. Figure has been translated from Norwegian to English (Norwegian Institute of Public Health [NIPH], 2019a https://www.fhi.no/nettpub/skadedyrveiledere n/edderkopper-og-midd/skogflatt/)

1.1.3 Transmission of tick-borne pathogens

Most tick-borne pathogens are transmitted to the vertebrate host via tick saliva. Ticks use the same canal to absorb blood from their host and inject tick saliva by alternating. Pathogens in the host's blood enter the tick's body and are later excreted through tick saliva into the host again (Šimo et al., 2017). However, not all pathogens are equally as efficiently transmitted (Ueti et al., 2007). Other transmission routes have also been suggested (Pospisilova et al., 2019), such as direct infection of the host via the tick's mouth parts, by regurgitating pathogens present in the midgut (Benach et al., 1987).

A common transmission route for the TBE-virus is by co-feeding; this involves the transmission of pathogens from one tick to another while both are feeding close to each other on the same host (Randolph, 2011). The two pathogens *Rickettsia* spp. and *Borrelia miyamotoi,* are transmitted transovarially (Burgdorfer & Brinton, 1975; Richter et al., 2012) which means that

the pathogens are transmitted from the female adult tick to her offspring/eggs (Hauck et al., 2020). However, the most typical way of transmission is during a blood meal (Randolph, 2011) which is usually how *Borrelia* is transmitted (Richter et al., 2012). This transmission cycle starts with a small or medium-sized mammal carrying pathogens that infects tick larvae (Figure 2). Next, the infected larvae molt into infected host-seeking nymphs, which further transmit the pathogen to other small mammals. The transmission cycle starts again when larvae feed on the infected small mammals. Adult ticks will usually feed on bigger mammals such as deer, but these are believed to not transmit *Borrelia* efficiently to ticks (Gern, 2008; Thompson et al., 2001).

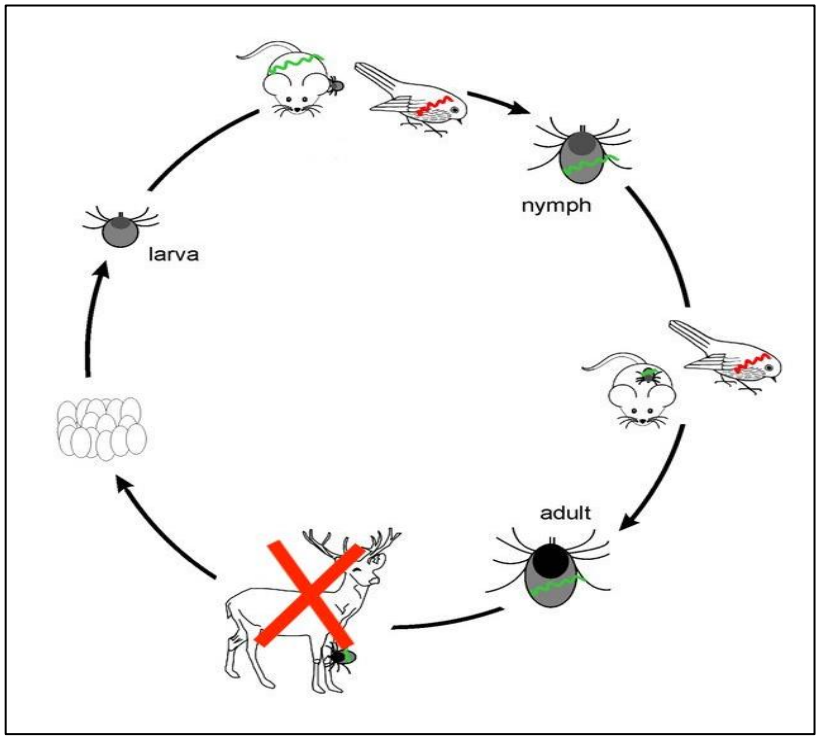

Figure 2: Transmission of Borrelia from small mammals to I. ricinus ticks (Ruyts, 2017). The X on the deer means that the pathogen is not efficiently transmitted to other species. Permission to use figure from Sanne Ruyts. Figure has been modified.

1.1.4 Tick-host-pathogen associations

For a tick-borne pathogen to spread, survive and persist in the environment, the tick needs to be a vector, and it needs to feed on a reservoir host (Kahl et al., 2002). The transmission then

depends on the ability of the reservoir host to carry the infection and the pathogen to infect the tick vector (Medlock et al., 2013). According to Kahl et al. (2002), a tick is a vector when pathogens acquired from an infected vertebrate host during a blood meal remain in the tick for one or several stages (transstadial survival), then are transmitted to a new host when the tick is feeding again. A vertebrate is a reservoir host when pathogens acquired from an infected feeding tick successfully multiply in the host's body and transmit subsequently to other feeding ticks (Kahl et al., 2002).

What makes ticks and vertebrate species competent vectors and reservoir hosts varies by the pathogen. *I. ricinus* is a suitable vector for most *Borrelia* genospecies, but not all (Kahl et al., 2002) implying that there is a difference in the tick's susceptibility to the various genospecies (Kahl et al., 2002; Kurtenbach et al., 2002). Furthermore, vectorial competence might also be affected if there is competition among pathogens from multiple infections within a tick vector (de la Fuente et al., 2017). Just as vectorial competence, reservoir competence might vary among vertebrate species (McCoy et al., 2013). For example, even though the two pathogens, *B. afzelii* and *B. garinii,* are closely related genetically, in Europe, *B. afzelii* is mainly associated with rodents, while *B. garinii* is primarily associated with birds (Heylen et al., 2014; Kurtenbach et al., 2002). Ungulates (especially deer) serve as the main host for feeding adult *I. ricinus* ticks and play an essential role in maintaining their population (Hofmeester et al., 2016; Medlock et al., 2013). Some studies imply that deer might be important reservoir hosts for *A. phagocytophilum,* and on the contrary, they do not seem to be susceptible to *Borrelia* infections (Rosef et al., 2009). Despite being able to acquire *Borrelia* pathogens from feeding ticks, deer do not seem to transmit the pathogen further. As a result, they are called non-reservoir hosts for *Borrelia* (Kahl et al., 2002) or "incompetent reservoir hosts" which is a term also often used in studies. Because of the inefficient transmission, it has been suggested that deer can have a diluting effect on the *Borrelia* spirochete, resulting in a lower prevalence in reservoir hosts (Jaenson & Tälleklint, 1992).

These tick-host-pathogen associations are complex, and the basic biology behind them is not yet fully understood (Wikel, 2021). Pathogens might have developed strategies to manipulate the immune response of tick vectors (de la Fuente et al., 2017). It is believed that pathogens use similar mechanisms to infect vertebrate hosts. Because these strategies have no

significant destructive effects on reservoir hosts and vectors, survival and transmission of pathogens are ensured (de la Fuente et al., 2016). An insight into tick-host-pathogen associations might be beneficial for understanding, if there is, the association between the two tick-borne pathogens, *B. afzelii*, and *N. mikurensis*, that are the focus of this master thesis.

1.2 Bacteria

1.2.1 *Neoehrlichia mikurensis*

Neohrlichia mikurenisis is a pathogen first discovered between 1998 and 2003 in ticks and spleen samples from rats on the Mikura Island in Japan. At that time, it had not yet been cultivated. It was therefore given the name *Candidatus* (Latin for candidate) *Neoehrlichia mikurensis*, where mikurensis referred to the Mikura Island (Kawahara et al., 2004). The study by Kawahara et al. (2004) revealed that the pathogen was a pleomorphic round-shaped gram-negative bacterium and that it belonged to the family of Anaplasmataceae, order Rickettsiales. In previous studies, several names have been used for the pathogen such as *Ehrlichia*-like "Schotti-varient", *Ehrlichia*-like Rattus variant and *Candidatus* Ehrlichia walkerii (Wennerås, 2015). In Norway, it was described for the first time by Jenkins et al. as an *Ehrlichia*-like organism found in *I. ricinus* ticks which later was identified as *N. mikurensis* (Jenkins et al., 2001). Today the bacterium has been cultivated, and the name *Candidatus* can therefore be removed from its title (Wass et al., 2019). The disease caused by an *N. mikurensis* infection is called neoehrlichiosis and usually affects patients with a weak immune system, leaving them with high fever and possible thromboembolic complications. However, other studies show that patients with a regular immune system also have been infected and developed milder symptoms (Wass et al., 2019; Wennerås, 2015). The first known case of disease caused by *N. mikurensis* was detected in the blood of a patient in Sweden in 2010 by PCR assays (Welinder-Olsson et al., 2010). The only known case of human neoehrlichiosis in Norway was reported in 2017 by Frivik et al. (2017). However, several asymptotic cases have also been reported; a recent study by Quarsten et al. (2021) showed that *N. mikurensis* was the predominant pathogen in blood samples taken from immunosuppressed patients living in a tick-endemic area of southern Norway. The study concluded that people living in these types of areas had

a higher risk of developing disease from *N. mikurensis* infections (Quarsten et al., 2021). In addition to Norway and Sweden, other countries that have reported human neoehrlichiosis are Germany, Switzerland, the Czech Republic (Fehr et al., 2010; Pekova et al., 2011; von Loewenich et al., 2010), with recent cases from France and Slovenia (Boyer et al., 2021; Lenart et al., 2021). *I. ricinus* is the primary vector for *N. mikurensis* and has a prevalence that varies from 0.1% to 24.3% in Europe (Portillo et al., 2018). Unlike humans, rodents do not seem to develop disease when infected with *N. mikurensis* (Wennerås, 2015); in fact, bank voles (*Myodes glareolus*) and field mice (*Apodemus flavicollis* and *A. sylvaticus)* have proven to be the main reservoir hosts for the pathogen given their ability to transmit the pathogen to ticks (Burri et al., 2014). On the other hand, studies have failed to detect *N. mikurensis* in shrews (*Sorex araneus, S. minutus* and *Neomys fodiens*) and concluded that they are most likely incompetent vertebrate hosts (Andersson & Råberg, 2011).

Previous studies have shown that *N. mikurensis* is considered the second most common tick-borne pathogen after *B. afzelii* in Norway (Jenkins et al., 2019; Kjelland et al., 2018). The pathogen is abundant mainly along the coast in the south, east, and north, but the prevalence diminishes along the southwestern part of Norway and is sometimes entirely absent (Jenkins et al., 2019; Kjelland et al., 2018; Larsson et al., 2018; Pedersen et al., 2019). The prevalence of *N. mikurensis* in *I. ricinus* ticks along the coast of Norway was reported to vary from 0% to 25.5% in nymphal and adult ticks (Pedersen et al., 2019).

1.2.2 *Borrelia afzelii*

The tick-borne pathogen *B. afzelii and* the other *Borrelia* genospecies (see section 1.1 *Ixodes ricinus*; vector of tick-borne diseases) make up the *Borrelia* species complex named after the scientist Willy Burgdorfer. In 1982, Burgdorfer and colleagues were the first to discover the causative agent(s) of Lyme borreliosis/disease, which is today recognized as the most common tick-borne disease in the northern hemisphere (Burgdorfer et al., 1982), with more than 360.000 reported cases in Europe over the last two decades (World Health Organisation [WHO], 2014). The *Borrelia* genus belongs to the family Spirochaetaceae, order Spirochaetales (Stanek et al., 2012) and are thin, elongated, gram-negative bacteria (Burgdorfer et al., 1982;

Takayama et al., 1987). The different *Borrelia* genospecies are usually connected with various symptoms (Stanek et al., 2012), where *B. afzelii* is known to cause a skin rash called acrodermatitis chronica atrophicans (ACA) in patients (Canica et al., 1993). Unfortunately, the Norwegian Surveillance System for Communicable Diseases (MSIS) does not have a complete and reliable dataset concerning reported ACA cases in the country (Eirik Olsen, Pers. comm.). In 2021, MSIS reported 536 cases of systemic *Borrelia* infections in Norway, which is higher than previous annual registrations (Norwegian Surveillance System for Communicable Diseases [MSIS], 2021). That same year, the two counties, Vestland and Agder, had the highest number of reported cases (MSIS, 2021). *I. ricinus* is the main vector of *B. afzelii* in Norway, and southern/southeastern Norway is an *I. ricinus* endemic area (Norwegian Institute of Public Health [NIPH], 2019b). People living in these areas could thereby be at a higher risk of developing a *Borrelia* infection (Kjelland et al., 2018).

Rodents are the main reservoir hosts for most *Borrelia* genospecies in Scandinavia (Rizzoli et al., 2014). Bank voles and field mice are proven to transfer *B. afzelii* to *I. ricinus* ticks (Burri et al., 2014; Humair et al., 1999; Pérez et al., 2012) making them the most important reservoir hosts for the pathogen. Various research has linked *Borrelia* to the common shrew (*Sorex araneus*), suggesting even that they are compatible reservoir hosts for the pathogen (Tälleklint & Jaenson, 1994). On the other hand, Humair et al. (1993) stated that the status of the common shrew and its role as a reservoir for *Borrelia* species is unclear. Moreover, studies that focus on the genospecies *B. afzelii* in relation to shrews as possible reservoir hosts are unfortunately lacking. Although several studies have shown that shrews have been infected with *B. afzelii* (Hellgren et al., 2011; Mysterud et al., 2019a; Zhong et al., 2019), the presence of a pathogen in a vertebrate host does not confirm that it meets the criteria of being a reservoir host as described by Kahl et al. (2002).

The genospecies *B. afzelii* is mainly distributed in Europe and parts of Asia (Stanek et al., 2012). Prevalence studies on *Borrelia* genospecies in Norway have been done in the south, the east, and near the arctic circle (Hvidsten et al., 2014, 2015, 2020; Jenkins et al., 2001; Kjær et al., 2020; Kjelland et al., 2010, 2018). Current data from these areas have shown that *B. afzelii* is the most common borrelia genospecies found in *I. ricinus* ticks, with a prevalence that varied

from 14 to 19%[1] in nymphal and adult ticks (Hvidsten et al., 2015; Jenkins et al., 2001; Kjelland et al., 2010). Still, nearly no prevalence studies on *B. afzelii* in *I. ricinus* ticks have been done in southwestern Norway. Further studies are therefore necessary since the vector *I. ricinus* is already known to exist along the west coast.

1.2.3 Co-infections

Various pathogens can simultaneously infect an individual tick, such as *I. ricinus* (Ginsberg, 2008; Swanson et al., 2006). Therefore, these pathogens might interact, creating either positive or negative associations with each other (Andersson et al., 2014a). Positive associations are described as pathogens supporting each other, while negative associations are seen as pathogens competing (Ginsberg, 2008). Positive associations can intensify symptoms in humans and animals (Belongia, 2002; Thomas et al., 2001). This is seen in patients co-infected with *B. burgdorferi* and *A. phagocytophilum*, having more symptoms and longer disease duration compared to patients infected with only *B. burgdorferi* (Krause et al., 2002). Co-infections with *Borrelia* and *A. phagocytophilum* in vertebrate hosts and ticks are more frequent than expected by random chance (Nieto & Foley, 2009). The thought behind this might be that *A. phagocytophilum* manipulates immune responses and thereby makes the host more susceptible to a *B. burgdorferi* infection (Holden et al., 2005; Thomas et al., 2001). On the other hand, negative associations between pathogens have been studied in field voles *(Microtus agrestis)*, showing that *A. phagocytophilum* infections reduced the risk of acquiring a *Bartonella* infection (Telfer et al., 2010). Interactions between pathogens can be complicated and difficult to demonstrate, considering that positive and negative associations can also be affected by the host's susceptibility to infections (Andersson et al., 2014a).

In 2014, Andersson et al., (2014b) reported that an *I. ricinus* tick that had bitten a human in Romania was infected with both *N. mikurensis* and *B. afzelii*. In addition, several other studies have reported that *I. ricinus* ticks have been co-infected with the two pathogens, with a prevalence higher than expected by random chance (Andersson et al., 2013; Jenkins et al.,

[1] The prevalence was not stated in the articles by Hvidsten et al., 2015 and Kjelland et al., 2010. The prevalence was therefore calculated using data in the articles. Prevalence calculations for individual ticks are mentioned in the method section of this study.

2001; Kjelland et al., 2018). Taking these facts into consideration together with that the prevalence of *N. mikurensis* is low along the southwest coast, it is thus natural to wonder whether the low prevalence of *N. mikurensis* is paralleled with a low prevalence of *B. afzelii* that hints toward a possible co-occurrence of the two pathogens along the west Norwegian seaboard.

1.3 Laboratory methods

1.3.1 Real-time PCR

Polymerase chain reaction (PCR) is a widely used molecular biology technique that serves to amplify a region of interest on a DNA molecule strand and replicate it into thousands to millions of copies (Joshi & Deshpande, 2010). The method allows the detection of viruses, fungi, parasites, and bacteria (Evans, 2009) such as tick-borne pathogens. Real-time PCR is a method based on conventional PCR but is known to be more accurate, sensitive, and less time-consuming (Navarro et al., 2015). The basic principle behind real-time PCR is that the DNA products can be analyzed in "real-time" meaning that the results are visualized on a screen while the PCR reaction is ongoing (Joshi & Deshpande, 2010). This process is accomplished by using fluorescence emitters (such as SYBR Green or probes) that interact with the amplified DNA product and emits signals detectible by an instrument (Joshi & Deshpande, 2010). In addition to DNA product and fluorescence emitters, some of the other components needed for a successful real-time PCR reaction are: forward/reverse primers, DNA polymerase, and dNTPs (consists of the DNA base pairs adenine (A), guanine (G), cytosine (C) and thymine (T)) (Evans, 2009). The fluorescent emitter used in this master thesis is SYBR Green. Therefore, the steps in a real-time PCR reaction will be explained with the last mentioned and not probes.

Denaturation, annealing, and extension are the three basic steps in a PCR reaction; in the first step, the double-stranded DNA is separated into two single-stranded DNA templates at high temperature (90-97°C). During the second step, temperature decreases (40-65°C), primers attach to each of the two separated strands of the DNA template, and DNA polymerase

subsequently binds to the primers. In the last step, the temperature rises (70-72°C) to activate the DNA polymerase, which starts to create a new DNA strand in 5'3' direction, using dNTPs as building blocks that are complementary to the DNA template (Evans, 2009; Joshi & Deshpande, 2010). In the case of real-time PCR, the extension step will have the same temperature as the annealing step, which is usually 60°C (Thermo Fisher Scientific, 2014).

In the last step (Figure 3), SYBR Green will attach to the newly synthesized double-stranded DNA and emit fluorescence signals detectible by the instrument. When the three steps of denaturation, annealing, and extension are repeated from 30-50 cycles, the amplified DNA products can be measured by detecting the intensity of fluorescence generated at the end of each PCR cycle (Evans, 2009).

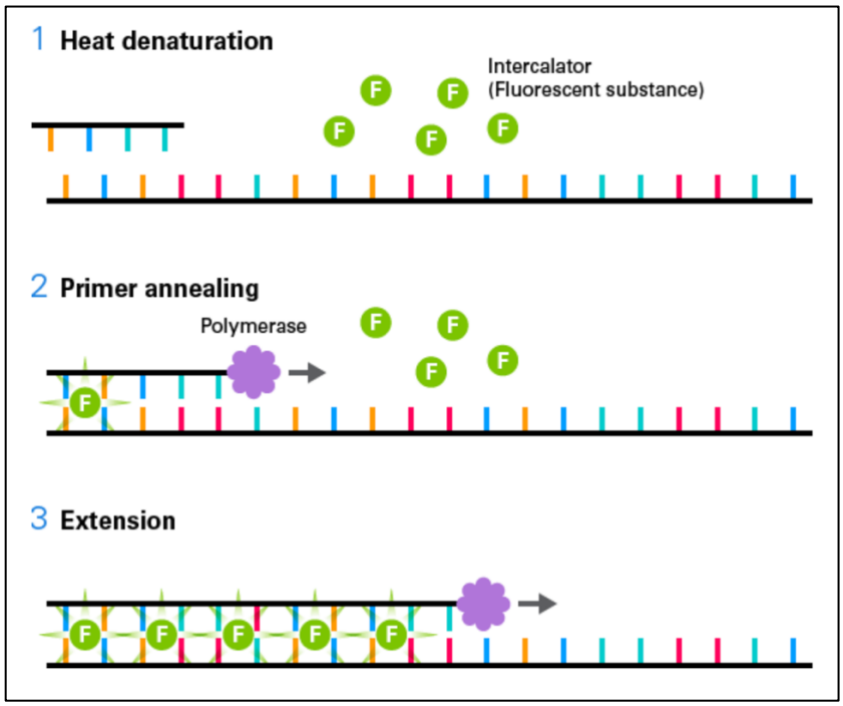

Figure 3: Real-time PCR with intercalator/fluorescence emitter SYBR Green as detection method (Takara Bio, https://www.takarabio.com/learning-centers/real-time-pcr/overview/tb-green-based-qpcr-kits)

1.3.2 Specificity and sensitivity of real-time PCR

Optimization of parameters affecting the specificity and sensitivity of a real-time PCR assay is highly needed to provide robust and reliable data (Zhao et al., 2021). Well-designed primers ensure the specificity of the real-time PCR assay by amplifying only the target organism (Ye et al., 2012). However, a primer's specificity depends also on choosing the right annealing temperature (Evans, 2009). As described in the previous section, primers bind to the two complementary DNA strands during the annealing step (Evans, 2009; Joshi & Deshpande, 2011). An annealing temperature that is too low or too high might amplify undesired products and lead to the formation of primer-dimers. Therefore, optimization of the annealing temperature is needed for the target-specific primers to anneal with the sequence of interest (Evans, 2009). Another parameter that can affect the specificity of a real-time PCR assay is the detection method (Dye-or probe-based). As previously stated, SYBR Green binds to double-stranded DNA (Evans, 2009). Although SYBR Green is a highly sensitive detection method (Espy et al., 2006), it is non-specific and binds to all double-stranded DNA sequences (such as primer-dimers), which can increase the risk of false-positive signals (Applied biosystems, 2010 ; Yin et al., 2001). Since non-specific products can potentially form during a real-time PCR assay (Chou et al., 1992), a melt curve analysis should be conducted to check for the specificity of the amplified products, especially when SYBR Green is used (Applied biosystems, 2010; Navarro et al., 2015). Melt curves are plots of data collected during the real-time PCR process displayed as peaks on a screen. These peaks can identify the target organism's melting temperature (Tm), non-specific amplification products, and/or other unexpected peaks such as contamination and primer-dimers (Applied Biosystems, 2010). Since non-specific products and primer-dimers are denatured at lower temperatures than specific products (Ririe et al., 1997), it is possible to distinguish between specific and non-specific amplification (Thermo Fisher Scientific, 2014).

The analytical sensitivity of a real-time PCR assay needs to be high to detect the lowest amount of target product in a sample (Bustin et al., 2009; Evans, 2009). The analytical sensitivity might depend on the nature of the template used in the assay; the analytical sensitivity was higher when using DNA than when using cDNA, regardless of the target gene used (Lager et al., 2017). Furthermore, using multiple positive controls in a real-time PCR setup is essential to

ensure appropriate detection sensitivity of the target and thereby reduce the risk of obtaining false-negative data (Evans, 2009). When using the same positive controls in the same type of experiments, similar Ct values are favorable to indicate consistency between real-time PCR runs (Moldovan & Moldovan, 2020). On the contrary, inconsistency might lead to an underestimation of prevalence. In addition, inhibitors (such as blood) present in the sample material can interfere with PCR amplification. Inhibitors may also affect the prevalence estimate due to false-negative results (Sidstedt et al., 2018, 2020).

Aims of study

1. Analyze samples for *B. afzelii* with real-time PCR and calculate its prevalence in *I. ricinus* ticks.

2. Investigate whether *B. afzelii* and *N. mikurensis* in *I. ricinus* co-occur along the Norwegian coast by comparing their prevalence at different locations.

2 Materials and methods

2.1 Tick samples and study area

In total, 42 samples were included in establishing the species-specific PCR for the detection of *B. afzelii.* The samples contained genomic DNA (gDNA) extracted from *I. ricinus* ticks, collected by flagging (Hillyard, 1996) on Langøya, Bamble county, in 1998, by Andrew Jenkins and co-workers (Andrew Jenkins, Pers. Comm.) Individual nymphal and adult ticks were extracted by heating in ammonium hydroxide. The method has previously been described by Guy & Stanek, (1991) and Morán-Cadenas et al., (2007).

To investigate the co-occurrence of *B. afzelii* and *N. mikurensis*, 856 samples were analyzed (185 adult ticks and 671 nymph pools). The samples had been extracted prior to this study. They contained a mixture of gDNA, RNA, and cDNA (hereafter called DNA extracts) extracted from questing *I. ricinus* ticks collected from 10 locations along the coast of Norway. The locations were: Spjærøy (59.1°N 10.9°E), Hille (58.0°N 7.4°E) , Kjosavik (58.9°N 5.9°E) , Talgje (59.1°N 5.8°E), Einevika (60.7°N 5.6°E), Florø (61.6°N 5.3°E), Lote (61.9°N 6.1°E), Kanestraum (63.1°N 8.1°E), Rørvik (64.9°N 11.1°E) and Brønnøysund (65.4°N 12.1°E). The ticks were collected by flagging (Hillyard, 1996) between 2014 and 2016. The vegetation of the locations was described as “moist deciduous forests with rich undergrowth, where traces of rodents and cervids were often observed” (Pedersen et al., 2019). Nymphs were extracted and then analyzed in pools of ten, while adult ticks, both male and female, were extracted then analyzed individually (Pedersen et al., 2019). The collection method, the storage, and processing of ticks have previously been described by Andreassen et al. (2012) and Paulsen et al. (2015). The samples were collected, extracted, and kindly provided by the Norwegian Institute of Public Health (NIPH).

The positive controls used in this study were gDNA extracted from cultured *B. afzelii*. The negative control was water.

2.2 Real-time PCR

2.2.1 Optimization of real-time PCR

To establish a species-specific PCR for detecting *B. afzelii*, gDNA samples from Langøya were analyzed for *B. afzelii* with real-time PCR using SYBR Green and GIII forward and reverse primers targeting the OspA gene (Table 1) (Demaerschalck et al., 1995). The PCR reaction mix used for the real-time PCR setup was developed by Andrew Jenkins and contained 100 µl of each GIII primer mix, 625 µl of SYBR Green, and 175 µl of nuclease-free water. The reaction volume was 20 µl of PCR reaction mix and 5 µl of sample material. Five different annealing temperatures were tested to determine the optimal annealing temperature for primer specificity. The annealing temperatures tested were 60°C, 62°C, 63.5°C, 64°C, and 65°C, where 65°C was rechecked for a second time. The real-time PCR conditions are described in Table 2. The same samples were used for each real-time PCR run when testing the annealing temperatures. Positive and negative controls were included in each setup.

A sample was considered positive when the threshold cycle (Ct) was under 45 cycles and the melting temperature (Tm) was within a range of ±1.5°C from the Tm of the positive control. Since SYBR Green can bind inaccurately, a melt curve analysis was done to confirm the Tm of the target gene. Real-time PCR was conducted on Applied Biosystems StepOne™ real-time PCR System, and the results were viewed in StepOne™ Software v2.3.

The PCR setup was always performed in three separate rooms while using gloves to avoid contamination. See Appendix 8.1 for a detailed description of the method in laboratory protocol.

Table 1: Primers used in OspA real-time PCR for detection of B.afzelii (Demaerschalck et al., 1995).

Primer	Sequence
GIII forward primer	5'TAAAGACAAAACATCAACAGATGAAATG
GIII reverse primer	5'TTCCAATGTTACTTTATCATTAGCTACTT

Table 2: OspA real-time PCR conditions 60℃, 62℃, 63.5℃, 64℃, 65℃.

Stages	Cycles	Temperature (°C)	Time (min)
Holding stage	1	50.0 95.0	02:00 10:00
Cycling stage	45	95.0 60.0/62.0/63.5/64.0/65.0*	00:15 01:00
Melt curve stage	1	95.0 60.0/62.0/63.5/64.0/65.0* 0.2 (temperature increment) 90	00:15 01:00 00:15

*65°C was the annealing temperature used to detect *B. afzelii* in ticks from the coast of Norway.

2.2.2 Detection of *B. afzelii*

DNA extracts from ticks collected at 10 different locations along the coast of Norway were analyzed with a *B. afzelii*-specific real-time PCR. Since most of the tubes contained little sample material, 10 µl of water was added to each tube. The real-time PCR method (see Table 1 for primer sequences), the components of the PCR reaction mix, and the reaction volume are mentioned in section 2.2.1. Based on the optimization of annealing temperature, 65°C was

chosen as the annealing temperature for detecting *B. afzelii* (see Table 2, section 2.2.1 for real-time PCR conditions). Positive and negative controls were again included in each setup. The criteria by which the sample was considered positive, and the real-time PCR instrument used are the same as described in section 2.2.1. A melt curve analysis was performed for each sample. The PCR setup was as usual conducted in three separate rooms while using gloves to avoid contamination.

2.3 Statistics

2.3.1 Prevalence calculations

Epitools epidemiological calculator (Cowling et al., 1999) was used for calculating an estimated pooled prevalence (EPP) with confidence intervals for pooled nymphs.

The prevalence for individual ticks was calculated with 95% confidence intervals, using the formulae:

$$P_L = \frac{\left(2np + z_{\alpha/2}^2 - 1\right) - z_{\alpha/2} \cdot \sqrt{z_{\alpha/2}^2 - \{2 + (1/n)\} + 4p(nq + 1)}}{2\left(n + z_{\alpha/2}^2\right)}$$

and

$$P_U = \frac{\left(2np + z_{\alpha/2}^2 + 1\right) + z_{\alpha/2} \cdot \sqrt{z_{\alpha/2}^2 + \{2 - (1/n)\} + 4p(nq - 1)}}{2\left(n + z_{\alpha/2}^2\right)}$$

where P_L and P_U are the upper and lower confidence limits, respectively. n is the number of samples, p and q are the proportions of positive and negative samples, and $z_{\alpha/2}$ is the critical value of the normal distribution for α/2, in this case, 1.96. If p or q $\leq$ 5/n, the confidence limit was not valid (Fleiss, 1981; Jenkins et al., 2019).

2.3.2 Chi-square test

A chi-square test was performed to look for statistical differences in *B. afzelii* prevalence between areas with high prevalence and areas with low prevalence. Locations were combined as seen in Table 3. Because the total number of pooled samples from Spjærøy was much higher than that from the other locations, 50/356 pooled nymph samples from Spjærøy were randomly selected and included in the test. A probability of ≤ 0.05 was considered as statistically significant for every chi-square test in this study.

Table 3: Locations of high and low B. afzelii prevalence in adult ticks and pooled nymphs.

	High prevalence area	**Positive ticks/total ticks analyzed**	**Low prevalence area**	**Positive ticks/total analyzed ticks**
Adult ticks	Hille and Spjærøy.	25/140	Kanestraum, Florø and Einevika	3/45
Pooled nymphs	Brønnøysund, Rørvik, Hille and Spjærøy	111/132	Lote, Florø, Einevika, Talgje, Kjosavik	96/233

2.3.3 Co-infections

132/185 individual adult ticks were investigated for co-infections. Positive *B. afzelii* samples were checked if they had previously been positive with *N. mikurensis*. Real-time PCR results from the *N. mikurensis* study by Pedersen et al. (2019) were kindly provided by Benedikte Pedersen. A chi-square test was performed to look for over-or underprevalence of co-infections.

3 Results

3.1 Optimization of real-time PCR

The species-specific real-time PCR assay with an annealing temperature of 65°C had higher Ct values (Ct= 30-42) than the assays with an annealing temperature below 65°C (Ct=15-31). The assay with the highest annealing temperature was viewed as less sensitive than the other assays. On the other hand, it showed greater specificity to the target gene as it was the only assay with no false-positive results; the number of false-positive samples registered when using an annealing temperature of 60°C, 62°C, 63.5°C, 64°C, and 65°C was 34/42 (81%), 29/42 (69%), 20/42 (48%), 13/42 (31%) and 0/42 (0%) respectively. 65°C was thereby chosen as the annealing temperature for detecting *B. afzelii*. The Tm of the positive controls was 74°C ±1.5°C (Figure 4).

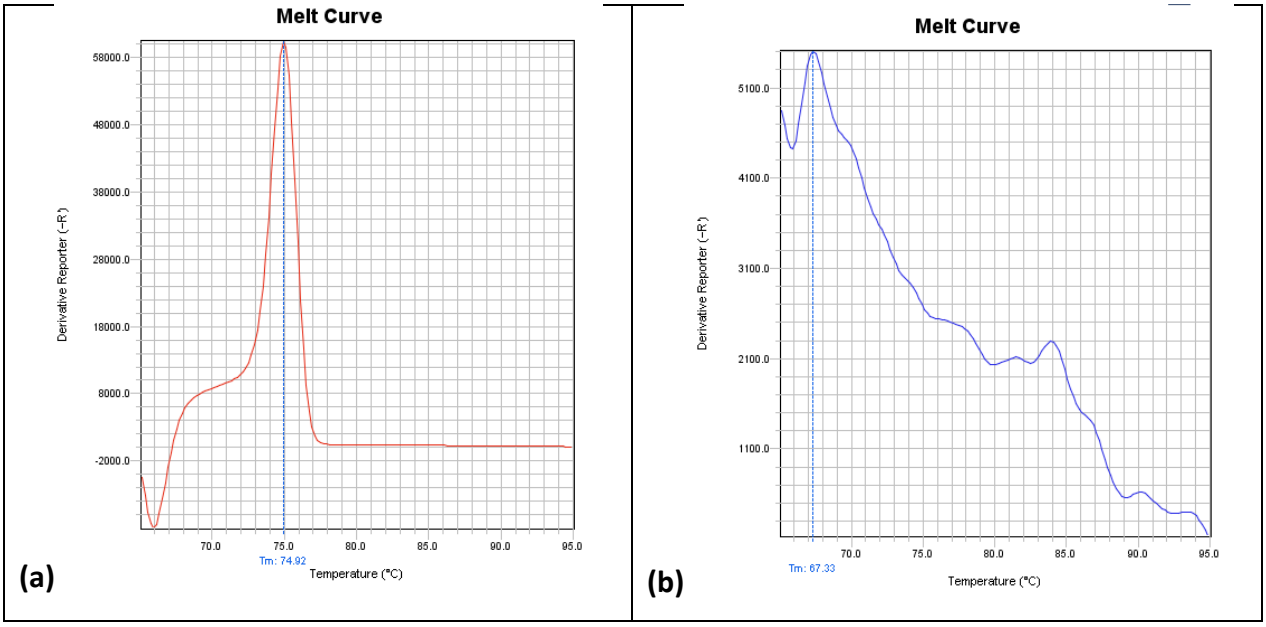

Figure 4: Melt curves of two samples in a real-time PCR analysis with an annealing temperature of 65℃. (a) Melting temperature of B. afzelii (Tm= 74.92), corresponding to a positive sample or control. (b) Melting temperature different to that of the positive control (Tm= 67.33), corresponding to a negative sample or control.

3.2 Prevalence of *B. afzelii*

In total, 28/185 adult ticks (15%) and 472/671 nymph pools (EPP 11.4%) tested positive for *B. afzelii* with real-time PCR using SYBR Green (Table 4; Figure 5). Figure 5 was made to visualize the results at each location. The highest prevalence of *B. afzelii* was found in ticks along the coast in the northwest (Brønnøysund and Rørvik), south (Hille), and southeast (Spjærøy) of Norway; the EPP of *B. afzelii* in nymphs was 19.7% in Brønnøysund (L1) and 18.8% in Rørvik (L2). The prevalence at Hille (L9) was 20% in adult ticks and 18.6% (EPP) in nymphs. Spjærøy (L10) had a prevalence of 17% in adult ticks and 17.5% (EPP) in nymphs. The intermediate area, from Kanestraum (L3) to Kjosavik (L8), had a prevalence that varied from 0 to 4% in adult ticks and from <0,6 to 10,2% (EPP) in nymphs. The lowest prevalence was measured in nymphs from Talgje (L7) and Kjosavik (L8), with an EPP of <0,6% and 1%, respectively.

The chi-square test revealed that there was a significant difference in *B. afzelii* prevalence for pooled nymphs between high and low prevalence areas (χ^2 = 63.1; *df* = 1; $p < 0.05$). Statistical significance was not achieved for adult ticks (χ^2= 3.2; *df* = 1; $p > 0.05$). See Appendix 8.2.1 for chi-square tests.

Table 4: Prevalence of Borrelia afzelii in Ixodes ricinus ticks

Seaboard	Location number	Location name	*Borrelia afzelii* in adult ticks		*Borrelia afzelii* in nymphs	
			Positive ticks/total adult ticks analyzed	Prevalence % (confidence intervals)	Positive pools of nymphs/total pools analyzed[b]	EPP%[a] (confidence intervals)
Northwest	L1	Brønnøysund	-	-	24/27	19.7 (11.6 - 31.3)
	L2	Rørvik	-	-	7/8	18.8 (6.2 - 43.8)
Southwest	L3	Kanestraum	1/24	4[c]	-	-
	L4	Lote	-	-	40/72	7.8 (5.5 - 10.6)
	L5	Florø	2/19	11[c]	35/53	10.2 (7.0 - 14.2)
	L6	Einevika	0/2	0	19/39	6,5 (3.8 – 10.0)
	L7	Talgje	-	-	0/48	<0,6%
	L8	Kjosavik	-	-	2/21	1.0 (0.1 - 3.5)
South	L9	Hille	10/51	20 (8-31)	41/47	18.6 (12.7 - 26.1)
Southeast	L10	Spjærøy	15/89	17 (9-25)	304/356	17.5 (15.4 - 19.7)
		Total	28/185	15 (10-21)	472/671	11.4 (10.4 – 12.5)

[a]Abbreviation: EPP, estimated pooled prevalence.
[b]Each pool consists of 10 nymphs.
[c]Confidence intervals could not be calculated.
"-" means no samples available.

3.1 Comparing *B. afzelii* and *N. mikurensis* prevalence

The total prevalence of *B. afzelii* and *N. mikurensis* was 15% and 13% in adult ticks, and 11.4% and 5% (EPP) in pooled nymphs, respectively (Table 5). The total prevalence of *N. mikurensis* was recalculated using data from (Pedersen et al., 2019) for the purpose to compare it with the total prevalence of *B. afzelii.* See Figures 5 and 6 for a visualized comparison of the results.

B. afzelii and *N. mikurensis* co-occurred in *I. ricinus* ticks at seven out of ten locations (Brønnøysund, Kanestraum, Florø, Einevika, Talgje, Hille and Spjærøy). *B. afzelii* and *N.*

mikurensis were present in ticks from Børnnøysund (L1) with a respective prevalence of 19.7% (EPP) and 7.9% (EPP). The prevalence of *B. afzelii* in nymphs from Rørvik (L2) was 18.8% (EPP), while that of *N. mikurensis* was 0% (EPP). From Rørvik (L2) to Kjosavik (L 8), the prevalence of *N. mikurensis* was <5% (EPP) in nymphs (Pedersen et al., 2019) whereas the prevalence of *B. afzelii* varied from <0.6% to 18.8% (EPP). The prevalence of *N. mikurensis* in adult ticks was higher than that of *B. afzelii* at two locations; Einevika (L6; *N. mikurensis*: 15.4%; *B. afzelii*: 0%) and Hille (L9; *N. mikurensis*: 25.5%; *B. afzelii*: 20%). At Spjærøy (L10), the prevalence of *B. afzelii* in adult ticks (17%) and pooled nymphs (EPP 17.5%) was higher than that of *N. mikurensis* (14.6% and EPP 10.2%).

Table 5: Prevalence of B. afzelii and N. mikurensis at the 10 locations investigated. The prevalence of N. mikurensis is illustrated with data from Pedersen et al. (2019)

Seaboard	**Location number**	**Location name**	**Prevalence % in adult ticks (confidence intervals)**		**EPP %[a] in nymphs (confidence intervals)**	
			B. afzelii	***N. mikurensis*[b]**	***B. afzelii***	***N. mikurensis*[b]**
Northwest	L1	Brønnøysund	-	-	19.7 (11.6 - 31.3)	7.8 (2.4-18.0)
	L2	Rørvik	-	-	18.8 (6.2 - 43.8)	0
Southwest	L3	Kanestraum	4[c]	3.3[c]	-	-
	L4	Lote	-	-	7.8 (5.5 - 10.6)	0
	L5	Florø	11[c]	6.5[c]	10.2 (7.0 - 14.2)	4.7 (2.9-7.0)
	L6	Einevika	0	15.4[c]	6,5 (3.8 – 10.0)	3.1 (1.7-5.0)
	L7	Talgje	-	-	<0,6%	0.4 (0.1-1.5)
	L8	Kjosavik	-	-	1.0 (0.1 - 3.5)	0
South	L9	Hille	20 (8-31)	25.5 (14.8-39.9	18.6 (12.7 - 26.1)	9.9 (6.1-14.0)
Southeast	L10	Spjærøy	17 (9-25)	14.6 (9.6-21.4)	17.5 (15.4 - 19.7)	10.2(8.9-11.5)
		Total	15 (10-21)	13 (9-17)[d]	11.4 (10.4 – 12.5)	5 (4.5-5.6)[d]

[a]Abbreviation: EPP, estimated pooled prevalence (each pooled consists of 10 nymphs).
[b]Prevalence data from the article by Pedersen et al., (2019).
[c]Confidence intervals could not be calculated.
[d]The total prevalence of *N. mikurensis* from the article was recalculated.
"-" means no data.

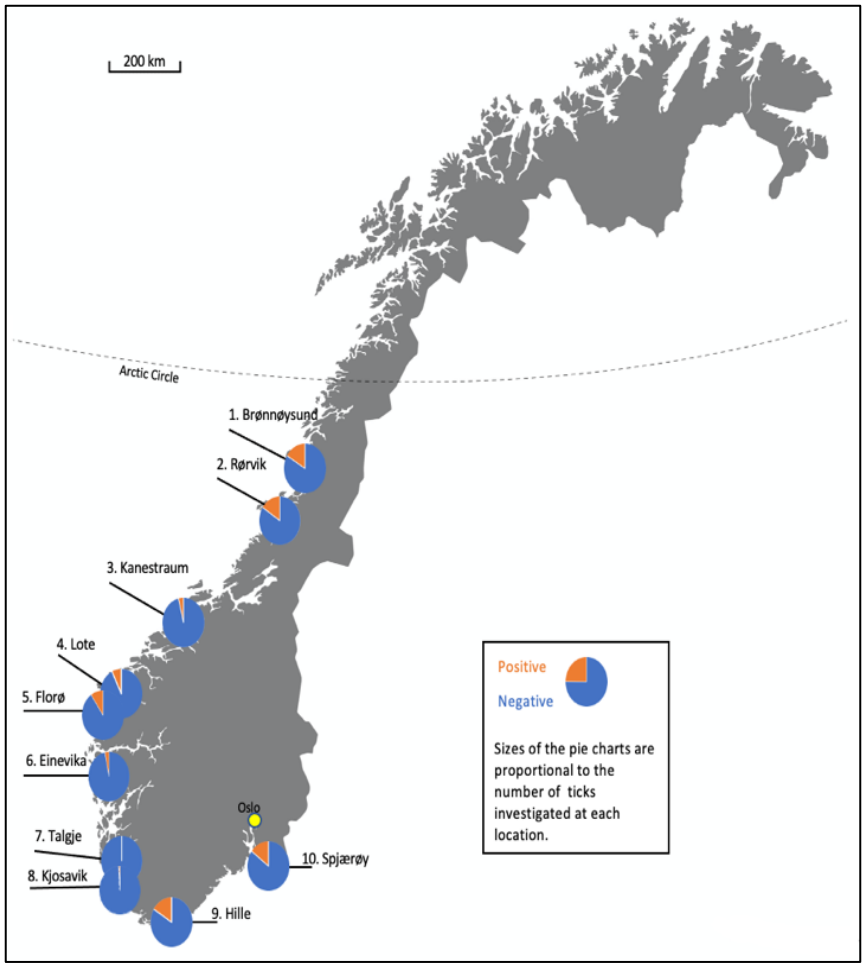

Figure 5: Map of Norway showing the 10 locations where Ixodes ricinus was collected. The positive parts of the pie charts are the mean prevalence of B. afzelii in adult ticks and nymph pools. The prevalence was added without calculating the mean if a location had a single prevalence. Illustration: Jamila Synnøve Saber.

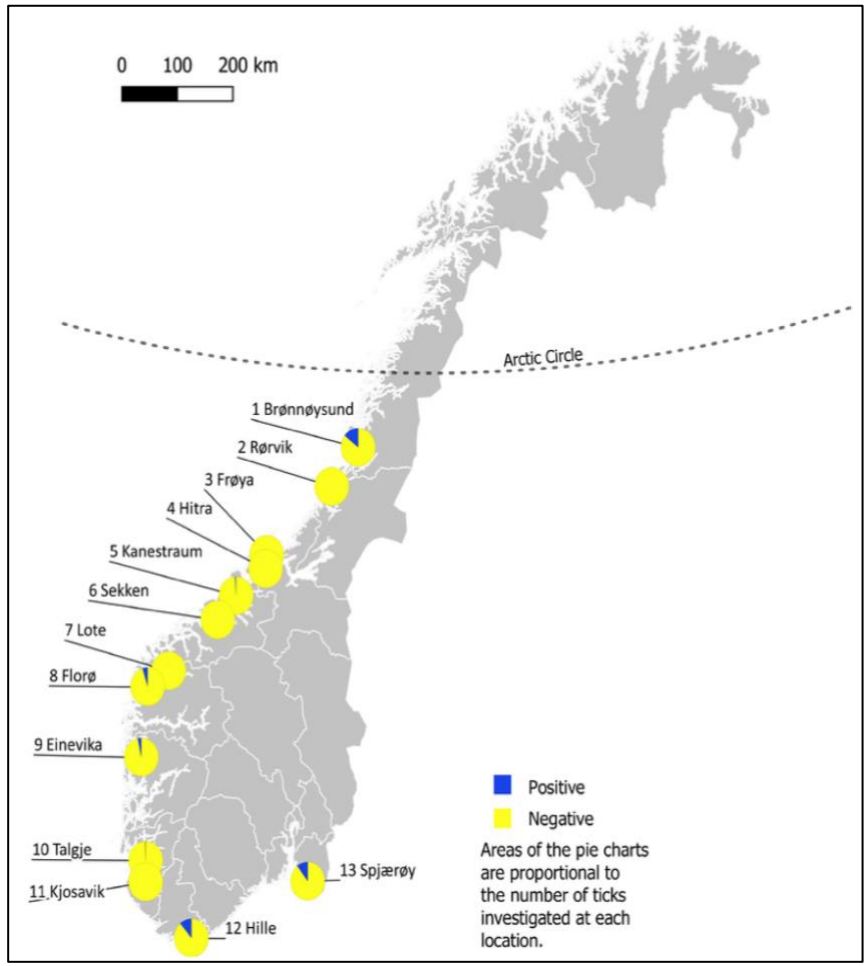

Figure 6: Map of Norway showing the prevalence of N. mikurensis in I. ricinus ticks collected at different sites along the Norwegian coast (Pedersen et al., 2019). The figure was first illustrated in the article by Pedersen et al., 2019. Permission to use figure by Andrew Jenkins and Benedikte Pedersen.

3.2 Co-infections

6/132 (5%) individual adult ticks were co-infected with *B. afzelii* and *N. mikurensis.* The chi-square test revealed that the co-infections occurred more than twice than expected under random chance (expected 2%) and with a significant result (χ^2= 4.7; *df* = 1; $p < 0.05$). See Appendix 8.2.2 for chi-square test.

4 Discussion

This study investigated the prevalence of *B. afzelii* in *I. ricinus* ticks at 10 locations along the Norwegian coast. Then the prevalence of *B. afzelii* was compared to that of *N. mikurensis* to investigate whether the pathogens co-occurred and whether the low prevalence of *N. mikurensis* was paralleled with a low prevalence of *B. afzelii* along the southwest coast.

4.1 Prevalence of *B. afzelii*

The study's first aim was to investigate the prevalence of *B. afzelii* in *I. ricinus* ticks collected along the Norwegian coast. The prevalence of *B. afzelii* was the highest in the northwest (Brønnøysund and Rørvik) and the south/southeast (Hille and Spjærøy). Previous studies reported that the Brønnøysund area had a high abundance of *Borrelia* genospecies in adult and nymphal ticks, with *B. afzelii* being the dominant one (Hvidsten et al., 2014, 2015). In this study, the prevalence of *B. afzelii* in nymphal ticks from Brønnøysund was 19.7% (EPP), which is similar to what was reported by Hvidsten et al. (2015). The pathogen has previously been found in ticks from Rørvik (Hvidsten et al., 2020), but its prevalence was lower compared to this study's results. The prevalence of *B. afzelii* in nymphs from Rørvik (EPP 18.8%) was almost equal to the prevalence in nymphs from Brønnøysund (EPP 19.7 %), which could mean that factors influencing the prevalence of *B. afzelii* might have been similar at these locations.

The south of Norway is an *I. ricinus* endemic area (Norwegian Institute of Public Health [NIPH], 2019b), with several reported instances of systemic Lyme disease each year (Norwegian Surveillance System for Communicable Diseases [MSIS], 2021). The prevalence of *Borrelia* in ticks in southern Norway is thus high, and *B. afzelii* has been marked as the dominant genospecies (Kjelland et al., 2018). In this study, the prevalence in nymphs from Hille and Spjærøy was 18.6% (EPP) and 17.5% (EPP), respectively. This matches the findings from a previous study by Jenkins et al. (2001) and Kjelland et al. (2010). However, the prevalence of *B. afzelii* in adult ticks from Hille and Spjærøy in this study was higher than what has earlier been reported from southern Norway (Jenkins et al., 2001; Kjelland et al., 2010). Tick

collection dates vary between studies, and a difference in reported prevalence may be due to year-to-year seasonal and climatic conditions (Estrada-Peña et al., 2012). An additional reason could be a difference in the sensitivity of the detection method.

A drop in prevalence was seen between Rørvik and Kanestraum, from 18.8% (EPP) to 4%. The drop in prevalence may indicate that conditions affecting the prevalence at these locations differed when ticks were collected. However, this comparison is statistically not optimal because it is between pooled nymphs and individual adult ticks. In the lower-prevalence area (from Kanestraum to Kjosavik), the prevalence in nymphs varied between <0,6 and 10,2% (EPP), which is significantly lower (χ^2 = 63.1; *df* = 1; $p < 0.05$) compared to the northern (Brønnøysund and Rørvik) and the southern locations (Hille and Spjærøy) which had a much higher prevalence (from 17.5% to 19.5% (EPP)). The prevalence in adult ticks in the lower-prevalence area varied from 0 to 4%, which was also lower compared to the high prevalence locations Hille (20%) and Spjærøy (17%). Although the prevalence of adult ticks had a similar trend to that of pooled nymphs, the chi-square test did not meet statistical significance (χ^2= 3.2; *df* = 1; $p > 0.05$). The three locations Lote, Florø and Einevika can be described as a *B. afzelii* pocket within the low-prevalence area; the prevalence of *B. afzelii* in nymphs was higher in Lote (EPP 7.8%), Florø (EPP 10.2%), and Einevika (EPP 6.5%) compared to Talgje (EPP <0,6%) and Kjosavik (EPP 1.0%). The different prevalence between locations could have been affected by climate, seasonal variation, and availability of key hosts, as these factors are known to have an impact on tick activity (Medlock et al., 2013; Mysterud et al., 2016; Qviller et al., 2016; Randolph & Storey, 1999; Ribeiro et al., 2019; Soleng et al., 2018).

4.2 Prevalence concerning climate

The lower prevalence of *B. afzelii* between Kanestraum and Kjosavik may be linked to climatic conditions as the west of Norway receives more rain than any other area in Norway (Moen et al., 1999). Randolph & Storey (1999) conducted a semi-natural microclimatic experiment that declared a difference in ticks' questing height in wet and dry conditions. The study showed that some larvae quested higher up in the vegetation during warm and humid conditions (Randolph & Storey, 1999). It is usually stated that larvae feed on smaller mammals such as

rodents, and nymphs on rodents and medium hosts (Hofmeester et al., 2016), but ticks at these stages have also been found on larger mammals, such as deer who are believed to be incompetent vertebrate hosts for *Borrelia* (Kurtenbach et al., 2002; Tälleklint & Jaenson, 1997). If the rainy and humid weather along the west coast has had an impact on ticks' questing height remains unknown, but if so, then larvae and nymphs may reach higher up in the vegetation and feed on incompetent reservoir hosts. These types of hosts may then cause a reduction in pathogen prevalence (dilution effect) if a large number of ticks are fed (Jaenson & Tälleklint, 1992; LoGiudice et al., 2003). Moreover, it is important to note that the ticks from the area between Kanestraum and Kjosavik in this study were collected in either 2014, 2015, or 2016 over one or a few days in the summertime. Ticks should be collected over extended periods to determine how seasonal variation and microclimatic conditions may impact the prevalence. In addition, weather loggers at each location can be used to monitor the climate.

In the same study by Randolph & Storey (1999), nymphs under dry conditions quested lower in the vegetation, which allowed them to come in greater contact with rodents. On the other hand, larvae showed decreased questing activity under dry conditions, but once the humidity increased, so did their questing activity (Randolph & Storey, 1999). These climatic effects may force larvae and nymphs to quest at the same level, leading them to co-feed on the same reservoir hosts such as rodents and subsequently molt into infected nymphs and adult ticks. This can lead one to wonder whether a drier climate and co-feeding of larvae and nymphs could be a reason for a high prevalence of *B. afzelii* in the southern locations of this study; southeast Norway has the fewest days of precipitation annually and the most elevated measured temperatures from May to September (Moen et al., 1999) notably within tick activity season (Norwegian Institute of Public Health [NIPH], 2021).

Brønnøysund and Rørvik in the northwest had almost as high a prevalence as in the south/southeast (Hille and Spjærøy). However, Brønnøysund and Rørvik belong to an area that receives more rainfall than the south (Moen et al., 1999) which could mean that climate alone may not have been the only reason for the high prevalence in the northern locations of this study. *Borrelia* has a long duration of infectivity in vertebrates (weeks to months), making the opportunity for transmission greater (Randolph, 2008). The presence of vertebrate hosts might therefore be more important than seasonal and climatic conditions when it comes to

the persistence of *B. afzelii* in the tick-host-pathogen transmission cycle (Randolph, 2008). Furthermore, hosts' responses to climate and seasonal variations should also be considered.

4.3 Prevalence concerning hosts

4.3.1 Incompetent vertebrate hosts

Reservoir competence varies between vertebrate species (McCoy et al., 2013), and the biology behind why some species are more competent than others is still unclear (Wikel, 2021). The lower prevalence of *B. afzelii* along the southwest coast (from Kanestraum to Kjosavik) might be linked to an abundance of incompetent vertebrate hosts. It is well known that deer are an essential asset in the survival of tick populations as ticks usually feed on deer (Hofmeester et al., 2016; Medlock et al., 2013; Mysterud et al., 2021). Even so, deer do not seem susceptible to *Borrelia* infections (Rosef et al., 2009) and are believed to insufficiently transfer the pathogen to feeding ticks (Kahl et al., 2002). However, nymphs that have been feeding on roe deer can contain low bacterial loads of *B. burgdorferi* s.l. (Kjelland et al., 2011). A possible explanation for why deer are incompetent vertebrate hosts for the pathogen could be that their immune system can lyse *Borrelia* spirochetes in the tick's midgut, eliminating them before they are transmitted to the vertebrate host (Kurtenbach et al., 2002). The two species that are suggested to be incompetent vertebrate hosts for *Borrelia* are red deer (*Cervus elaphus*) (Matuschka et al., 1993; Telford et al., 1988) and roe deer (*Capreolus capreolus*) (Jaenson & Tälleklint, 1992). Red deer are highly abundant in Norway along the west coast, notably within this study's lower *B. afzelii*-prevalence area. Roe deer have been found in the eastern, southern (Mysterud et al., 2019b) and the western parts of the country (Rosef et al., 2009). In this study, the *B. afzelii* prevalence was high in ticks from the south/southeast and lower in ticks from the southwest. Other studies showed similar trends when investigating tick infections in relation to the distribution of wild cervids; the infection rate of *Borrelia* in *I. ricinus* ticks was lower in western areas with high densities of wild cervids (Mysterud et al., 2013; Rosef et al., 2009) whereas the infection rate was higher in eastern areas with low densities of wild cervids (Rosef et al., 2009). Moreover, a recent paper by Mysterud et al. (2019a) failed

to detect *Borrelia* spirochetes in red deer and roe deer collected in the west. These findings agree with the statement that deer are incompetent vertebrate hosts for *Borrelia* spirochetes. Still, the fact that high deer densities can reduce the prevalence of *Borrelia* has been controversial (Ogden & Tsao, 2009; Randolph & Dobson, 2012). Counter effects have been suggested implying that high deer densities naturally will harbor more adult ticks and thereby increase the number of larvae which will later feed on reservoir hosts such as rodents (Mannelli et al., 2012). This could mean that the prevalence of *B. afzelii* in ticks from the western locations of this study (Lote, Florø, Einevika, Brønnøysund, and Rørvik) is due to the availability of incompetent hosts (deer) combined with reservoir hosts (rodents).

4.3.2 Reservoir hosts

Rodents are the main transmission route for *B. afzelii* and are a key to its persistence in the environment (van Duijvendijk et al., 2016). *B. afzelii*-prevalence has been positively correlated with rodent densities (Krawczyk et al., 2020; Tälleklint & Jaenson, 1994). An abundance of rodents as reservoir hosts in the northern (Brønnøysund and Rørvik), the southern (Hille and Spjærøy), and the southwest locations (Lote, Florø, and Einevika) could therefore be a reason for the *B. afzelii* infections in the ticks of this study. In that case, areas with a high pathogenic prevalence (north and south/southeast) would naturally have higher rodent densities than areas with a lower pathogenic prevalence (Lote, Florø, and Einevika). The species that have been confirmed to be reservoir hosts for *B. afzelii* are bank voles and field mice (Burri et al., 2014; Humair et al., 1999; Pérez et al., 2012). In multiple studies, a third species, the common shrew, has also been infected with the pathogen (Hellgren et al., 2011; Mysterud et al., 2019a; Zhong et al., 2019). However, its role as a reservoir host remains to be demonstrated. These vertebrate species are widely distributed in Norway in the east, along the west coast, and bank voles have been found up to Nordland in the north (Norwegian Biodiversity Information Center [NBIC], 2022; Soleng, 2016). The western locations Lote (EPP 7.8%) and Florø (EPP 10.2%) belong to the county of Sogn and Fjordane; a study showed that bank voles and common shrews collected from the county were infected with *B. afzelii* (Mysterud et al., 2019a), confirming their distribution in that area but also their importance as vertebrate hosts for the pathogen (Mysterud et al., 2015). Moreover, all three species were infected with the

pathogen when collected in southeastern Norway (Mysterud et al., 2019a), which is a high-prevalence area in this study. The main factor for rodent distribution is food availability (Ostfeld et al., 2006). Seeds are an essential food source for rodents (Wilson et al.., 2017). Some tree species (e.g., beech and oak) do not produce seeds every year but will periodically produce enormous amounts of seeds depending on climate (Kelly & Sork, 2002). This phenomenon is called masting (Selås, 2016), and a high seed production one year has been linked to high bank vole densities the year after (Reil et al., 2015). Annual fluctuations of rodent densities are driven by seed production and climatic variations (H. P. Andreassen et al., 2021; Selås et al., 2019) and could thereby influence pathogen prevalence. These elements merit more study to understand better the role of reservoir hosts in the matter of *B. afzelii* transmission and other tick-borne pathogens.

4.4 Co-occurrence of *B. afzelii* and *N. mikurensis*

The second aim of this study was to compare the prevalence of *N. mikurensis* reported by Pedersen et al. (2019) with the prevalence of *B. afzelii* to investigate whether they co-occur along the Norwegian coast with the intention to look for whether the low prevalence of *N. mikurensis* is paralleled with a low prevalence of *B. afzelii*. The two pathogens co-occurred at seven out of 10 locations (see section 3.1, Table 5). The highest prevalence of both *B. afzelii* and *N. mikurensis* was measured in ticks collected from Brønnøysund, Hille, and Spjærøy. Rørvik was an additional high-prevalence location for *B. afzelii* (EPP 18.8%) but not for *N. mikurensis* (EPP 0%). The prevalence pattern appeared to be the same for both pathogens; their prevalence was highest in this study's most southern and northern locations and lowest at the locations in-between situated in the southwest (see section 3.1, Figure 5 and 6). The lower prevalence of the pathogens along the southwest coast could have been impacted by factors such as climate and seasonal variation, as previously discussed in section 4.2 "Prevalence concerning climate", or by less availability or absence of reservoir hosts. Because *B. afzelii* and *N. mikurensis* share common reservoir hosts, which are bank voles and field mice (Burri et al., 2014), it would be interesting to investigate the availability of these vertebrate species along the Norwegian coast and especially in the low-prevalence area. However, in the

low-prevalence area, *B. afzelii* was present in nymphs from Lote (EPP 7.8%) and Kjosavik (1.0%) whereas *N. mikurensis* was not (EPP 0%) (Pedersen et al., 2019). These findings indicate that *B. afzelli* and *N. mikurensis* co-occur at some locations along the Norwegian coast, but *B. afzelii* seems to be present at locations where no *N. mikurensis* is found. It would thereby be interesting to investigate whether *B. afzelii* has an additional reservoir host. The common shrew is widely distributed all over Norway (Wilson et al., 2018), including Brønnøysund (Hvidsten et al., 2020), and it has previously been infected with *B. afzelii* (Hellgren et al., 2011; Mysterud et al., 2019a; Zhong et al., 2019). However, studies have failed to detect *N. mikurensis* in this species and concluded that shrews were probably not reservoir hosts for the pathogen (Andersson & Råberg, 2011); moreover, when a species is not infected with a pathogen, the chance of it being a reservoir host is little (Kahl et al., 2002). Since the prevalence of *Borrelia* positively correlates with rodent densities (Krawczyk et al., 2020; Tälleklint & Jaenson, 1994), maybe the prevalence of *B. afzelii* would be higher if it was compatible with three reservoir hosts instead of two as the effective host density is then higher.

The overall prevalence of *B. afzelii* in adult ticks and pooled nymphs was higher than that of *N. mikurensis* (see section 3.1, Table 5). A Swedish study by Andersson et al. (2013) also found a higher prevalence of *B. afzelii* than *N. mikurensis* in *I. ricinus* ticks. An explanation for this could be that *B. afzelii* has a broader spectrum of reservoir hosts, as previously discussed or that the reservoir competence in vertebrate hosts is different for the two pathogens (McCoy et al., 2013). Some species are more competent reservoir hosts than others; van Duijvendijk et al. (2016) showed that bank voles had a higher bacterial load of *B. afzelii* compared to wood mice *(Apodemus sylvaticus)* and concluded that bank voles were better reservoir hosts for the pathogen. In a similar study, bank voles and shrews had higher bacterial loads than yellow-necked-mice (Apodemus falvicollis) (Zhong et al., 2019), which could mean that shrews, together with bank voles, are better reservoir hosts for *B. afzelii* than mice. Moreover, due to differences in reservoir competence between voles and mice, the infection rate of nymphal ticks who fed on bank voles was higher than that of nymphal ticks who fed on wood mice (van Duijvendijk et al., 2016). Andersson et al. (2014a) found a slightly higher prevalence of *B. afzelii* than *N. mikurensis* in bank voles. Therefore, it would be interesting to investigate whether voles, mice, and shrews have different susceptibility to *B. afzelii* and *N. mikurensis* infections.

Likewise, ticks' susceptibility to pathogen infections should also be considered, as the vectorial competence of ticks may not be the same for all tick-borne pathogens (Kahl et al., 2002; Kurtenbach et al., 2002).

Ticks will most likely acquire pathogens during a blood meal when feeding on an infected vertebrate host (Randolph, 2011). However, they can also acquire pathogens when feeding close to other infected ticks (co-feeding), even if the vertebrate host is not infected (Randolph, 2011). As a result, ticks can interact with several bacterial species, possibly leading to multiple infections within the vertebrate host and the tick (Andersson et al., 2014a; Ginsberg, 2008; Swanson et al., 2006). In this study, 6/132 (5%) of the individual adult ticks were co-infected with *B. afzelii* and *N. mikurensis.* This is higher than previously reported (Andersson et al., 2013; Kjelland et al., 2018; Jenkins et al., 2001). However, these studies investigated co-infections in individual nymphs and not in adult ticks. Comparing nymphal to adult tick co-infections is not ideal because nymphs and adult ticks differ in how many blood meals they have had (Apanaskavich & Oliver, 2014) and may feed on different vertebrate hosts (Hofmeester et al., 2016). Unfortunately, nymphs could not be investigated for co-infections in this study because they were pooled. Nonetheless, co-infections in ticks with *B. afzelii* and *N. mikurensis* should be of concern, as infections with multiple pathogens can intensify symptoms in humans and animals (Belongia, 2002; Thomas et al., 2001). This was, for instance, seen in patients infected with *A. phagocytophilum* and *B. burgdorferi* which had a more severe infection than patients with a single *B. burgdorferi* infection (Krause et al., 2002). So far, there has only been one reported case of neoehrlichiosis in the country (Frivik et al., 2017), and most Lyme disease cases have been reported in patients from southern Norway (Norwegian Surveillance System for Communicable Diseases [MSIS], 2021). It is thereby possible that infections in the rest of the country may have been overlooked or underdiagnosed as the prevalence of both *B. afzelii* and *N. mikurensis* is also high in the Brønnøysund-area, as seen in this study and in Pedersen et al. (2019). Even though pathogen associations can be difficult to demonstrate (Andersson et al., 2014a), co-infections with these pathogens in ticks should be of clinical concern and investigated in future research.

Studies showed that co-infections in ticks with *B. afzelii* and *N. mikurensis* were higher than expected by random chance (Andersson et al., 2013; Jenkins et al., 2001; Kjelland et al., 2018),

suggesting that there may be an ecological link between the two pathogens (Andersson et al., 2013, 2014a). This study, combined with the prevalence results of Pedersen et al. (2019), showed that *B. afzelii* and *N. mikurensis* in *I. ricinus* ticks co-occurred in seven out of 10 locations, confirming a possible link. The pathogens seemed to follow each other as their prevalence was highest in the most southern and northern locations of this study and lower along the southwest coast. However, the exact mechanisms behind their co-occurrence and non-co-occurrence remain to be explored.

4.5 Evaluation of method

Although the prevalence of adult ticks and pooled nymphs showed a similar trend between high and low prevalence areas, chi-square results for individual adult ticks did not achieve statistical prevalence ($p= 0.06 > 0.05$). This could be because the sample size of adult ticks at most locations was usually smaller than that of pooled nymphs. A bigger sample size of adult ticks from each location could have given better results. Unfortunately, samples used in the study by Pedersen et al. (2019) were either lost or destroyed prior to this study. Next, extracted samples can become contaminated with inhibitors if the tick contains blood from its host. Components in the blood may interfere with the real-time PCR reaction and thereby give false-negative data, resulting in an underestimated prevalence of the studied pathogen (Sidstedt et al., 2018, 2020). It is important to keep this in mind as the samples investigated for *B. afzelii* were extracted prior to this study. However, when working with the samples, further measures were taken to avoid contamination of inhibitors by keeping laboratory surfaces clean and using gloves as hands naturally contain enzymes than can degrade DNA and RNA (Invitrogen, 2019). Adding water to a sample may dilute inhibitors (Pedersen, 2020). This was necessary for this study because the sample tubes contained very little sample material. On the other hand, the amount of DNA present in the real-time PCR reaction can be reduced when diluted. Consequently, the bacterial load can become too low and undetectable by the instrument (Pedersen, 2020).

Five positive controls were used for each real-time PCR run. Unfortunately, the instrument failed to detect *B. afzelii* in some of the positive controls. The number of positive controls that were undetectable by the instrument varied between runs. In addition, there was a slight variation in the Ct values of the positive controls when comparing them between the 17 real-time PCR runs of this study. To avoid false-negative results and ensure high specificity, multiple positive controls with consistent Ct values are necessary (Evans, 2009; Moldovan & Moldovan, 2020). The sensitivity of the real-time PCR method in this study may thereby not have been ideal because of the variation in Ct values between PCR runs. On the bright side, most positive samples had Ct values between 30 and 34, which is well below the 45 Ct maximum for a positive sample. If a less sensitive PCR assay resulted in increased Ct values, the positive samples would probably still be under 45 cycles, meaning that the samples would remain positive. See Appendix 8.3 for Ct values of positive samples.

None of the 42 samples from Langøya gave a false-positive result when the annealing temperature was 65°C. Unfortunately, out of the 856 samples from the coast of Norway, two showed a false-positive result. The two false-positive samples were discovered during melt curve analysis. They were then considered negative for *B. afzelii* because they had a melting temperature (Tm) under the melting temperature of the positive controls. There was an attempt to test the specificity of the primers with a probe, but due to maintenance trouble with the real-time PCR instrument and shortage of time, this was not accomplished. A search in BLAST for primer cross-reactions was also done, and luckily no significant homology to non-afzelii sequences was found (Andrew Jenkins, Pers. comm.). Furthermore, material fatigue, caused by continuous and sudden changes in temperature in a running real-time PCR instrument, is a common source of error that can lead to inaccurate real-time PCR results (Navarro et al., 2015).

5 Conclusion

This study's first aim was to investigate the distribution of *B. afzelii* in *I. ricinus* ticks along the Norwegian coast. The highest prevalence of *B. afzelii* was found in *I. ricinus* ticks collected from Brønnøysund (19.7% EPP), Rørvik (18.8% EPP), Hille (20%; 18.6% EPP), and Spjærøy (17%; 17.5% EPP). The locations in-between (from Kanestraum to Kjosavik) had a lower prevalence that varied from <0.6 to 10% EPP in nymphs and from 0 to 11% in adult ticks. The locations Lote, Florø, and Einevika, were described as a *B. afzelii* pocket within the low-prevalence area. The exact reason for the differences in prevalence is unknown. Still, availability and compatibility of vertebrate hosts, climate, and seasonal variation were suggested and discussed as possible factors that could have influenced the prevalence of *B. afzelii* in ticks.

The second aim of the study was to investigate whether *B. afzelii* and *N. mikurensis* co-occurred along the Norwegian coast with the intention to look for whether the low prevalence of *N. mikurensis* in the southwest was paralleled with a low prevalence of *B. afzelii.* This study showed that *B. afzelii* and *N. mikurensis* in *I. ricinus* ticks co-occurred in seven out of 10 locations. The prevalence of the two pathogens appeared to have a similar trend as the prevalence of both was the highest in the southernmost and northernmost locations and lower at the locations in-between, in the southwest. On the other hand, *B. afzelii* was found in ticks at locations in the southwest where *N. mikurensis* was absent, implying that *B. afzelii* may have a broader specter of reservoir hosts than *N. mikurensis.* This study confirms that *B. afzelii* and *N. mikurensis* co-occur along the Norwegian coast, with both having a lower prevalence in the southwest. However, the exact mechanisms behind their high and low prevalence along the Norwegian coast, whether related to the availability of common reservoir hosts or climate, require further study.

6 Future perspectives

This master thesis has been a small contribution to the still ongoing research on tick-borne pathogens. Much is yet to learn. Further investigations around tick-hosts-pathogen associations could shed light on the infectious cycle of *B. afzelii* and *N. mikurensis* and may help with disease control.

Futures studies should aim to:

- Perform transmission tests to uncover if shrews are reservoir hosts for *B. afzelii.*
- Investigate rodent/shrew distribution in relation to food availability along the Norwegian coast.
- Test if there is a difference in the compatibility/susceptibility of reservoir hosts towards infections with *B. afzelii* and *N. mikurensis.*
- Shed light on possible clinical consequences from co-infections with these pathogens.

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8 Appendices

8.1 Laboratory protocol for *B. afzelii* detection with real-time PCR

8.1.1 Materials and reagents

Checklist of materials and reagents used in each step of the protocol		
Step 1	**Step 2**	**Step 3**
GIII forward and reverse primers.	Positive and negative controls.	Real-time PCR instrument (Applied Biosystems StepOne™ real-time PCR Systems).
1 x TE Buffer containing 10 mM Tris-Cl (pH 8) and 1 mM EDTA (pH 8).	Sample material for analysis.	Software to read real-time PCR results (StepOne™ Software).
Nuclease-free water.	MicroAmp™ Optical 96, or 48-Well Reaction Plate.	
1 x 5 mL *Power* SYBR™ Green PCR Master Mix*	MicroAmp™ Optical Adhesive Film.	
Microfuge Tubes (1.5 mL).	MicroAmp™ Adhesive Film Applicator.	

*See thermofisher.com for components.

8.1.2 Procedure

Each step should be performed in separate clean rooms. Gloves should always be used and changed between the steps or when necessary.

Step 1: Preparation of primer mix and PCR reaction mix

Primer mix

- In a microfuge tube, dilute 100 µl of GIII forward primer with 900 µl of TE Buffer.
- Repeat the step above with the GIII reverse primer in a second microfuge tube.

PCR reaction mix

- Into a third microfuge tube, transfer 100 µl of each GIII primer mix, 175 µl of nuclease-free water, and 625 µl of *Power* SYBR™ Green PCR Master Mix. Mix up and down three times. Avoid air bubbles.

Step 2: Preparation of reaction plate

- With a repetitive pipette, distribute 20 µl of the PCR reaction mix into each well of a MicroAmp™ Optical Well Reaction Plate.
- Transfer 5 µl of positive and negative controls into the first wells of the reaction plate.
- Transfer 5 µl of sample material into the remaining wells of the reaction plate.
- Seal the reaction plate with a MicroAmp™ Optical Adhesive Film. Apply firmly using a MicroAmp™ Adhesive Film Applicator. Avoid air bubbles.

Step 3: Preparation of real-time PCR template and data analysis

Preparation of real-time PCR template

- Turn on the Applied Biosystems StepOne™ real-time PCR instrument.
- Turn on the computer and log into the StepOne™ Software.
- Click on “Template”

- In “Experiments Properties”, chose “SYBR®Green Reagents” as the target sequence. Tick box: “Include Melt Curve”.
- In “Plate Setup”, chose “SYBR” as the target name and reporter. Assign the positive and negative controls to given wells.
- In “Run Method” enter the desired real-time PCR conditions of holding, cycling, and melt curve stage.
- Click on “Save As Template…” and give a name based on annealing temperature, e.g., «B. afzelii 65C».

Real-time PCR data analysis

- Place the sealed reaction plate into the Applied Biosystems StepOne™ real-time PCR instrument.
- Click on “Template” in StepOne™ Software.
- Chose premade template with desired annealing temperature, e.g., «B. afzelii 65C».
- Save the run in a file and give it a name.
- Click on “Run”, then “START RUN”.
- When the analysis is completed, check each sample’s Ct value, and melt curve temperature (including controls).
- Note down positive and negative samples.
- Save changes.
- Dispose of the reaction plate in the trash bin.

8.2 Chi-square tests

8.2.1 High and low prevalence areas

Table 8.2.1-1: Chi-square results from testing if there is a difference in the prevalence between high and low prevalence areas for adult ticks.

		Observed			
		Positive	Negative	Row totals	Description
	Positive	25	115	140	0x
	Neg	3	42	45	1x
		28	157	185	Column totals
		Expected			
		21,18918919	118,8108108		
		6,810810811	38,18918919		
	p=	0,068437486	Chisq= 3,20104		p-verdi

Table 8.2.1-2: Chi-square results from testing if there is a difference in the prevalence between high and low prevalence areas for pooled nymphs.

		Observed			
		Positive	Negative	Row totals	Description
	Positive	111	21	132	0x
	Neg	96	137	233	1x
		207	158	365	Column totals
		Expected			
		74,86027397	57,13972603		
		132,139726	100,860274		
	p=	1,92717E-15	Chisq= 63,13803		p-verdi

8.2.2 Co-infections

Table 8.2.2: Chi-square results of over-or underprevalence of co-infections.

		Observed			
		Positive	Negative	Row totals	Description
	Positive	6	12	18	0x
	Neg	15	99	114	1x
		21	111	132	Column totals
		Expected			
		2,863636364	15,13636364		
		18,13636364	95,86363636		
	p=	0,029642041	Chisq= 4,72993294		p-verdi

8.3 Ct values of positive samples

Table 8.3: Ct values of the positive samples of the study.

Range of Ct values	**Number of positive samples**
25-29	99
30-34	283
35-39	89
40-45	10