Hosts and pathogens of *Ixodes ricinus* in Norway

Master of Science Thesis in Ecology and Evolution

Susanna Emmika Lybæk

Natural History Museum

University of Oslo

2012

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Abstract

Ticks are vectors for a number of infectious diseases, and an understanding of their ecology is valuable for minimising the risk of human and livestock infections. The aim of this study was to identify the previous hosts of questing ticks, and to investigate the pathogen prevalence of ticks in the south-east of Norway. A new method of analysing tick bloodmeals for the study of vector-host ecology has been developed, based on a reamplified real-time qPCR protocol targeting the *cytochrome b* region of the mitochondrial genome of vertebrates. The use of this method allowed for the identification of the previous bloodmeal hosts of 49% of 91 nymphal and adult *Ixodes ricinus* collected from vegetation and from hosts between 2010 and 2012. This is the first analysis of bloodmeals for questing ticks to have results confirmed by sequencing. *Borrelia burgdorferi sensu lato* infection was not detected in ticks collected from vegetation at Tomb (Østfold, Norway) and a 2% *Borrelia afzelii* prevalence was found in ticks from Nesodden (Akershus, Norway). Ticks collected at Tomb and Nesodden, as well as ticks from some other sources were also tested for presence of *Babesia* spp. infections; infections of *Babesia microti* (0.9%) and a *Babesia venatorum/capreoli/divergens* cluster (5%) were detected.

The reamplified real-time qPCR bloodmeal analysis method described in this thesis allows for an accurate estimation of host importance. The further use and development of this method will allow for the identification of additional tick hosts, which will give increasing insights into the ecology of this disease vector. Understanding the epidemiology of tick-borne diseases can contribute to the development of disease control measures, making the study of vector-host ecology an important tool in the prevention of infectious diseases.

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1 Introduction

Ticks (Acari: Ixodidae) are an increasing problem in Norway, and tick-infested habitats are expanding (Jore *et al.* 2011). In Norway, 12 species of ticks have been recorded, 11 of which belong to the family Ixodidae – the hard ticks (Mehl 1979). The hard tick *Ixodes ricinus*, known as the castor bean tick or the sheep tick, is the primary vector of Lyme disease in Norway today (Mehl *et al.* 1987). *I. ricinus* is a vector of many pathogens that cause infectious diseases such as boutonneuse fever (*Rickettsia conori*), Q-fever (*Coxiella burneti*), tick-borne fever (*Cyoectes phagocytophila*), tularemia (*Francisella tularensis*), anaplasmosis (*Anaplasma* spp.), Lyme disease (*Borrelia burgdorferi*), babesiosis (*Babesia* spp.), tick-borne encephalitis (TBE) and louping-ill. It also causes tick paralysis, a relatively rare toxicosis caused by a reaction to toxins in the tick saliva (Hillyard 1996). *I. ricinus* has mainly been a vector of livestock pathogens such as *Babesia* of cattle and *Rickettsia* of sheep in Norway, in addition to the human diseases such as Lyme disease, tularemia and TBE (Mehl *et al.* 1987). Lyme borreliosis is the most common vector-borne disease in Europe (Killilea *et al.* 2008), and TBE is an emerging tick-borne virus in Norway. The first case of TBE was diagnosed in 1997, and reports suggest 0-2 human cases have occurred yearly since (Skarpaas *et al.* 2004; Skarpaas *et al.* 2006). While the virus is still considered rare in Norway, TBE antibodies have been found in 16.4% of dogs in Aust-Agder county in southern Norway (Csango *et al.* 2004).

A profound knowledge of vector and host ecology is important in order to minimise the risk of human infection. The diversity of a community of hosts may dilute the risk of zoonoses transmission to humans by providing hosts that are less competent disease reservoirs for the vectors (Ostfeld and Keesing 2000b). Given the severity and prevalence of the illness, a better understanding of the transmission of Lyme disease by studying both the tick vectors and their mammalian hosts is crucial.

Ticks rely heavily on rodent hosts during parts of their life cycles, so rodents play a fundamental part in the transmission of tick-borne diseases (Killilea *et al.* 2008; Paulauskas *et al.* 2009; Harrison *et al.* 2010; Paziewska *et al.* 2010). With forest fragmentation and urbanisation, it appears that the prevalence of ticks and their infection rates, as well as human proximity to tick-infested areas, increase. Fragmentation seems to favour those species of tick hosts which provide the most competent *B. burgdorferi* reservoirs, causing a rise in the

density of infected ticks (Ostfeld and Keesing 2000b; Allan *et al.* 2003; Brownstein *et al.* 2005; Brunner and Ostfeld 2008).

Host trapping alone is not a viable method of assessing host importance (Humair *et al.* 2007), as *I. ricinus* is a generalist parasite capable of feeding on reptiles, birds and mammals (Mehl *et al.* 1987). Therefore, the most efficient and accurate method of investigating host importance is to analyse the remains of the previous bloodmeal in ticks that have successfully survived to the next life stage: the questing nymphs and adults. This host DNA is highly degraded and is present in very small amounts, requiring specific and sensitive methods of analysis (Kirstein and Gray 1996; Humair *et al.* 2007).

In particular, we are interested to find which hosts are utilised by the ticks and how they are distributed across the different life stages of the ticks. The present study aims to investigate this by developing a new method of identifying the previous hosts of questing *I. ricinus* based on remnants of bloodmeals. This work will also focus on the tick-borne diseases which pose the largest threats to humans and animals in Norway. We are interested in the rate of *B. burgdorferi* and *Babesia* spp. encountered in the tick population and whether pathogen presence or host identity affect the size of the ticks.

1.1 The life cycle and ecology of *I. ricinus*

The life cycle of *I. ricinus* consists of three stages: the larva, the nymph and the adult. They take one large bloodmeal per life stage, adding up to three meals in total. While adult male hard ticks are able to feed, their feeding is sporadic, and their blood intake is low (Moorhouse 1966; Shipley *et al.* 1993; Bior *et al.* 2002). For this reason, adult male ticks are generally considered to be non-feeding.

I. ricinus larvae usually feed on small mammals such as rodents and shrews. Nymphs feed on hosts such as birds and hares (*Lepus timidus)*, while the adult tick mainly feeds on larger mammals such as cervids (Talleklint and Jaenson 1994; Gray 2002), see Figure 1. While all three life stages of *I. ricinus* can feed on humans, nymphs are responsible for the majority of human tick-borne diseases (Radolf *et al.* 2012).

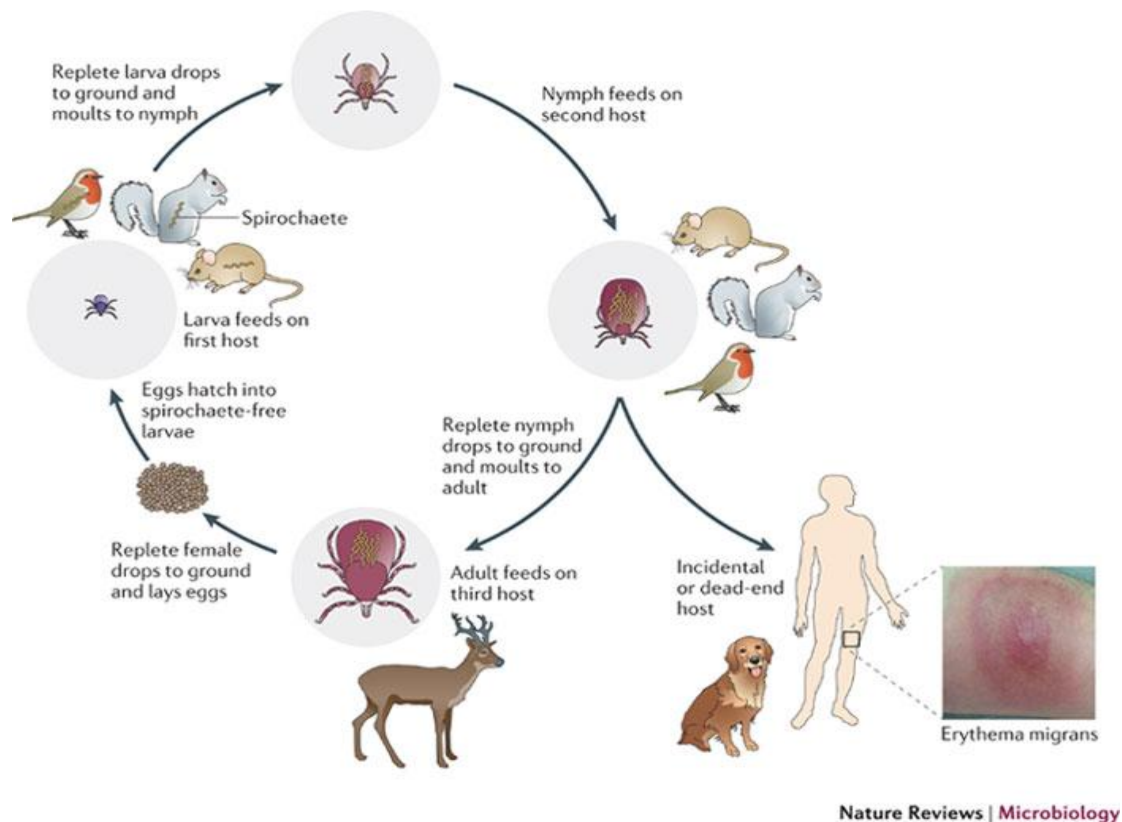


Figure 1. The life cycle of a tick. Figure from: Radolf *et al.* (2012)

The life cycle of *I. ricinus* in a seasonal environment requires at least one and a half years to complete, with slow development in winter, and questing and feeding behaviour in spring, summer and autumn. Depending on climate and the success of the tick in finding a host, this cycle may take up to four and a half years or longer to complete (MacLeod 1936). An important factor contributing to the role of *I. ricinus* as a disease vector is the ability of some pathogens to be passed from the adult female into the oviposited eggs, a phenomenon termed transovarial transmission.

1.2 The effects of hosts on ticks

While the ticks may affect their hosts through the spread of pathogens, the choice of host may also have a significant impact on the tick. There are several ways in which the host may affect the ticks that are feeding on them, as species or individuals may vary in their quality as hosts.

Moulting success after feeding is often used as a measurement of host quality. Moulting success can differ between species, as was found experimentally by Brunner *et al.* (2011).

Approximately 90% of *Ixodes scapularis* larvae that had engorged on white-footed mice (*Peromyscus leucopus*), veeries (*Catharus fuscescens*) and gray catbirds (*Dumetella carolinensis*) moulted successfully. Short-tailed shrews (*Blarina brevicauda*), robins (*Turdus migratorius*) and wood thrushes (*Hylocichla mustelina*) were considered to be lower quality hosts as only approximately 70% of the larvae moulted successfully after feeding on these species. The authors concluded that host quality differs between species, and that it does not follow taxonomic groups. Dizij and Kurtenbach (1995) found that *I. ricinus* larvae experience a higher moulting success after feeding on yellow-necked mice *(Apodemus flavicollis)* than after feeding on bank voles *(Myodes glareolus)*. The same was observed by Humair *et al.* (1999) who found that *I. ricinus* larvae and nymphs collected from *A. flavicollis* and wood mice (*Apodemus sylvaticus*) displayed a higher moulting success than those fed on *M. glareolus*. Another determinant of host quality is the feeding success of the ticks, measured as the weight of fully engorged ticks. The engorged weight of a nymph is a significant determinant of the length of the adult female scutum (Ogden *et al.* 2002). Larger adult females have also been found to be more likely to find a host (Ogden *et al.* 2002), and so the feeding success and scutum sizes of ticks appear to be closely interlinked.

There is evidence for intraspecific variation in quality between individual hosts, as measured by tick feeding and moulting success (Hazler and Ostfeld 1995; Brunner *et al.* 2011). It is not clear what causes this difference, although it is clear that certain host species exhibit a higher level of individual variation than others. Brunner *et al.* (2011) found that *B. brevicauda*, gray squirrels (*Sciurus carolinensis*) and *T. migratorius* display a high level of individual variation in their host quality for *I. scapularis*. However, the most competent hosts found in the study – *P. leucopus*, *C. fuscescens* and *D. carolinensis* – displayed low individual variation and all ticks feeding on individuals of these species experienced a high moulting success. One factor which might explain this individual variation in host quality is an acquired resistance to ticks developed by the hosts after repeated infestations. Davidar *et al.* (1989) found that while *Ixodes dammini* larvae fed on naïve rodent hosts were larger when engorged than those that had fed on previously exposed animals, this difference was not reflected in moulting success. Randolph (1994) found that *M. glareolus*, but not *A. sylvaticus*, could acquire resistance to ticks after successive infestations. This was supported by Dizij and Kurtenbach (1995), who found that *M. glareolus,* but not *A. flavicollis* acquired a resistance. This was estimated from a decreasing feeding success with consecutive infestations in *M. glareolus*, and no change in

feeding success in ticks feeding on *A. flavicollis*. A similar trend, although statistically insignificant, was seen in the proportion of engorged *I. scapularis* and moulting success on *P. leucopus* by Hazler and Ostfeld (1995).

Talleklint and Jaenson (1997) found that both larvae and nymphs which had fed on *Apodemus* spp. had higher mean weights at full engorgement than those fed on *M. glareolus*. This supported the findings of Davidar *et al.* (1989), as the differences in engorged weights were not reflected by the moulting success of the larvae. The *Apodemus*-fed ticks did, however, have a 13% larger nymphal scutum after moulting than those that had fed on *M. glareolus* (Talleklint and Jaenson 1997). The differences in engorgement weights and scutum sizes were explained by the acquired resistance developed by *M. glareolus*, but not by *Apodemus* spp., as noted by Randolph (1994) and Dizij and Kurtenbach (1995).

It has been discussed whether a high tick density on hosts may facilitate or suppress tick feeding success. On the one hand, the tick saliva may trigger immune responses by the host, and thus a higher concentration of ticks may trigger an increased response. On the other hand, tick saliva contains anti-inflammatory and immunosuppressive agents, and a high tick density increases the concentrations of these agents within the host. This may lead to a facilitation of feeding at high tick densities (Ribeiro *et al.* 1985; Davidar *et al.* 1989; Hazler and Ostfeld 1995). Support for this type of density-dependent intraspecific facilitation has been documented on several occasions, e.g. with *I. dammini* on *P. leucopus* (Davidar *et al.* 1989) and with *I. scapularis* on deer mice (*Peromyscus maniculatus bairdii*) (Hazler and Ostfeld 1995), while other studies find no relationship between tick density and moulting success (Brunner *et al.* 2011). An interaction of acquired resistance and tick density is also a possibility: Ogden *et al.* (2002) found that while nymphal *I. ricinus* fed on naïve sheep (*Ovis aries*) weighed significantly more at engorgement than nymphs fed on sheep previously exposed to ticks, this acquired resistance effect vanished at high tick densities.

1.3 The pathogen transmission cycle

Pathogen transmission from ticks to their hosts depends on many factors. The tick must have acquired the pathogen, either transovarially, or while feeding on an infected host in a previous life stage. Previously uninfected larvae or nymphs may become infected when feeding on an infected host. They may then transfer the infection to a healthy host when they feed as

nymphs or adults (Ostfeld and Keesing 2000b). There is also some evidence that co-feeding transmission may occur, in which a tick can become infected by feeding near an infected tick, on an uninfected host (Gern and Rais 1996). The likelihood of an uninfected tick becoming infected depends on the biodiversity, as well as the density of available hosts in an area. The infected tick must then successfully moult and survive to the next life stage, and it must maintain its infection. This is dependent on biotic and abiotic factors such as host species and climatic conditions (Ostfeld and Keesing 2000b). Hosts differ in their competence as pathogen reservoirs. The reservoir competence of a host species can be measured by a combination of three factors: the susceptibility of the host to a pathogen following a tick bite, the ability of the pathogen to survive and multiply within the host, and the readiness and duration of the passage of infection on to further ticks feeding on the host (Richter *et al.* 2000). In order to have an impact on the disease transmission within a system, the host must also be relatively common within the ecosystem.

The most competent reservoirs for Lyme disease in Europe are generally considered to be rodents, such as *Apodemus* spp., *M. glareolus* and squirrels (*Sciurus* spp.) (Matuschka *et al.* 1992b; Kurtenbach *et al.* 1995; Humair and Gern 1998). Cervids are common incompetent reservoirs, and appear to play a zooprophylactic role by removing pathogens from the tick population (Jaenson and Talleklint 1992; Matuschka *et al.* 1992b). This places cervids in a unique position as they are important blood sources for all stages of ticks, but apparently poor reservoirs for *Borrelia* (Telford *et al.* 1988; Jaenson and Talleklint 1992). They are particularly important hosts for adult ticks, and can thus contribute to the density of host-seeking ticks, an effect called vector augmentation (Mannelli *et al.* 2012).

It has been suggested that an increased biodiversity in an area may reduce the risk of transmission of Lyme disease when the most competent reservoirs of *Borrelia* are common. An increased level of biodiversity could cause the tick vector to also feed on other host species, as its relative encounter rate with the reservoir competent host decreases. For this ‘dilution effect’ to occur, four features must be present. First, it is required that the vector is a generalist, as is the case with *I. ricinus*. Second, oral acquisition must be the main transmission pathway for ticks, and therefore transovarial transmission must be relatively rare. Third, available hosts must vary in their reservoir competence for the *Borrelia* pathogen. And finally, the most competent host must be a common species in the system (Ostfeld and

Keesing 2000a). In the case of *I. ricinus* and its transmission by rodents in Norway, all of these attributes appear to be present.

This model has primarily been based on an American system in which *P. leucopus* is a highly abundant and reservoir competent host for *B. burgdorferi s.s.* carried by *I. scapularis* ticks (Mannelli *et al.* 2012). Some European studies have found evidence for a dilution effect from cervids (Gray *et al.* 1992; Matuschka *et al.* 1993), although others have found that the role of cervids is more likely one of vector augmentation (Talleklint and Jaenson 1994; Rizzoli *et al.* 2002). While the dilution-effect model was originally created for the study of *Borrelia* transmission, it has been suggested that it may also apply to other tick-borne pathogens such as *Anaplasma* and *Babesia* (Ostfeld and Keesing 2000b).

1.4 Lyme borreliosis

Lyme disease is the most common vector-borne disease in Europe (Killilea *et al.* 2008). Human infection can result in severe illness or death if not treated in time (Burgdorfer *et al.* 1982; Ostfeld and Keesing 2000b). Over 200 cases of chronic and disseminated Lyme disease are reported annually in Norway (Anon 2012b), and the tick-infested habitats in Norway are expanding (Jore *et al.* 2011). While the reasons for this expansion remain unclear (Jore *et al.* 2011), climate change, increasing deer (Cervidae) populations and agriculture-related changes in habitat structure have been suggested as possible causes (Randolph 2004a).

Different species of *Borrelia* cause different symptoms, making Lyme disease notoriously difficult to diagnose. This wide range of possible symptoms is the reason why Lyme disease has been referred to as “the new great imitator” (claiming the title from another disease caused by a spirochaete: syphilis), as it often mimics other diseases (Pachner 1989; Rudenko *et al.* 2011). *Borrelia afzelii*, *Borrelia garinii* and *Borrelia burgdorferi sensu stricto* (*B. burgdorferi s.s.*) are the main causes of human disease, although *Borrelia valaisiana* has been found in patients on some occasions in Europe (Rijpkema *et al.* 1997; Schaarschmidt *et al.* 2001; Hulinska *et al.* 2009). When isolating *Borrelia* strains from patients with borreliosis in Sweden, Ornstein *et al.* (2001) found that *B. afzelii* was associated with cases of Lyme disease skin symptoms, and *B. garinii* dominated in cases of neuroborreliosis. These results are supported by Ryffel *et al.* (1999), who also found that *B. burgdorferi s.s.* appears to be the main cause of Lyme arthritis.

Transovarial transmission appears to be rare for *Borrelia*. The infection prevalence of unfed larvae rarely exceeds 5%, and it is usually much lower than the nymphal and adult infection rates (Matuschka *et al.* 1992a; Kurtenbach *et al.* 1998b; Stunzner *et al.* 2006). This suggests that transovarial transmission is a relatively inefficient pathway in the spread of Lyme disease throughout an ecosystem, and that the infection is maintained primarily through feeding on infected vertebrates. Only when few competent hosts are available, does transovarial transmission become important.

1.5 Babesiosis

Babesia spp. are protozoan parasites which require a vertebrate and an invertebrate host to complete their life cycles. They belong to the phylum Apicomplexa, and are closely related to the genus *Theileria* (Homer *et al*. 2000). *Babesia* spp. reproduce sexually in a tick vector, generally an ixodid tick (Homer *et al.* 2000; Kjemtrup and Conrad 2000). While feeding, the tick transfers *Babesia* parasites to its vertebrate host, where they divide asexually. Division occurs within the erythrocytes, which are destroyed in the process, causing anemia in the host. In some species, the parasites are also transmitted transovarially to tick larvae (Kjemtrup and Conrad 2000). The disease has a broad spectrum of manifestations, ranging from almost silent cases of mild flu-like symptoms, to a malaria-like and sometimes lethal disease. Severe disease and complications are considerably more frequent in those who are immunocompromised, such as in asplenic or elderly patients (Gorenflot *et al.* 1998; Homer *et al.* 2000).

Traditionally, *Babesia* spp. have been divided into morphologically small and large genospecies, and this division generally fits well with phylogenetic trees created from nuclear small subunit ribosomal DNA (Homer *et al*. 2000). Such trees reveal a closer relation of small *Babesias* to *Theileria* than to large *Babesia* (Figure 2). It appears that only large *Babesia* species such as *B. divergens* display transovarial transmission in their tick vectors (Joyner *et al.* 1963; Lewis and Young 1980; Homer *et al.* 2000).

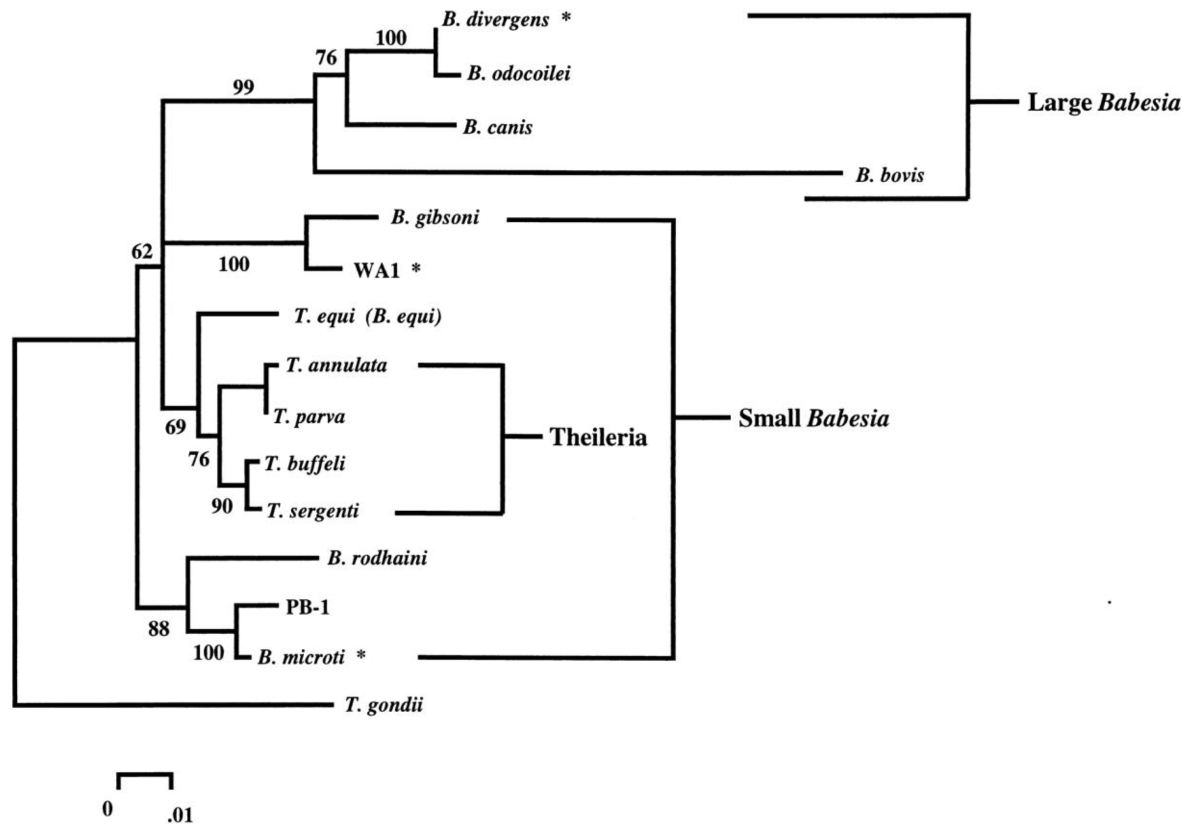


Figure 2. Phylogenetic tree from a neighbour-joining analysis of several species of *Babesia* and *Theileria*. The percentage of neighbour-joining bootstrap replications (>50%) is shown above each node. Asterisks mark species that are infective to humans. Figure from Homer *et al*. (2000).

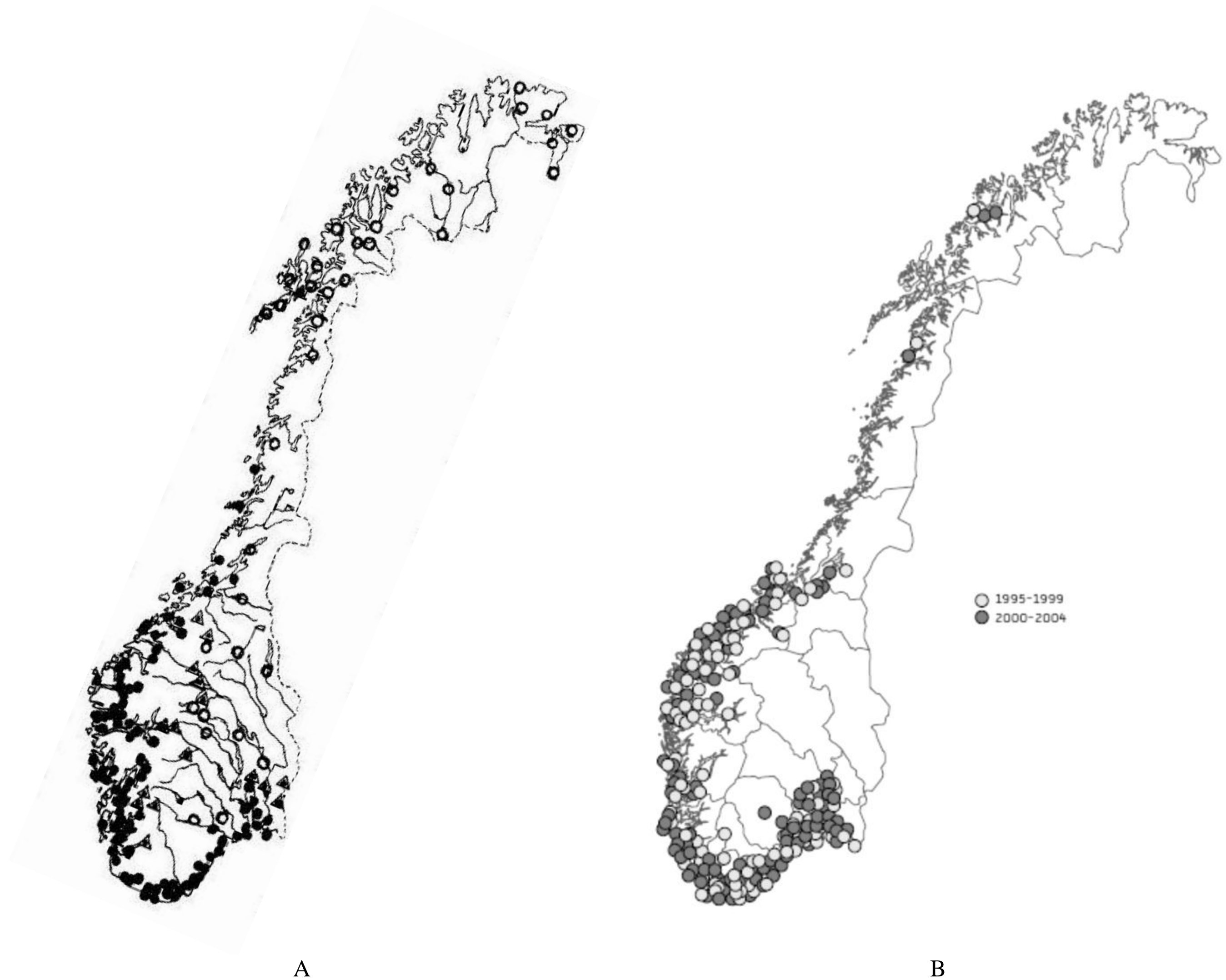
There are two main species of *Babesia* that are known to cause human illness: *Babesia divergens* and *Babesia microti* (Hildebrandt *et al.* 2007). *B. microti* is the main agent of human babesiosis in the United States (Meldrum *et al.* 1992), and *B. divergens* in Europe (Homer *et al.* 2000). In addition, three newer forms have been discovered, two in the United States (*Babesia duncani* and *Babesia* sp. MO1) and one in Europe (*B. venatorum*). Initially referred to as *Babesia* sp. WA1, *B. duncani* was discovered in an apparently immunocompetent person in Washington state in the United States (Quick *et al.* 1993; Conrad *et al.* 2006). It was later found in 2% of blood donor specimens from a diverse geographic area of the United States (Prince *et al.* 2010). The MO1 organism was identified in an elderly asplenic patient in Missouri, United States, and found to be similar to, but distinct from *B. divergens* (Herwaldt *et al.* 1996; Gray *et al.* 2010). The EU1 organism is a relatively recent discovery that was later named *Babesia venatorum*, and has been detected in three asplenic individuals in Italy, Austria and Germany (Herwaldt *et al.* 2003; Häselbarth *et al.* 2007).

In general, human babesiosis is rarer in Europe than in America. However, it more often has a fatal outcome in Europe (Homer *et al.* 2000). It is believed that most *Babesia* infections are mild or asymptomatic in immunocompetent individuals, and thus are never reported (Meldrum *et al.* 1992). Most *B. divergens* infections may be so much milder than *B. microti* infections that comparatively few infected individuals seek medical treatment.

In Norway, the disease has primarily been a bovine problem, and most research into *Babesia* has focused on cattle. In the 1930s, *B. divergens* was prevalent in cows along the southern Norwegian coast from Østfold to Trøndelag (Tambs-Lyche 1943). Today the problem is much more local, and the reduction is thought to be due to a change in pasture use (Hasle *et al.* 2010). Only one case of human babesiosis has been reported in Norway. An asplenic patient from the west of Norway was in 2007 diagnosed with *B. divergens* (Hasle *et al.* 2010).

1.6 Phenology and activity of *I. ricinus* in Norway

While 12 species of ticks have been recorded in Norway, *I. ricinus* is the main vector responsible for the transmission of Lyme disease (Mehl 1979; Nygård *et al.* 2005). *I. ricinus* is present in a large area of Norway, covering most of the coastal areas reaching from the east of the Oslo fjord, around the entire south coast, and up the west coast to Nordland county (Mehl 1983; Mehl 1999; Jore *et al.* 2011). This belt corresponds well with the reported cases of *Borrelia* infections, see Figure 3. Tick-infested habitats are also expanding in Norway. Jore *et al.* (2011) found that the northern distribution limit of *I. ricinus* has experienced a 400 km shift, and that *I. ricinus* is now present at 69°N, as opposed to the 66°N reported by Tambs-Lyche (1943) and Mehl (1983). There is some debate regarding the cause of this range expansion. Climate, increased soil moisture, altered human activities, larger numbers of cervids and higher vegetation density have been discussed as possible causes for the increased tick ranges (Nygård *et al.* 2005; Mehl 2006).



A B

Figure 3. The geographical distribution of Lyme disease in Norway is fairly consistent with the areas of high tick prevalence. A: The recorded distribution of *I. ricinus* ticks in Norway, collected between 1968-1983. ● = areas where *I. ricinus* have been collected and identified, ○ = areas in which *I. ricinus* hosts are present, but no ticks have been found, Δ = areas where *I. ricinus* has been found outside its usual range, attributed to transportation by birds or other animals. Figure from Mehl (1983). B: The distribution of reported cases of chronic and disseminated Lyme disease in Norway 1995-2004. Figure from Nygård *et al.* (2005).

In Norway, only disseminated and chronic cases of Lyme disease are reported (Mehl 1999), and no cases showing only erythema migrans are reportable. Thus, while there are numbers available for disseminated and chronic cases, it is not known how many cases of Lyme disease occur annually in total. In 1993, all cases were reportable. Figure 4 displays the relative percentages of the manifestations of Lyme disease reported during 1993.

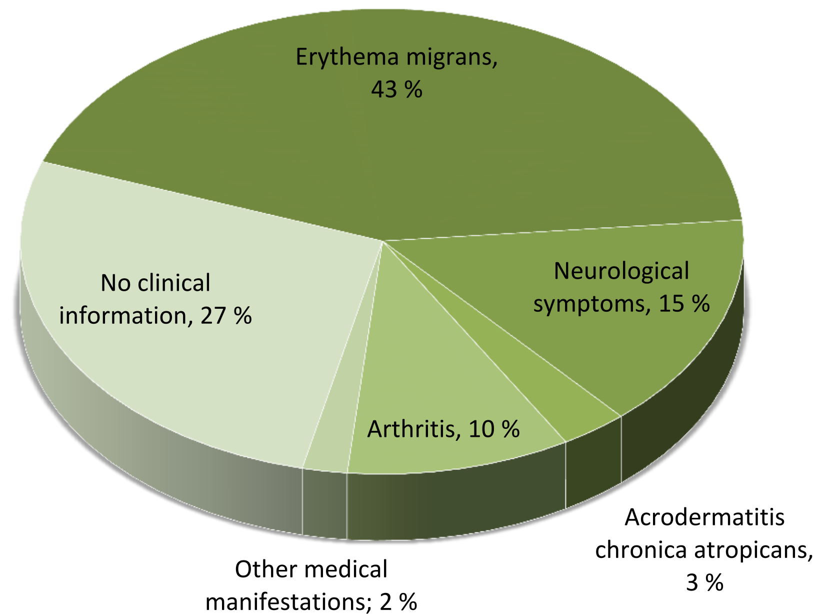


Figure 4. The relative proportions of different manifestations of Lyme borreliosis reported in 1993. Data from Mehl (1999).

1.7 Bloodmeal analysis of ticks

In order to prevent disease, an understanding of the vector-host ecology is essential. This includes the investigation of the hosts utilised by the ticks, and an analysis of the relative contributions of the hosts in terms of tick population growth and pathogen transmission. Studies purely utilising host trapping as a method of determining the relative tick burdens of different host species may overestimate the importance of rodent hosts, as these are more easily trapped than many other animals (Humair *et al.* 2007). In order to report accurate host numbers, such studies would require a sampling method that gives findings representative of the potential hosts available to the ticks, something which is difficult to accomplish (Kirstein and Gray 1996). Host trapping also does not give an accurate estimate of the survival of the ticks post-feeding. Analysis of the host blood remains inside questing ticks may allow for the identification of hosts that are rarely trapped (Cadenas *et al.* 2007). The methods are flexible, and can be applied to a range of tick hosts (Kirstein and Gray 1996).

Bloodmeal analysis has been successfully used to analyse the hosts of arthropods such as fleas, mosquitoes and tsetse flies (*Glossina* spp.) (Kent and Norris 2005; Franklin *et al.* 2010;

Muturi *et al.* 2011), and a range of different methods have been applied. Protein mass spectrometry has been utilised to analyse tick bloodmeals, however the lack of a good reference database makes this a less viable option for host identification (Wickramasekara *et al.* 2008). The use of DNA in bloodmeal identification is a more promising method, with large databases available for comparison.

The analysis of bloodmeals from ticks poses particular problems relative to other haematophagous arthropods, as any DNA left in the tick midgut after moulting is degraded and available only in small quantities (Kirstein and Gray 1996; Humair *et al.* 2007). The probability of finding the identity of the host from the remnants of blood in questing ticks diminishes with time after moulting (Cadenas *et al.* 2007; Kent 2009), and varies throughout the year (Humair *et al.* 2007; Kent 2009) and possibly with temperature (Cadenas *et al.* 2007). The type of host animal may also affect the results, as blood from hosts with nucleated red blood cells (e.g. birds) may be identified more easily (Kent 2009). The process of finding an optimal tick bloodmeal analysis method includes both comparing available molecular markers and reviewing the existing techniques of analysing DNA, with a strong focus on developing a highly sensitive method.

1.8 Molecular methods of bloodmeal analysis

There are seven main methods of bloodmeal analysis that will be discussed here, and these are summarised in Table 1.

With DNA sequences for many organisms available from databases like GenBank or the Barcode of Life Datasystem, DNA sequencing of PCR products can be a reliable method of identifying the host from a parasite bloodmeal. However, the method is time-consuming and costly if a high number of samples are to be analysed (Kent 2009). While the detection limit is low, the success of DNA sequencing depends on the ability of the PCR method to amplify the target fragment. The use of a single pair of universal primers, as in barcoding, has the potential of making the host identification process more efficient. The Barcode of Life Data System offers a large database of already identified gene fragments with which to compare results, and the project aims to have a barcode library for all eukaryotic life within the year 2027 (Ratnasingham and Hebert 2007).

Table 1. Summary of molecular methods of bloodmeal identification in ticks.

Advantages	Disadvantages	Sensitivity	References
DNA Sequencing			
Reliable, good reference databases available.	Expensive, time-consuming.	Depends on PCR method. As low as 0.2 pg	Mansfield et al. (1996)
Barcoding			
Large database, only one set of primers.	Expensive, time-consuming.	Depends on PCR/sequencing method.	Ratnasingham and Hebert (2007) Stoeckle and Hebert (2008)
Capillary fragment analysis			
Allows quantification, can analyse several reactions per lane, reliable.	Expensive, time-consuming.	0.2 pg	Mansfield et al. (1996)
Experion™ Automated Electrophoresis System			
Fast, simple, uses low amounts of PCR product.	Requires PCR, low sensitivity, risk of cross-reactions.	80 pg	Oto and Suda (2010)
Heteroduplex Analysis			
Simple, clear result if negative.	Difficult if likely host is not known. Requires large quantities of product and specific equipment.	Depends on PCR/sequencing method.	Kent (2009)
Reverse Line-Blot Hybridisation			
Well-known, cost-effective.	Moderate sensitivity, time-consuming. Product is not available for further analysis. Risk of cross-reactions.	61 pg	Wahl et al. (1987) Kim et al. (2010) O'Sullivan et al. (2011)
Real-Time Quantitative PCR			
Heightened sensitivity, gives starting concentrations and amplification curve. Product is available for melt curve and further analysis.	Primer dimer and non-specific product can be an issue.	0.02-0.8 pg	DeGraves *et al*. (2003) Valasek and Repa (2005) Lopez-Andreo *et al*. (2005) Camma *et al*. (2012)

The barcoding initiative uses the 648 bp *cytochrome c oxidase 1* (*CO1*) mitochondrial gene, which allows for the identification of 98% of the species recognised through previous taxonomic methods (Stoeckle and Hebert 2008). However, as the method relies on the use of traditional PCR and sequencing, it has no higher sensitivity than these methods, and it may be an expensive option. Moreover, the degradation of DNA in the tick gut requires the use of

short target fragments (Kirstein and Gray 1996), so a full barcode may be impossible to obtain from a questing nymph or adult tick.

Fluorescent primers can be used for fragment size analysis during capillary sequencing, and this allows for quantification of signals, and several different reactions can be analysed per lane (Herbergs *et al.* 1999). However, this method is also time-consuming and expensive, and the sensitivity of the approach will be divided between all simultaneous reactions.

The Experion™ Automated Electrophoresis System offers a high-speed microfluidics system for electrophoresis, making it an efficient and simple method of analysing DNA fragments (Oto and Suda 2010). Oto and Suda (2010) found the absolute limit of detection of the Experion™ Automated Electrophoresis System to be 80 pg for a 100 bp fragment, far superior to the >400 pg of a 3% agarose gel. However, only 1 µl of PCR product per lane can be used in an Experion™ DNA chip. An agarose gel can be loaded with a much higher volume, giving it a higher actual sensitivity.

In a heteroduplex analysis, unknown host DNA is denatured together with that of a known and closely related sequence and then re-annealed to create homoduplex and heteroduplex strands of DNA. If the two sequences turn out to be different, two bands – a homoduplex and a heteroduplex – will be visible after electrophoresis. If the two samples come from the same species, only one homoduplex band will be seen (Kent 2009). While the technique provides an elegant method of analysing an unknown sample for the DNA of a known species, it may be difficult to utilise in studies where the parasites may have fed on one of a multitude of hosts. As this is the usual situation encountered when working with field-collected ticks, the method has been of limited use in this area of research.

Traditionally, reverse line blot hybridisation (RLB) has been the method of choice when identifying tick bloodmeals, and it has proved to be a useful tool. RLB was originally used for pathogen diagnosis, and the method utilises species-specific DNA sequences attached to a nylon membrane. Biotin-labelled PCR products are then hybridised to the probe sequences, and chemiluminescence for positive samples is detected using photosensitive film (Figure 5). RLB is cost-effective, allows for the detection of up to 43 targets in 43 samples, and the nylon membrane can be washed and re-used (O'Sullivan *et al.* 2011). The method originally used radioactive probes, giving it a very high sensitivity of > 1 pg (Wahl *et al.* 1987). The

detection limit with biotin-labelled probes used today is lower, at approximately 61 pg for a 100 bp fragment (Kim *et al.* 2010), which gives it a good level of sensitivity, comparable to that of the Experion™ Automated Electrophoresis System. This method does not, however, give any information regarding the size or sequence of the fragment, other than its hybridisation with a species or group specific probe. It also does not allow for further analysis of the PCR product, such as sequencing or electrophoresis. RLB use for bloodmeal analysis of unfed ticks has never been quality controlled in the form of product sequencing. The advent of new DNA analysis methods and the improvement of others now make it relevant to question whether a better option may be available.

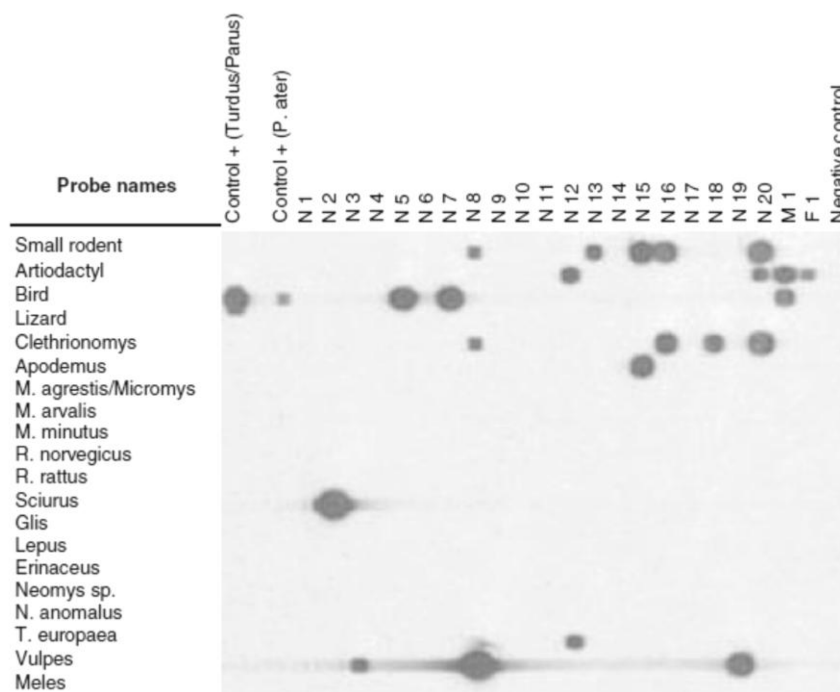


Figure 5. RLB results for host DNA identification in field-collected ticks. Probe names are listed on the left. N = nymphs, M = males, F = females. Figure from Cadenas *et al.* (2007).

Real-time quantitative PCR (also known as real-time qPCR) increases the value of traditional PCR by including a fluorescent signal that heightens the sensitivity of the method while at the same time adding the benefit of calculating the starting concentrations of the target sequence within the samples. The technology is of particular use when attempting to analyse partially digested material, due to its increased sensitivity (Kent 2009). The sensitivity of real-time qPCR gives it detection limits ranging from 0.02 to 0.80 pg (Lopez-Andreo *et al.* 2005; Camma *et al.* 2012). The method allows for a theoretical detection limit as low as one target per 100 µl (DeGraves *et al.* 2003; Valasek and Repa 2005), although 10-100 copies per

reaction is more realistic (Lockey *et al.* 1998). While the use of real-time qPCR may cause certain issues with primer dimer and the production of non-specific product, it allows for further analysis, as the product is available for melt curve analysis and sequencing. The method also gives a higher efficiency than traditional PCR due to its high speed. As the real-time qPCR reaction is performed and read within a sealed capsule, there is a reduced contamination risk associated with this method.

1.9 Suitable diagnostic markers

Tick bloodmeal digestion is an intracellular process which occurs in the midgut (Tarnowski and Coons 1989). The digestion of DNA occurs more quickly in ticks than in other arthropods (Alcaide *et al.* 2009). As ticks feed only once during each life stage, finding a fresh bloodmeal containing unfragmented DNA in a questing tick is unlikely, and it is necessary to take great care when choosing a diagnostic marker. From work on ancient DNA, it has been found that when working with highly degraded DNA, the efficiency of the PCR amplification is inversely related to the length of the target fragment (Pääbo *et al.* 1989). Thus, the ideal marker is short, so as to not be degraded within the tick, and it should be species specific (Kirstein and Gray 1996; Humair *et al.* 2007).

Mitochondrial genes are good diagnostic markers due to the large quantity of the organelles present in each cell and the lack of recombination (Humair *et al.* 2007; Kent 2009). The main mitochondrial genes used in tick bloodmeal analyses are the *12S ribosomal RNA* gene, *CO1* and *cytochrome b* (*cyt b*) (Figure 6). *Cyt b* is a thoroughly studied mitochondrial gene which is well covered in Genbank, making it a good candidate for sequencing. It has been used for bloodmeal identification through heteroduplex analyses and real-time qPCR (Kent 2009). It has, however, been criticised as a diagnostic marker due to its high inter- and intraspecific variability (Humair *et al.* 2007). While this is unlikely to cause difficulties when it is known which species are most likely to have been hosts for the ticks, it is a problem when the local vertebrate fauna is unfamiliar. *CO1* is another well-characterised mitochondrial gene which has been selected for DNA Barcoding. This makes it a good option for bloodmeal analysis, as The Barcode of Life Data System may provide matches for DNA sequences from unknown hosts (Kent 2009). However, *CO1* barcodes are, as mentioned previously, too long for the bloodmeal analysis of unfed ticks due to DNA degradation in the tick gut (Kirstein and Gray

1996). Another of the most commonly used molecular markers is the *12S rRNA* gene. It has proven to be an excellent diagnostic marker, and it is one of the most conserved parts of the mitochondrial genome (Humair *et al.* 2007). This does, however, increase the chances of issues related to sample contamination with other vertebrate DNA.

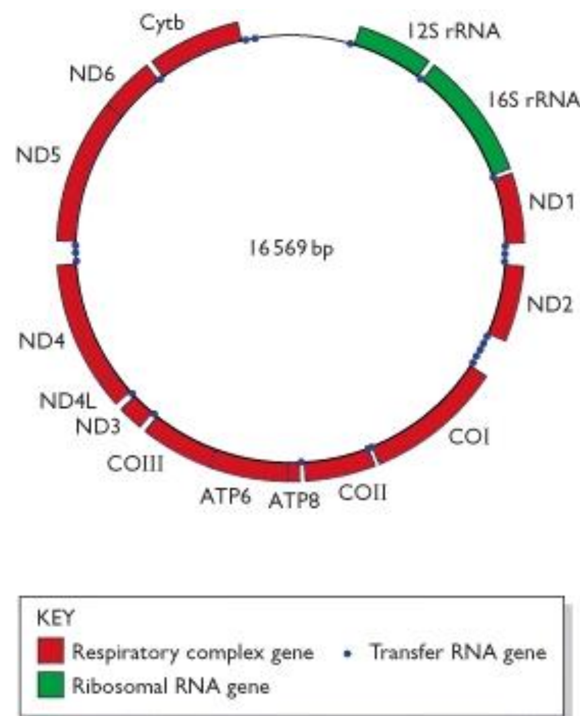


Figure 6. The mitochondrial genome, showing the loci of the *12S rRNA* gene, *cyt b* and *CO1*. Figure from Brown (2002).

Ribosomal RNA (rRNA) genes such as the *18S cytoplasmic rRNA* gene have been targeted for tick bloodmeal analysis, and the relatively low evolutionary rate of *18S rRNA* ensures low intraspecific variability while showing reliable interspecies diagnostics (Humair *et al.* 2007; Kent 2009). It is, however, more conserved than the *12S rRNA* gene, and is therefore not an optimal marker for identifying the genus and species of a host (Humair *et al.* 2007).

While it is possible to identify a parasite bloodmeal host using nuclear genes, bloodmeals from ticks are particularly degraded, making the use of single copy nuclear genes unreliable (Kent 2009). It appears that the use of mitochondrial genes which are present in high numbers in cells is the best option for tick bloodmeal analysis, and *12S rDNA* and *cyt b* have been used in a number of previous investigations on the topic (Kirstein and Gray 1996; Estrada-Peña *et al.* 2005; Humair *et al.* 2007).

2 Methods

2.1 Sampling of *I. ricinus*

Ticks were collected from vegetation by blanket dragging and from rodents by live-trapping. All ticks collected were identified morphologically as either larva, nymph, adult male or adult female using descriptions by Hillyard (1996). No other species of tick was encountered during these collections. All ticks were preserved in 70% ethanol and stored at -20°C.

2.1.1 Rodent trapping

The methodology used was similar to that of Paziewska *et al.* (2010). Rodent trapping was carried out for three consecutive nights each month from June to October 2011 in the Nesodden area of Akershus, Norway (59°50'43.38"N 10°39'19.21"E). This peri-urban area consists of woodland and mixed vegetation and is adjacent to a small lake. The area is utilised by small rodents, mallards (*Anas platyrhynchos)*, graylag geese (*Anser ferus*) and cats *(Felis catus)*, and 2-3 roe deer (*Capreolus capreolus)* visit the site daily during the summer.

Rodents were live-trapped using maize, apple and peanut butter as bait. Tissue paper was provided as bedding material. The wooden box traps (Figure 7) were set in the evening and collected the following morning.

Figure 7. Traps were made from wood, and were covered with leaves for additional insulation during cold nights.

Animals were anesthetised using Isoba™ isoflurane (Merck Animal Health, Boxmeer, The Netherlands). The method of administration was similar to that described by Parker *et al*. (2008) for use on *S. carolinensis*. A perforated metal ball was filled with tissue soaked in approximately 1 ml of isoflurane and dropped into a large glass bowl covered by a lid. The rodent was removed from its trap and placed in the chamber and observed for signs of anaesthesia. Once unconscious, the rodents could be handled for approximately 1-2 minutes before requiring additional doses. Each animal was anesthetised between 1-3 times. The total number of doses was based on the welfare of the animal and the time required to complete sampling. Once fully anesthetised, the rodents were checked for ticks, weighed to the nearest gram, sexed, their length was measured (nose-anus, anus-tail tip), and they were marked by injection of Trovan® 9 mm transponders (BTS ID, Helsingborg, Sweden). Their age groups were noted as 'weanling', 'juvenile' or 'adult'. Ticks were removed from the animals using forceps. All rodents were released at their site of capture after a short recovery period.

2.1.2 Blanket dragging

Actively questing ticks will climb vegetation to a preferred questing height and grab any object that passes, a behaviour which is exploited in cloth dragging (Milne 1943; Mehl 1970; Daniels *et al.* 2000; Randolph 2004b; Brownstein *et al.* 2005). In order to estimate tick density and infection rates, ticks were collected using this method by dragging a 150 cm x 90 cm piece of cloth attached to a handle (Figure 8) through the vegetation. The blanket was searched and all ticks were removed using forceps.

Figure 8. Blanket dragging at the Nesodden field site.

Drags were performed between April-November 2011, at approximately 2 or 3-week intervals, depending on weather conditions. Optimal weather for dragging was considered to be little or no precipitation, and drags were performed in the afternoon or evening at the Nesodden field site. Dragging at Tomb school of agriculture in Råde, Østfold, in Norway (59°17'38.77"N 10°48'24.47"E) was performed for one week of June, 2010-2012. This site is a managed area of coniferous forest with some clearings, and it is regularly visited by cervids and moose *(Alces alces),* as well as red foxes *(Vulpes vulpes),* hares *(L. timidus)* and small rodents.

2.1.3 Nymph scutum measurements

To investigate whether nymph size was related to larval host and/or infection prevalence, nymph scutum size as a proxy for nymph size was measured in 30 nymphs collected from vegetation at Tomb. This was performed as described by Talleklint and Jaenson (1997). The width and length of the scutum was measured as shown in Figure 9, by overlaying the ticks with a measuring slide. The measurements were made at 12x magnification under a dissecting microscope. These two measurements were then added together and divided by four to estimate the scutum radius. The radius was used to calculate the total area of the scutum according to the formula πr^2.

Figure 9. The radius of the nymph scutal area was calculated based on the width and length of the scutum.

2.1.4 Other tick collections

Ticks were also provided from other sources. Some were collected from pets at the Røyken og Omegn veterinary clinic near Oslo, and some were collected from humans, dogs and cats in Oslo and Arendal.

2.2 DNA extraction

2.2.1 Development of DNA extraction technique

Two extraction methods were tested in preliminary experiments: the ammonium extraction method described by Guy and Stanek (1991) and a proteinase K column-based kit method (E.Z.N.A.® Tissue DNA Kit, Omega Bio-Tek, Georgia, United States).

In order to simulate ticks at different stages after feeding, five questing larval ticks were mixed with 5 µl of different dilutions of *M. glareolus* blood and then extracted by one of the two methods. The blood contained 1mM EDTA and had been stored at -20°C until use. Serial dilutions (0.5, 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-10} and 10^{-12}) were prepared with distilled water, and a positive control (0.5 blood dilution, no larvae) and a negative control (five larvae, no blood) was used. These samples were extracted using ammonium and the E.Z.N.A.® Tissue DNA Kit.

The E.Z.N.A.® Tissue DNA Kit was used as per the instructions except that only half the suggested volumes of TL and OB proteinase were used (100 µl and 12 µl, respectively) due to small sample sizes. Samples were lysed overnight at 55°C after mechanical crushing using a sterilized toothpick. Due to evaporation during incubation, distilled H_2O was added to each sample to a total volume of 200 µl. DNA was eluted in two aliquots of 100 µl elution buffer, giving a final volume of 200 µl.

For the ammonium extractions, the samples were extracted using a standard method (Guy and Stanek 1991). Each pool of ticks was homogenised using sterile toothpicks in 100 µl 0.7M ammonium hydroxide, and then incubated at 100°C for 15 min. The tubes were then opened and allowed to incubate for a further 10 min in 100°C (after which the volume had reduced to between 10-60 µl), before centrifugation at 11 290 RCF for 5 min. The supernatant was removed and diluted to 200 µl with distilled H_2O. DNA was then precipitated by addition of 0.1 volumes 3M sodium acetate (20 µl) and 2 volumes absolute ethanol (400 µl, -20°C). Samples were then incubated at -20°C for a minimum of 30 minutes before centrifugation at 11 290 RCF for 15 min. The pellet was rinsed with 100 µl of 70% ethanol and centrifuged at 11 290 RCF for 5 min. The samples were incubated at 37°C for 15 min to evaporate the surplus ethanol, after which the DNA was reconstituted with 20 µl of distilled H_2O. All DNA

samples were stored at -20°C until further use. Once extracted, a PCR was run using *M. glareolus* primers MyoCytbF and MyoCytbR targeting the *cytochrome b* gene within the mitochondrial genome, see Appendix 1.

2.2.2 Tick extractions

Routine extractions of nymphs and adults were performed using the ammonium method described above, except that samples were extracted individually and reconstituted with 20 µl distilled H_2O for nymphs and 40 µl distilled H_2O for adults.

2.2.3 Control DNA samples

DNA samples used as controls were collected from various sources, see Table 2. Vertebrate DNA was extracted from blood or tissue using the E.Z.N.A.® Tissue DNA Kit and the E.Z.N.A. ® Blood DNA Kit.

Table 2. DNA sources and extraction methods.

DNA Sample	**Source**	**Obtained from**
Wood mouse *Apodemus sylvaticus*	Tissue	Killed by cat at Nesodden
Cat *Felis catus*	Tick gut contents	Cordelia Bracht, Bærum Dyreklinikk
Squirrel *Sciurus vulgaris*	Tissue	Arild Johnsen, NHM, University of Oslo
Common redstart *(Phoenicurus phoenicurus)*	Blood	Arild Johnsen, NHM, University of Oslo
Brown rat *Rattus norvegicus*	Tissue	Ida Fløystad, NHM, University of Oslo
Roe deer, *Capreolus capreolus*	Tissue	Oslo Vet Institute
Field vole *Myodes glareolus*	Tissue	NHM sample from Øvre Heimdalen
Shrew *Sorex* sp.	Tissue	Killed by cat at Nesodden
Dog *Canis lupus familiaris*	Blood	Cordelia Bracht, Bærum Dyreklinikk

2.3 DNA amplification

Vertebrate primers were designed to amplify species or group specific fragments of DNA from the *12S rDNA* or *cyt b* region of the mitochondrial genome. See Appendix 1 for a full list and description of primers. All primers were manufactured by Eurofins.

Standard PCR amplifications were performed in BioRad T100 thermal cyclers. DNA extraction, pre-PCR, PCR amplification, post-PCR work and electrophoresis was all performed in five separate rooms in the DNA laboratory at the Zoological Museum, Oslo. Sterile filter tips were used for all laboratory work. Electrophoresis of PCR products was performed on 1% agarose gels using GelRed™ nucleic acid gel staining reagent (Biotium, Hayward, USA).

2.3.1 Controls for primer specificity and sensitivity

Primer specificity testing

Primer pairs for each species and each gene used were compared against PCR target DNA and the DNA of all other species (including tick DNA, extracted from unfed larvae). Positive (target host) controls and negative (water) controls were included with every set of primers tested. For a full list of primers tested, see Table 4, p. 33.

The total reaction volume of 20 µl was made up of: 1 µl DNA template, 1 µl forward primer (10 pmol), 1 µl reverse primer (10 pmol), 10 µl AmpliTaq Gold® Fast PCR Master Mix (Life Technologies, Paisley, United Kingdom) and 7 µl distilled H_2O. DNA amplification used the following PCR protocol: 94°C for 4 min, then 35 cycles of 94°C for 45 s, primer specific annealing temperature for 45 s, 72°C for 45 s, and finally 72°C for 7 min. The annealing temperatures, sequences and positions for the different primers are shown in Appendix 1, and the primer placements within the genomes are displayed in Appendix 2.

Bloodmeal analysis by PCR

It was explored whether standard PCR using the primers listed in Table 4 had a sufficiently high level of sensitivity and specificity to identify the previous bloodmeal hosts of questing ticks. Initially, a single round of PCR was used (35 cycles), and subsequently re-amplification

and nested (using generic mammal primers *12S*oF and *12S*oR, Appendix 1) approaches were attempted for identifying tick bloodmeals. Both *12S rDNA* and *cyt b* primers were tested, and the PCRs followed the same protocol as for the primer specificity testing.

2.3.2 Real-time qPCR bloodmeal analysis

Due to the superior sensitivity and data output of real-time qPCR, bloodmeal analysis was performed using a reamplified real-time qPCR. 89 ticks, consisting of 29 adult females, 31 adult males and 29 nymphs, were tested to identify the previous bloodmeal host using real-time qPCR. The outer *cyt b* primer pairs for the four most likely hosts were used: *Apodemus* spp. (wood mouse/yellow-necked mouse; CytboutFApo and CytbRoutApo), *Capreolus capreolus* (roe deer; CytboutFRoe and CytboutRdeercat), cat (*Felis catus;* CytboutFcat and CytboutRdeercat) and *Myodes glareolus* (bank vole; CytboutFMyo and CytboutRMyo). The positions, annealing temperatures and sequences of these primers can be found in Appendix 1, and their placements within the *cyt b* gene in the mitochondrial genome are shown in Appendix 2.

The protocol followed a reamplification approach. In the first round, a mix of all of the outer *cyt b* primers was used. In the second round, only one pair of *cyt b* outer primers was used for each host. This approach increased the sensitivity of the method, while still limiting the time required to run each test. In the primary amplification, the PCR reaction mix of 10 µl included 1 µl DNA template, 0.25 µl of each forward primer (10 pmol), 0.25 µl of each reverse primer (10 pmol), 5 µl AmpliTaq Gold® Fast PCR Master Mix and 2 µl UltraPure™ DEPC-treated Water (Life Technologies, Paisley, United Kingdom). The primary amplification was performed as a traditional PCR, using the following protocol: 95°C for 3 min, then 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, and finally 72°C for 5 min. In the second amplification, the total reaction volume of 10 µl was made up of: 1 µl DNA template, 1 µl forward primer (10 pmol), 1 µl reverse primer (10 pmol), 5 µl SsoFast™ EvaGreen® Supermix (BioRad, Hercules, United States) and 2 µl UltraPure™ DEPC-treated Water. The reamplified real-time qPCR followed this protocol: 95°C for 3 min, then 45 cycles of 95°C for 10 s, primer specific annealing temperature for 20 s, and finally 72°C for 15 s. This was followed by a melt curve step with 5 s increments of 0.5°C, from 65°C to 95°C. All real-time qPCR work was performed using the Bio-Rad MiniOpticon Real-Time PCR System.

The use of qPCR allowed for a visual display of the melting curves of the products. The melt peaks have individual shapes and melting temperatures depending on the species, allowing for an accurate identification, determined by two independent observers. Results were confirmed by sequencing or by use of the Experion™ Automated Electrophoresis System.

2.3.3 Pathogen detection; *Borrelia*

A total of 120 unfed ticks, consisting of nine adult females and 111 nymphs, were tested for *Borrelia*. The amplification of *Borrelia* DNA followed a heminested procedure (Michel *et al.* 2004), with some modifications. Following the original procedure resulted in multiple unclear bands on the gel, so only the R1 reverse primer was used, the annealing temperature was raised to 58°C, and the amplification was extended to 40 cycles. The primers (see Appendix 1) target an approximately 800 bp fragment of the bacterial DNA, coding for the outer surface protein (Osp) A.

The primary amplification carried out in 10 µl was made up of 2 µl DNA template, 1 µl V1 forward primer (10 pmol), 1 µl R1 reverse primer (10 pmol), 5 µl AmpliTaq Gold® Fast PCR Master Mix and 1 µl distilled H_2O. The reaction volume of the second amplification was 20 µl, which was made up of 4 µl first-round product, 1 µl V3 forward primer (10 pmol), 1 µl R1 reverse primer (10 pmol), 10 µl AmpliTaq Gold® Fast PCR Master Mix and 4 µl distilled H_2O. The *Borrelia* PCR protocol was as follows: 95°C for 5 min, then 40 cycles of 94°C for 45 s, 58°C for 45 s, 72°C for 1 min, followed by 72°C for 7 min. Negative controls were included with every run. No positive control was used as none was available.

2.3.4 Pathogen detection; *Babesia*

A total of 234 unfed ticks, made up of 173 nymphs, 30 females and 31 males, were tested for *Babesia* spp. by PCR. The primers (see Appendix 1) target a 403 or 427 bp fragment of the *Babesia 18S rRNA* gene.

The reaction mixtures were made up of 2 µl DNA template, 0.5 µl forward primer (10 pmol), 0,5 µl reverse primer (10 pmol), 5 µl AmpliTaq Gold® Fast PCR Master Mix and 2 µl distilled H_2O, adding up to a total reaction volume of 10 µl. This amplification followed a similar protocol to that described by Armstrong *et al.* (1998): 94°C for 5 mins, followed by 45

cycles of 94°C for 45 s, 64°C for 45 s and 72°C for 45 s, and finally 72°C for 10 mins. Negative and positive controls were included with each run. A clear result could not be obtained from 12 samples (two adult males and ten nymphal ticks), and these were excluded from further analysis.

All products giving a positive band after gel electrophoresis were additionally tested using the Experion™ Automated Electrophoresis System to allow an estimation of product concentration. All samples giving a clear signal after this were sequenced to determine the *Babesia* species, see Appendix 3 for sequence alignment. The samples displaying low product concentrations were treated as *Babesia* positive, but were not sequenced.

2.4 DNA sequencing

As the real-time qPCR products were difficult to sequence, all second-round amplifications from the bloodmeal analyses were repeated using traditional PCR. When electrophoresis results of this PCR were unclear using the outer *cyt b* primers, and inner reverse pair was used with the outer forward primers in a heminested second round (CytbinnRRoe and CytbinnRMyo in Appendix 1). This was the case for two ticks which had fed on *C. capreolus* and one which had fed on *M. glareolus*. These traditional PCR products were then sequenced, after ExoSAP-IT® treatment (USB, High Wycombe, United Kingdom). Sequencing was performed using Sanger dideoxy sequencing on an Applied Biosystems capillary sequencer at the Norwegian Sequencing Centre at the University of Oslo.

FinchTV© version 1.4.0 (Geospiza Inc, Seattle, United States) was used for proof reading of the sequences. Sequences were aligned using MEGA version 4 (Tamura *et al.* 2007), and a BLAST search (NCBI, http://blast.ncbi.nlm.nih.gov) was performed to confirm sequence identity.

2.5 Statistical analysis

All statistical analysis was performed using the R program version 2.14.0 (R-Development-Core-Team 2011).

3 Results

3.1 Rodent trapping

A total of only 16 individual rodents were trapped, consisting of 15 *A. sylvaticus* and one *A. flavicollis*. Table 3 displays the sexes and age groups of the mice.

Table 3. Mice (*Apodemus* spp.) trapped during the summer 2012.

Sex	Adult	Juvenile	Weanling
Male	7	2	1
Female	5	0	1

359 ticks were found on the mice, made up of 353 larvae and six nymphs. The tick burden varied from one to 43 between individual mice. On average, adult mice had a higher tick burden at first capture than juveniles and weanlings, and males were more heavily infested than females. A Mann-Whitney U two-sample rank-sum test revealed that there was no statistical significance between the tick burdens of males (median=15) compared to females (median=10) ($U = 22.5$, $n_1 = 10$, $n_2 = 6$, $P > 0.05$ two-tailed; Figure 10). No significant difference was found in the tick burdens of adults (median=15) and immature rodents (median=5.5) ($U = 12.5$, $n_1 = 12$, $n_2 = 4$, $P > 0.05$ two-tailed; Figure 11).

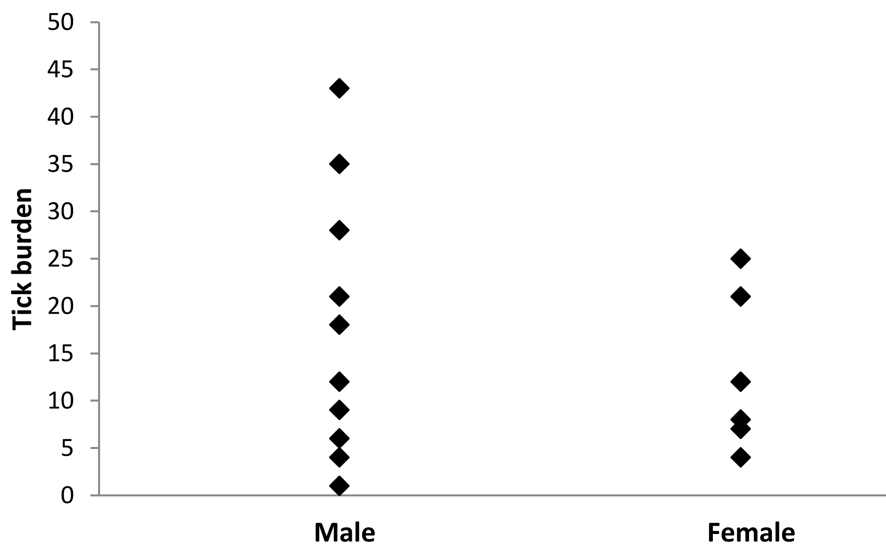


Figure 10. Distribution of tick burdens in male and female rodents at first capture.

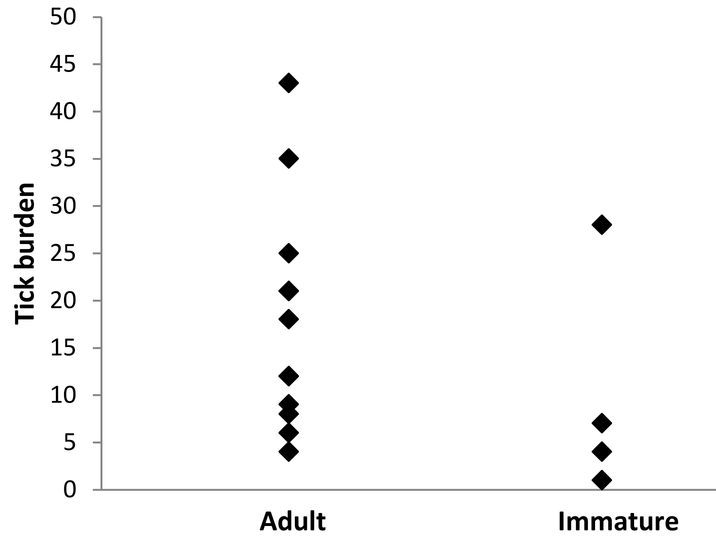


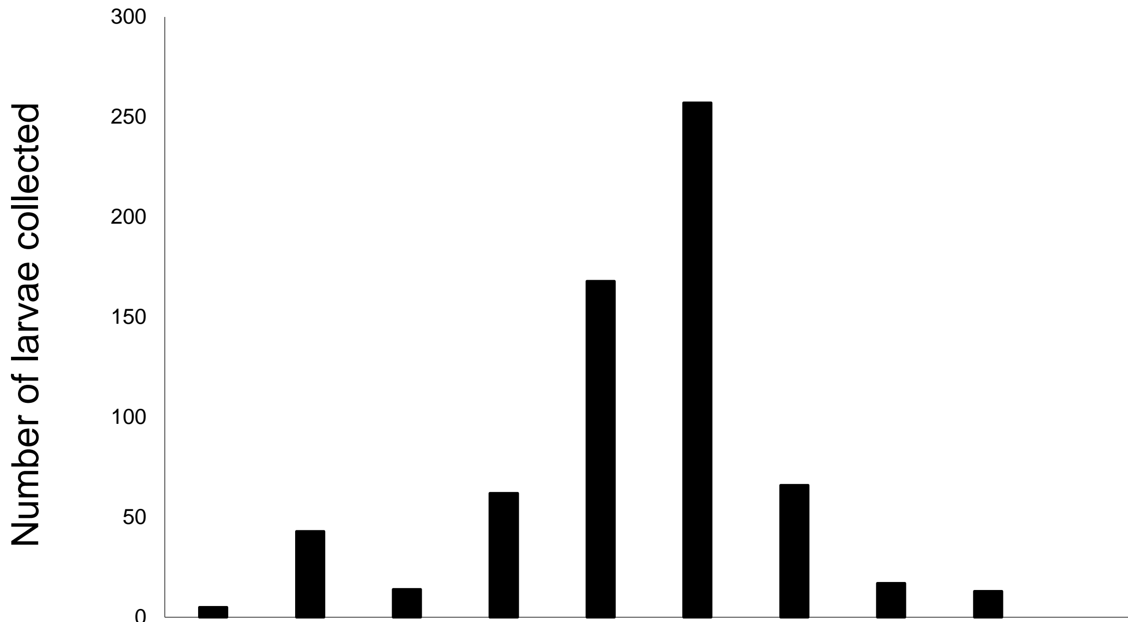
Figure 11. Distribution of tick burdens in adult and immature rodents at first capture.

Larvae were found on the rodents throughout the trapping season, whereas nymphs were only found in May and June (Figure 12).

3.2 Blanket dragging

During the summer of 2011, 780 ticks were collected by dragging at Nesodden, consisting of 647 larvae, 126 nymphs, six females and one male. The larvae appear to peak in May and August and the nymphs in May (Figure 12).

A



B

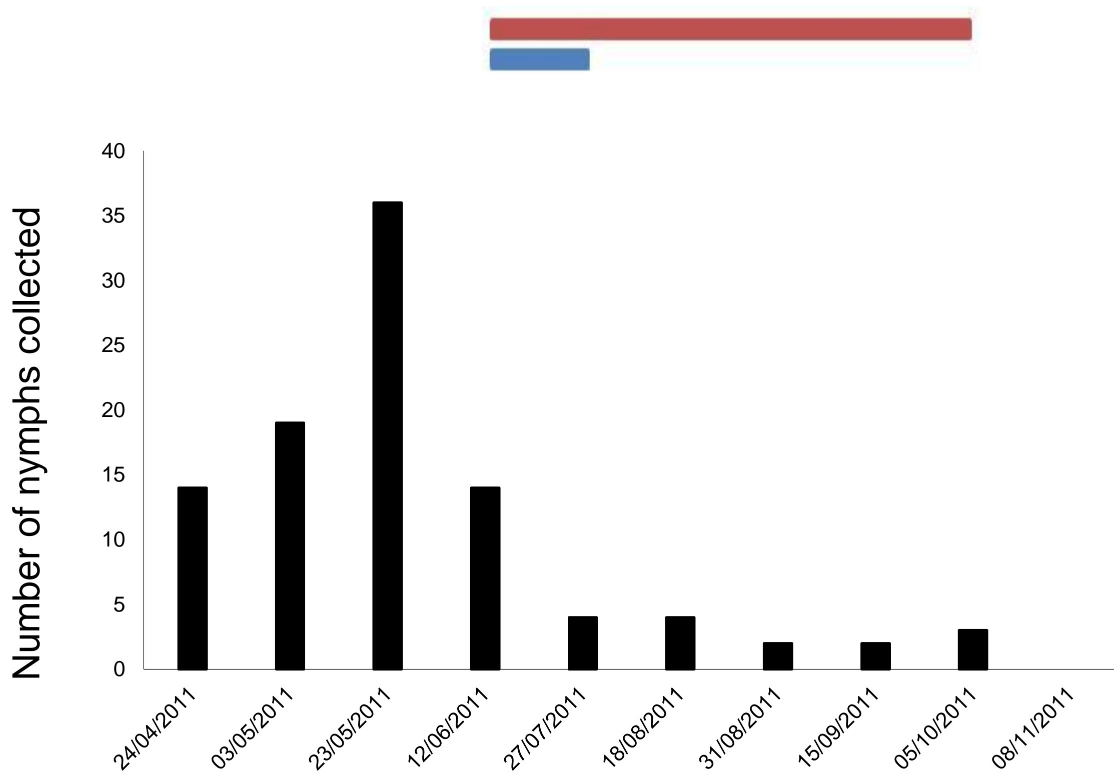


Figure 12. Ticks collected by blanket dragging at the Nesodden field site between April – November 2011. A: larvae, B: nymphs. Red bar: larvae present on trapped rodents, Blue bar: nymphs present on trapped rodents.

3.3 Nymph scutum measurements

A Kolmogorov-Smirnov (K-S) test revealed that the scutum sizes of the measured nymphs appear to not follow a normal distribution (D =1, $P < 0.001$). This was further supported by a Q-Q plot (Figure 13). The data points do not follow the normal distribution line closely, and they do not appear to display a linear pattern.

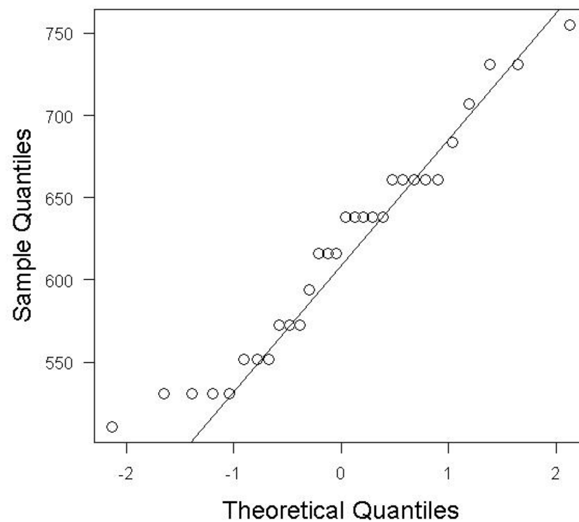


Figure 13. Q-Q plot of scutum sizes of measured nymphs, which do not appear to follow a normal distribution.

The scutum sizes appear to follow a polymodal distribution, see Figure 14.

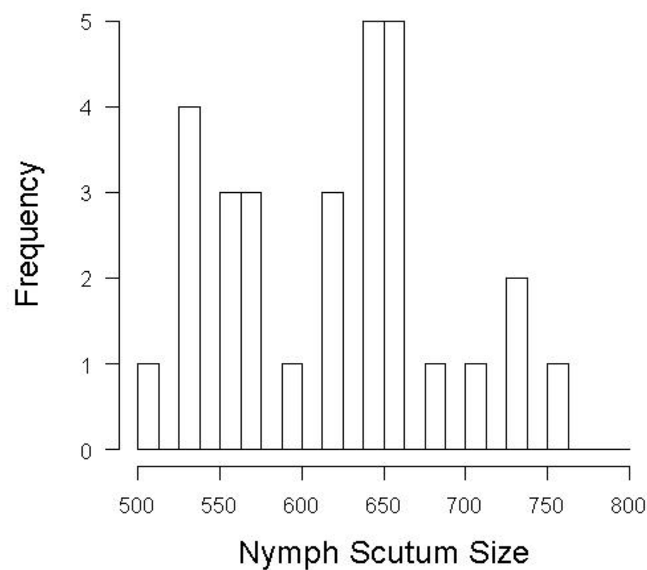


Figure 14. The scutum sizes of the nymphal ticks appear to display a polymodal distribution.

3.4 Development of bloodmeal analysis method

3.4.1 DNA extraction

Both ammonium and proteinase K protocols gave clear bands for the positive control, the 0.5 and 10^{-2} dilutions (see Figure 15). The ammonium appeared to also find a very weak DNA signal in the 10^{-4}, 10^{-6} and 10^{-10} dilutions, but not at the 10^{-8} or 10^{-12} dilutions. The E.Z.N.A.® Tissue DNA protocol appeared to give very faint bands in the 10^{-4} and 10^{-6} dilutions, but not at weaker concentrations. Given the comparable sensitivity of the method, ammonium extraction was used throughout this study.

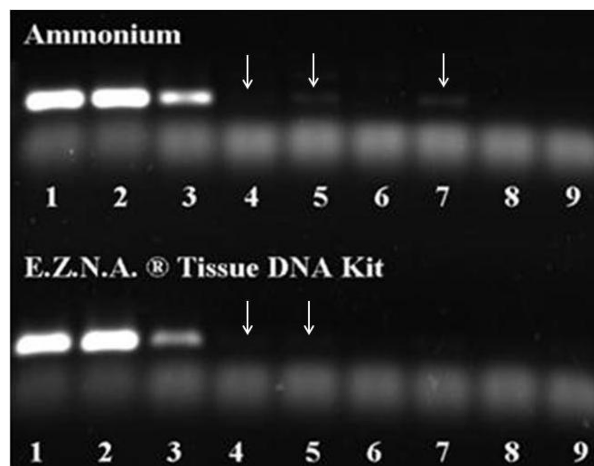


Figure 15. Comparison of DNA extraction methods; sensitivity of PCR for M. glareolus DNA after ammonium and E.Z.N.A.® Tissue DNA Kit extraction. Lanes: 1 = positive control, 2 = 0.5, 3 = 10^{-2}, 4 = 10^{-4}, 5 = 10^{-6}, 6 = 10^{-8}, 7 = 10^{-10}, 8 = 10^{-12} and 9 = negative control. Bands show fragments of approximately 100 bp. Arrows locate the positions of weak bands.

3.4.2 Bloodmeal analysis by PCR

Primer specificity

All tested primer pairs allowed accurate identification of vertebrate DNA, see Table 4. In general, the primer pairs were specific for the host species they were designed for, with the exception of *R. norvegicus 12S rDNA* primers, which cross-reacted with *A. sylvaticus* DNA, *S. araneus* primers, which showed a range of cross-reactivities, and the *F. catus 12S rDNA* primers, which reacted with *C. lupus familiaris* DNA.

Table 4. Specificity tests of primers with vertebrate and tick DNA. + = strong positive reaction, (+) = weak positive reaction, - = negative reaction

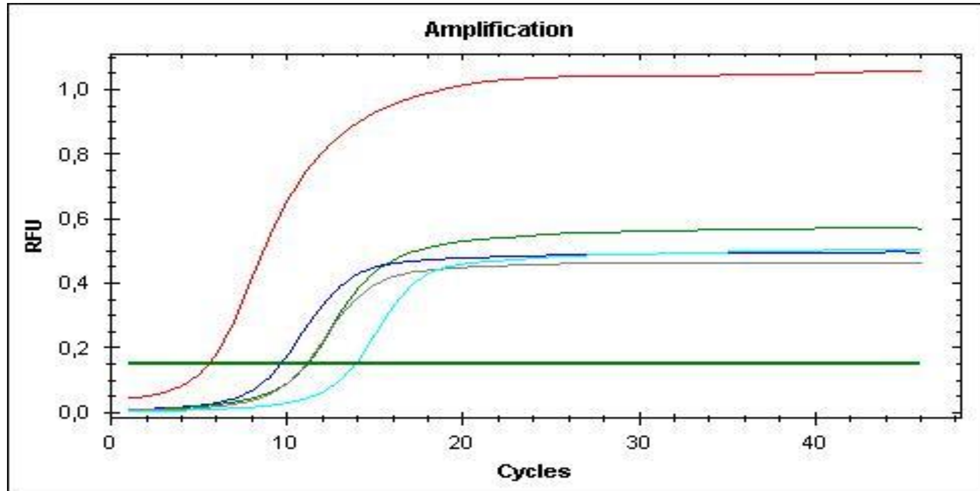
		Primer pair										
		***Apodemus* spp.**		***F. catus***		***S. vulgaris***	**Bird**	***R. norvegicus***	***C. capreolus***	***M. glareolus***		***Sorex* spp.**
		12S	***Cyt b***	***12S***	***Cyt b***	***12S***	***Cyt b***	***12S***	***Cyt b***	***12S***	***Cyt b***	***12S***
DNA	***A. sylvaticus***	+	+	-	-	-	-	+	-	-	-	+
	F. catus	-	-	+	+	-	-	(+)	-	-	-	-
	S. vulgaris	-	-	-	-	+	-	-	-	-	-	-
	P. phoenicurus	-	-	-	-	-	+	-	-	-	-	-
	R. norvegicus	(+)	-	-	-	-	-	+	-	-	-	-
	C. capreolus	-	-	(+)	-	-	-	-	+	-	-	+
	M. glareolus	(+)	(+)	-	-	-	-	-	-	+	+	+
	***Sorex* sp.**	-	-	-	-	-	-	-	-	-	-	+
	C. lupus familiaris	-	-	+	-	-	-	-	-	-	-	(+)
	I. ricinus	-	-	-	-	-	-	-	-	-	-	-

Bloodmeal analysis

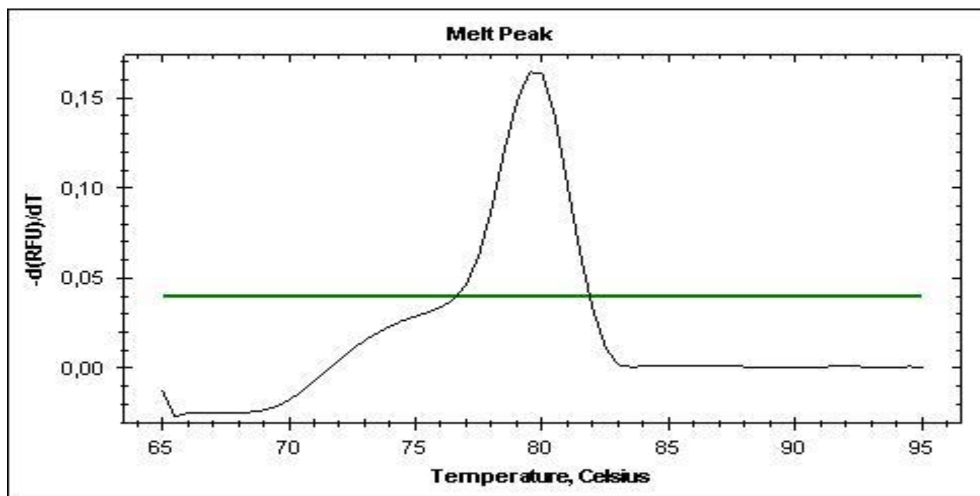
A high number of amplification cycles were required in order to amplify the degraded target DNA using *12S rDNA* and *cyt b* primers on nymphs and adult ticks. This led to the amplification of non-target DNA, which could not be differentiated from target DNA in both single-round and nested traditional PCR (data not shown).

3.5 Real-time qPCR bloodmeal analysis

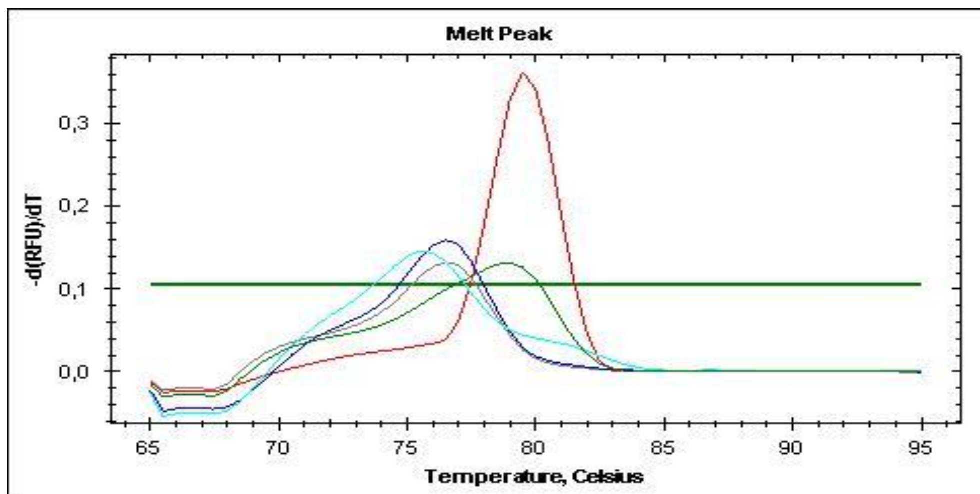
Real-time qPCR included the benefits of an amplification curve and a melt curve, allowing for the identification of genuine, target DNA signals. DNA of five separate ticks, marked in Figures 16-19 by individual colours, were amplified using real-time qPCR. The DNA of the tick marked in red amplified with *F. catus* primers (Figure 16), the DNA of the tick marked in green with *C. capreolus* primers (Figure 17), the DNA of the tick marked in dark blue with *Apodemus* spp. primers (Figure 18) and the DNA of the tick marked in light blue amplified with *M. glareolus* primers (Figure 19). The DNA of the tick marked in grey does not appear to amplify with any of the primers presented.



F. catus qPCR amplification curve

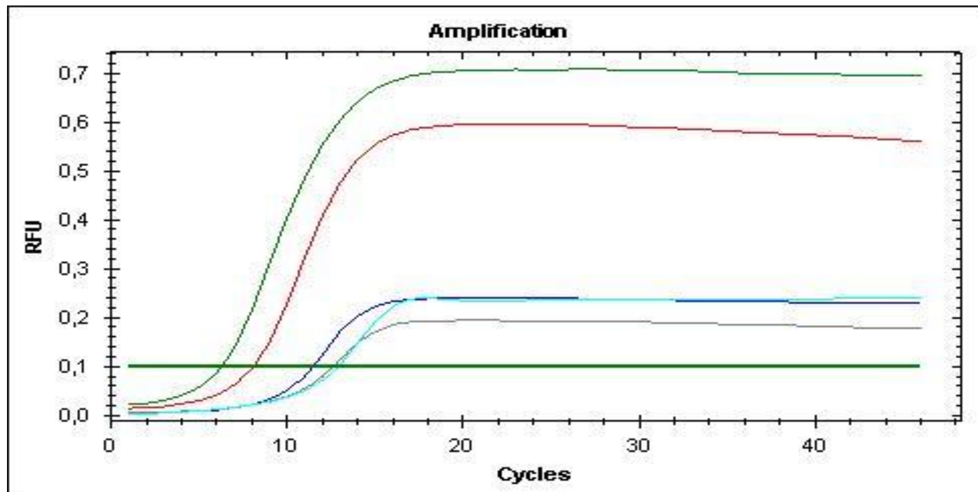


Standard *F. catus* melt curve

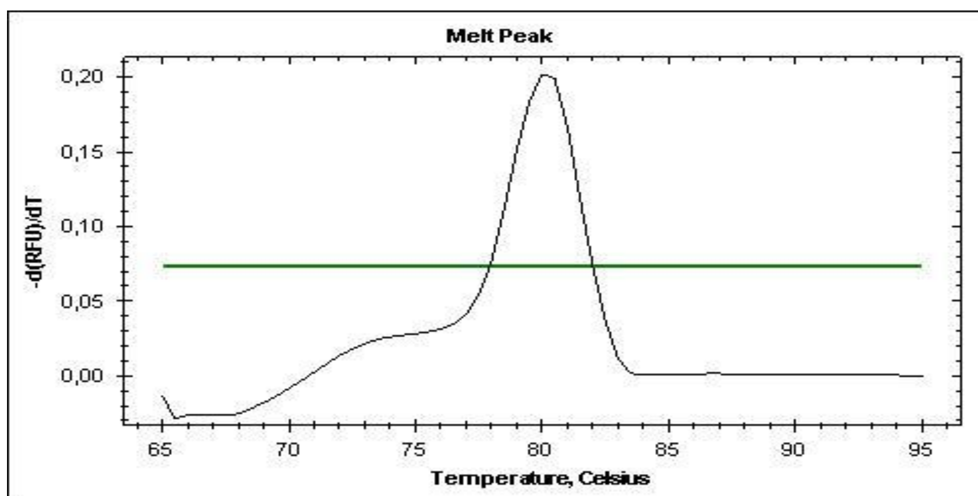


Bloodmeal analysis, *F. catus* primers

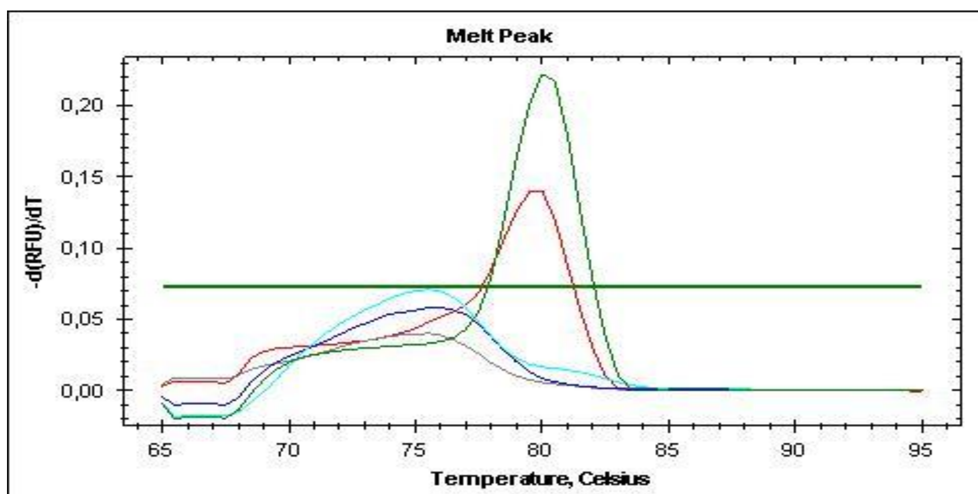
Figure 16. Real-time qPCR amplification and melt curves of ticks amplified with *F. catus* primers. The standard melt curve displays the melt curve of *F. catus* control product amplified with *F. catus* primers.



C. capreolus qPCR amplification curve

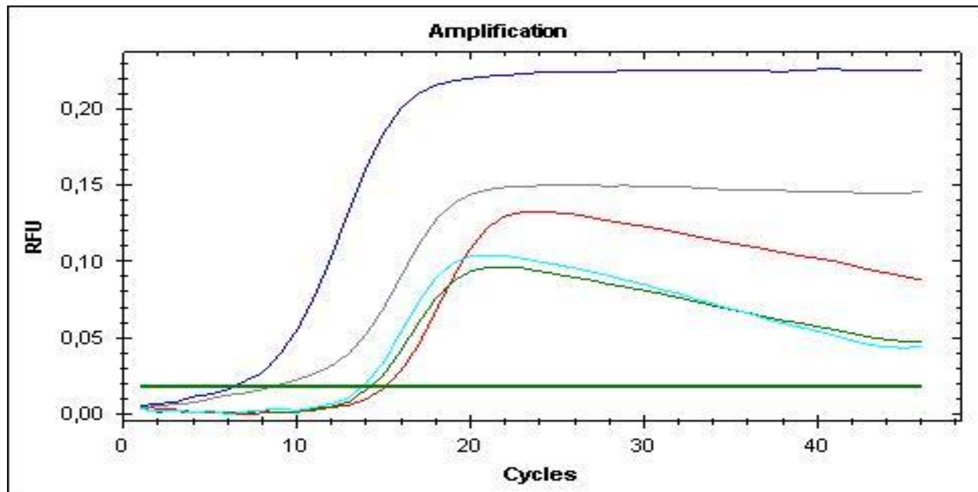


Standard *C. capreolus* melt curve

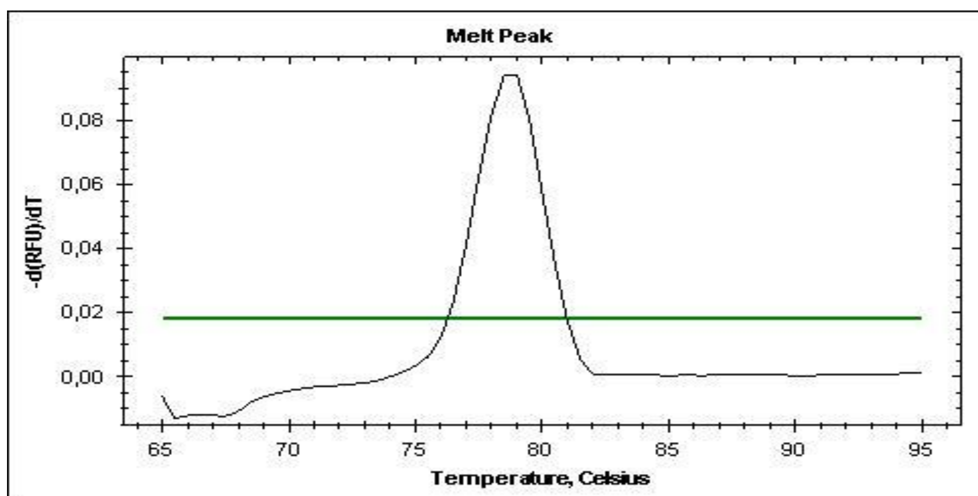


Bloodmeal analysis, *C. capreolus* primers

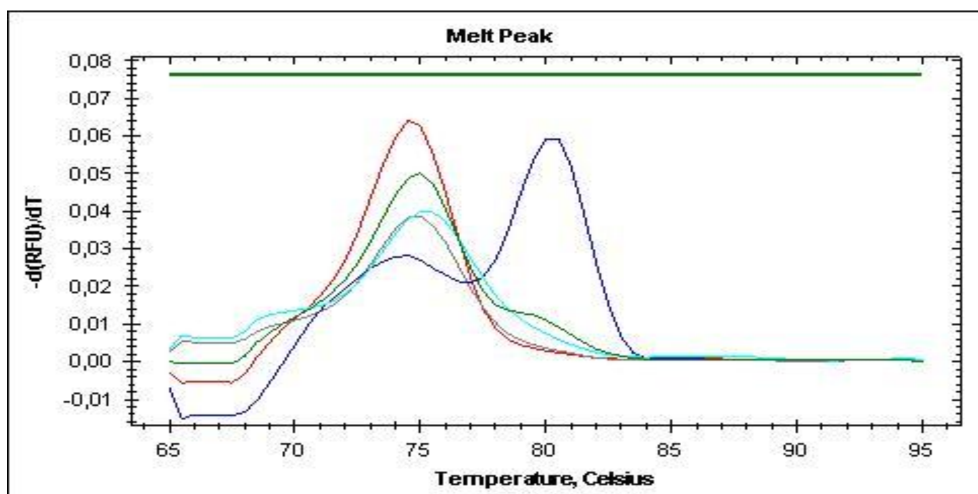
Figure 17. Real-time qPCR amplification and melt curves of ticks amplified with *C. capreolus* primers. The standard melt curve displays the melt curve of *C. capreolus* control product amplified with *C. capreolus* primers.



Apodemus spp. qPCR amplification curve

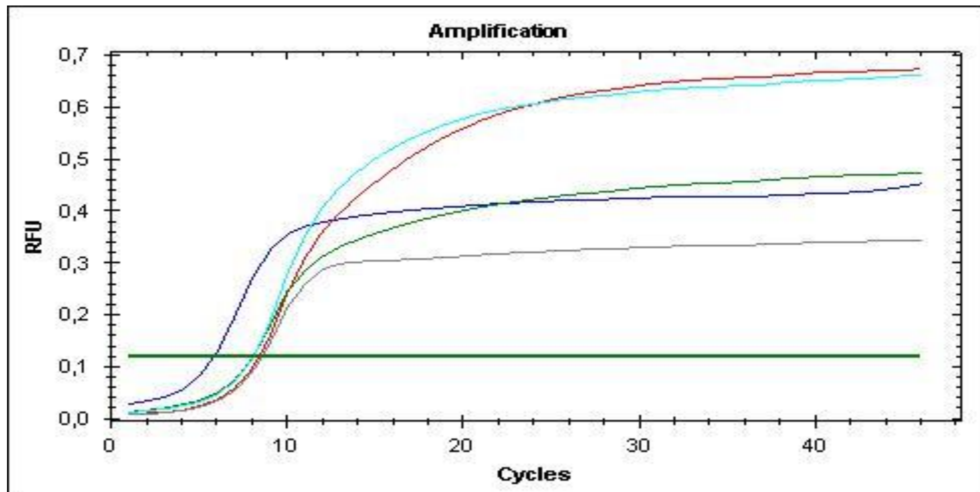


Standard *Apodemus* spp. melt curve

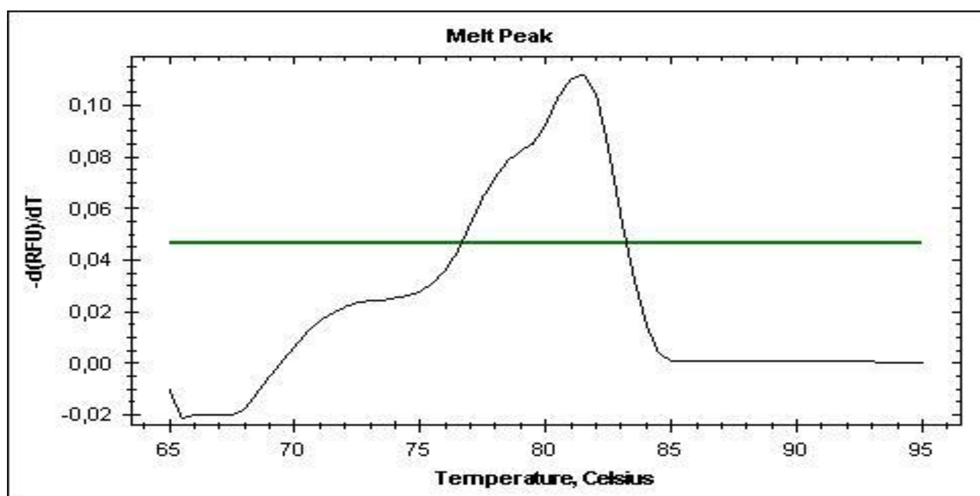


Bloodmeal analysis, *Apodemus* spp. primers

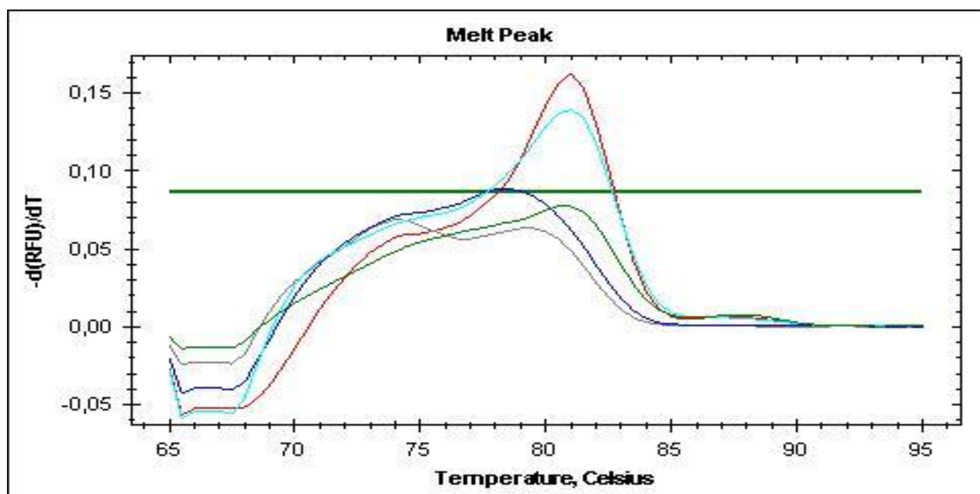
Figure 18. Real-time qPCR amplification and melt curves of ticks amplified with *Apodemus* spp. primers. The standard melt curve displays the melt curve of *A. sylvaticus* control product amplified with *Apodemus* spp. primers.



M. glareolus qPCR amplification curve



Standard *M. glareolus* melt curve



Bloodmeal analysis, *M. glareolus* primers

Figure 19. Real-time qPCR amplification and melt curves of ticks amplified with *M. glareolus* primers. The standard melt curve displays the melt curve of *M. glareolus* control product amplified with *M. glareolus* primers.

The identities of hosts based on bloodmeal analysis were determined by two independent observers, and later confirmed by the Experion™ Automated Electrophoresis System and/or sequencing. Figure 20 displays ticks which tested positive (lanes 1-6) or negative (lanes 7-9) for *F. catus* using *F. catus* primers in real-time qPCR. An approximately 250 bp long band is visible in the Experion™ Automated Electrophoresis System. The product in lane 1 gave a strong signal in the real-time qPCR amplification and melt curves (Figure 20 B and C), as well as in the electrophoresis (Figure 20 A). The product in lane 4 gave weaker, yet specific signals in the real-time qPCR amplification and melt curves, and produced many non-target bands in the electrophoresis in addition to a strong *F. catus* band. The product in lane 8 gave no signal using either method.

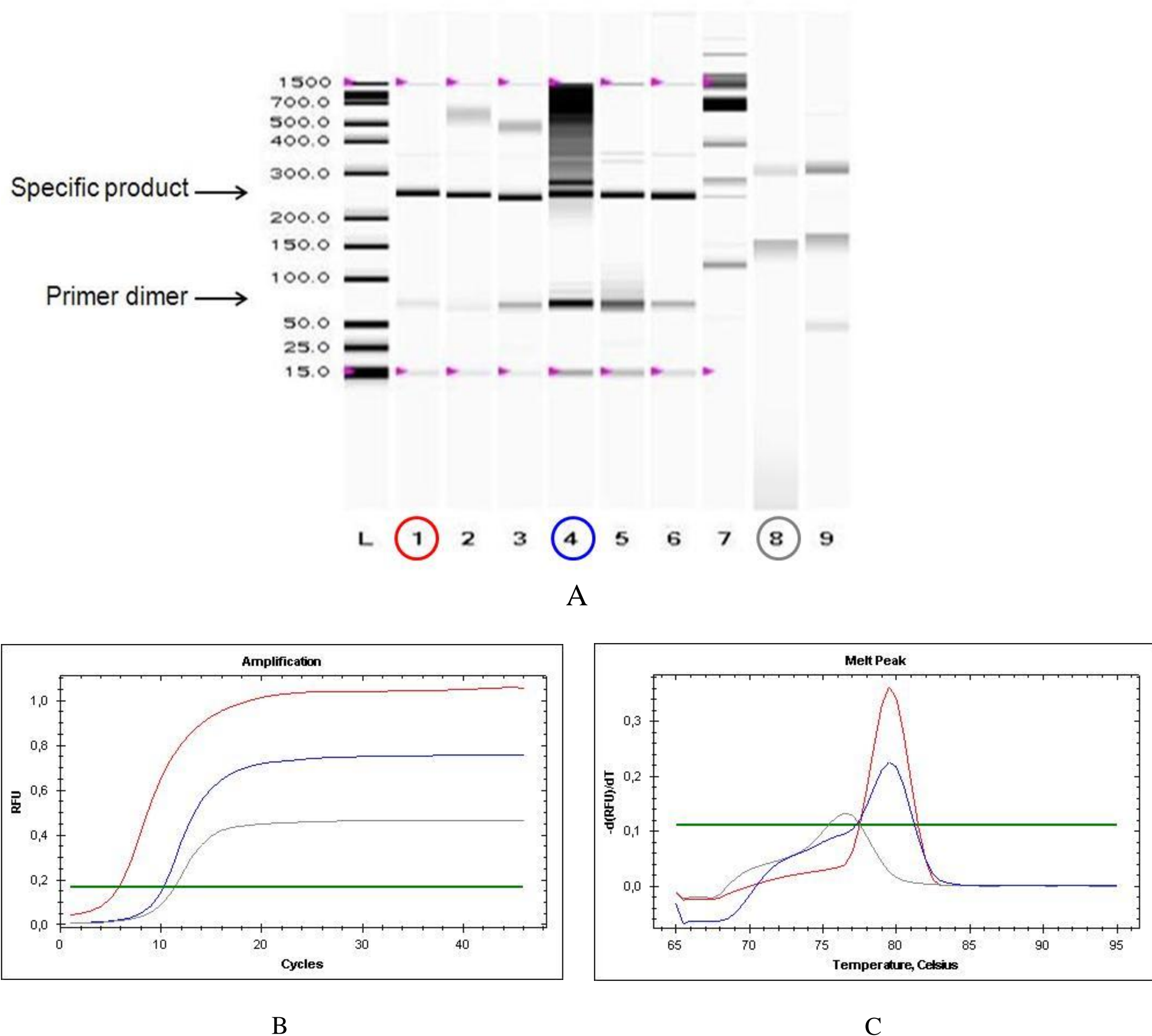


Figure 20. A: Electrophoresis of products amplified by real-time qPCR from tick samples using *F. catus* primers. Lanes: L = molecular size marker, 1-9 = tick products. B: Amplification curves of products from lane 1 (red), lane 4 (blue) and lane 8 (grey). C: Melt curves of products from lane 1 (red), lane 4 (blue) and lane 8 (grey).

Some cross-reactions were observed, in particular between the *F. catus* and *C. capreolus* primers. These were distinguishable through the amplification curve, as well as by the strength of the melt curve signal. One tick was misidentified through observation of the real-time qPCR amplification curves and melt curves. This isolate amplified strongly with roe deer primers, however, subsequent sequencing revealed the bloodmeal source to be *F. catus*. One reason for the cross-reactivity of the *F. catus* and *C. capreolus* primers may be the use of a common reverse primer for these two host species. A specific product with a distinct melt curve was amplified from some ticks with both *C. capreolus* and *Apodemus* spp. primers. As controls did not display any pattern of cross-reaction between these species and primer pairs, this was believed to represent DNA from an unknown host species.

The previous bloodmeal hosts of 45 of 91 nymphal and adult *I. ricinus* (49%) were successfully identified using the amplification curves and melt curves of real-time qPCR, see Table 5.

Table 5. Identities of the previous bloodmeal hosts of adults and nymphs tested by real-time qPCR. Numbers in brackets represent identities confirmed by sequencing.

	F. catus	***M. glareolus***	***Apodemus* spp.**	***C. capreolus***	**No host identified**	**Total**
Females						
Nesodden	1	1	0	0	3	5
Tomb	0	3 (2)	5	1 (1)	14	23 (3)
Arendal	1 (1)	0	0	1	0	2 (1)
Males						
Nesodden	2 (1)	1	0	1 (1)	1	5 (2)
Tomb	2 (2)*	1	1	0	6	10 (2)
Vet clinic	7 (4)	1	1	0	3	12 (4)
Other	1 (1)	0	0	0	3	4 (1)
Nymphs						
Tomb	4 (3)	3	5	2	16	30 (3)
Total	18 (12)	10 (2)	12	5 (2)	46	

*One tick was amplified using *C. capreolus* primers, but was revealed by sequencing to be *F. catus*.

In total, 18 ticks were identified as having fed on *F. catus*, 12 bloodmeals were identified as *Apodemus* spp., ten as *M. glareolus* and five as *C. capreolus*, see Figure 21. The bloodmeals of 46 ticks were not identifiable. As some males were collected directly from hosts (most of these from *F. catus*), it is possible that these had already fed prior to sampling, or had been contaminated during collection. However, out of 17 cat-collected male ticks, only nine of the

bloodmeals were identified as *F. catus*. Four of the tick bloodmeals were unidentifiable, two had fed on *M. glareolus*, one on *A. sylvaticus*, and one had fed on *C. capreolus*. Three males, one from Arendal (Aust-Agder) and two from Oslo, were also collected from *C. lupus familiaris*, a species which we did not test for. None of these ticks gave positive results with real-time qPCR.

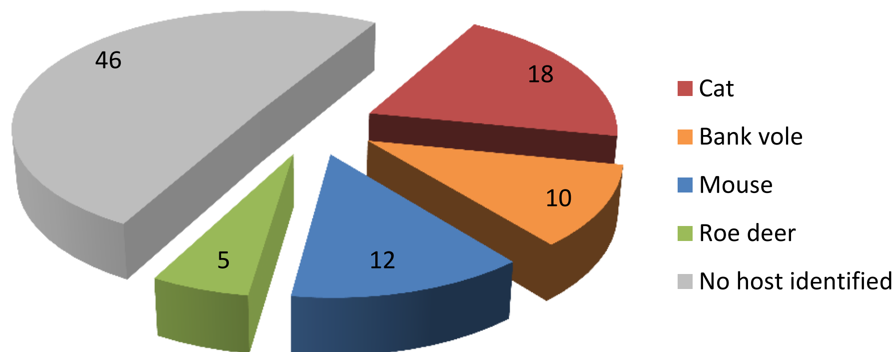


Figure 21. The total number of bloodmeal hosts identified as cat, bank vole, mouse or roe deer across all tick stages and localities. 46 tick bloodmeals were not identifiable.

The prevalence of the different hosts varied between the localities sampled and the life stages of the ticks. Interestingly, the most commonly identified bloodmeal host of females was *Apodemus* spp., followed by *M. glareolus* (Figure 22). The bloodmeal DNA extracted from males was mainly identified as *F. catus* (Figure 23), and nymphs had mainly fed on *Apodemus* spp. and *F. catus* (Figure 24).

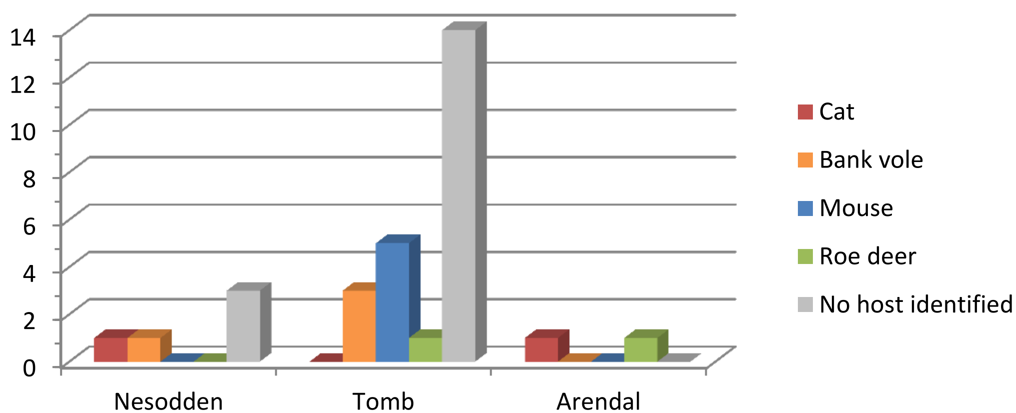


Figure 22. The identified bloodmeal hosts of female *I. ricinus* collected at different localities.

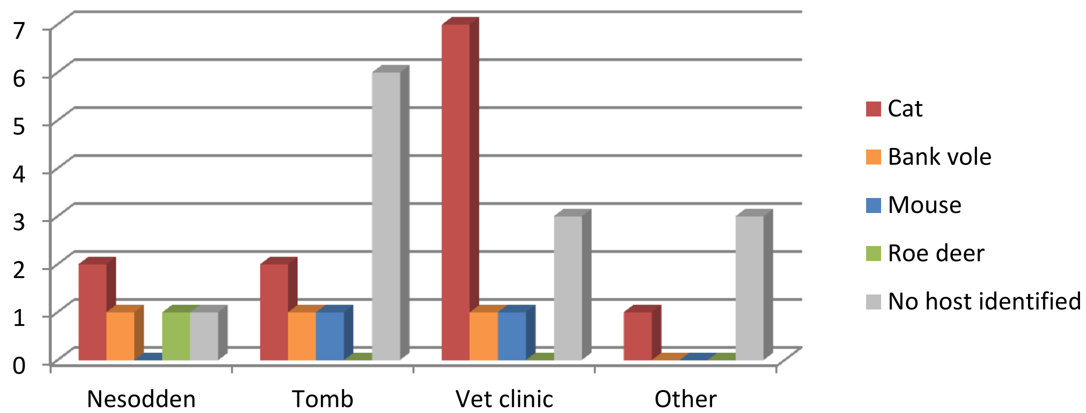


Figure 23. The identified bloodmeal hosts of male *I. ricinus* collected at different localities.

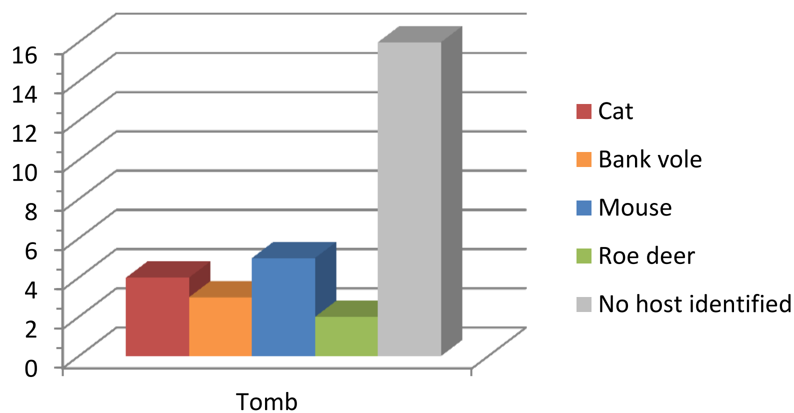


Figure 24. The identified bloodmeal hosts of nymphal *I. ricinus* collected at Tomb.

3.5.1 Bloodmeal host and scutum size

The scutum sizes of the measured nymphs did not appear to be closely related to the previous bloodmeal hosts of the ticks, see Figure 25. However, known hosts seem to be over-represented in the small scutum sizes, and ticks which failed to amplify a previous host meal are more highly represented in the medium and larger scutum sizes.

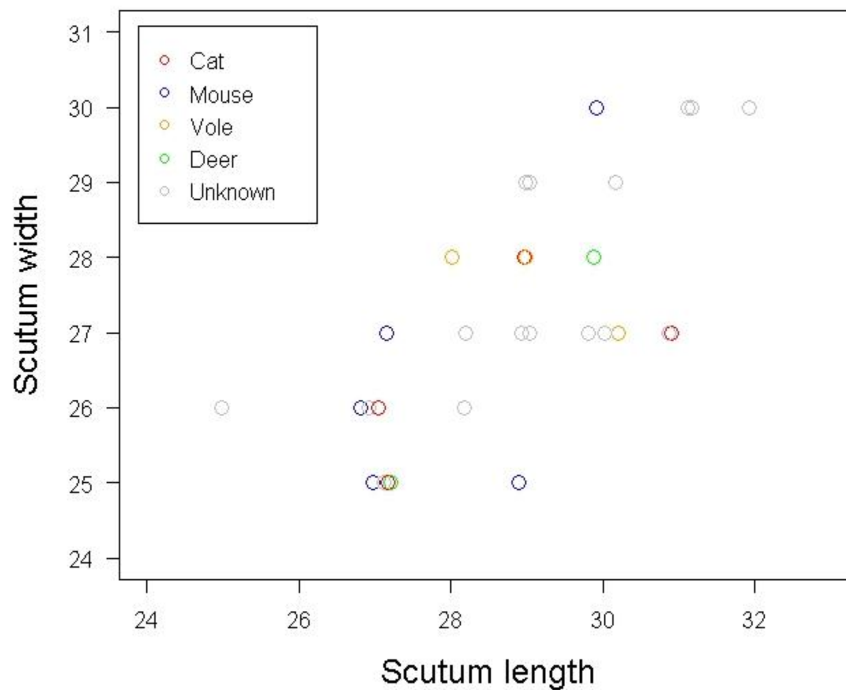


Figure 25. Scutum sizes and bloodmeal hosts of the measured nymphs.

3.6 Prevalence of *Borrelia*

Two out of 122 ticks (2%) tested positive for *Borrelia*, see Table 6. Sequencing revealed that the ticks, both nymphs collected by blanket dragging at Nesodden, were infected with *B. afzelii*.

Table 6. *Borrelia* prevalence in unfed *I. ricinus* in different localities.

Site	Infected/examined (infection prevalence)	
	Nymphs	**Females**
Tomb	0/0	0/9 (0%)
Nesodden	2/113 (2%)	0/0

3.7 Prevalence of *Babesia*

19 out of 222 ticks (9%) tested positive for *Babesia* (see Figure 26 for example gel electrophoresis). The *Babesia* prevalence varied between different tick life stages and field sites, see Table 7. Despite habitat and biodiversity differences, the overall *Babesia* prevalences at Tomb and Nesodden were similar at 5% and 7%. The highest *Babesia*

prevalence was found in nymphs at Tomb, where 27% of 30 nymphs were infected. No infected males were found at Tomb.

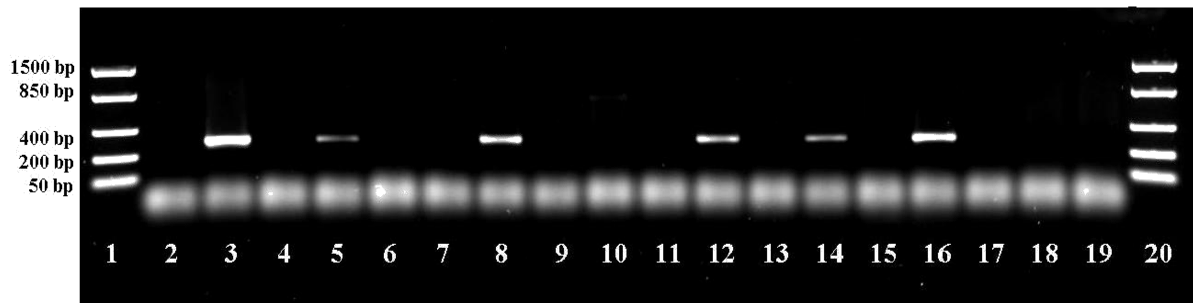


Figure 26. Gel electrophoresis of *Babesia* products amplified from nymphal ticks collected at the Tomb field site. Lanes: 1 and 20 = molecular size marker FastRuler™ Low Range (Thermo Scientific, Waltham, Massachusetts, USA), 2 = negative water control, 3 = positive control (from infected *Myodes* blood), 4-19 = DNA extracted from nymphal ticks. The resulting products in lanes 3, 5, 8, 12, 14 and 16 are ≈ 400 bp.

Table 7. *Babesia* prevalence in unfed *I. ricinus* in different localities.

Site	Infected/examined (infection prevalence)			Total
	Nymphs	Females	Males	
Tomb	8/30 (27%)	1/23 (4%)	0/9 (0%)	9/62 (5%)
Nesodden	8/133 (6%)	1/5 (20%)	1/4 (25%)	10/142 (7%)
Other (Røyken, Arendal, Oslo)	0/0	0/2 (0%)	0/16 (0%)	0/18 (0%)
Total	16/163 (10%)	2/30 (7%)	1/29 (3%)	19/222 (9%)

3.7.1 Nymph scutum sizes and *Babesia* infection

A Spearman's rank correlation revealed no association between the size of the nymphal scutum and *Babesia* infection presence in nymphal ticks at the Tomb study area ($\rho(28)$ = -0.15, P = 0.44; Figure 27 A). It is also apparent from Figure 27 B that there is no general pattern of *Babesia* prevalence associated with certain scutum sizes.

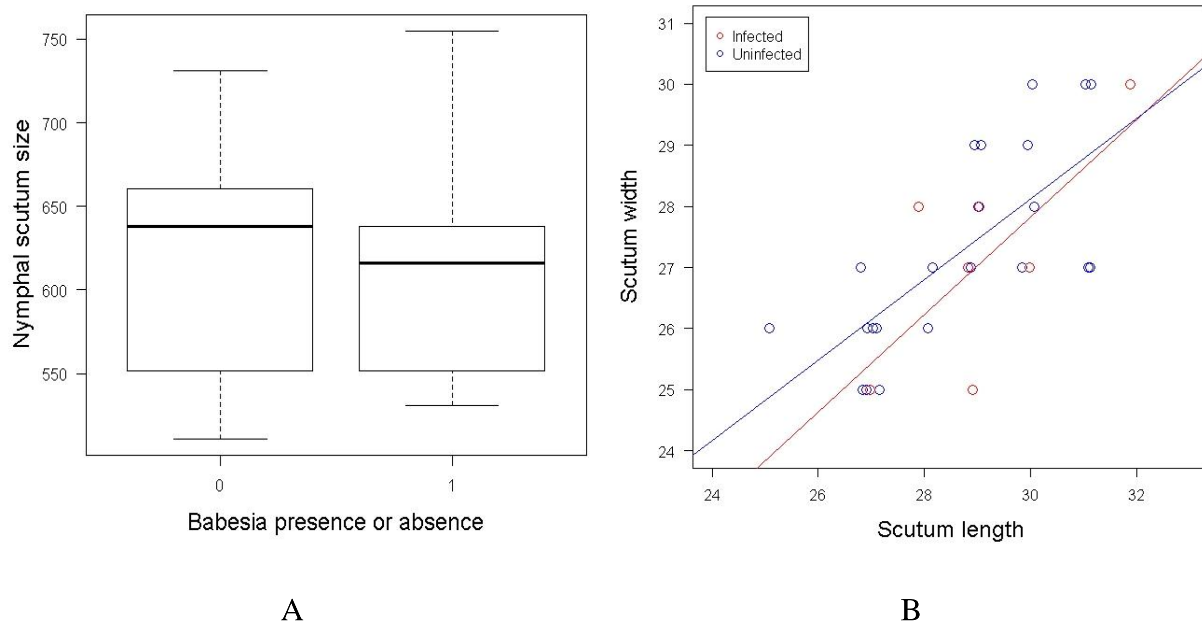


Figure 27. A: The nymphal scutum size is not correlated with *Babesia* presence (1) or absence (0). B: *Babesia* prevalence does not appear to be associated with certain scutum sizes. Regression lines are displayed in red (infected ticks) and blue (uninfected ticks).

3.7.2 Species identification of *Babesia*

12 of the 19 *Babesia*-infected ticks were successfully sequenced after PCR of the *18S rDNA* gene fragment of *Babesia*. Two sequences matched *B. microti* isolates, and the remaining ten matched a *B. venatorum/capreoli/divergens* cluster, see Table 8 and Appendix 3. As the region amplified is identical for *B. venatorum, B. capreoli* and *B. divergens*, a clear identification of these isolates was not possible. Six of the *Babesia*-positive samples did not yield enough product for sequencing, and were treated as *Babesia* spp. Any mismatch between the isolates and reference Genbank sequences are probably due to sequencing errors and not genuine differences in the DNA fragments.

For four ticks from Tomb, both bloodmeal host and *Babesia* species were identified. These consisted of three nymphs infected with *B. venatorum/capreoli/divergens,* one of which had fed on *Apodemus* spp., one had fed on *C. capreolus* and one on *M. glareolus*. One other nymph found to be infected with *B. microti* had fed on *M. glareolus*.

Table 8. *Babesia* species identified from tick isolates.

No. of isolates	***Babesia* spp.**	**Locality**	**Life stage**	**Extent of similarity**
1	*B. venatorum* *B. capreoli* *B. divergens*	Tomb	Nymph	99.5%
4	*B. venatorum* *B. capreoli* *B. divergens*	Tomb	Nymph	100%
1	*B. venatorum* *B. capreoli* *B. divergens*	Tomb	Nymph	99.3%
1	*B. venatorum* *B. capreoli* *B. divergens*	Tomb	Nymph	92.3%
1	*B. microti*	Tomb	Nymph	100%
1	*B. venatorum* *B. capreoli* *B. divergens*	Nesodden	Nymph	95.9%
1	*B. venatorum* *B. capreoli* *B. divergens*	Nesodden	Nymph	100%
5	*Babesia* spp.	Nesodden	Nymph	Not enough product for sequencing
1	*B. microti*	Tomb	Female	95.9%
1	*B. venatorum* *B. capreoli* *B. divergens*	Nesodden	Female	92.2%
1	*Babesia* spp.	Nesodden, cat	Male	Not enough product for sequencing

4 Discussion

An increased knowledge of the vector-host ecology of *I. ricinus* can aid our understanding of the risks associated with tick-borne diseases. As *I. ricinus* is considered a generalist parasite (Mehl *et al.* 1987), knowledge of the diversity of hosts and their relative tick burdens in an area can be used to minimise the risk of human disease.

4.1 Tick and rodent host ecology at Nesodden

Host trapping at Nesodden revealed a very small population of *Apodemus* spp., consisting mainly of *A. sylvaticus*. Although no other species were trapped, the area also holds other small rodents, such as the common shrew (*Sorex araneus*) the European water vole (*Arvicola amphibius*) and the field vole (*Microtus agrestis*) (personal observations). The small number of trapped rodents did not allow for an accurate population estimation. Although statistically insignificant due to a small sample size, older male *Apodemus* spp. appeared to suffer the highest tick burdens, corresponding well with other studies. It is commonly seen that the level of tick burden is associated with the movement patterns of the host. Male *Apodemus* spp. have greater home ranges than females, and mature males range further than sexually immature males (Randolph 1977; Matuschka *et al.* 1992b; Talleklint and Jaenson 1997), explaining the pattern found in this study.

In Norway, ticks are mainly active from April to September, with a peak in activity around May-June. This may, however, vary to a great extent between years depending on weather conditions (Talleklint and Jaenson 1997; Mehl 1999). The seasonality of reports of *Borrelia* infections reflects the seasonality of the ticks, with a short delay before medical attention is sought and a diagnosis is set. The number of reported cases usually starts to increase around May, peaks in August and slows down again in autumn, coinciding with the peak activity times of ticks (Nygård *et al.* 2005), see Figure 28.

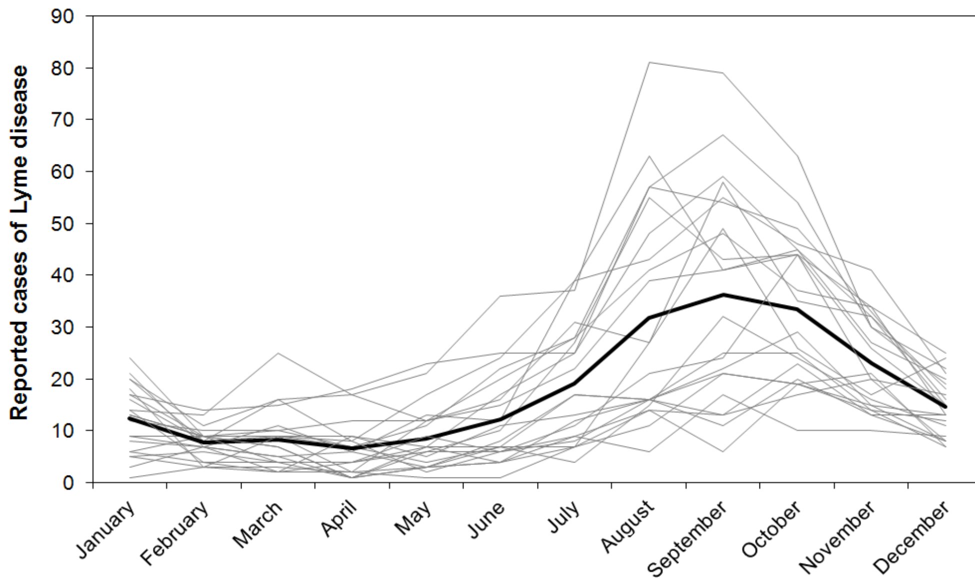


Figure 28. Seasonal variation in the number of reported cases of Lyme borreliosis in Norway from 1990-2011. The bold line shows the average number of cases reported during the 21-year period. Data from Anon (2012b).

In this study, larvae showed uni- or bimodal seasonality, with a small activity peak in May and a large peak in August (Figure 12 A, p. 30). Nymphal activity is unimodal with a large peak in May (Figure 12 B, p. 30).

Other European studies have found great variation in the seasonality of tick activity patterns between locations and between years. Both larvae and nymphs have been found to display unimodal and bimodal seasonal activity patterns in different locations and in different years. Larval unimodal peaks have been reported both in May (Vassallo *et al.* 2000) and in August (Nilsson 1988), and in the studies reporting bimodal larval activity distributions, the first peak usually appears in June, and the second peak appears between July and October (Gray 1985; Nilsson 1988).

Nymphs display either one peak in May/July, e.g. in Yvelines, France (Vassallo *et al.* 2000) or a bimodal activity pattern peaking in June and October (Nilsson 1988). Talleklint and Jaenson (1996) investigated the seasonality of questing nymphs in southern Sweden for three consecutive years. They found three different seasonal distribution patterns: two showing bimodal distributions peaking in May/June and September, and one showing a unimodal pattern peaking in May. Our findings resemble this latter seasonal distribution pattern.

The questing tick seasonality found in this study are fairly consistent with the mentioned reports, as one early and one late larval peak in a season is not uncommon. Interestingly, the unimodal nymphal peak is more similar to that found in France (Vassallo *et al.* 2000) than the patterns recorded by Nilsson (1988) in southern Sweden. It would be interesting to explore the activity patterns in the Nesodden population more closely, as some gaps between sampling sessions may prevent us from seeing the full picture. Some year-to-year variation can also be expected. In a 5-year study in one site in southern England, Randolph *et al.* (2002) discovered considerable variation in the annual activity patterns of questing *I. ricinus* larvae and nymphs. Larval peaks varied from June to September, and both unimodal and bimodal distribution patterns were seen. Nymphal activity first peaked between March and May, and displayed between one and three peaks before the end of the season.

A nymphal peak in May also corresponds well with the annual increase in reported cases of Lyme disease in Norway, as seen in Figure 28. The rise in disease incidence around July could be caused by an increase in nymphal activity around May, as nymphs are considered to be the main cause of human disease (Radolf *et al.* 2012).

Larval ticks commonly feed on rodent hosts (Gray 2002), and this is reflected by the relatively high abundance of larvae found on the trapped rodents. Larvae were found on rodents throughout the trapping season, indicating that these are important hosts for the larvae in Nesodden. Nymphs were rarer on rodents, as these also feed on a range of larger mammals (Gray 2002). In this study, nymphs were only found on rodent hosts in May and June, shortly after the peak in questing nymphal activity. It is possible that the drop in the number of questing nymphs between May/June is due to host attachment and feeding, so it is not surprising that nymphs were found on rodents at this time.

4.2 Development of technique

Tick bloodmeals contain small amounts of degraded DNA. Therefore, the efficiency of the extraction method is important, so that the highest possible amount of DNA can be recovered from each tick. Our extraction sensitivity analysis revealed that the standard ammonium extraction was marginally more sensitive than the E.Z.N.A.® Tissue DNA Kit. Ammonium extraction is cheap, extracts bacterial DNA effectively, is considered the gold standard of tick

extractions, and is widely used in the field (Kirstein and Gray 1996; Pichon *et al.* 2003; Allan *et al.* 2010).

The results of the E.Z.N.A.® Tissue DNA Kit may have been improved by eluting the DNA with two aliquots of 50 µl instead of 100 µl elution buffer in the final stage, as this would have created a higher DNA concentration. It is, however, worth noting that the ammonium hydroxide method is designed specifically to break down the chitinous tick exoskeleton. In this experiment, the blood was extracted together with larval ticks, ignoring this barrier. In a real life situation the bloodmeal would be inside the tick, and a method which can break down chitin, such as ammonium extraction, may prove to be advantageous.

The *12S rDNA* and *cyt b* primers used for primer testing worked well when applied to non-degraded vertebrate DNA. The *cyt b* primers tested displayed a slightly lower level of cross-specificity than the *12S rDNA* primers. When applied to degraded DNA from the tick gut, however, it became clear that a higher sensitivity was required. Increasing the number of amplification cycles led to the amplification of non-target DNA, and traditional PCR methods were substituted with real-time qPCR.

4.3 Real-time qPCR host identification

When testing with real-time qPCR, the previous bloodmeal hosts of 49% of 91 ticks were successfully identified. The data files from the real-time qPCR were verified individually by two observers before the results were compiled and validated using sequencing and electrophoresis. The use of *cyt b* as a molecular marker allowed for the identification to species level for three hosts (*F. catus, M. glareolus* and *C. capreolus*) and to genus level for two hosts (*Apodemus* spp.). The use of real-time qPCR allowed products to be readily available for confirmation by sequencing and electrophoresis. This was especially important in the case of *F. catus* and *C. capreolus* products as some cross-reactions were observed.

As larvae mainly feed on small mammals such as rodents, it was not surprising to find that the nymphs in this study had mainly fed on *Apodemus* spp. The male ticks had mainly fed on *F. catus*. Female ticks had mainly fed on *Apodemus* spp. and *M. glareolus*, which are unusual hosts for adult ticks. However, females were also the group of ticks with the highest level of

unidentifiable hosts (57%), indicating that other hosts than those tested for in this study may be important.

4.4 Previous tick bloodmeal analyses

Several previous studies have attempted to identify the previous bloodmeals of moulted ticks, primarily through RLB methods. None of these studies have confirmed their findings by sequencing.

In a laboratory study (Kirstein and Gray 1996), larval *I. ricinus* ticks were fed on *Mus musculus*. Once fully fed, they were frozen at ten day intervals. PCR was performed using primers targeting a 638 bp segment of the *cytochrome b* region of the mitochondrial genome. Host DNA was detectable by PCR and restriction fragment length polymorphism (RFLP) analysis in the fed larvae up to 10 days postengorgement. A nested amplification targeting a smaller 368 bp product within this segment allowed detection of DNA for up to 40 days postengorgement, i.e. until moulting. A different method targeting a 135 bp *cyt b* segment in the first round of PCR and a 95 bp segment in the second amplification, allowed detection of the bloodmeal of moulted nymphs for up to 200 days postengorgement. Reverse line blot (RLB) was used to identify these PCR products as *M. musculus*. While the authors confirmed the findings by sequencing when working with fed ticks, none of the moulted tick samples were sequenced.

In a similar study by Pichon *et al.* (2003), *I. ricinus* larvae were allowed to feed on gerbils (*Meriones unguiculatus*), *M. musculus* and a rabbit (*Oryctolagus cuniculus*). Once fully fed and moulted, the resulting nymphs were kept in tubes in a field location and sampled monthly. Nymphal ticks were collected from the vegetation for comparison. Vertebrate DNA was amplified by PCR targeting the nuclear *18S rRNA* gene, and sequences identified by RLB using vertebrate subgroup-specific probes. The bloodmeals of ticks fed in the lab were detectable for up to seven months after moulting. Furthermore, host identification was possible for 26 out of 49 field-collected nymphs (53%). 14 of these contained ruminant DNA, ten had fed on birds, and two nymphs tested positive for rodent DNA. The same RLB methods and *18S rDNA* probes were used on a number of occasions in different geographical locations. Estrada-Peña *et al.* (2005) collected unfed nymphs from a field location in the Rioja region of north-central Spain. A total of 61 nymphs were tested. A RLB was performed using

18S and *12S rDNA* probes. 22 of the 61 ticks (36%) gave an unambiguous host identification. 14 of these bloodmeals were from birds (Galliformes and Passeriformes), four were from wild boar (*Sus scrofa*), three were from *Apodemus* spp., and one was from *Cervus* spp.(deer).

Following this, Pichon *et al.* (2005) collected nymphs and adult ticks from vegetation in Ireland by flagging, Host identification by *18S rDNA* RLB was possible in half (49.4%) of nymphs tested, and the identification success varied through the season. 46.4% were identifiable in spring, 40.4% in summer, and 55.7% of bloodmeals were identified in nymphs collected in autumn. 76 nymphs were positive for bird DNA (64 Passeriformes, 12 Galliformes), 61 for ruminant DNA and 22 for rodent DNA (four *Apodemus* spp., four *M. glareolus* and 14 *Sciurus* spp.). Cross-reactivity, attributed to interrupted feeding, was identified in two nymphs that tested positive for *Sciurus* spp. and ruminant DNA. In a further study, Pichon *et al.* (2006) used the same methods to test nymphal *I. ricinus* ticks collected in Germany. Host DNA was amplified in 33% of the collected ticks. 11 of these were from rodents (*Apodemus* spp., *M. glareolus* and *Sciurus* spp.), eight were from birds (Passeriformes and Galliformes), and one tick tested positive for ruminant DNA.

Allan *et al.* (2010) used RLB and probes of their own design targeting the *18S rRNA* gene in order to identify the previous bloodmeal hosts of field-collected Amblyomma americanum nymphs from Missouri, United States. 62.8% of the 1 383 ticks tested were identifiable, and cross-reactivity was detected in 16.2% of the ticks. This was attributed to interrupted feeding. Most of the bloodmeals identified came from white-tailed deer (*Odocoileus virginianus* 44.8%*)*, birds (Galliformes 11.2%, Passeriformes 10.8%) and *Sciurus* spp. (10.9%). Humair *et al.* (2007) collected questing adult and nymphal *I. ricinus* ticks from vegetation in Switzerland. These were tested for previous host identity using a single-round PCR amplification of the *12S rRNA* region of the mitochondrial genome, followed by RLB. The *12S rDNA* primers and probes were of their own design. Approximately half (48.6%) of the 109 tested ticks were identifiable, and the detection rate in adults was significantly higher than in nymphs. One third of the identified ticks could only be determined to group level (small rodent, bird or artiodactyl), and the rest were identifiable to genus or species level. Mixed bloodmeals were detected in three tested ticks (0.03%). The same methods were used when Cadenas *et al.* (2007) collected questing *I. ricinus* nymphs and adults in a field location and used RLB in order to identify their previous hosts. Host DNA was found in 44% of the

1 326 ticks, and more hosts were identifiable in the adult ticks than in the nymphs. The most commonly identified hosts were *S. vulgaris* (18% of identified hosts), *S. scrofa* (15.7%), *V. vulpes* (8.4%) and *C. capreolus* (6.6%). 111 (8%) ticks appeared to contain the DNA of more than one host. This cross-reactivity was as common in nymphs as in adults, and was believed to be caused by interrupted feeding or external contamination due to unsuccessful host-encounters. Similar methods were also used by Scott *et al.* (2012) when testing for *A. americanum* and *I. scapularis* bloodmeals.

Grigoryeva and Markov (2012) used a heminested PCR approach with gel electrophoresis targeting the *12S rRNA* gene to identify the pervious bloodmeal hosts of field-collected nymphal *Ixodes persulcatus*. 30.8% of 143 hosts were identifiable, and multiple hosts were identified for 3.5% of the ticks.

None of these studies confirmed their findings of questing tick bloodmeal hosts by sequencing. RLB does not allow for direct sequencing of the product, in contrast to real-time qPCR. This, combined with the increased sensitivity of the real-time qPCR method, makes the latter a better option for identifying the previous bloodmeal hosts of ticks.

Some bloodmeals in the present study were not identifiable, and two possible explanations are suggested for this. With increasing time from feeding, the bloodmeal is increasingly degraded, and after a certain period it may not be possible to identify the remaining DNA fragments (Kirstein and Gray 1996). In field-collected ticks, we have no measure of the time passed since the last bloodmeal of the ticks, and this factor cannot be ruled out. Alternatively, the unidentified bloodmeals could originate from other hosts than the five species we tested for. As roughly half of the bloodmeals were identifiable using primer pairs reactive to only five host species (*F. catus, M. glareolus, C. capreolus, A. sylvaticus* and *A. flavicollis*), a wider range of primers would presumably reveal even more bloodmeal identities. This is supported by the appearance of unknown non-*C. capreolus* and non-*Apodemus* product being amplified by the *C. capreolus* and *Apodemus* spp. primers. The use of further primer pairs was not possible in this study due to time constraints. It would be interesting to test the remaining samples for other vertebrate DNA, in particular for bird DNA, hare *(L. timidus)*, moose *(A. alces)* and dog (*C. lupus familiaris),* as these hosts are abundant in some of our study sites. Tomb was the site with the most unidentifiable bloodmeals, with 58% of the bloodmeal hosts unknown. Only 40% of the Nesodden ticks were unidentifiable. As Tomb is

a forest location with a wider range of available hosts than Nesodden, it can be expected that many of the ticks would have fed on other hosts than the four groups that we tested for. *A. alces* and *L. timidus* are particularly abundant in the forest at Tomb (personal observations), and both species are expected to harbour a large number of ticks. Therefore, these two host species may contribute significantly to the tick population in the area.

Interrupted feeding has been documented on several occasions within the hard ticks. In one study, *Rhipicephalus appendiculatus* females fed successfully on a second guinea pig (*Cavia porcellus*) host when interrupted from feeding during the first four days of attachment (Wang *et al.* 1999). *I. dammini* nymphs fed on *M. musculus* were also able to successfully feed on a second host after removal from the first host within 48 hours (Shih and Spielman 1993). Similar findings have also been documented for *I. ricinus* nymphs fed on *M. musculus* (Výrosteková 1994). While interrupted feeding is possible in *I. ricinus* under laboratory conditions, we did not find any evidence for this occurring in the field-collected ticks in this study. Some results of the real-time qPCR were ambiguous, and did at first appear to display interrupted feeding. However, further testing revealed this to be caused by cross-reactivity of the primers. Several previous tick bloodmeal analyses using RLB have claimed to have found evidence for interrupted feeding in a small percentage of ticks tested, without having sequenced the products. Our primers worked well for identifying vertebrates from control DNA samples using traditional PCR, but not for degraded vertebrate DNA from ticks. This suggests that vertebrate DNA controls of the RLB method is insufficient for determining its specificity when analysing DNA from ticks. As this has been the only method of confirming the specificity of the RLB probes, it is still not clear that RLB produces genuine results when testing tick bloodmeals.

Some male ticks in this study were collected directly from hosts, and it is possible that some of these had fed. Of those collected from one of our five target hosts, *F. catus*, only 9 out of 17 ticks (53%) tested positive for this host. Furthermore, two had fed on *M. glareolus*, one on *A. sylvaticus* and one on *C. capreolus*, indicating that the vertebrate DNA from males also gives genuine product from nymphal bloodmeals, and not just the sporadic feeding of adult males or external contamination.

4.5 Pathogens

As the distribution limits of *I. ricinus* expand (Jore *et al.* 2011), new areas are exposed to tick-borne diseases such as Lyme disease and babesiosis. These diseases pose a threat to human and animal health, and they can also have a large economic impact through human healthcare and illness of livestock. Knowledge of the prevalence and risks associated with these diseases enable the development of pathogen control strategies such as public awareness campaigns and management of vector hosts.

In this study, 12 of 19 ticks which tested positive for *Babesia* spp. were successfully identified to species level. Two of the ticks carried *Babesia microti*. While other *Babesia* genospecies are relatively host-specific, *B. microti* appears to display a relatively low specificity to its hosts. It has mainly been found in small mammals such as the tundra vole (*Microtus oeconomus*), field vole (*Microtus agrestis*), *M. glareolus* and *A. sylvaticus*, as well as a range of other rodents and insectivores (Karbowiak and Sinski 1996; Karbowiak *et al.* 1999; Sinski *et al.* 2006). Rodents of the genus *Microtus* have usually been found to be the best reservoirs for *B. microti* (Sinski *et al. 2006)*. While present in mainland Norway, *M. oeconomus* are not found near our study sites at Nesodden or Tomb (Anon 2012a). *M. agrestis* is the only *Microtus* spp. present at Nesodden (personal observations). While the nearest official observations are some 10 km from the Tomb site, it is certainly present in this area as well, see Figure 29. One *B. microti*-infected nymph in our study was found to have fed on *M. glareolus*. This was expected as *M. glareolus* is a reservoir host for *B. microti*. Furthermore, as *B. microti* is not transovarially transmitted (Homer *et al.* 2000), the infection must have occurred during the larval feeding.

The remaining ten ticks which were species-determined for *Babesia* were identified as a *B. venatorum/divergens/capreoli* cluster. These species are commonly associated with cattle (*Bos bovis*) and cervid host species. Due to time constraints, sequencing of additional fragments of the *cyt b* gene for further identification as described by Øines *et al.* (2012) was not performed.

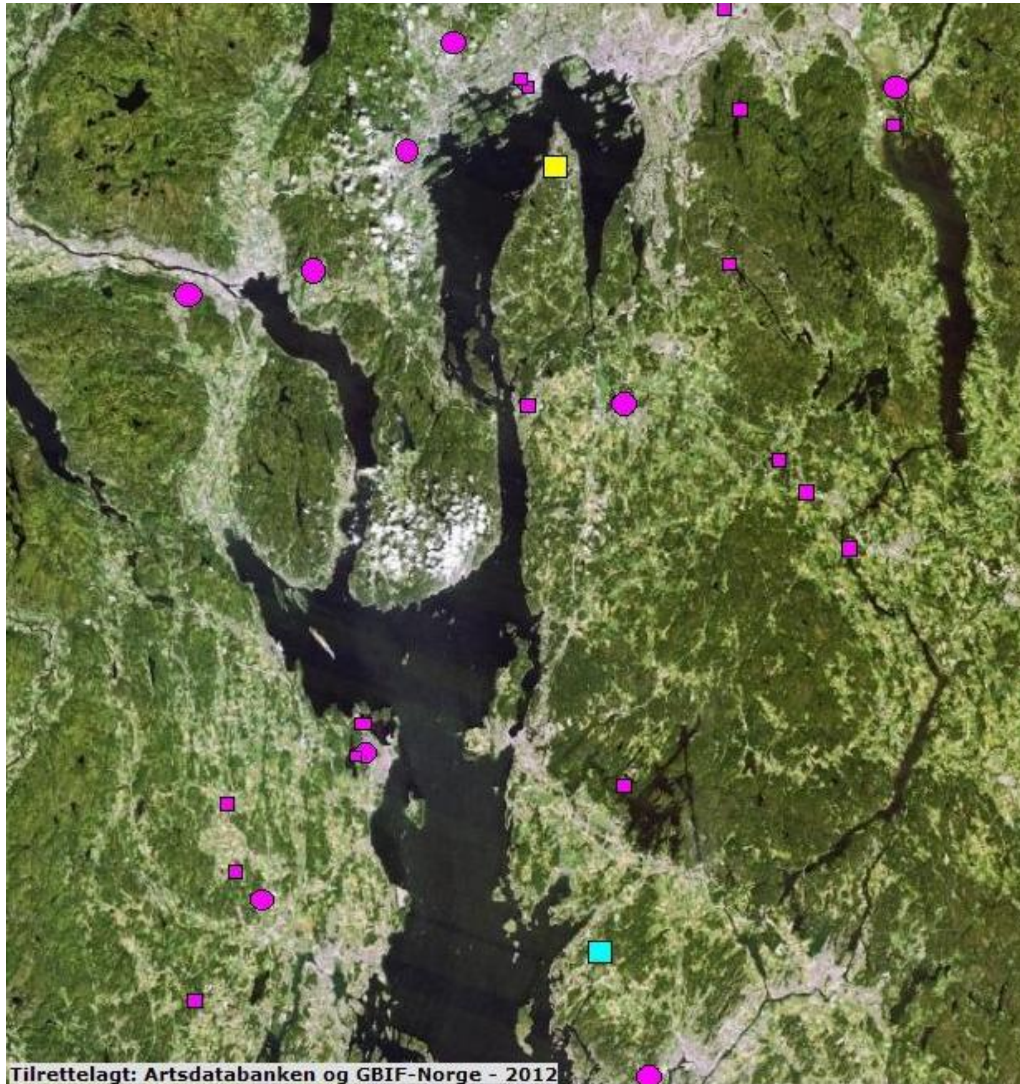


Figure 29. The prevalence of *M. agrestis* around the Oslo fjord area. Yellow square = Nesodden field site, blue square = Tomb field site, pink circle = *M. agrestis* recorded at municipality level, pink square = *M. agrestis* recorded at site. Scale: 1:750 000. Data from Anon (2012a).

B. bovis and red deer (*Cervus elaphus*) are the main reservoirs for *B. divergens*, and there is some debate regarding the presence of the pathogen in *C. capreolus* (Zintl *et al.* 2003; Duh *et al.* 2005; Zintl *et al.* 2011). Molecular studies show that *B. capreoli* is similar to, but distinct from, *B. divergens* (Malandrin *et al.* 2010). The main host of *B. capreoli* is *C. capreolus*, and the pathogen is not believed to be infective to humans or livestock (Malandrin *et al.* 2010). *C. capreolus* has also been regarded as the main host of *B. venatorum*, although the species has also been isolated from reindeer (*Rangifer tarandus*) and *C. elaphus* (Duh *et al.* 2005; Kik *et al.* 2011). As *C. capreolus* regularly frequent the Nesodden site, and both *C. capreolus* and *C. elaphus* are present at Tomb, either of the *B. venatorum/divergens/capreoli* species are likely candidates. The larval bloodmeals of three *B. venatorum/divergens/capreoli*-infected nymphs

collected from Tomb were identified as *C. capreolus, Apodemus* spp., and *M. glareolus*. While the *C. capreolus*-fed nymph may have acquired the infection during larval feeding, the rodent-fed nymphs would not have been able to, as rodents are not reservoirs for these *Babesia* species. Large *Babesia* species such as *B. venatorum, B. divergens and B. capreoli* are, however, known to transmit ovarially (Homer *et al.* 2000), and these nymphs would have acquired the infection through this pathway.

All of the identified species detected in this study have been recorded in Norway previously. One study found *B. microti* infections by Giemsa staining in 0.2% of *M. glareolus*, 0.8% of *A. sylvaticus* and 14.6% of *M. agrestis* trapped in the Kviteseid district of southern Norway from 1970-75 (Wiger 1979). When Hasle *et al.* (2011) investigated the diseases carried by ticks on migrating birds in southern Norway, it was found that 5 (1%) of 512 ticks investigated carried *Babesia*, and four of these were confirmed as *Babesia venatorum*. Radzijevskaja *et al.* (2008) tested questing nymphal and adult *I. ricinus* collected by flagging from four sites on the southern coast of Norway for *B. divergens.* PCR testing found only 0.9% of the 224 ticks to be infected. Only adult females were found to harbour the blood parasites (4% of 57 females). Øines *et al.* (2012) expanded this data set by using the original data from the four sites sampled, and adding tick collections up to a total of 22 sites, ranging from the south-east to the north-west of Norway. 0.9% of 1908 nymphal and adult ticks tested positive for *Babesia* by real-time PCR, representing nine of the sites. Sequencing revealed that 12 of the 17 *Babesia*-positive ticks carried *B. venatorum*, and two were infected with *B. divergens*. Two ticks were infected with *B. capreoli*, a species which had never been recorded in Norway previously. The final *Babesia*-positive tick collected by Øines *et al.* (2012) was infected by an apparently new variant of *Babesia*. The *18S rRNA* region of the sequences were similar to unknown strains found in Ireland, Switzerland and Austria which appear to be blood parasites of *C. elaphus*. Our results correspond well to the range of hosts which are expected to present the main *Babesia* reservoirs at our sites, as well as to the findings of previous studies in Norway.

Lyme disease is the most common tick-borne disease in Europe (Killilea *et al.* 2008), and over 200 cases of disseminated or chronic borreliosis are reported yearly in Norway (Anon 2012b). Knowledge of *Borrelia* prevalence can be utilised to raise public awareness of the risks associated with outdoor activities within certain areas. Furthermore, health workers in

high-risk areas can be informed of the local prevalence to aid a rapid diagnosis when faced with patients displaying symptoms of Lyme disease.

The prevalence of *Borrelia* in ticks can vary to a high degree from one location to another and is affected by many factors such as tick and host densities, host and vegetation type and climate (Mehl 1999). There is also a temporal change in *Borrelia* prevalence in ticks through the seasons (Kjelland *et al.* 2010). Mehl *et al.* (1987) found a mean *Borrelia* prevalence of 31% in adult ticks, and 3% in nymphs, although findings from individual localities range from 0-49% and 0-4% for adults and nymphs, respectively. In Råde, near our Tomb field site, no infection was detected in 36 ticks that were tested for *Borrelia* (Mehl *et al.* 1987). In the present study, nine ticks from the Tomb field site were tested for *Borrelia*, and no infection was detected. When sampling two tick populations in southern Norway, Jenkins *et al.* (2001) found an average *Borrelia* prevalence of 21% on an island location and 13% in a nearby mainland location. Paulauskas *et al.* (2009) collected questing *I. ricinus* from nine sites in Norway and detected *Borrelia* in 5.2% of 535 ticks, with local prevalences ranging from 0-17%. Kjelland *et al.* (2010) detected *Borrelia* in 25.2% of 398 questing nymphal and adult *I. ricinus* collected in southern Norway, with individual locations ranging from 22.1-31.3%.

The *B. burgdorferi s.l.* species complex consists of different serotypes, based on immunology to the *OspA* gene. *B. burgdorferi s.s.* belongs to serotype 1 and *B. afzelii* to serotype 2 (Wilske *et al.* 1993; Marconi *et al.* 1999; Hu *et al.* 2001). *B. garinii* is divided into *OspA* serotypes 3-7 (Wilske *et al.* 1993).

The link between *Borrelia* serotypes and tick hosts has been the topic of many studies. It has, however, proved to be difficult to investigate this due to the complexity of this relationship, local and regional differences in ecosystems and difficulty in testing ticks and hosts for infections. In a study by Kurtenbach *et al.* (1998b), uninfected tick larvae were allowed to feed on 21 wild-caught *A. sylvaticus* and 26 *M. glareolus*. 14 days after feeding was complete, ticks from four of the rodents had acquired a *B. burgdorferi s.s.* infection. However, in three of these infective rodents, no infection could be detected, despite tests being performed on the heart, bladder, kidney, brain and skin by PCR. Such studies underline the difficulty of *Borrelia* diagnostics and the elusive nature of *Borrelia* infections.

Several studies have found a close association between *Borrelia* serotype identity and tick host identity (Figure 30). Hanincova *et al.* (2003) trapped rodents near Bratislava, Slovakia, and found that among those that were infected with *Borrelia*, 100% of the *M. glareolus* and 91% of the *A. flavicollis* were infected with *B. afzelii*. Correspondingly, the infected larval and nymphal ticks feeding on these animals were mainly infected with *B. afzelii*. All of the infected larvae and nymphs feeding on the infected *M. glareolus*, and nearly all of those on infected *A. flavicollis* carried *B. afzelii.* Other studies support this association between *B. afzelii* and rodents (Humair *et al.* 1995; Hu *et al.* 1997a; Kurtenbach *et al.* 1998a; Humair *et al.* 1999). On the other hand, there is evidence for *B. garinii* being more easily transmitted to ticks by birds than by rodents (Kurtenbach *et al.* 1998b). It was found that while *A. sylvaticus* and *M. glareolus* sera destroyed *B. garinii*, pheasant (*Phasianus colchicus*) serum had little effect on the pathogen (Kurtenbach *et al.* 1998a). While this is the case for most serotypes within *B. garinii,* the main reservoir for serotype 4, now known as *Borrelia bavariensis* (Margos *et al*. 2009), appears to be rodents (Hu *et al.* 2001). While *B. burgdorferi s.s*. is a more generalist genospecies and no strong link to a particular host type has been found, it has been suggested that rodents may maintain this species at a low level (Kurtenbach *et al.* 1998b).

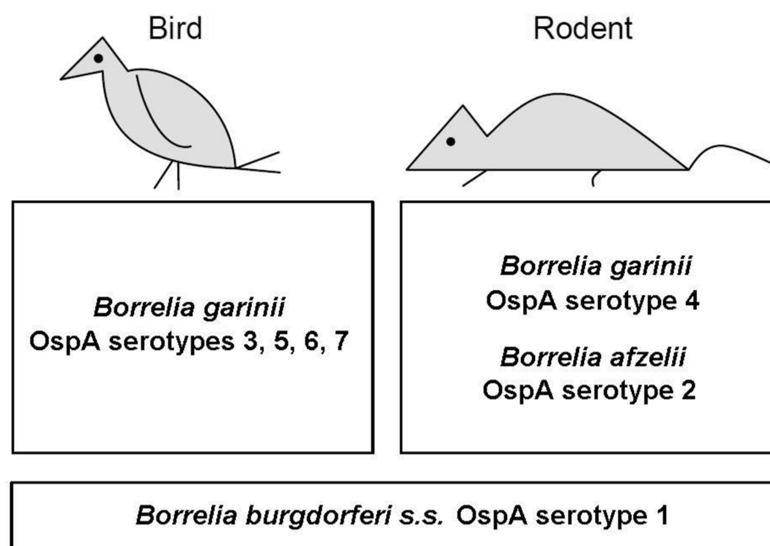


Figure 30. The three ecological reservoirs of *B. burgdorferi s.l.* Modified from Kurtenbach *et al.* (2002).

In the present study, two nymphal ticks infected with *B. afzelii* were detected. The bloodmeal hosts of these ticks were not identified, although *B. afzelii* is most commonly associated with

rodent hosts. Because the different life stages of ticks often exploit different host species, the *Borrelia* species infecting the ticks can differ between life stages. Nymphs have been found to carry higher levels of *B. afzelii* infections, while adults carry higher proportions of *B. garinii*, *B. burgdorferi s.s.* and *B. valaisiana* (Kjelland *et al.* 2010). As both of the *Borrelia*-positive ticks in this study were nymphs, the infection would most likely have been acquired during the larval stage, for which rodents are the most prominent hosts.

In the case of *Borrelia* prevalence, not enough data could be attained in order to estimate the possibility of a dilution effect or vector augmentation of this pathogen in our study sites. *Babesia*, on the other hand, was markedly more prevalent in Tomb (15%) than in Nesodden (7%). It would be interesting to investigate the biodiversity of these areas in order to find whether there is evidence for a dilution effect or vector augmentation in these sites.

4.6 The effects of hosts and pathogens on the ticks

As both larval host choice and pathogen infection may affect the size of the subsequent nymph, nymphal scutal areas were measured as a proxy for nymph size. The scutum sizes did not appear to follow a normal distribution. This suggested a possible impact of one or more environmental factors such as host or pathogen influence on nymph size. The scutum size of the nymphs however, did not appear to be related to the larval bloodmeal hosts found in this study. This is contrary to the study by Talleklint and Jaenson (1997), where larvae fed on *Apodemus* spp. had higher engorgement weights and larger nymphal scutum sizes than those fed on *M. glareolus*. Larger sample sizes and the use of primer pairs targeting further vertebrate species may reveal a clearer pattern, as many of the larger nymphs in the present study had fed on unidentifiable hosts. Ogden *et al.* (2002) found that engorged female nymphs weighed less than engorged male nymphs, and that the engorged weight of a nymph is related to the size of the female scutum. As we have no measure of nymphal sex in our study, it cannot be ruled out that the sex of the ticks also affect the size of the nymphal scutum. If divided into male/female groups, the patterns in our data may change.

It is also probable that host species do vary in their quality to the ticks, although these differences may manifest themselves in effects other than the size of the nymphs. Other studies have found qualitative differences between hosts affecting feeding success and moulting success (Dizij and Kurtenbach 1995; Humair *et al.* 1999; Ogden *et al.* 2002;

Brunner *et al.* 2011). As the nymphs of the present study were collected in the field and only successfully moulted ticks were sampled, ruling out an effect of hosts on moulting success was not possible. In addition to interspecific differences, individual intraspecific differences in host quality may also occur, such as that of acquired resistance to ticks (Davidar *et al.* 1989; Randolph 1994; Dizij and Kurtenbach 1995). Any such individual differences between the hosts in our study may obscure any other patterns present.

Pathogen infection has also been found to affect the tick vectors. When allowing *I. scapularis* larvae and nymphs to feed on *B. microti*-infected and uninfected Syrian golden hamsters (*Mesocricetus auratus*), Hu *et al.* (1997b) found that *Babesia* infection had no significant effect on the engorged weight of larvae, although infection did have a positive effect on larval moulting success. An opposite pattern was found in nymphs: *Babesia* infection had a positive effect on the mean engorged weight, but no effect on nymph moulting success. This was interpreted as a possible mutualistic relationship between *B. microti* and *I. scapularis*, enhancing the survival of both tick and pathogen. In a similar experiment, Randolph (1991) found a positive effect of *B. microti* on the weight of *Ixodes trianguliceps* larvae engorged on *M. musculus* and *M. glareolus*. In the Tomb study site, *Babesia* infection did not appear to be associated with the size of the tick, as measured by the scutal area. This corresponds better with the results of Hu *et al.* (1997b) than Randolph (1991), as the engorged weight of a tick appears to be related to its size in the next life stage (Ogden *et al.* 2002). While this was documented only in the case of nymphs moulting to females, it is possible that the same effect may occur in larvae moulting to nymphs. While no one factor could be determined as the cause of the seemingly polymodal size distribution of the nymphal scutal areas, an interaction of host and pathogen effects may obscure our perception. In addition, other factors which may affect the nymphal scutum size cannot be ruled out, such as feeding time, abiotic factors during feeding/moulting, sex and genetics.

The development of a new method of tick bloodmeal analysis enabled the identification of 49% of *I. ricinus* nymphs and adults. This information, combined with information on pathogen prevalence, form a basic knowledge which can be used for disease control in Norway. With further studies and increased knowledge of pathogen-vector-host ecology, additional control measures may be developed and implemented.

5 Conclusions and further prospects

This study suggests a new method of tick bloodmeal analysis, performed as a reamplified real-time qPCR. This method allows for an accurate identification of tick bloodmeals through the use of real-time qPCR amplification and melt curves, and it offers the possibility of confirmation of the results through electrophoresis and sequencing. This is the first time the results of a tick bloodmeal analysis have been confirmed through sequencing of products. The sensitivity of the method and the specificity of the primers used, allowed for an accurate identification of the previous hosts of unfed field-collected ticks. Analyses of tick bloodmeals suggested *Apodemus* spp. and *F. catus* as the main hosts of *I. ricinus* nymphs at our Tomb field site. Most males had been removed from cats, and were primarily found to contain *F. catus* DNA; either having become contaminated from them, or having fed on the same host as larvae or nymphs. The use of a higher number of species-specific primer pairs would reveal other, perhaps more significant host species. 2% of ticks tested were infected with *B. afzelii*. These were nymphs collected at Nesodden, suggesting a rodent infection reservoir at this field site. *Babesia* infection was detected in 9% of ticks collected from vegetation at Nesodden and Tomb. These were identified as *B. microti* and a *B. venatorum/divergens/capreoli* cluster. Despite habitat differences, no significant difference in *Babesia* infection prevalence was noted between the Nesodden and Tomb field sites. It is recommended that any future investigations into the ecology and prevalence of *Babesia* infections in ticks take into account the similarities of the genomes of *Babesia* species when selecting primer regions.

The analysis of tick bloodmeals through real-time qPCR as described in this study offers a heightened sensitivity compared with the traditional use of reverse line-blot hybridisation. The possibility of sequencing and electrophoresis to confirm results is a significant advantage of this technique. As tick ranges in Norway are expanding (Jore *et al.* 2011), an increased understanding of the host community utilised by ticks is crucial for the prevention of tick-borne diseases such as Lyme disease and babesiosis (Ostfeld and Keesing 2000b). The analysis of tick bloodmeals allow for an estimation of tick feeding success and survival from different hosts in an area, as the relative contributions of different hosts to the nymphal and adult life stages can be quantified. This method facilitates ecological analyses of tick feeding and tick population ecology, which are instrumental for increasing our knowledge of the epidemiology of tick-borne diseases.

6 References

ALCAIDE, M., RICO, C., RUIZ, S., SORIGUER, R., MUNOZ, J. AND FIGUEROLA, J. (2009). Disentangling vector-borne transmission networks: A universal DNA barcoding method to identify vertebrate hosts from arthropod bloodmeals. *Plos One* 4(9): 1-6.

ALLAN, B. F., GOESSLING, L. S., STORCH, G. A. AND THACH, R. E. (2010). Blood meal analysis to identify reservoir hosts for *Amblyomma americanum* ticks. *Emerging Infectious Diseases* 16(3): 433-440.

ALLAN, B. F., KEESING, F. AND OSTFELD, R. S. (2003). Effect of forest fragmentation on Lyme disease risk. *Conservation Biology* 17(1): 267-272.

ANON (2012a). Artskart 1.6. Artsdatabanken and GBIF-Norge. Retrieved 13.08.2012, from http://artskart.artsdatabanken.no.

ANON (2012b). Lyme borreliose. The Norwegian surveillance system for communicable diseases. The Norwegian Institute of Public Health: Norway. Retrieved 06.07.2012, from http://www.msis.no/.

ARMSTRONG, P. M., KATAVOLOS, P., CAPORALE, D. A., SMITH, R. P., SPIELMAN, A. AND TELFORD, S. R. (1998). Diversity of *Babesia* infecting deer ticks (*Ixodes dammini*). *American Journal of Tropical Medicine and Hygiene* 58(6): 739-742.

BIOR, A. D., ESSENBERG, R. C. AND SAUER, J. R. (2002). Comparison of differentially expressed genes in the salivary glands of male ticks, *Amblyomma americanum* and *Dermacentor andersoni*. *Insect Biochemistry and Molecular Biology* 32(6): 645-655.

BROWN, T. (2002). *Genomes*. Wiley-Liss: Oxford.

BROWNSTEIN, J. S., SKELLY, D. K., HOLFORD, T. R. AND FISH, D. (2005). Forest fragmentation predicts local scale heterogeneity of Lyme disease risk. *Oecologia* 146(3): 469-475.

BRUNNER, J. L. AND OSTFELD, R. S. (2008). Multiple causes of variable tick burdens on small-mammal hosts. *Ecology* 89(8): 2259-2272.

BRUNNER, J. L., CHENEY, L., KEESING, F., KILLILEA, M., LOGIUDICE, K., PREVITALI, A. AND OSTFELD, R. S. (2011). Molting success of *Ixodes scapularis* varies among individual blood meal hosts and species. *Journal of Medical Entomology* 48(4): 860-866.

BURGDORFER, W., BARBOUR, A. G., HAYES, S. F., BENACH, J. L., GRUNWALDT, E. AND DAVIS, J. P. (1982). Lyme disease - A tick-borne spirochetosis. *Science* 216(4552): 1317-1319.

CADENAS, F. M., RAIS, O., HUMAIR, P. F., DOUET, V., MORET, J. AND GERN, L. (2007). Identification of host bloodmeal source and *Borrelia burgdorferi* sensu lato in field-collected *Ixodes ricinus* ticks in Chaumont (Switzerland). *Journal of Medical Entomology* 44(6): 1109-1117.

CAMMA, C., DI DOMENICO, M. AND MONACO, F. (2012). Development and validation of fast Real-Time PCR assays for species identification in raw and cooked meat mixtures. *Food Control* 23(2): 400-404.

CONRAD, P. A., KJEMTRUP, A. M., CARRENO, R. A., THOMFORD, J., WAINWRIGHT, K., EBERHARD, M., QUICK, R., TELFORD, S. R. AND HERWALDT, B. L. (2006). Description of *Babesia duncani* n.sp (Apicomplexa : Babesiidae) from humans and its differentiation from other piroplasms. *International Journal for Parasitology* 36(7): 779-789.

CSANGO, P. A., BLAKSTAD, E., KIRTZ, G. C., PEDERSEN, J. E. AND CZETTEL, B. (2004). Tick-borne encephalitis in southern Norway. *Emerging Infectious Diseases* 10(3): 533-534.

DANIELS, T. J., FALCO, R. C. AND FISH, D. (2000). Estimating population size and drag sampling efficiency for the blacklegged tick (Acari : Ixodidae). *Journal of Medical Entomology* 37(3): 357-363.

DAVIDAR, P., WILSON, M. AND RIBEIRO, J. M. C. (1989). Differential distribution of immature *Ixodes dammini* (Acari, Ixodidae) on rodent hosts. *Journal of Parasitology* 75(6): 898-904.

DEGRAVES, F. J., GAO, D. Y. AND KALTENBOECK, B. (2003). High-sensitivity quantitative PCR platform. *Biotechniques* 34(1): 106-115.

DIZIJ, A. AND KURTENBACH, K. (1995). *Clethrionomys glareolus*, but not *Apodemus flavicollis*, acquires resistance to *Ixodes ricinus* L, the main european vector of *Borrelia burgdorferi*. *Parasite Immunology* 17(4): 177-183.

DUH, D., PETROVEC, M., BIDOVEC, A. AND AVSIC-ZUPANC, T. (2005). Cervids as babesiae hosts, Slovenia. *Emerging Infectious Diseases* 11(7): 1121-1123.

ESTRADA-PEÑA, A., OSÁCAR, J. J., PICHON, B. AND GRAY, J. S. (2005). Hosts and pathogen detection for immature stages of *Ixodes ricinus* (Acari : Ixodidae) in North-Central Spain. *Experimental and Applied Acarology* 37(3-4): 257-268.

FRANKLIN, H. A., STAPP, P. AND COHEN, A. (2010). Polymerase chain reaction (PCR) identification of rodent blood meals confirms host sharing by flea vectors of plague. *Journal of Vector Ecology* 35(2): 363-371.

GERN, L. AND RAIS, O. (1996). Efficient transmission of *Borrelia burgdorferi* between cofeeding *Ixodes ricinus* ticks (Acari: Ixodidae). *Journal of Medical Entomology* 33(1): 189-192.

GORENFLOT, A., MOUBRI, K., PRECIGOUT, E., CARCY, B. AND SCHETTERS, T. P. M. (1998). Human babesiosis. *Annals of Tropical Medicine and Parasitology* 92(4): 489-501.

GRAY, J. S. (1985). Studies on the larval activity of the tick *Ixodes ricinus* L. in Co. Wicklow, Ireland. *Experimental & Applied Acarology* 1: 307--316.

GRAY, J. S. (2002). Biology of *Ixodes* species ticks in relation to tick-borne zoonoses. *Wiener Klinische Wochenschrift* 114(13-14): 473-478.

GRAY, J. S., KAHL, O., JANETZKI, C. AND STEIN, J. (1992). Studies on the ecology of Lyme disease in a deer forest in county Galway, Ireland. *Journal of Medical Entomology* 29(6): 915-920.

GRAY, J., ZINTL, A., HILDEBRANDT, A., HUNFELD, K. P. AND WEISS, L. (2010). Zoonotic babesiosis: Overview of the disease and novel aspects of pathogen identity. *Ticks and Tick-Borne Diseases* 1(1): 3-10.

GRIGORYEVA, L. A. AND MARKOV, A. V. (2012). PCR identification of the host DNA in the taiga tick (Ixodinae: *Ixodes persulcatus*) nymphs in St. Petersburg and its environs. *Entomological Review* 92(4): 466-470.

GUY, E. C. AND STANEK, G. (1991). Detection of *Borrelia burgdorferi* in patients with Lyme disease by the polymerase chain reaction. *Journal of Clinical Pathology* 44(7): 610-611.

HANINCOVA, K., SCHAFER, S. M., ETTI, S., SEWELL, H. S., TARAGELOVA, V., ZIAK, D., LABUDA, M. AND KURTENBACH, K. (2003). Association of *Borrelia afzelii* with rodents in Europe. *Parasitology* 126: 11-20.

HARRISON, A., SCANTLEBURY, M. AND MONTGOMERY, W. I. (2010). Body mass and sex-biased parasitism in wood mice *Apodemus sylvaticus*. *Oikos* 119(7): 1099-1104.

HÄSELBARTH, K., TENTER, A. M., BRADE, V., KRIEGER, G. AND HUNFELD, K. P. (2007). First case of human babesiosis in Germany - Clinical presentation and molecular characterisation of the pathogen. *International Journal of Medical Microbiology* 297(3): 197-204.

HASLE, G., BJUNE, G. A., CHRISTENSSON, D., ROED, K. H., WHIST, A. C. AND LEINAAS, H. P. (2010). Detection of *Babesia divergens* in southern Norway by using an immunofluorescence antibody test in cow sera. *Acta Veterinaria Scandinavica* 52: 1-9.

HASLE, G., LEINAAS, H. P., ROED, K. H. AND OINES, O. (2011). Transport of *Babesia venatorum*-infected *Ixodes ricinus* to Norway by northward migrating passerine birds. *Acta Veterinaria Scandinavica* 53: 1-5.

HAZLER, K. R. AND OSTFELD, R. S. (1995). Larval density and feeding success of *Ixodes scapularis* on two species of *Peromyscus*. *Journal of Parasitology* 81(6): 870-875.

HERBERGS, J., SIWEK, M., CROOIJMANS, R., VAN DER POEL, J. J. AND GROENEN, M. A. M. (1999). Multicolour fluorescent detection and mapping of AFLP markers in chicken (*Gallus domesticus*). *Animal Genetics* 30(4): 274-285.

HERWALDT, B. L., PERSING, D. H., PRECIGOUT, E. A., GOFF, W. L., MATHIESEN, D. A., TAYLOR, P. W., EBERHARD, M. L. AND GORENFLOT, A. F. (1996). A fatal case of babesiosis in Missouri: Identification of another piroplasm that infects humans. *Annals of Internal Medicine* 124(7): 643-650.

HERWALDT, B. L., CACCIO, S., GHERLINZONI, F., ASPOCK, H., SLEMENDA, S. B., PICCALUGA, P. P., MARTINELLI, G., EDELHOFER, R., HOLLENSTEIN, U., POLETTI, G., PAMPIGLIONE, S., LOSCHENBERGER, K., TURA, S. AND PIENIAZEK, N. J. (2003). Molecular characterization of a non-*Babesia divergens* organism causing zoonotic babesiosis in Europe. *Emerging Infectious Diseases* 9(8): 942-948.

HILDEBRANDT, A., HUNFELD, K. P., BAIER, M., KRUMBHOLZ, A., SACHSE, S., LORENZEN, T., KIEHNTOPF, M., FRICKE, H. J. AND STRAUBE, E. (2007). First confirmed autochthonous case of human *Babesia microti* infection in Europe. *European Journal of Clinical Microbiology & Infectious Diseases* 26(8): 595-601.

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

HOMER, M. J., AGUILAR-DELFIN, I., TELFORD, S. R., KRAUSE, P. J. AND PERSING, D. H. (2000). Babesiosis. *Clinical Microbiology Reviews* 13(3): 451-469.

HU, C. M., HUMAIR, P. F., WALLICH, R. AND GERN, L. (1997a). *Apodemus* sp. rodents, reservoir hosts for *Borrelia afzelii* in an endemic area in Switzerland. *Zentralblatt Fur Bakteriologie-International Journal of Medical Microbiology Virology Parasitology and Infectious Diseases* 285(4): 558-564.

HU, R. J., HYLAND, K. E. AND MARKOWSKI, D. (1997b). Effects of *Babesia microti* infection on feeding pattern, engorged body weight, and molting rate of immature *Ixodes scapularis* (Acari: Ixodidae). *Journal of Medical Entomology* 34(5): 559-564.

HU, C. M., WILSKE, B., FINGERLE, V., LOBET, Y. AND GERN, L. (2001). Transmission of *Borrelia garinii OspA* serotype 4 to BALB/c mice by *Ixodes ricinus* ticks collected in the field. *Journal of Clinical Microbiology* 39(3): 1169-1171.

HULINSKA, D., VOTYPKA, J., VANOUSOVA, D., HERCOGOVA, J., HULINSKY, V., DREVOVA, H., KURZOVA, Z. AND UHERKOVA, L. (2009). Identification of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* sensu lato in patients with erythema migrans. *Folia Microbiologica* 54(3): 246-256.

HUMAIR, P. F. AND GERN, L. (1998). Relationship between *Borrelia burgdorferi* sensu lato species, red squirrels (*Sciurus vulgaris*) and *Ixodes ricinus* in enzootic areas in Switzerland. *Acta Tropica* 69(3): 213-227.

HUMAIR, P. F., PETER, O., WALLICH, R. AND GERN, L. (1995). Strain variation of Lyme disease spirochetes isolated from *Ixodes ricinus* ticks and rodents collected in two endemic areas in Switzerland. *Journal of Medical Entomology* 32(4): 433-438.

HUMAIR, P. F., RAIS, O. AND 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: 33-42.

HUMAIR, P. F., DOUET, V., CADENAS, F. M., SCHOULS, L. M., VAN DE POL, I. AND GERN, L. (2007). Molecular identification of bloodmeal source in *Ixodes ricinus* ticks using 12S rDNA as a genetic marker. *Journal of Medical Entomology* 44(5): 869-880.

JAENSON, T. G. T. AND TALLEKLINT, L. (1992). Incompetence of roe deer as reservoirs of the Lyme borreliosis spirochete. *Journal of Medical Entomology* 29(5): 813-817.

JENKINS, A., KRISTIANSEN, B. E., ALLUM, A. G., AAKRE, R. K., STRAND, L., KLEVELAND, E. J., VAN DE POL, I. AND SCHOULS, L. (2001). *Borrelia burgdorferi* sensu lato and *Ehrlichia* spp. in *Ixodes* ticks from southern Norway. *Journal of Clinical Microbiology* 39(10): 3666-3671.

JORE, S., VILJUGREIN, H., HOFSHAGEN, M., BRUN-HANSEN, H., KRISTOFFERSEN, A. B., NYGARD, K., BRUN, E., OTTESEN, P., SAEVIK, B. K. AND YTREHUS, B. (2011). Multi-source analysis reveals latitudinal and altitudinal shifts in range of *Ixodes ricinus* at its northern distribution limit. *Parasites & Vectors* 4.

JOYNER, L. P., DAVIES, S. F. M. AND KENDALL, S. B. (1963). The experimental transmission of *Babesia divergens* by *Ixodes ricinus*. *Experimental Parasitology* 14: 367-373.

KARBOWIAK, G. AND SINSKI, E. (1996). The finding of *Babesia microti* in bank vole *Clethrionomys glareolus* in the district of Mazury Lakes (Poland). *Acta Parasitologica* 41(1): 50-51.

KARBOWIAK, G., STANKO, M., RYCHLIK, L., NOWAKOWSKI, W. AND SIUDA, K. (1999). The new data about zoonotic reservoir of *Babesia microti* in small mammals in Poland. *Acta Parasitologia* 44(2): 142-144.

KENT, R. J. (2009). Molecular methods for arthropod bloodmeal identification and applications to ecological and vector-borne disease studies. *Molecular Ecology Resources* 9(1): 4-18.

KENT, R. J. AND NORRIS, D. E. (2005). Identification of mammalian blood meals in mosquitoes by a multiplexed polymerase chain reaction targeting cytochrome B. *American Journal of Tropical Medicine and Hygiene* 73(6): 147-147.

KIK, M., NIJHOF, A. M., BALK, J. A. AND JONGEJAN, F. (2011). *Babesia* sp EU1 Infection in a forest reindeer, the Netherlands. *Emerging Infectious Diseases* 17(5): 936-938.

KILLILEA, M. E., SWEI, A., LANE, R. S., BRIGGS, C. J. AND OSTFELD, R. S. (2008). Spatial dynamics of Lyme disease: A review. *Ecohealth* 5(2): 167-195.

KIM, S. W., LI, Z. H., MOORE, P. S., MONAGHAN, A. P., CHANG, Y., NICHOLS, M. AND JOHN, B. (2010). A sensitive non-radioactive northern blot method to detect small RNAs. *Nucleic Acids Research* 38(7): 1-7.

KIRSTEIN, F. AND GRAY, J. S. (1996). A molecular marker for the identification of the zoonotic reservoirs of lyme borreliosis by analysis of the blood meal in its European vector *Ixodes ricinus*. *Applied and Environmental Microbiology* 62(11): 4060-4065.

KJELLAND, V., STUEN, S., SKARPAAS, T. AND SLETTAN, A. (2010). Prevalence and genotypes of *Borrelia burgdorferi* sensu lato infection in *Ixodes ricinus* ticks in southern Norway. *Scandinavian Journal of Infectious Diseases* 42(8): 579-585.

KJEMTRUP, A. M. AND CONRAD, P. A. (2000). Human babesiosis: an emerging tick-borne disease. *International Journal for Parasitology* 30(12-13): 1323-1337.

KURTENBACH, K., KAMPEN, H., DIZIJ, A., ARNDT, S., SEITZ, H. M., SCHAIBLE, U. E. AND SIMON, M. M. (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. *Journal of Medical Entomology* 32(6): 807-817.

KURTENBACH, K., SEWELL, H. S., OGDEN, N. H., RANDOLPH, S. E. AND NUTTALL, P. A. (1998a). Serum complement sensitivity as a key factor in Lyme disease ecology. *Infection and Immunity* 66(3): 1248-1251.

KURTENBACH, K., PEACEY, M., RIJPKEMA, S. G. T., HOODLESS, A. N., NUTTALL, P. A. AND RANDOLPH, S. E. (1998b). Differential transmission of the genospecies of *Borrelia burgdorferi* sensu lato by game birds and small rodents in England. *Applied and Environmental Microbiology* 64(4): 1169-1174.

KURTENBACH, K., DE MICHELIS, S., ETTI, S., SCHAFER, S. M., SEWELL, H. S., BRADE, V. AND KRAICZY, P. (2002). Host association of *Borrelia burgdorferi* sensu lato - the key role of host complement. *Trends in Microbiology* 10(2): 74-79.

LEWIS, D. AND YOUNG, E. R. (1980). The transmission of a human strain of *Babesia divergens* by *Ixodes ricinus* ticks. *Journal of Parasitology* 66(2): 359-360.

LOCKEY, C., OTTO, E. AND LONG, Z. (1998). Real-time fluorescence detection of a single DNA molecule. *Biotechniques* 24: 744-746.

LOPEZ-ANDREO, M., LUGO, L., GARRIDO-PERTIERRA, A., PRIETO, M. I. AND PUYET, A. (2005). Identification and quantitation of species in complex DNA mixtures by real-time polymerase chain reaction. *Analytical Biochemistry* 339(1): 73-82.

MACLEOD, J. (1936). *Ixodes ricinus* in relation to its physical environment - IV. An analysis of the ecological complexes controlling distribution and activities. *Parasitology* 28(3): 295-319.

MALANDRIN, L., JOUGLIN, M., SUN, Y., BRISSEAU, N. AND CHAUVIN, A. (2010). Redescription of *Babesia capreoli* (Enigk and Friedhoff, 1962) from roe deer (*Capreolus capreolus*): Isolation, cultivation, host specificity, molecular characterisation and differentiation from *Babesia divergens*. *International Journal for Parasitology* 40(3): 277-284.

MANNELLI, A., BERTOLOTTI, L., GERN, L. AND 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 Microbiology Reviews* 36(4): 837-861.

MANSFIELD, E. S., VAINER, M., ENAD, S., BARKER, D. L., HARRIS, D., RAPPAPORT, E. AND FORTINA, P. (1996). Sensitivity, reproducibility, and accuracy in short tandem repeat genotyping using capillary array electrophoresis. *Genome Research* 6: 893-903.

MARCONI, R. T., HOHENBERGER, S., JAURIS-HEIPKE, S., SCHULTE-SPECHTEL, U., LAVOIE, C. P., ROSSLER, D. AND WILSKE, B. (1999). Genetic analysis of *Borrelia garinii OspA* serotype 4 strains associated with neuroborreliosis: Evidence for extensive genetic homogeneity. *Journal of Clinical Microbiology* 37(12): 3965-3970.

MARGOS, G., VOLLMER, S. A., CORNET, M., GARNIER, M., FINGERLE, V., WILSKE, B., BORMANE, A., VITORINO, L., COLLARES-PEREIRA, M., DRANCOURT, M. AND KURTENBACH, K. (2009). A new *Borrelia* species defined by multilocus sequence analysis of housekeeping genes. *Applied and Environmental Microbiology* 75(16): 5410-5416.

MATUSCHKA, F. R., FISCHER, P., HEILER, M., BLUMCKE, S. AND SPIELMAN, A. (1992a). Stage-associated risk of transmission of the Lyme disease spirochete by European *Ixodes* ticks. *Parasitology Research* 78(8): 695-698.

MATUSCHKA, F. R., FISCHER, P., HEILER, M., RICHTER, D. AND SPIELMAN, A. (1992b). Capacity of European animals as reservoir hosts for the Lyme disease spirochete. *Journal of Infectious Diseases* 165(3): 479-483.

MATUSCHKA, F. R., HEILER, M., EIFFERT, H., FISCHER, P., LOTTER, H. AND SPIELMAN, A. (1993). Diversionary role of hoofed game in the transmission of Lyme disease spirochetes. *American Journal of Tropical Medicine and Hygiene* 48(5): 693-699.

MEHL, R. (1970). Om innsamling av insekter og midd på fugler og pattedyr. *Fauna* 23: 237-252.

MEHL, R. (1979). Checklist of Norwegian ticks and mites (Acari). *Fauna Norvegica Ser. B* 26: 31-45.

MEHL, R. (1983). The distribution and host relations of Norwegian ticks (Acari Ixodides). *Fauna Norvegica Series B* 30(1): 46-51.

MEHL, R. (1999). Ticks and borreliosis in Norway - epidemiology. *The Norwegian Medicines Control Authority* 22; Suppl 1: 15-16.

MEHL, R. (2006). Hjortedyr og flått. *Hjorteviltet*: 98-99.

MEHL, R., SANDVEN, P. AND BRAATHEN, L. R. (1987). Skogflåtten *Ixodes ricinus*. *Tidsskrift for den norske lægeforening* 107: 1642-1644, 1651.

MELDRUM, S. C., BIRKHEAD, G. S., WHITE, D. J., BENACH, J. L. AND MORSE, D. L. (1992). Human babesiosis in New York state - an epidemiologic description of 136 cases. *Clinical Infectious Diseases* 15(6): 1019-1023.

MICHEL, H., WILSKE, B., HETTCHE, G., GOTTNER, G., HEIMERL, C., REISCHL, U., SCHULTE-SPECHTEL, U. AND FINGERLE, V. (2004). An *ospA*-polymerase chain reaction/restriction fragment length polymorphism-based method for sensitive detection and reliable differentiation of all European *Borrelia burgdorferi* sensu lato species and *OspA* types. *Medical Microbiology and Immunology* 193(4): 219-226.

MILNE, A. (1943). The comparison of sheep-tick populations (*Ixodes ricinus* L). *Annals of Applied Biology* 30(3): 240-250.

MOORHOUSE, D. E. (1966). Observations on copulation in *Ixodes holocyclus* Neumann and the feeding of the male. *Journal of Medical Entomology* 3(2): 168-171.

MUTURI, C. N., OUMA, J. O., MALELE, I. I., NGURE, R. M., RUTTO, J. J., MITHOFER, K. M., ENYARU, J. AND MASIGA, D. K. (2011). Tracking the feeding patterns of tsetse flies (*Glossina* genus) by analysis of bloodmeals using mitochondrial cytochromes genes. *Plos One* 6(2): 1-6.

NILSSON, A. (1988). Seasonal occurrence of *Ixodes ricinus* (Acari) in vegetation and on small mammals in southern Sweden. *Holarctic Ecology* 11(3): 161-165.

NYGÅRD, K., BROCH BRANTSÆTER, A. AND MEHL, R. (2005). Disseminated and chronic Lyme borreliosis in Norway, 1995-2004. *Eurosurveillance* 10(10-12): 235-238.

O'SULLIVAN, M. V. N., ZHOU, F., SINTCHENKO, V., KONG, F. AND GILBERT, G. L. (2011). Multiplex PCR and reverse line blot hybridization assay (mPCR/RLB). *Journal of Visualized Experiments* 54(e2781): 1-5.

OGDEN, N. H., CASEY, A. N. J., FRENCH, N. P., ADAMS, J. D. W. AND WOLDEHIWET, Z. (2002). Field evidence for density-dependent facilitation amongst *Ixodes ricinus* ticks feeding on sheep. *Parasitology* 124: 117-125.

ORNSTEIN, K., BERGLUND, J., NILSSON, I., NORRBY, R. AND BERGSTROM, S. (2001). Characterization of Lyme borreliosis isolates from patients with erythema migrans and neuroborreliosis in southern Sweden. *Journal of Clinical Microbiology* 39(4): 1294-1298.

OSTFELD, R. S. AND KEESING, F. (2000a). Biodiversity and disease risk: The case of lyme disease. *Conservation Biology* 14(3): 722-728.

OSTFELD, R. AND KEESING, F. (2000b). The function of biodiversity in the ecology of vector-borne zoonotic diseases. *Canadian Journal of Zoology-Revue Canadienne De Zoologie* 78(12): 2061-2078.

OTO, M. AND SUDA, W. (2010). Application of the Experion automated electrophoresis system DNA assays to VNTR analysis, SNP typing, and bacterial 16S rRNA gene analysis. *Bio-Rad Tech Note* 5909(Rev A).

PÄÄBO, S., HIGUCHI, R. G. AND WILSON, A. C. (1989). Ancient DNA and the polymerase chain reaction - the emerging field of molecular archaeology. *Journal of Biological Chemistry* 264(17): 9709-9712.

PACHNER, A. R. (1989). Neurologic manifestations of Lyme disease, the new great imitator. *Reviews of Infectious Diseases* 11: S1482-S1486.

PARKER, W. T., MULLER, L. I., GERHARDT, R. R., O'ROURKE, D. P. AND RAMSAY, E. C. (2008). Field use of isoflurane for safe squirrel and woodrat anesthesia. *Journal of Wildlife Management* 72(5): 1262-1266.

PAULAUSKAS, A., RADZIJEVSKAJA, J., ROSEF, O., TURCINAVICIENE, J. AND AMBRASIENE, D. (2009). Infestation of mice and voles with *Ixodes ricinus* ticks in Lithuania and Norway. *Estonian Journal of Ecology* 58(2): 112-125.

PAZIEWSKA, A., ZWOLINSKA, L., HARRIS, P. D., BAJER, A. AND SINSKI, E. (2010). Utilisation of rodent species by larvae and nymphs of hard ticks (Ixodidae) in two habitats in NE Poland. *Experimental and Applied Acarology* 50(1): 79-91.

PICHON, B., EGAN, D., ROGERS, M. AND GRAY, J. (2003). Detection and identification of pathogens and host DNA in unfed host-seeking *Ixodes ricinus* L. (Acari : Ixodidae). *Journal of Medical Entomology* 40(5): 723-731.

PICHON, B., ROGERS, M., EGAN, D. AND GRAY, J. (2005). Blood-meal analysis for the identification of reservoir hosts of tick-borne pathogens in Ireland. *Vector-Borne and Zoonotic Diseases* 5(2): 172-180.

PICHON, B., KAHL, O., HAMMER, B. AND GRAY, J. S. (2006). Pathogens and host DNA in *Ixodes ricinus* nymphal ticks from a German forest. *Vector-Borne and Zoonotic Diseases* 6(4): 382-387.

PRINCE, H. E., LAPE-NIXON, M., PATEL, H. AND YEH, C. (2010). Comparison of the *Babesia duncani* (WA1) IgG detection rates among clinical sera submitted to a reference laboratory for WA1 IgG testing and blood donor specimens from diverse geographic areas of the United States. *Clinical and Vaccine Immunology* 17(11): 1729-1733.

QUICK, R. E., HERWALDT, B. L., THOMFORD, J. W., GARNETT, M. E., EBERHARD, M. L., WILSON, M., SPACH, D. H., DICKERSON, J. W., TELFORD, S. R., STEINGART, K. R., POLLOCK, R., PERSING, D. H., KOBAYASHI, J. M., JURANEK, D. D. AND CONRAD, P. A. (1993). Babesiosis in Washington state - a new species of *Babesia*. *Annals of Internal Medicine* 119(4): 284-290.

R-DEVELOPMENT-CORE-TEAM (2011). R: A language and environment for statistical computing. Vienna, Austria, R Foundation for Statistical Computing. ISBN 3-900051-07-0, http://www.R-project.org/.

RADOLF, J. D., CAIMANO, M. J., STEVENSON, B. AND HU, L. D. T. (2012). Of ticks, mice and men: understanding the dual-host lifestyle of Lyme disease spirochaetes. *Nature Reviews Microbiology* 10(2): 87-99.

RADZIJEVSKAJA, J., PAULAUSKAS, A. AND ROSEF, O. (2008). Prevalence of *Anaplasma phagocytophilum* and *Babesia divergens* in *Ixodes ricinus* ticks from Lithuania and Norway. *International Journal of Medical Microbiology* 298: 218-221.

RANDOLPH, S. E. (1977). Changing spatial relationships in a population of *Apodemus sylvaticus* with onset of breeding. *Journal of Animal Ecology* 46(2): 653-676.

RANDOLPH, S. E. (1991). The effect of *Babesia microti* on feeding and survival in its tick vector, *Ixodes trianguliceps*. *Parasitology* 102: 9-16.

RANDOLPH, S. E. (1994). Density-dependent acquired resistance to ticks in natural hosts, independent of concurrent infection with *Babesia microti*. *Parasitology* 108: 413-419.

RANDOLPH, S. E. (2004a). Evidence that climate change has caused 'emergence' of tick-borne diseases in Europe? *International Journal of Medical Microbiology* 293: 5-15.

RANDOLPH, S. E. (2004b). Tick ecology: processes and patterns behind the epidemiological risk posed by ixodid ticks as vectors. *Parasitology* 129: S37-S65.

RANDOLPH, S. E., GREEN, R. M., HOODLESS, A. N. AND PEACEY, M. F. (2002). An empirical quantitative framework for the seasonal population dynamics of the tick *Ixodes ricinus*. *International Journal for Parasitology* 32(8): 979-989.

RATNASINGHAM, S. AND HEBERT, P. D. N. (2007). BOLD: The Barcode of Life Data System (www.barcodinglife.org). *Molecular Ecology Notes* 7(3): 355-364.

RIBEIRO, J. M. C., MAKOUL, G. T., LEVINE, J., ROBINSON, D. R. AND SPIELMAN, A. (1985). Antihemostatic, antiinflammatory, and immunosuppressive properties of the saliva of a tick, *Ixodes dammini*. *Journal of Experimental Medicine* 161(2): 332-344.

RICHTER, D., SPIELMAN, A., KOMAR, N. AND MATUSCHKA, F. R. (2000). Competence of American robins as reservoir hosts for Lyme disease spirochetes. *Emerging Infectious Diseases* 6(2): 133-138.

RIJPKEMA, S. G. T., TAZELAAR, D. J., MOLKENBOER, M. J. C. H., NOORDHOEK, G. T., PLANTINGA, G., SCHOULS, L. M. AND SCHELLEKENS, J. F. P. (1997). Detection of *Borrelia afzelii*, *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* and group VS116 by PCR in skin biopsies of patients with erythema migrans and acrodermatitis chronica atrophicans. *Clinical Microbiology and Infection* 3(1): 109-116.

RIZZOLI, A., MERLER, S., FURLANELLO, C. AND GENCH, C. (2002). Geographical information systems and bootstrap aggregation (bagging) of tree-based classifiers for Lyme disease risk prediction in Trentino, Italian Alps. *Journal of Medical Entomology* 39(3): 485-492.

RUDENKO, N., GOLOVCHENKO, M., GRUBHOFFER, L. AND OLIVER, J. H. (2011). Updates on *Borrelia burgdorferi* sensu lato complex with respect to public health. *Ticks and Tick-Borne Diseases* 2(3): 123-128.

RYFFEL, K., PETER, O., RUTTI, B., SUARD, A. AND DAYER, E. (1999). Scored antibody reactivity determined by immunoblotting shows an association between clinical manifestations and presence of *Borrelia burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. valaisiana* in humans. *Journal of Clinical Microbiology* 37(12): 4086-4092.

SCHAARSCHMIDT, D., OEHME, R., KIMMIG, P., HESCH, R. D. D. AND ENGLISCH, S. (2001). Detection and molecular typing of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks and in different patient samples from southwest Germany. *European Journal of Epidemiology* 17(12): 1067-1074.

SCOTT, M. C., HARMON, J. R., TSAO, J. I., JONES, C. J. AND HICKLING, G. J. (2012). Reverse line blot probe design and polymerase chain reaction optimization for bloodmeal analysis of ticks from the eastern United States. *Journal of Medical Entomology* 49(3): 697-709.

SHIH, C. M. AND SPIELMAN, A. (1993). Accelerated transmission of Lyme disease spirochetes by partially fed vector ticks. *Journal of Clinical Microbiology* 31(11): 2878-2881.

SHIPLEY, M. M., DILLWITH, J. W., BOWMAN, A. S., ESSENBERG, R. C. AND SAUER, J. R. (1993). Changes in lipids of the salivary glands of the lone star tick, *Amblyomma Americanum* during feeding. *Journal of Parasitology* 79(6): 834-842.

SINSKI, E., BAJER, A., WELC, R., PAWELCZYK, A., OGRZEWALSKA, M. AND BEHNKE, J. M. (2006). *Babesia microti*: Prevalence in wild rodents and *Ixodes ricinus* ticks from the Mazury Lakes district of north-eastern Poland. *International Journal of Medical Microbiology* 296: 137-143.

SKARPAAS, T., LJOSTAD, U. AND SUNDOY, A. (2004). First human cases of tickborne encephalitis, Norway. *Emerging Infectious Diseases* 10(12): 2241-2243.

SKARPAAS, T., GOLOVLJOVA, I., VENE, S., LJOSTAD, U., SJURSEN, H., PLYUSNIN, A. AND LUNDKVIST, A. (2006). Tickborne encephalitis virus, Norway and Denmark. *Emerging Infectious Diseases* 12(7): 1136-1138.

STOECKLE, M. Y. AND HEBERT, P. D. N. (2008). Barcode of life. *Scientific American* 299(4): 82-88.

STUNZNER, D., HUBALEK, Z., HALOUZKA, J., WENDELIN, I., SIXL, W. AND MARTH, E. (2006). Prevalence of *Borrelia burgdorferi* sensu lato in the tick *Ixodes ricinus* in the Styrian mountains of Austria. *Wiener Klinische Wochenschrift* 118(21-22): 682-685.

TALLEKLINT, L. AND JAENSON, T. G. T. (1994). Transmission of *Borrelia burgdorferi* S1 from mammal reservoirs to the primary vector of Lyme borreliosis, *Ixodes ricinus* (Acari, Ixodidae), in Sweden. *Journal of Medical Entomology* 31(6): 880-886.

TALLEKLINT, L. AND JAENSON, T. G. T. (1996). Seasonal variations in density of questing *Ixodes ricinus* (Acari: Ixodidae) nymphs and prevalence of infection with *B. burgdorferi* sl in south central Sweden. *Journal of Medical Entomology* 33(4): 592-597.

TALLEKLINT, L. AND JAENSON, T. G. T. (1997). Infestation of mammals by *Ixodes ricinus* ticks (Acari: Ixodidae) in south-central Sweden. *Experimental & Applied Acarology* 21(12): 755-771.

TAMBS-LYCHE, H. (1943). *Ixodes ricinus* og piroplasmosen i Norge. *Norsk Veterinær-Tidsskrift* 55(10): 401-441.

TAMURA, K., DUDLEY, J., NEI, M. AND KUMAR, S. (2007). MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24(8): 1596-1599.

TARNOWSKI, B. I. AND COONS, L. B. (1989). Ultrastructure of the midgut and blood meal digestion in the adult tick *Dermacentor variabilis*. *Experimental & Applied Acarology* 6: 263-289.

TELFORD, S. R., MATHER, T. N., MOORE, S. I., WILSON, M. L. AND SPIELMAN, A. (1988). Incompetence of deer as reservoirs of the Lyme disease spirochete. *American Journal of Tropical Medicine and Hygiene* 39(1): 105-109.

VALASEK, M. A. AND REPA, J. J. (2005). The power of real-time PCR. *Advances in Physiology Education* 29(3): 151-159.

VASSALLO, M., PAUL, R. E. L. AND PEREZ-EID, C. (2000). Temporal distribution of the annual nymphal stock of *Ixodes ricinus* ticks. *Experimental and Applied Acarology* 24(12): 941-949.

VÝROSTEKOVÁ, V. (1994). Transstadial transmission of *Francisella tularensis* by *Ixodes ricinus* ticks infected during the nymphal stage. *Epidemiologie, Mikrobiologie, Imunologie* 43(4): 166-170.

WAHL, G. M., BERGER, S. L. AND KIMMEL, A. R. (1987). Molecular hybridization of immobilized nucleic acids: Theoretical concepts and practical considerations. Methods in Enzymology: Guide to Molecular Cloning Techniques. Berger, S. L. and Kimmel, A. R. London, Academic Press. 152**:** 399-407.

WANG, H., HENBEST, P. J. AND NUTTALL, P. A. (1999). Successful interrupted feeding of adult Rhipicephalus appendiculatus (Ixodidae) is accompanied by reprogramming of salivary gland protein expression. *Parasitology* 119: 143-149.

WICKRAMASEKARA, S., BUNIKIS, J., WYSOCKI, V. AND BARBOUR, A. G. (2008). Identification of residual blood proteins in ticks by mass spectrometry proteomics. *Emerging Infectious Diseases* 14(8): 1273-1275.

WIGER, R. (1979). Seasonal and annual variations in the prevalence of blood parasites in cyclic species of small rodents in Norway with special reference to *Clethrionomys glareolus*. *Holarctic Ecology* 2(3): 169-175.

WILSKE, B., PREACMURSIC, V., GOBEL, U. B., GRAF, B., JAURIS, S., SOUTSCHEK, E., SCHWAB, E. AND ZUMSTEIN, G. (1993). An *OspA* serotyping system for *Borrelia burgdorferi* based on reactivity with monoclonal antibodies and *OspA* sequence analysis. *Journal of Clinical Microbiology* 31(2): 340-350.

ZINTL, A., MULCAHY, G., SKERRETT, H. E., TAYLOR, S. M. AND GRAY, J. S. (2003). *Babesia divergens*, a bovine blood parasite of veterinary and zoonotic importance. *Clinical Microbiology Reviews* 16(4): 622-636.

ZINTL, A., FINNERTY, E. J., MURPHY, T. M., DE WAAL, T. AND GRAY, J. S. (2011). Babesias of red deer (*Cervus elaphus*) in Ireland. *Veterinary Research* 42: 1-6.

ØINES, Ø., RADZIJEVSKAJA, J., PAULAUSKAS, A. AND ROSEF, O. (2012). Prevalence and diversity of *Babesia* spp. in questing *Ixodes ricinus* ticks from Norway. *Parasites & Vectors* 5(1): 156.

Appendix 1 Primers

Table 1. Primers used in the study. Vertebrate primer position was based on alignment with *M. musculus musculus* mitochondrial genome (from GenBank, NCBI Reference Sequence: NC_010339.1). *Borrelia burgdorferi sensu lato* primer position was based on alignment with *B. burgdorferi outer surface protein A* (*ospA*) gene (GenBank: HM756743.1). *Babesia spp*. primer position was based on alignment with *Babesia sp*. EU1 *18S ribosomal RNA* gene (GenBank: JQ929917.1). Table modified from Michel *et al*. (2004).

Target	Amplification round (if two rounds)	Region	Annealing temperature calculated (used, if different)	Primer name	Sequence (5'-> 3')	Position (bp)	Predicted fragment length (bp)
Vertebrates							
Generic mammal	1	*12S*	52 (55)	12SoF 12SoR	CAAACTGGGATTAGATACCC AGAACAGGCTCCTCTAG	484–631	147
Mouse *Apodemus* spp.	2	*12S*	56 (55)	12SifApo 12SiR	GCTTAGCCTTAAACTTAAATAA GGATATAAAGTACCGCCAAG	510–612	102
		Cyt b	44 (45)	ApocytbF ApocytbR	TTCACCTCTTGTTTCTC GGAAGGAAACTATTATT	14 744–14 875	131
		Cyt b	66 (62/66)	CytboutFApo CytbRoutApo	TTAACACGTTTTTTCGCTTTTTCACTT GTAGTTGTCCGGATCACCTAAAAGGTCT	14 683–14 928	245
Cat *Felis catus*		*12S*	62 (57)	12SFcF 12SFCSvR	GCTTAGCCCTAAACTTAGATAG GGATGTAAAGCACCGCCAAG	510–612	102
		Cyt b	46	CatcytbF CatcytbR	CTGAATCATCCGATATT TCATGTCTCTGAGAAG	14 338–14 499	161
		Cyt b	74 (64/66)	CytboutFcat CytboutRdeercat	CTAACACGATTCTTTGCCTTCCACTT GTAGTTATCTGGGTCTCC(T/A)AGCAGGTCT	14 683–14 928	245
Squirrel *Sciurus vulgaris*		*12S*	62 (60)	12SSvF 12SFCSvR	GCTTAGCCCTAAACATAGACACTC GGATGTAAAGCACCGCCAAG	510–612	102

Bird (not phasianid)		*Cyt b*	54 (56)	PasscytbF	CAACCCACTAGGAATCCC	14 778–14 939	161
				BirdcytbR	CACCGCCAAGTCCTTAG		
Bird (phasianid)		*Cyt b*	54	pheascytbF	CAATCCACTAGGCATCTC	14 778–14 939	161
				BirdcytbR	CACCGCCAAGTCCTTAG		
Rat *Rattus norvegicus*		*12S*	58 (55)	12SRnF	GCCCTAAACCTTAATAATTAAAC	515–612	97
				12SinR	GGATATAAAGTACCGCCAAG		
Roe deer *Capreolus capreolus*		*Cyt b*	54 (56)	RoecytbF	CAACCCGACAGGAATCCC	14 778–14 906	128
				RoecytbR	AGGTCTGGTGCGAATAAG		
	2	*Cyt b*	62	CytbinnRRoe	GAATATTAGGGAAAGAATTAAGAGT	14 859–14 883	145
	1	*Cyt b*	76 (64)	CytboutFRoe	CTGACCCGATTTTTCGCTTTCCACTT	14 683–14 928	245
				CytboutRdeercat	GTAGTTATCTGGGTCTCC(T/A)AGCAGGTCT		
Field vole *Myodes glareolus*	2	*12S*	58 (52)	12SifMyo	GCTTAGCCCTAAACTTCAACAT	510–612	102
				12SiR	GGATATAAAGTACCGCCAAG		
		Cyt b	48	MyoCytbF	CTATGGCTCCTATAACA	14 469–14 567	98
				MyoCytbR	CCTCATGGTAGGACGTAA		
	2	*Cyt b*	60	CytbinnRMyo	AATTATGAGACCTATTAATAAGATA	14 859–14 883	145
	1	*Cyt b*	81 (66)	CytboutFMyo	CT(C/T)ACACGATTCTTCGCATTCCACTT	14 689–14 928	239
				CytboutRMyo	GTAATTATC(T/C)GGGTCTCCGAGAACATCT		
Shrew *Sorex* spp.		*12S*	58 (57)	12SSaF	GCTTAGCCCTAAACTCAGG	510–612	102
				12SSaR	GGATATAAAGCACCGCCAAG		
Pathogens							
Borrelia burgdorferi s.l. (Michel *et al.* 2004)	1	*OspA*	56	V1	GGGRATAGGTCTAATATTAGC	18–38	798 - 807 bp, depending on the *OspA* type
	2	*OspA*	56 (58)	V3	GCCTTAATAGCATGYAAGC	37–55	
	1+2	*OspA*	56 (56/58)	R1	CATAAATTCTCCTTATTTTAAAGC	832–855	
Babesia sp. (Armstrong *et al.* 1998)		*18S rDNA*	55	Bab18SF	AATACCCAATCCTGACACAGGG	202–608	408 bp (*Babesia odocoilei*) and 437 bp (*Babesia microti*)
				Bab18SR	TTAAATACGAATGCCCCCAAC		

Appendix 2 Primer positions

The primers and their positions within the respective genomes.

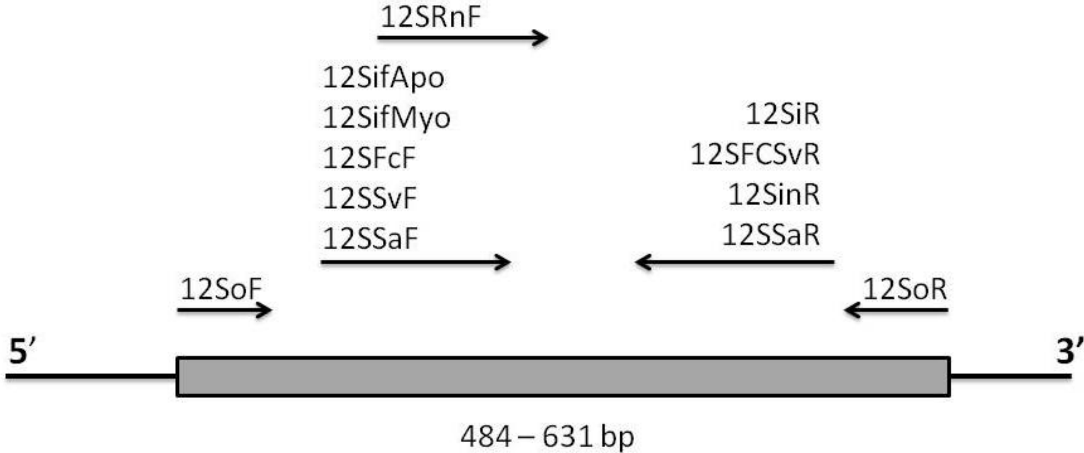


Figure 1. PCR primers in the *12S rDNA* fragment of the mitochondrial genome.

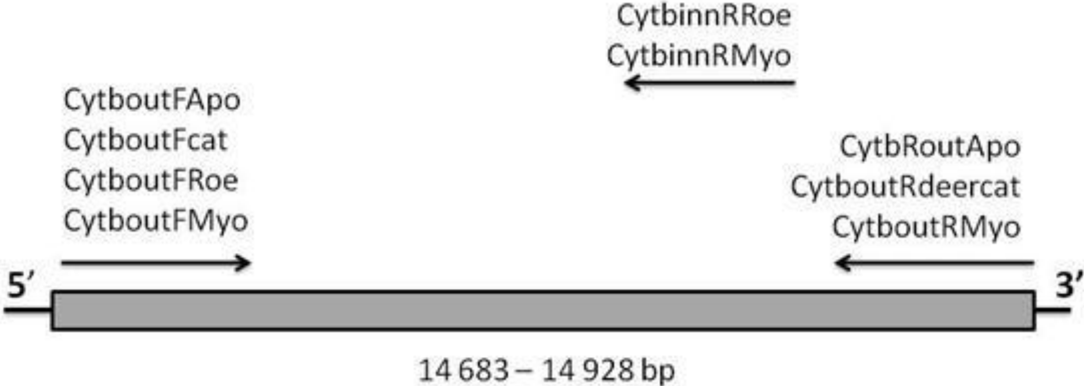


Figure 2. PCR primers in the *cytochrome b* fragment of the mitochondrial genome.

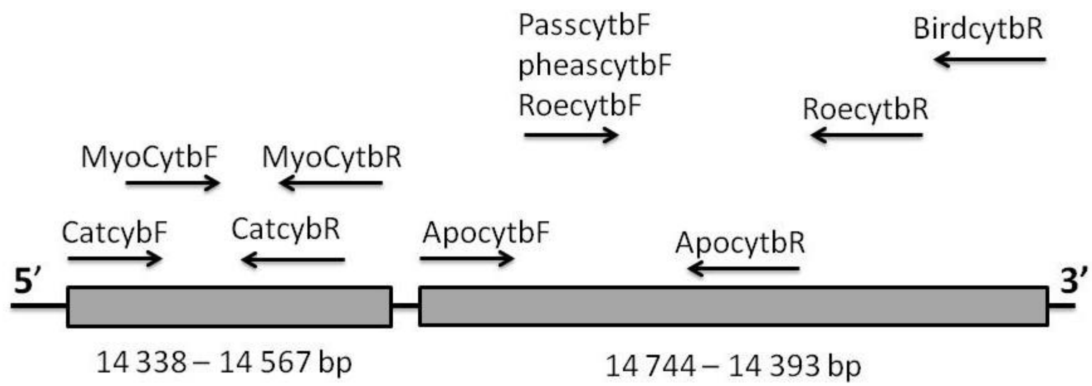


Figure 3. Single PCR primers in the *cytochrome b* fragment of the mitochondrial genome.

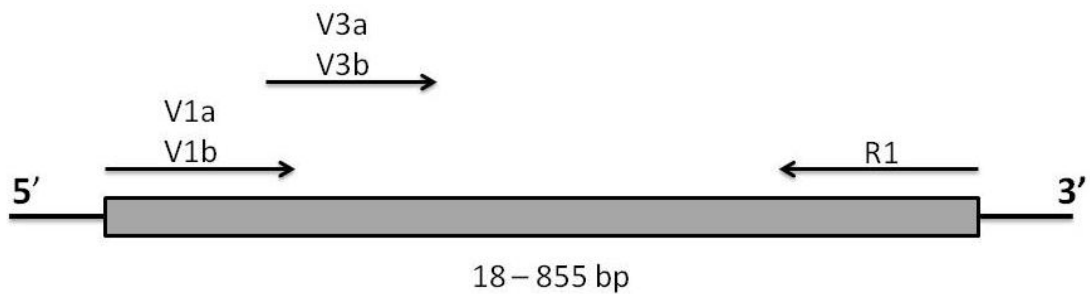


Figure 4. Heminested PCR primers in the *Borrelia OspA* gene.

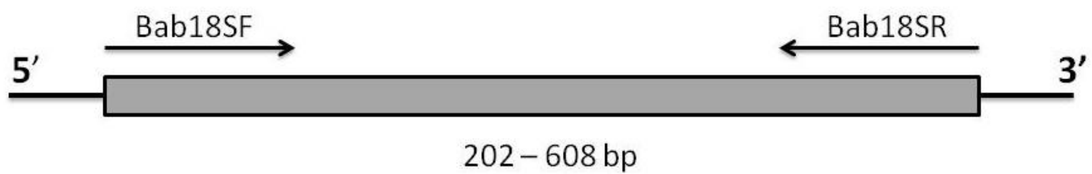


Figure 5. Single PCR primers in the *Babesia 18S rRNA* gene.

Appendix 3 Sequence alignment of *Babesia* spp.

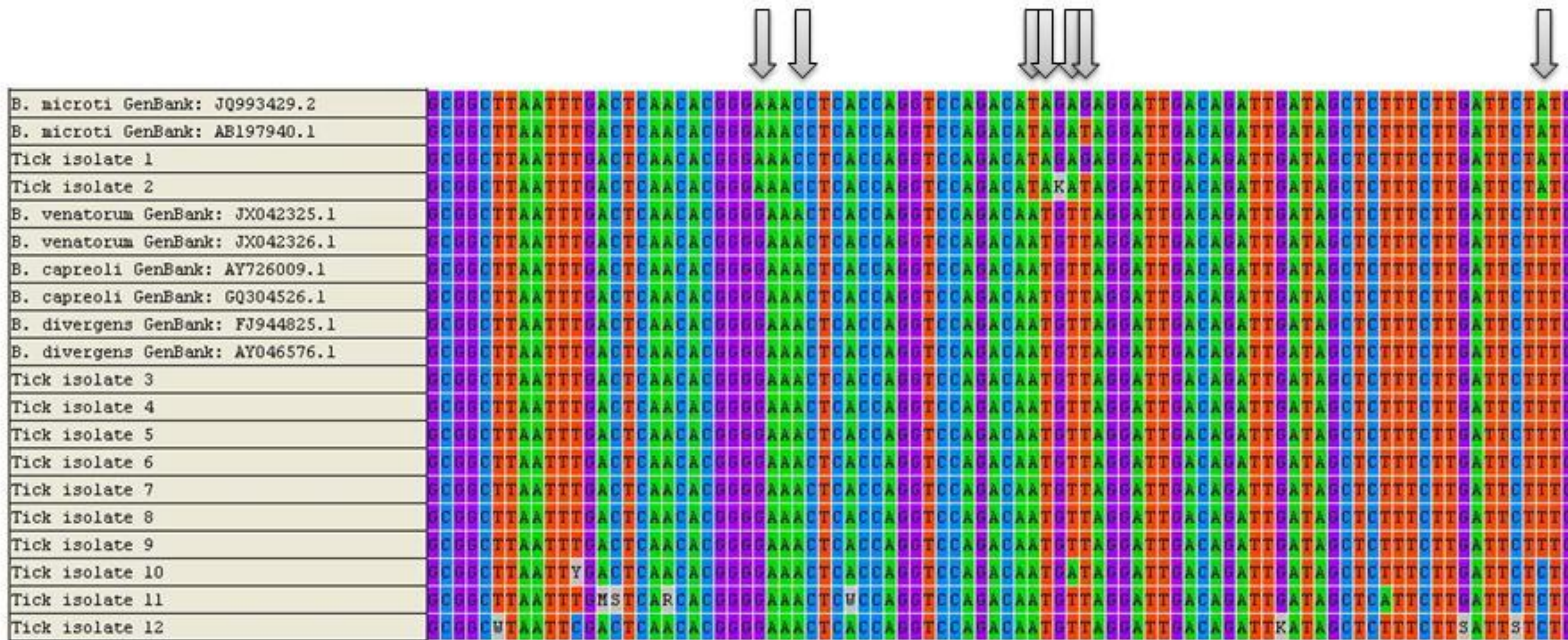


Figure 1. Alignment of *Babesia* spp. *18S rDNA* sequences with GenBank reference sequences. Arrows display base differences between *B. microti* and *B. venatorum/capreoli/divergens*. Tick isolates 1 and 2 are infected with *B. microti*, and tick isolates 3-12 are infected with *B. venatorum/capreoli/divergens*.