Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Forest Science

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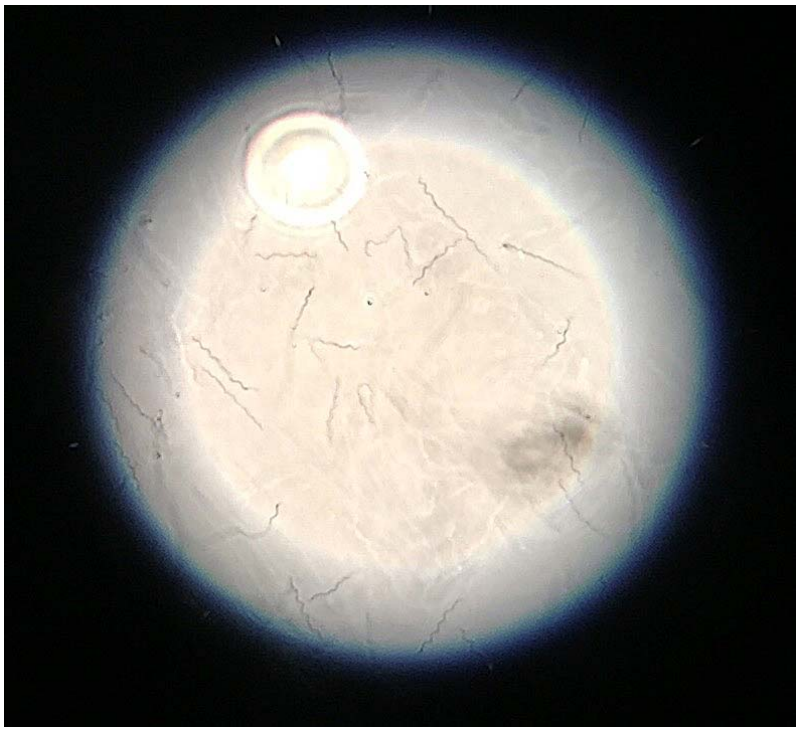

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Abstract

On a global scale, zoonotic vector-borne diseases pose an increasing threat towards human health. In Europe, the haemotophagous ectoparasite sheep tick (*Ixodes ricinus*) acts as a pathogen vector for diseases like tick-borne encephalitis and Lyme disease with increasing annual incidences during the last decades. In 2015, Lyme borreliosis was ranked in the top ten priority group of pathogens to guide resource allocation within the Public Health Agency of Sweden. *Borrelia burgdorferi* sensu lato (s.l) is the pathogen that causes Lyme disease in humans and animals and is maintained in the wild by a complex multi-species transmission process involving tick vectors and many different vertebrate hosts, including humans as dead-end hosts. In Sweden, the relation between *B. burgdorferi* s.l. prevalence in rodents and ungulate abundance is unknown. In this study, the prevalence of *B. burgdorferi* s.l. in rodents (Yellow-necked mouse (*Apodemus flavicollis)*, wood mouse (*Apodemus* sylvaticus) and bank vole (*Myodes glareolus*), was evaluated in Gnesta/Nyköping (G/N) and Växjö/Vetlanda (V/V), with varying abundances of wild ungulates as moose (*Alces alces)*, roe deer (*Capreolus capreolus),* red deer *(Cervus elaphus) and* fallow deer (*Dama dama*). Rodent data and ungulate population density data were based on data collected by the National Environmental and Wildlife Monitoring and Assessment program at SLU. Prevalence of *B. burgdorferi* in rodents were analysed with real-time quantitative polymerase chain reaction. The results show equal *B. burgdorferi* s.l. prevalence in the two study regions (22%), but between species, *M. glareolus* showed a significantly higher prevalence (26%) compared to *Apodemus spp.* (15%). *B. burgdorferi* s.l.. Overall, the infection probability analysis revealed a higher risk for *M. glareolus* of getting infected than *Apodemus spp.* However, when comparing infection probability between species in each region, these same results were found for the V/V area, but were contradictory within the G/N reg. The probability of infection also increased with body mass in rodents. Most importantly, the infection probability analysis revealed that deer species such as roe deer and fallow deer did not significantly influence infection probability in rodents. In conclusion, the dataset for prevalence and infection probability analysis, should have been larger to obtain greater statistical power. Also, the method using external oligonucleotides as standard curve reference, are in need of a scientific evaluation.

Technical terms and Acronyms

AGE (Agarose Gel Electrophoresis)

B31-strain (*Borrelia* genome originated from a *Ixodes Dammini* tick collected in North America)

clpA (Specific primer-set for MLST and nPCR)

C_t (Cycle threshold value, used in RT-qPCR analysis)

Dead end host (Host in which the pathogen cannot fulfil its life-cycle)

MLST (Multi Locus Sequence Typing)

nifS (Specific primer-set for MLST and nPCR)

nPCR (Nested Polymerase Chain Reaction)

Osp (Outer Surface Protein)

pyrG (Specific primer-set for MLST and nPCR)

Questing (Tick activity in search for blood meal host)

RT-qPCR (Real-Time Quantitative Polymerase Chain Reaction)

SEON (Synthetic External OligoNucleotide)

Taq (-Polymerase, -man) (Polymerase enzyme, originated from the bacteria *Thermus aquatis*)

Transovarial transmission, vertical transmission (Pathogen transmission from adult to offspring)

Transstadial transmission (The pathogen survives between each tick instar)

Introduction

Globally, zoonotic vector-borne diseases pose an increasing threat towards human health, and almost one-third of all emerging infectious diseases identified the last decade was vector-borne (Jones et al. 2008). In northern Europe, hard ticks of the genus *Ixodes*, act as pathogen vector for human diseases such as tick-borne encephalitis and Lyme borreliosis (Fraenkel *et al.*, 2002; Jongejan & Uilenberg, 2004). The annual incidences of tick-borne infections in Europe have increased since the 1980s (Lundkvist *et al.*, 2011; Rizzoli *et al.*, 2011). In Sweden, the sheep tick (*Ixodes ricinus*), which is the main vector of the pathogen agent of Lyme borreliosis, have during the last three decades become much more abundant in the Central and Southern parts of Sweden and also expanded its range in the North (Jaenson *et al.*, 2012). The risk for humans getting infected by *Borrelia* spirochetes are related to both infection prevalence among ticks, tick abundance (Glass *et al.*, 1995) and host abundance (Randolph, 2004; Gilbert *et al.*, 2012). However, these factors are also in vary in relation to the composition, size and abundance of the host vertebrate community, and whether these species are suitable for the ticks' different life stages respectively competent reservoir host for the pathogen (Ostfeld & Keesing, 2001; LoGiudice *et al.*, 2003). In a recent paper by Dahl, Tegnell & Wallensten (2015), Lyme borreliosis was ranked among the top ten of prioritized communicable diseases to guide resource allocation within the Public Health Agency of Sweden. This underpins the importance of an in depth understanding in the dynamics and ecology of the *Borrelia* complex.

The Borrelia pathogen

There are several different pathogens of the genus *Borrelia* (Fraenkel *et al.*, 2002). These spirochete bacteria are responsible for two main groups of human diseases: relapsing fever, which are rare in Europe (Anda *et al.*, 1996), and Lyme borreliosis, which is the most prevalent tick-borne zoonosis in Europe and North America (Steere, 2001). The endemic distribution comprises countries from Portugal in the Western parts of Europe to East Asian regions like Japan and also covers large parts of the American continent (Schüler *et al.*, 2015). However, the pathogen causing Lyme borreliosis in humans is the bacteria within the *B. burgdorferi* sensu lato (s.l.) complex (Burgdorfer, 1984; Fraenkel *et al.*, 2002). *B. burgdorferi* s.l. spirochetes belong to the eubacterial phylum and is characterized by its spiral shape and motile abilities (Steere *et al.*, 2004). The *B. burgdorferi* spirochete, has a cell envelope consisting of two membranes between which is a peptidoglycan layer. The outer membrane of *B. burgdorferi* contains several lipoproteins, designated Osp (Outer Surface Proteins). Unlike many other bacteria, the flagellum is not extended outside the bacterium, but instead they are wrapped around the bacterium between cytoplasmic membrane and outer membrane, promoting the corkscrew and vibrating motion. The *B. burgdorferi* spirochete is unusually long and thin, about 20-30μm in its whole extent and 0.2μm in diameter (Salyers & Whitt, 1994). The genomic sequence of the *B. burgdorferi* strain contains 1 chromosome (≈1 megabase) and 21 circular and linear plasmids (Stewart *et al.*, 2005).

The life-cycle of *B. burgdorferi* s.l spirochetes is characterized by complex interactions between bacteria, vector and hosts, but is also affected by abiotic factors such as landscape and climate, having impact on vector and host ecology (Kurtenbach *et al.*, 2006). *B. burgdorferi* s.l. bacterial spirochetes exist in several different genospecies (Fraenkel *et al.*, 2002), where *B. burgdorferi* sensu stricto (s.s.), *B. garinii* and *B. afzelii* are the three main genospecies causing Lyme borreliosis in western Europe (Gern *et al.*, 1998; Grubhoffer *et*

al., 2005) and in Sweden (Fraenkel *et al.*, 2002). Each genospecies is also associated with varying clinical expressions and enzootic lifecycles (Kurtenbach *et al.*, 1998), where *B. burgdorferi* s.s. demonstrates arthritogenic expressions, *B. garinii* is considered the most neurotrophic, and *B. afzelii* mostly associated with skin manifestations (Stanek *et al.*, 2012). Each genospecies is also associated with main vertebrate hosts, where *B. garinii* is mostly associated with birds and *B. afz*elii is associated with rodents (Kurtenbach *et al.*, 1998; Grubhoffer *et al.*, 2005). Rodents act as a competent reservoir host of *B. afzelii* and also act as main blood host for larvae (Matuschka *et al.*, 1991). This does not mean that *B. garinii* cannot be found in rodents, but the specific strain is not transferred to a vector and thereby the rodent become a dead-end host for this specific genospecies (Kurtenbach *et al.*, 1998). The pathogen survives between the different life-stages of the tick through trans-stadial maintenance of infection (Randolph, 2004).

To be able to initiate an infection, the pathogen must be able to evade the innate immune system of the host. *B. burgdorferi* spirochetes have the ability to produce a surface of seven different lipoproteins – OspA to OspF – as defence mechanisms against innate defences within the host, where the OspC is the most abundant of lipoproteins. *B. burgdorferi* also seem to maintain the level of lipoprotein expression during its enzootic lifecycle travelling through vector, hosts and infection phase (Xu *et al.*, 2008). A very unusual feature of the *Borrelia* spirochete is that it does not requires iron for its ability to grow, which enables the spirochete to circumvent the usual host defence of limiting the iron availability (Steere *et al.*, 2004).

Vector ecology

Ixodes ricinus is a hematophagous ectoparasite and due to its ubiquity, abundance and ability to use different vertebrate hosts, it is an effective vector for several bacterial, protozoan and viral infections in Europe (Dobson *et al.*, 2011). In Europe *I. ricinus* is the most common tick (Lane *et al.*, 1991) and act as the main vector species for the *B. burgdorferi s.l.* pathogen (Steere *et al.*, 2004; van Duijvendijk *et al.*, 2015). The *I. ricinus* lifecycle involves four life stages – egg, larva, nymph, and adult – and one blood meal in respectively stage larva, nymph, and adult (ECDC, 2017) (Figure 1). Larvae and nymphs uses a wide range of different sized host during its lifecycle, but adult females mostly prefer larger vertebrates (>1kg) for reproduction (Mysterud *et al.*, 2016). Rodents, birds and reptiles are fed upon by larvae and nymphs whereas medium to large-sized wild or domestic mammals act as blood meals to adult ticks (Lane *et al.*, 1991). This ontogenetic niche shifts from small to large vertebrate hosts complicates the completion of the tick life-cycle since it depends on the abundance of both small (larvae and nymph) and large (for reproduction) hosts (Laurenson *et al.*, 2003; Sonenshine & Roe, 2014).

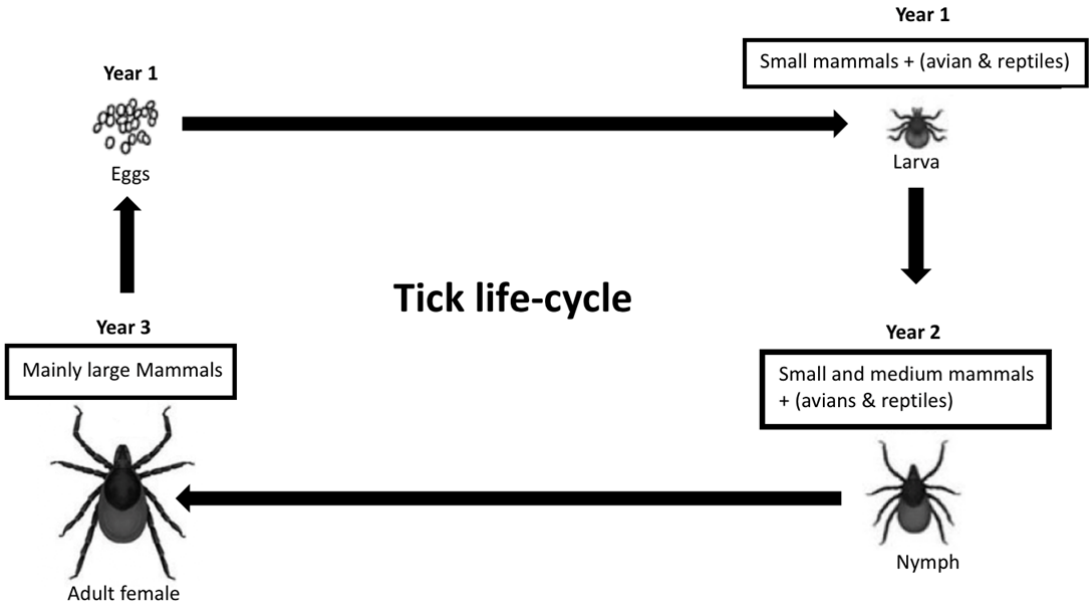

Figure 1. The temporal lifecycle of I. ricinus comprising a 3-year cycle involving 3 instar and different host niche-shifts.

following each blood meal, the tick moults into its next stage or, if it is an adult female egg laying phase, hence adult males do not blood feed (van Duijvendijk *et al.*, 2015). During each instar, the tick enters a host-finding stage and quests, i.e. climb up a grass stem or onto the edge of a leave, for passing-by suitable hosts. The questing height in the vegetation was higher for nymphs compared to larvae (van Duijvendijk *et al.*, 2015) and adult ticks quest even higher (Mejlon & Jaenson, 1997). *I. ricinus* can locate blood meal hosts by scent (Berret & Voordouw, 2015), but specific host preferences has not yet been proven experimentally (van Duijvendijk *et al.*, 2015). Ticks are sensitive to desiccation during questing and moulting phase, and needs to obtain water (Gray, 1998). The tick obtains water from air humidity by secreting and thereafter re-ingesting hydroscopic fluids that are produced in the salivary glands (Kahl & Knülle, 1988). This mechanism enables the tick to maintain a stable water balance, but only as long as the humidity in their micro-climate stays over 80% during the driest period (Gray, 1998). There are variations in typical tick habitats across the European landscape, but mostly it includes deciduous and coniferous forests, heathlands, moors, rough pastures and urban parks (Medlock *et al.*, 2013).

In southern Sweden (Uppland/Södermanland), the tick infestation rate on mammal hosts exhibits seasonal changes. *I. ricinus* tick infestation by larvae and nymphs is bimodal with peaks in May-June and August-September (Mejlon & Jaenson, 1993), but unimodal peaks have been recorded in certain areas (Nilsson, 1988). A decline in host-seeking activity during midsummer period may partly reflect the relatively dry conditions at this particular time during the season (Mejlon & Jaenson, 1993). The host-seeking seasonality among adult ticks did not show a bimodal pattern, probably because adult ticks have a greater resistance to relatively lower humidity, compared to sub-adult ticks (Knülle & Rudolph, 1982). Mejlon & Jaenson (1993), suggest that there are no seasonal variations respect to spirochaete prevalence in *I. Ricinus* in their study. In contrast, Kurtenbach *et al.*, (1995) showed that ticks feeding on wild rodents had a lower infection rate in April (1.2-10.5%) compared to June and July (15.1-17.5).

Pathogen transmission

The spirochete bacteria *B. burgdorferi s.l.* use *I. ricinus* as the main vector for transmission between vertebrate hosts (Grubhoffer *et al.*, 2005; Jaenson *et al.*, 2012). When a *I. ricinus* larva for the first time feeds on an infected competent reservoir host for *B. burgdorferi* s.l., the vector transmission route is initiated (Caimano *et al.*, 2016). The *Borrelia*e spirochetes remains confined in the midgut lumen by transstadial maintenance of the infection when the larva moults from larva to nymph and nymph to adult. Then, when the nymph feeds on a host, the spirochetes migrate to the salivary glands and thereby the pathogen persists between different life-stages of the tick and is transmitted to the blood meal host (Burgdorfer *et al.*, 1982; Burgdorfer, 1984; Caimano *et al.*, 2016) (Figure 2). Larval and nymphal blood-feeding are crucial to maintaining the spirochete in the wild (Tilly *et al.*, 2008).

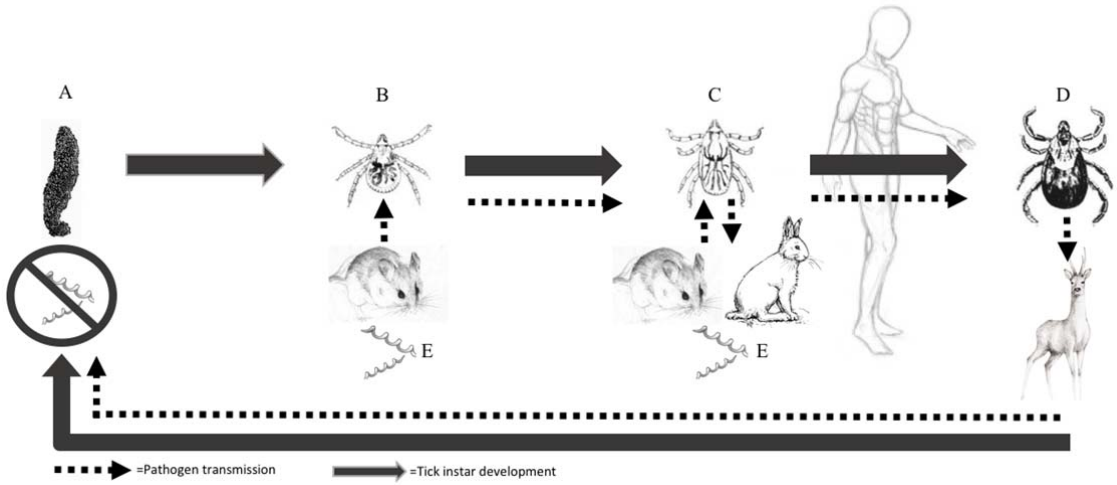

Figure 2. Pathogen transmission route, involving small- medium- and large mammals between each tick instar. A) tick egg cluster, B) tick larva, C) tick nymph, D) adult female tick and E) *B. burgdorferi* spirochetes.

Sub-adult stages of *I. ricinus* are suggested to be the main vector phase of *B. burgdorferi* transmission in Europe and feeds on various species of small mammals as squirrels, lagomorphs (*L. europaeus, L. timidus*), and rodents, but also on birds and reptiles (Gern *et al.*, 1998). The chance that questing sub-adult ticks encounter a host is related to host activity and abundance of the host species (Jones *et al.*, 1998; Rosà *et al.*, 2007) and in North-western Europe the tick sub-adults feed preferably on the most common rodent species in Europe: yellow-necked mice (*Apodemus flavicollis),* wood mice *(Apodemus sylvaticus)*, and bank voles *(Myodes glareolus)* (van Duijvendijk *et al.*, 2015).

Transovarial transmission, i.e. vertical transmission from adult female to eggs, of *B. burgdorferi* s.l. within *I. ricinus* is rare, with <1% of the hatched larvae infected vertically (Richter *et al.*, 2012, Gray 1998). Although it has been suggested that transovarial transmission must have an considerable role in maintaining the circulation of spirochetes in nature (DeBoer *et al.*, 1993), experimental approaches failed to prove inheritance of *Borrelia*e spirochetes to *I. ricinus* eggs (Gray, 1998). Instead, larvae become infected with *B. afzelii* through a blood meal from an infected rodent, but also in some cases when an uninfected rodent co-feeds close to another infected tick (Randolph *et al.*, 1996; Voordouw, 2015).

Van Duijvendijk et al. (2015) argue that the chance for a larva getting infected by a blood meal is determined by four factors: 1) the overall *Borrelia*e prevalence in the blood meal host community, which is influenced by 2) the probability that an infected nymph has fed on host, respectively 3) the host's susceptibility of the pathogen and 4) ability of the host to maintain the infection. Climatic conditions also favours the *B. burgdorferi* s.l. prevalence in nymphs, where mild winters, high summer temperature amplitudes and low seasonal variations (Estrada-Peña *et al.*, 2011).

Tälleklint & Jaenson (1996) suggest that there is a relationship between density of *I. ricinus* nymphs and *B. burgdorferi* s.l. prevalence, with increased nymph infection prevalence with increased nymph density, and that infection prevalence increased with nymphal densities between 0-20 nymphs per $100m^2$ and decreased at higher densities >20 ticks per $100m^2$. However, the development from uninfected larvae to infected nymph seems to be a key-factor in the enzootic cycle of *B. burgdorferi* (van Duijvendijk *et al.*, 2015) and today's knowledge suggests that nymphs are responsible for infecting rodents hence larvae rarely are infected, and adult *I ricinus* ticks less often feed on rodents (Gassner *et al.*, 2013; van Duijvendijk *et al.*, 2015). Other studies also show differences in tick burden between rodent species (van Duijvendijk *et al.*, 2015) and demographic factors such as gender, size, and age (Tälleklint & Jaenson, 1997). Yet also other factors such as warm seasons, early springs and high relative humidity also influence the tick population positively (Mejlon & Jaenson, 1993; Randolph & Storey, 1999; Lindgren *et al.*, 2000; Lindgren & Gustafson, 2001).

Tick blood meal hosts

Small blood meal hosts

I. ricinus feed on a wide range of small vertebrates, such as reptiles, birds and rodents (Lane *et al.*, 1991). However, rodents act as the main hosts for *I. ricinus* larvae and nymphs, and the tick burden varies between the most frequent rodent species in Europe; *A. sylvaticus*, *A. flavicollis* and *M. glareolus* (Matuschka *et al.*, 1991; van Duijvendijk *et al.*, 2015). Variations between the different rodent species on tick infestation rate, *Borrelia* infection rate, and contribution to tick moulting success can be seen, where tick larvae burden is higher on *A. sylvaticus* than *M. glareolus* (Tälleklint & Jaenson, 1997), *Borrelia* infections are higher in *M. glareolus* and *Apodemus spp.* contributed to higher moulting success (Kybicová *et al.*, 2008). Differences in rodent species' tick burden may be influenced by species specific variations in their ecological niche, habitat, home range, activity pattern and immune responses (Kaufman, 1989; Wikel, 1996; Hughes & Randolph, 2001). It has also been shown that the size of the actual blood meal and number of fully engorged sub-adults are larger for sub-adults feeding on *A. sylvaticus* than on *M. glareolus* (Nilsson & Lundqvist, 1978; Matuschka *et al.*, 1992; Tälleklint & Jaenson, 1997; Hughes & Randolph, 2001). But, *B. burgdorferi* s.l. infection was more frequently observed in *Myodes* than *Apodemus*, the infection rate in ticks feeding on *Myodes* was higher, but *Apodemus* yielded more infected ticks than *Myodes* due to higher moulting success (Humair *et al.*, 1999). Lagomorphs are also competent as a reservoir host and in fact the only known species that acts as both reservoir competent host and a blood meal source for all three stages of *I. ricinus* (Tälleklint & Jaenson, 1993) and therefore this species is able to maintain the pathogen without a sympatric rodent population. Ectoparasites such as the tick can affect the fitness of their host in many ways, and may consume up to 65% of the blood resource from a rodent, which affects the activity level and fitness of the host (Tälleklint & Jaenson, 1997). In a paper by Duijvendijk *et al.*, (2015), they suggest that the

largest impact on nymphal density derived from the probability that a larva encounters a rodent which is correlated with rodent density, which is also are affected by spatial and temporal variations (DeBoer *et al.*, 1993; Kurtenbach *et al.*, 1995).

Large blood meal hosts
In Europe, the most important blood meal host for maintaining *I. ricinus* in the wild are deer species (Ruiz-Fons & Gilbert, 2010), but are incompetent for *B. burgdorferi* s.l. spirochete transmission due to observed anti-*Borrelia* immune responses (Jaenson & Tälleklint, 1992; Pacilly *et al.*, 2014; van Duijvendijk *et al.*, 2015) and the ratio of incompetent and competent transmission hosts is an important factor for the prevalence of *B. burgdorferi* s.l. in the tick population (Mysterud *et al.*, 2016). This was supported by a study from Holland, which reveals that ticks feeding on red deer (*Cervus elaphus*) and wild boar (*Sus scrofa*), lose their infectious spirochetes (Pacilly *et al.*, 2014). Therefore it is suggested that deer species mainly act as blood meal hosts to reproducing female adult ticks (Rand *et al.*, 2004). However, even if the pathogen inhabits a dead-end host, deer abundance may, as blood meals to adult female ticks, affect pathogen transmission and dynamics in nature by increasing the number of larvae feeding on reservoir-competent hosts (Tälleklint & Jaenson, 1996).

Differences in number of questing ticks were reported from studies performed inside or outside deer exclosures, with results of reduced tick abundance inside the deer exclosure sites (Rand *et al.*, 2004; Gilbert *et al.*, 2012), probably hence the tick population were limited by the absence of reproduction hosts (Rand *et al.*, 2004). Similarly, a study from Ireland showed that tick abundances were higher inside deer enclosures than outside (Gray *et al.*, 1992). When it comes to the *Borrelia* pathogen, a Norwegian study conducted on islands showed that high abundance of wild ungulates (red deer and roe deer), reduced the *B burgdorferi* s.l. prevalence in host-seeking *I ricinus* (Rosef *et al.*, 2009). In contrast, Millins *et al.*, (2016), found no relationship between deer abundance index and abundance of *I. ricinus*, *B. burgdorferi* s.l. prevalence or density of infected nymphs. However, in a public health context, a study by Mysterud *et al.*, (2016) in Norway, found that high deer populations increased the incidence of Lyme borreliosis in humans, which is in contrast to these previously-mentioned pathogen studies.

Mysterud *et al.*, (2016) suggest that if there are few competent reservoir blood meal hosts and the ticks are forced to switch to a non-competent host species, there will be a higher proportion of sub-adult's ticks feeding on non-transmission hosts and thereby lower the proportion of pathogen in the tick population (dilution effect). In contrast, Ostfeld and Keesing, (2001), suggests that a dilution effect is less likely in Europe because of a larger base of reservoir hosts (Gern *et al.*, 1998). This was supported in a previous study in Bogesund, Sweden, showing that reservoir-incompetent ungulates served as host for >50% of adult female ticks, whilst only <5% of larvae fed on ungulates and were suggested to be one possible reason for increased infection prevalence in ticks within areas with higher ungulate densities (Tälleklint & Jaenson, 1994).

Aim and hypotheses
The aim of this study is to investigate the prevalence of *B. burgdorferi* s.l. in rodent populations within two areas (Gnesta/Nyköping (henceforth G/N) and Växjö/Vetlanda (henceforth V/V) representing areas of different ungulate abundances, where G/N shows higher ungulate abundance than V/V. Based on ungulate faecal pellet counts, rodent

trappings, and subsequent *Borrelia* analysis of rodent samples, the prevalence of *B. burgdorferi* s.l. was investigated in rodents.

Specifically, the following questions will be addressed:

1) Prevalence hypotheses:

1.1) Does *B. burgdorferi* s.l. prevalence differ between the two regions in all rodents and species specific? H_0 assumes that there are no differences.

1.2) Does *B. burgdorferi* s.l. prevalence differ between rodent species in whole and between the two regions? H_0 assumes that there are no differences.

1.3) Does *B. burgdorferi* s.l. prevalence differ between male and female rodents in whole and between the two regions? H_0 assumes that there are no differences.

1.4) Does *B. burgdorferi* s.l. prevalence differ between spring and fall? H_0 assumes that there are no differences.

2) Infection probability hypotheses:

2.1) Does the prevalence and infection probability of *B. burgdorferi* s.l. differ at regional level? H_0 assumes that there are no differences.

2.2) Does the prevalence and infection probability of *B. burgdorferi* s.l. differ between *Apodemus spp.* and *M. glareolus* in fall and spring? H_0 assumes that there are no differences.

2.3) Does the prevalence and infection probability of *B. burgdorferi* s.l. differ between males and females? H_0 assumes that there are no differences.

2.4) Does the infection probability of *B. burgdorferi* s.l differ in relation to rodent mass? H_0 assumes that there are no differences.

2.5) Does the infection probability of *B. burgdorferi* s.l in rodents differ in relation to rodent density? H_0 assumes that there are no differences.

2.6) Does the infection probability of *B. burgdorferi* s.l in relation to roe deer and fallow deer density? H_0 assumes that there are no differences.

Material and methods

Study areas

Rodent sampling and wild ungulate faecal pellet counts (Bergström *et al.*, 2011; Bergström & Wallin, 2017)were conducted in two regions within the Environmental monitoring and assessment program by the Swedish University of Agricultural Sciences (SLU) between 2012 to 2016. One sampling region, within Gnesta and Nyköping municipalities (henceforth G/N), are located in the County of Södermanland and the other, within Växjö

and Vetlanda municipalities (henceforth V/V), in the County of Jönköping/Kronoberg, both in southern Sweden (Figure 3).

Each region is ~ 58 km^2 and consists of 50 quadrats in a systematic grid (1 km^2) with 2 km spacing between. Each area contains 16 sampling sites dispersed along the outer edges of the area with 200m spacing (Figure 4). The field set-up regarding rodent snap-trapping and ungulate faecal pellet count is described in detail below.

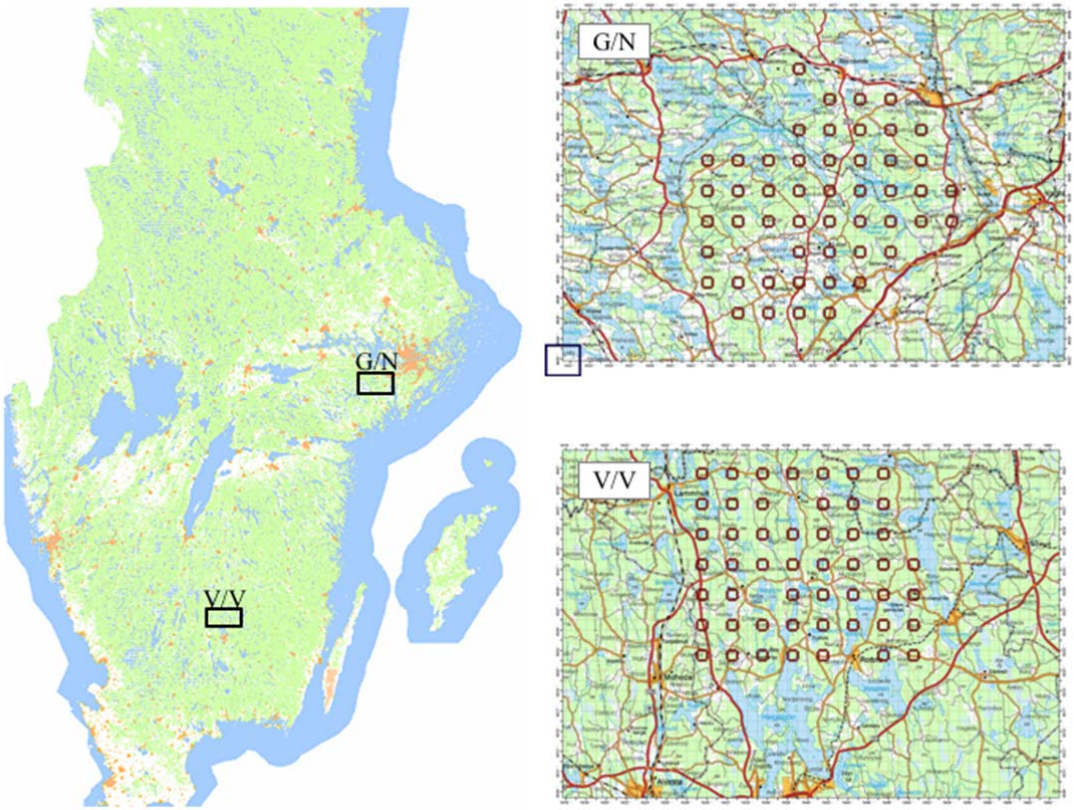

Figure 3. The left map show southern Sweden and the two Environmental monitoring and assessment sites (black squares). The red squares distributed on the maps to the right are the respectively areas' layout of the environmental monitoring and assessment sites.

Wild ungulate faecal pellet sampling

At each sampling site, all over-winter accumulated ungulate faecal pellet piles were counted in spring within the two FoMA regions. To meet the criteria "over-winter accumulated", only pellet piles found on top of previous year's fallen leaves, withered annual vegetation (or similar) were counted. The faecal pellet count was conducted directly after snow melt and lasted to the leafing or sprouting period (Edénius, 2012). Ungulate species taken in consideration were moose (*Alces alces*), red deer (*Cervus elaphus*), fallow deer (*Dama dama*) and roe deer (*Capreolus capreolus*). The definition of a fresh faecal pellet pile is piles that lies on top of the litter layer descended from the trees during last fall to the time of observation and the colouration. The definition of a faecal pellet pile is that it consists of at least 20 pellets from moose or red deer, or 10 pellets from fallow deer or roe deer. When counting faecal pellets from moose and red deer, the size of the sample area is

≈100m^2 (5,64m∅). The pellet count area for fallow deer and roe deer is 10m^2 (1,78m∅) (Figure 4).

Rodent trapping

Within each site selected to sample rodents (n= x), four 15x15m "small quadrats" (Myllymäki *et al.*, 1971) where placed in one corner each within a 80x80m square with 50m spacing. The small quadrat holds three snap-traps in each corner (Figure 4). The snap-traps were baited with hemp seed sandwiched between two pieces of beeswax. Rodents were trapped biannually from autumn 2012 to spring 2016. Each small quadrat site endured 2 trap nights per season. Trapped rodents were placed in a ZIP-lock bag with a unique ID number, immediately placed on ice, and at end of the day frozen to -20°C.

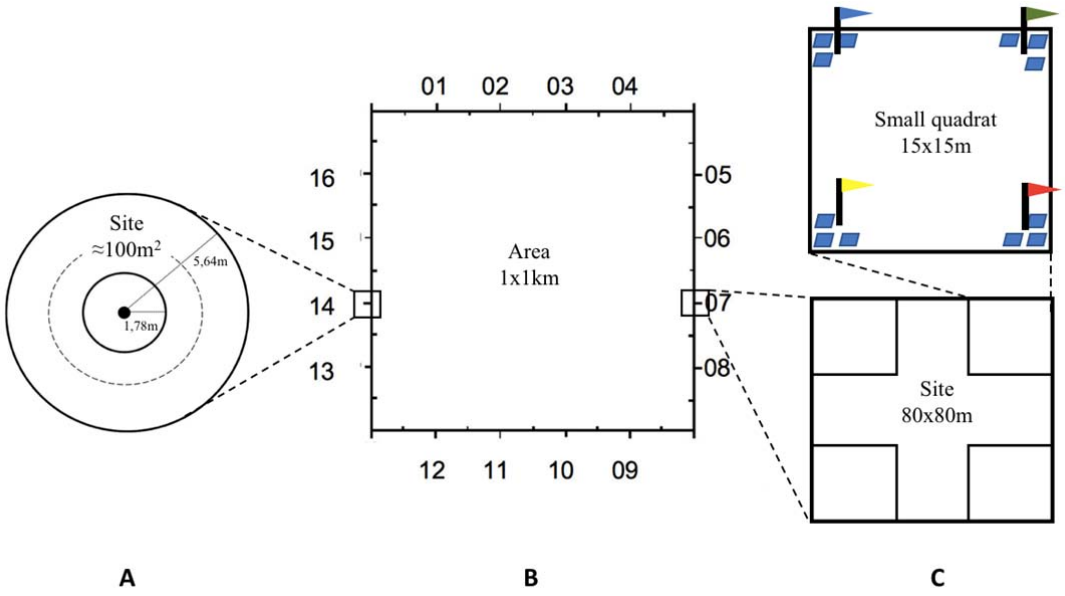

Figure 4. Spatial field set-up regarding faecal pellet count and rodent snap-traps within the FoMA regions G/N and V/V. In the middle (B): area set-up with 16 sites along the square sides, to the left (A): sampling set-up regarding faecal pellet count in each site. 5,64m radius are for moose and red deer pellet count, and 1,78m radius are for roe deer and fallow deer. Finally, to the right (C): the rodent snap-trap sites and small quadrat set-up. The three blue squares in each corner illustrates snap-traps and the coloured flags enables field staff to locate the traps. Dotted lines act as guidelines for magnify directions. Both faecal pellet count and rodent trapping were conducted at the same sites.

Laboratory processes

All rodents used were processed in a bio safety level 2 laboratory under a fume-hood to prevent aerosol exposure from samples. To avoid cross sample contamination, all instruments were autoclaved and cleaned in a Rely+On™ Virkon® (DUPONT, Wilmington/Delaware, USA) solution between dissections. A suitable number of rodents were brought out of the freezer and let to thaw at room temperature inside the fume-hood and after that dissection and blood sampling could begin. Molecular processes and DNA assays were also processed in a bio safety level 2 laboratory.

Species determination
Three target rodent species (*A. flavicollis, A. sylvaticus,* and *M. glareolus*) were specified based on morphometric and biometric data. Specific factors as length, tail length and weight was measured. Also, fur pattern and colour were used in species determination. *Apodemus flavicollis* and *A. sylvaticus* Overlaps were merged in to *Apodemus spp. (Henceforth Apodemus)* due to possible overlap on morphological measures (Figure 5) (De Jong & Lundberg, 1995; Walburg, 2015).

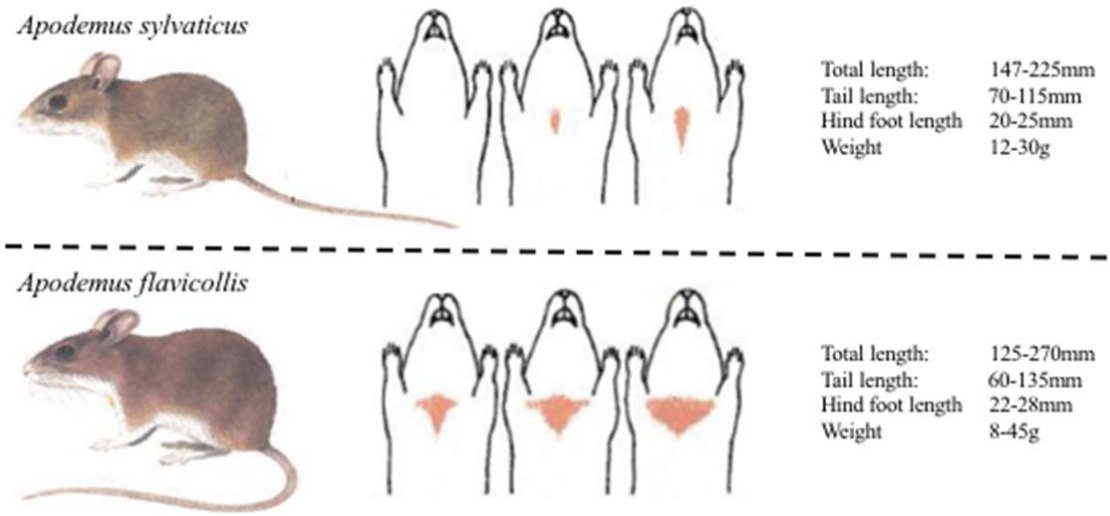

Figure 5. Modified drawing specifying the morphological differences between A. sylvaticus and A. flavicollis. Adopted from (De Jong & Lundberg, 1995; Walburg, 2015).

Rodent dissection
Before dissecting, an ear tissue biopsy was taken. On dissected rodents from previous years, an ear biopsy had to be taken after dissection. Before ear skin for this biopsy could be obtained, a batch of 20 rodents were thawed for 20 minutes. The biopsy was taken from left or right ear, depending on ear quality. The ear was cut with a scissor and held with a tweezer. The cut was located as near the skull as possible to ensure that ear cartilage tissues followed with the ear specimen. Snipped ear specimens were cut in half and put in an Eppendorf 2.0ml Safe-Lock Tube. The specimen was immediately put in a Styrofoam box with freezer blocks (frozen to -80°C) during the snipping-round. Thereafter the specimen was put in a -20°C freezer. After each use, the scissor and tweezer were cleaned in Virkon® and rinsed in two H_2O baths before let to hang dry. Between each dissection level, used scissors and tweezers were soaked for >10 minutes and cleaned in Virkon® (Virkon Disinfectant Technologies, Suffolk, UK), thereafter rinsed in two H_2O baths before hanging to dry. A toothbrush was used to remove denaturized proteins and other solid dried tissues from the tools. During dissection, the sex of the rodents was determined.

Borrelia burgdorferi detection methods
Ear biopsy is an efficient method to obtain skin tissues for *B. burgdorferi* detection and can be taken from, for example, spleen, urine-bladder or ear tissues (Radzijevskaja *et al.*, 2011). In a study conducted on white-footed mice (*Peromyscus leucopus*) in the United States, ear tissue had an almost equal sensitivity to bladder biopsy samples for detecting *B. burgdorferi*. The usage of ear skin tissues for *B. burgdorferi* detection is also supported by a study conducted by Radzijevskaja *et al.*, (2011). To determine which analysis to use for

B. burgdorferi s.l. detection in rodents, two analyse methods were tested; Nested Polymerase Chain Reaction (nPCR) and Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR). Both detection methods were using DNA purified from rodent ear tissues. The reason why two methods are tested is that if nPCR can successfully analyse the ear tissues, it would give the opportunity to run Multi Locus Sequence Typing on the positive samples to be able to genotype which B. burgdorferi genospecies that are present in the rodents, a function that will be lost in the use of RT-qPCR analysis. Serological blood sampling was also conducted on all rodents to facilitate future serological analysis but were not used in this thesis.

Ear tissue biopsy
On dissected rodents from previous years, an ear biopsy had to be taken. Before an ear skin biopsy could be obtained, a batch of 20 rodents were thawed for 20 minutes. The biopsy was taken from left or right ear, depending on ear quality. The ear was cut with a scissor and held with a tweezer. The cut was located as near the skull as possible to ensure that ear cartilage tissues followed with the ear specimen. Snipped ear specimens was cut in halves and put in an Eppendorf 2.0ml Safe-Lock Tube. The specimen was immediately put in a Styrofoam box with freezer blocks (frozen to -80°C) during the snipping-round. Thereafter the specimen was put in a -20°C freezer. Some ear biopsies were taken during the dissection process. After each snipped ear, used scissor and tweezer were cleaned in Virkon® and rinsed in two H_2O baths before let to hang dry.

Figure 6. A) Ear tissue biopsy with nymph infestation (red circles), B) *I. ricinus* larva collected from a rodent ear and C) I. ricinus larva from a rodent ear with higher magnification. Photo: Nyman (2016, 2017)

DNA analysis

Sample preparations (ear tissue)
Prior the actual DNA assay, a purification of total DNA was done using DNeasy® Blood & Tissue Kit (QIAGEN®, Hilden, Germany). Because I deviated from the suggested extraction protocol to get better DNA yield, the preparation process was performed as following: Initially, fur from the ear half was shaved off to avoid jam in the flow through tubes. The ear tissue was cut into small pieces and placed in a 1,5ml microcentrifuge tube adding 180µl Buffer ATL. Thereafter, 20µl proteinase K (14.4 µg/µl water, Roche Diagnostics, Germany) was added to the sample and mixed thoroughly by vortexing and incubated at 60°C with 300rpm shaker overnight until the tissue was completely lysed.

Thereafter vortexed for 15 seconds and 200 µl Buffer AL was added to the sample and again mixed thoroughly by vortexing for 15 seconds. The lyses was incubated at 70°C for 10 minutes and 200µl ethanol (96-100%) were added and again vortexed for 15 seconds. The supernatant was pipetted into a DNeasy Mini spin column placed in a 2ml collection tube and thereafter centrifuged at 8000 rpm (6800*g) for 1 minute. The flow-through precipitant and collection tubes was discarded and DNeasy Mini spin column was placed in a new 2ml collection tube and 500µl Buffer AW1 was added. The spin column tube was centrifuged for 1 minute at 8000 rpm and the flow-through precipitant and collection tube was discarded. 500µl Buffer AW2 was added to the spin column and centrifuged for 3 minutes at 13.000rpm (17.900*g). The procedure with Buffer AW2 was repeated for better DNA yield. After the washing process, the spin column was spun for 3 minutes at 13000rpm to let dry. The final step of the purification was to place the DNeasy mini spin column in a clean 1.5 or 2ml microcentrifuge tube and pipet 200µl Buffer AE (pre-heated to 57.5°C) directly onto the DNeasy membrane. The membrane was let to incubate at room temperature for 5 minutes and thereafter centrifuged for 1 minute at 8000 rpm to elute. If sample was not used directly, it was frozen and stored in -20°C. The DNA eluate was randomly tested to see the DNA concentration (x ng/ml ($x*10^{-9}$/ml)).

Nested Polymerase Chain Reaction (screening method 1)
The first screening attempt to detect *B. burgdorferi* s.l. DNA, was done with a Nested Polymerase Chain Reaction (nPCR) (Margos *et al.*, 2008). Briefly, the PCR copies and magnify short segments of the target double helix DNA repeatedly with exponential increase rate. As Crick & Watson, (1953) suggested in the early 50s, the DNA structure has the shape of a double helix with two strands bonded together with hydrogen bonds. To amplify DNA segments, the double helix DNA strain needs to be separated into two strains of single stranded DNA. Thereafter, a Taq-Polymerase enzyme synthesizes each single stranded DNA strain into two new strains of DNA, using the original DNA as a template. The cycle of denaturation and synthesis are repeated 30-40 times, leading to an exponential reaction, giving more than a million copies of the target DNA (NIH, 2015).

The nPCR protocol are described as following: The nPCR assay were based on three primer-set, nifS, clpA and pyrG (Table 1). The housekeeping genes (genomic segments that are required to maintain basic cellular functions) that the primers attach to are all located at the linear chromosome of the spirochete.

Table 1. Primer sequences for all three primer-sets and its No. base-pairs product.

Primer-set	Inner/outer	Primer sequence 5' to 3'	Primer name	Product bp	Annealing temp
nifS BB0084	Inner forward	ATGGATTTCAAACAAATAAAAG	nifF1	629	52°C
	Inner reverse	TCACAGCCAATTTTTTTAAC	nifR680		
	Outer forward	ATGGATTTCAAACAAATAAAAG	nifF1		
	Outer reverse	GTTGGAGCAAGCATTTTATG	nifR719		
clpA BB0369	Inner forward	GACAAAGCTTTTGATATTTTAG	clpAF1255	706	52°C
	Inner reverse	CAAAAAAAACATCAAATTTTCTATCTC	clpAR2104		
	Outer forward	GATAGATTTCTTCCAGACAAAG	clpAF1240		
	Outer reverse	TTCATCTATTAAAAGCTTTCCC	clpAR2214		
pyrG BB0575	Inner forward	GATATGGAAAATATTTTATTTATTG	pyrF448	687	52°C
	Inner reverse	AAACCAAGACAAATTCCAAG	pyrR1154		
	Outer forward	GATTGCAAGTTCTGAGAATA	pyrF391		
	Outer reverse	CAAACATTACGAGCAAATTC	pyrR1190		

The overall process per housekeeping gene, traversed through two reaction set-ups and two nPCR machine programs in which give six primer preparations and six nPCR machine programs for the whole nPCR setup per sample (Table 2). Positive Template DNA (ref+) had a concentration of 1pg/µl *B. burgdorferi* DNA B31-strain. The B31 isolate has its origin from a *I. Dammini* tick collected in New York (Marconi & Garon, 1992). The B31 isolate was diluted to a concentration of 100pg/µl.

On the very first assay attempt, 1µl of DNA template and 5µl MQ-Water was used. Thereafter, the DNA template concentration was increased to 5µl DNA sample and 1µl MQ-Water due to increase sensitivity in the nPCR process.

Table 2. Overarching table display the nPCR template preparations and program processes for each sample.

Primer-set	Outer/inner	PCR Master Mix	Water (MQ)	Forward	Reverse	DNA prep.	Prev. PCR
nifS	Outer PCR	10µl	1µl	2µl nifF1	2µl nifR719	5µl	
	PCR PROGRAM						
	Inner PCR	10µl	3.5µl	2µl nifF1	2µl nifR680		2.5µl
	PCR PROGRAM						
clpA	Outer	10µl	1µl	2µl	2µl	5µl	

	PCR			clpAF1240	clpAR2214		
	PCR PROGRAM						
	Inner PCR	10µl	3.5µl	2µl clpAF1255	2µl clpAR2104		2.5µl
	PCR PROGRAM						
pyrG	Outer PCR	10µl	1µl	2µl pyrF391	2µl pyrR1190	5µl	
	PCR PROGRAM						
	Inner PCR	10µl	3.5µl	2µl pyrF448	2µl pyrR1154		2.5µl
	PCR PROGRAM						

After sample preparations, each sample was run in a BioER Life Touch PCR Thermal Cycler (BIOER, Hangzhou Bioer Technology Co., Ltd, Japan). Each PCR program passes through three main phases: 1) 1st holding, 2) cycling (35x) and 3) 2nd holding phase (Figure 7). The 1st holding phase is an initiating denaturation process and the cycling phase involves three steps (denaturation, annealing and extension), which are cycled 35 times. The 2nd holding phase, ensures that the remaining single strands are fully extended and thereafter the storing phase which continuing until the samples were removed and put in 4°C storage until next PCR or agarose gel electrophoresis (AGE).

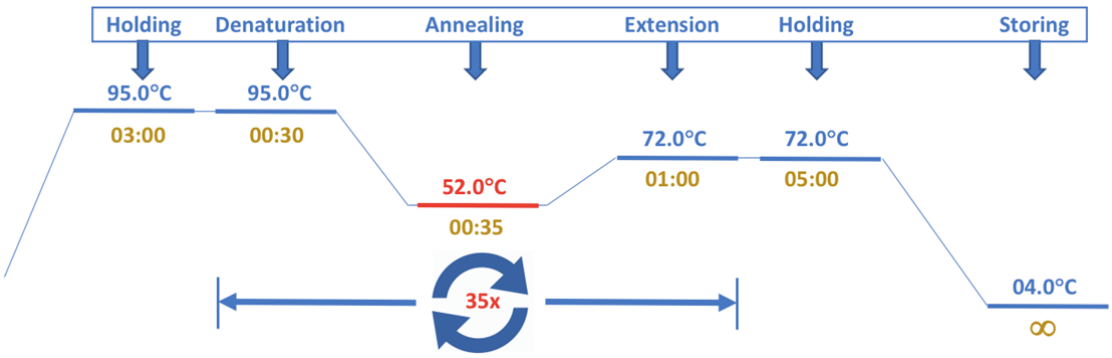

Figure 7. The nPCR program setup for all PCR primers on *B. burgdorferi* detection.

Agarose gel electrophoresis

To detect amplified *B. burgdorferi* s.l. DNA, the samples needed to be analysed with agarose gel electrophoresis (AGE). Before the amplified DNA samples could be placed in the electrophoresis chamber, it requires some preparations. Initially, 5µl of each finished nPCR sample was pipetted into a mix of 2µl orange loading dye and 5µl water. Thereafter the sample can be put onto the agarose gel. The gel was made by agarose powder mixed with TBE (1:10) in a 1/100 concentration, boiled and cooled down to at least 65°C, and then mixed with GelRed™ indicator. Thereafter the agarose gel was poured on a gel plate with a sample fork installed. When solidified, the gel was placed in the AGE chamber and drenched in Tris Borate EDTA buffer (1:10) solution until the gel plate was fully covered. On the first gel well, 3µl of 100bp DNA ladder plus was added as scale bar and thereafter 11µl of the sample mix was added separately in each gel well. Then a 130v current for 60 seconds and thereafter 100v for 60 minutes. Finally, the gel plate was photographed in a

transilluminator for forthcoming analysis. The expected number of base pairs (bp) within the three primer sets are: nifS (629bp), clpA (706bp) and pyrG (687bp) (Figure 7).

Real-Time Quantitative Polymerase Chain Reaction (screening method 2)
The second screening method on ***B. burgdorferi*** s.l. detection in rodents was performed with Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR). The RT-qPCR process is suggested to be more sensitive than nPCR and able to pick up smaller amounts of DNA in the templates (Fu ***et al.***, 2012). Additionally, in RT-qPCR, a probe with a fluorescent reporter dye and a quencher attaches to the specific target DNA and then releases when the DNA polymerase pass that specific location. Finally, the RT-qPCR machine reads the amount of released fluoresced molecules in the template and thereby the amount of amplified DNA can be measured. Another advantage with this method is that there is no post RT-qPCR analysis, and the outcome of the analysis can be analysed directly after the program was finished.

The results of the RT-qPCR are presented as a plot with three phases; exponential, linear and plateau phase. The section used in RT-qPCR is the exponential phase, since that is where the analysis gives the most precise and accurate analysis. A threshold line is drawn through the exponential phase, and from this intersection, another line can be drawn down to the X axis and assign a cycle-threshold value (C_t). The C_t value is inversely related to the starting amount of target DNA and this property means a higher amount of target DNA gives a smaller C_t value due to the cause that it requires fewer RT-qPCR cycles for the fluorescence signal to rise above the threshold line.

The RT-qPCR in this assay was based on a 16s primer-set and a TaqMan® Probe associated with Ribosomal DNA (rDNA) (Table 3). Initially a master stock was prepared for 96 wells (Table 4). Thereafter, 8µl master stock was pipetted into the number of desired wells on the well plate and additionally 2µl of DNA template was pipetted in each well (total amount of 10µl/well). In the first round, a standard reference curve, 8 wells of B31 isolate concentration ladder (10^{6}-10^{-1}) was added to the plate. Due to lack of prepared standards and limited time to cultivate and count new B31 spirochetes, an external oligonucleotide was used as standard (Table 5). Also, one well in each assay, a nailed reference template was added to detect any problems in the DNA purification process. The nailed template was made by adding B31 isolate with an ear tissue sample and thereafter let be prepared as described above, to ensure that the DNA quality was not lowered or destroyed during the DNA purification process.

Table 3. Primer sequence properties for the probe and primer locating 16s.

Primer-set	For/rev/probe	Sequence
16s	16s forward	5´-GCTGTAAACGATGCACACTTGGT-3´
	16s reverse	5´-GGCGGCACAGTTAACACGTTAG-3´
	16s TaqMan Probe	6-FAM TTCGGTACTAACTTTTAGTTA A-MGB

Table 4. RT-qPCR master stock properties and pipette process for a 96 well (10µl) set-up.

Primer-set	16s forward	16s reverse	PCR Master Mix	LD-probe	DEPC-water	ROX LOW
16s	130µl	130µl	650µl	65µl	64,8µl	0,1µl
Pipetted 8µl master stock and 2µl DNA template in each well.						

Table 5. Design properties for the 16s external oligonucleodtide.

	Length	Weight	TM	GC	Purification	Synth. scale
Properties	88bp	27308.6g/mol	81°C	46.6%	HPSF	0.05µl
Sequence	GGT AGT CTA CGC TGT AAA CGA TGC ACA CTT GGT GTT AAC TAA AAG TTA GTA CCG AAG CTA ACG TGT TAA GTG TGC CGC CTG GGG AGT A					

Following that sample precipitates were pipetted on to the plate, the plate was sealed with optical tape. Thereafter the plate was carefully vortexed and centrifuged at 3000rpm (1000g), and the optical tape was subsequently wiped with a clean paper tissue. After template and standard curve preparations, the RT-qPCR program was run at a QuantStudio™ 7 Flex System (Life Technologies Corporation, Massachusetts, USA). The software used in this RT-qPCR analysis was QuantStudio™ 7 Flex Real-Time PCR System Software (version?). The RT-qPCR program set-up was performed as shown in Figure 8.

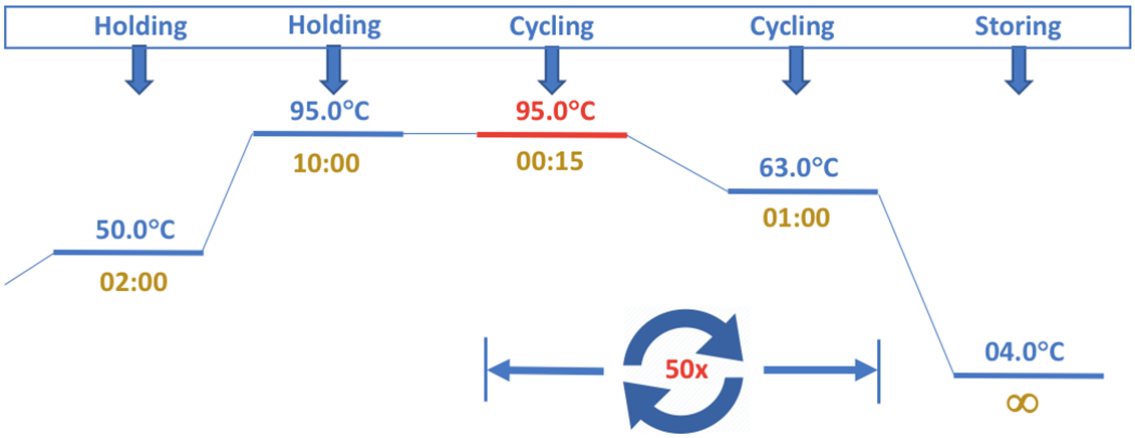

Figure 8. The program cycling setup for the RT-qPCR analysis, involving holding, cycling and storing phase.

Data analysis

Ungulate faecal pellet index

To be able to use the faecal pellet count as a determinant of wild ungulate densities within the two FoMA regions, the pellet count data needs to be transformed into a Faecal Pellet Index (FPI). The FPI value was used in forthcoming infection probability analysis. The formula of the FPI follows as:

$$FPI = \frac{number\ of\ faecal\ pellet\ piles}{number\ of\ visited\ plots} * 10\ (for\ roe\ deer\ and\ fallow\ deer)$$

Regarding roe deer and fallow deer, the FPI value were multiplied with the factor 10 due to that the sampling plot for these species was 10 times smaller. The formula was applied to respectively ungulate species.

Absolute ungulate densities

By recalculating the faecal pellet count data into absolute densities per 1000 hectare, it increases the ability to put the densities in relation to a more common format of ungulate presence. To calculate individuals/1000 hectare, the following formula was used (Bergström *et al.*, 2011; Bergström & Wallin, 2017):

$$Individuals\ per\ 1000\ hectare = \frac{S * k}{P * D * T}$$

Where S= the sum of all faecal pellet piles counted in all plots, k=the constant for /1000 hectare, P= the number of visited plots, D=defecation rate, and T=time of deposition. The defecation rate for each species was calculates as an average of a defecation rate range and was for moose (18,5), red deer (12,5), roe deer (19,5), fallow deer (21) and the time of deposition was set to 180 days (Edénius, 2012). The constant for moose and red deer was set to 100000 and 1000000 for roe deer and fallow deer, dependent on the size of the sampling plot.

Rodent trapping index

To be able to use the rodent trapping assay as an abundance reference, the trapping data was calculated into a Trap Index (TI). The TI formula was constructed as following:

$$TI = \frac{(N)\ number\ of\ trapped\ rodents}{(TN)\ number\ of\ trap\ nights} * 100$$

The TI value was multiplied with the factor 100 to avoid low values and have no impact of the results. The formula was applied to respectively rodent species.

Sample size needed for *B. burgdorferi* s.l. prevalence determination

To be able to calculate and determine the adequate sample size needed for *B. burgdorferi* s.l. prevalence (Naing *et al.*, 2006), the following formula was used (Daniel & Cross, 2013):

$$n = \frac{Z^2 P(1-P)}{d^2}$$

Where Z= indicates level of confidence, P=expected prevalence and d=precision. For a conventional level of confidence (95%), the (Z) value was set to 1.96. The expected proportion (P) of prevalence were set to 0.34 (34%) based on earlier *B. burgdorferi* s.l prevalence studies on rodents (Humair *et al.*, 1993; Tschirren *et al.*, 2013). The precision value (d) was set to 0.05 (5%).

Prevalence calculation
Prevalence, sometimes also referred to as prevalence rate, is the proportion of specimens in a population who have a specific disease, presence of antigen/antibodies or similar, at a specified point in time or over a specified time-period. When calculating the prevalence (P) of a pathogen or a disease within a population (N) the following equation are suggested (Bonita *et al.*, 2006):

$$P = \frac{(N+)\ positive\ B.burgdorferi\ rodents}{(N_{pop})\ rodents\ in\ the\ sample} * 10$$

Prevalence data is often expressed in percentage, and (P) may therefore be multiplied by the appropriate factor 10.

Statistical analysis
Statistical analyses were performed using the statistical program R version 3.3.0, EXCEL (Microsoft EXCEL for Mac, version 15.3, Microsoft Corporation 2017). The data material was analysed with Generalized Linear Mixed model fit by maximum likelihood (Laplace Approximation) (GLMER), t-tests and prevalence functions.

Results

Wild ungulate faecal count index
Between year 2012-2015, the results of the faecal pellet count show after transformation FPI that the G/N region had a higher ungulate density value than the V/V region within all species except for roe deer (Table 6).

Table 6. Calculated FPI value for each species in respective area. The G/N area show a higher FPI compared V/V.

Area	Moose	Red deer	Roe deer	Fallow deer	Total FPI	Visited plots	N piles
G/N	0,31	0,20	1,03	2,64	4,17	2290	2006
V/V	0,26	0,01	1,43	0,31	2,01	2608	1152

Looking at the FPI in area resolution, a t-test show that there is a significant difference in FPI (all species) between the G/N and V/V, with higher FPI in G/N (P<0,01, LCI=3,9924, UCI=6,0523). Some sites show a very high FPI and are viewed as outliers in the boxplot (Figure 9)

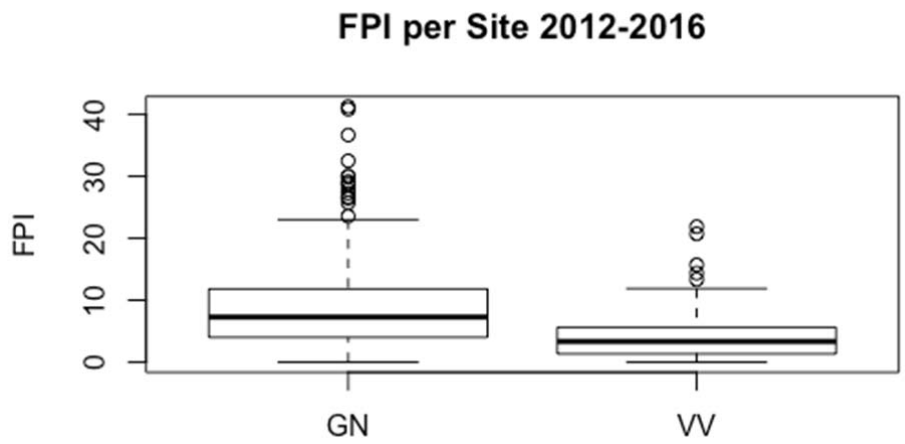

Figure 10. Calculated FPI at each site within G/N and V/V 2012-2015. The outliers show the sites with higher FPI than the average sites in respective region.

Absolute ungulate density

Between 2012-2015, the absolute wild ungulate density (individuals/1000 hectare) differs between G/N and V/V, with a higher ungulate density in G/N compared to V/V, except for roe deer, where the density was higher in V/V compared to G/N (Table 7), (Figure 10).

Table 7. The absolute density (individuals/1000hectare), for each species in respective area. The G/N area show a higher absolute density compared to V/V.

Species/1000hectare	G/N	V/V
Moose	9,3	7,8
Red deer	8,9	0,3
Roe deer	29,2	40,6
Fallow deer	69,8	8,3
Total/1000ha	117,2	57,1

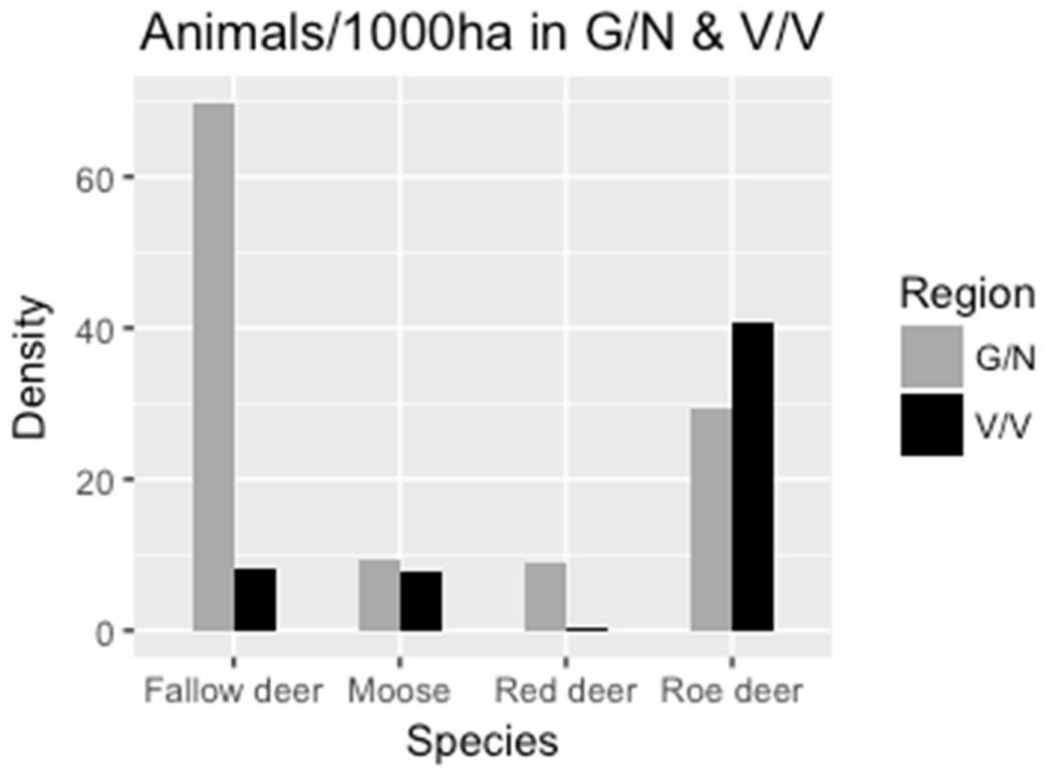

Figure 9. Calculated absolute density/1000 hectare for each species in respective region between 2012-2015. The G/N area show a higher density regarding fallow deer, moose and red deer compared to V/V. Roe deer show a higher density in V/V compared to G/N.

Rodent trapping

Between year 2012-2015 (7 seasons), 1052 *Apodemus spp.* and *M. glareolus* were trapped during 9918 trap nights. The results from the rodent trapping show after calculating the trap data into a trap index (TI) that rodent abundances are higher in V/V compared to G/N (all species) (Table 8). (Figure 11).

Table 8. Calculated TI for each species in respective region (2012-2015), show a higher rodent abundance in V/V compared to G/N regarding both species.

Area	M. glareolus TI	Apodemus Spp. TI	Tot. TI	Trap-nights	Total trapped
G/N	3,1	1,5	4,5	8782	396
V/V	4,0	3,2	7,1	9918	709

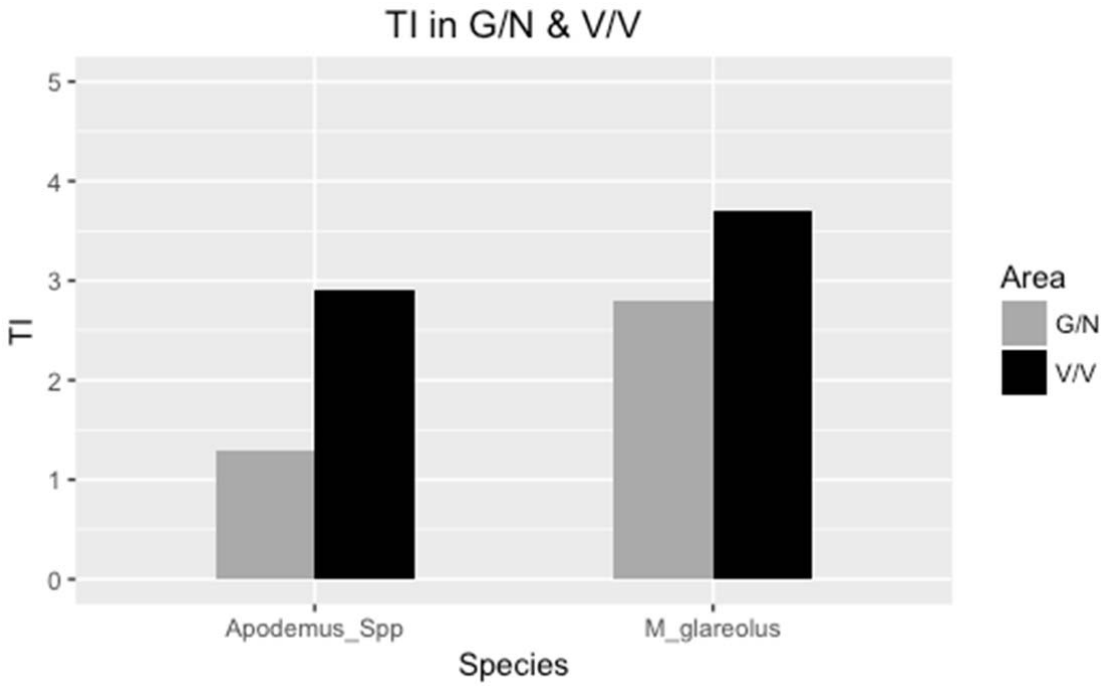

Figure 11. Rodent TI in V/V and G/N between 2012-2015, with a higher rodent abundance in V/V compared to G/N.

Nested PCR and agarose gel electrophoresis (Screening 1)

After screening the DNA templates in PCR and agarose gel electrophoresis, none (Pos+=0) of the screening templates (N=44) were *B. burgdorferi* s.l. positive (Figure 12). The expected number of base pairs was around 629-706. The positive *B. burgdorferi s.l.* reference (ref+) performed in all trials and therefore errors in the nPCR and agarose gel electrophoresis analysis was excluded. According to these results, nPCR were not suitable under current sample conditions.

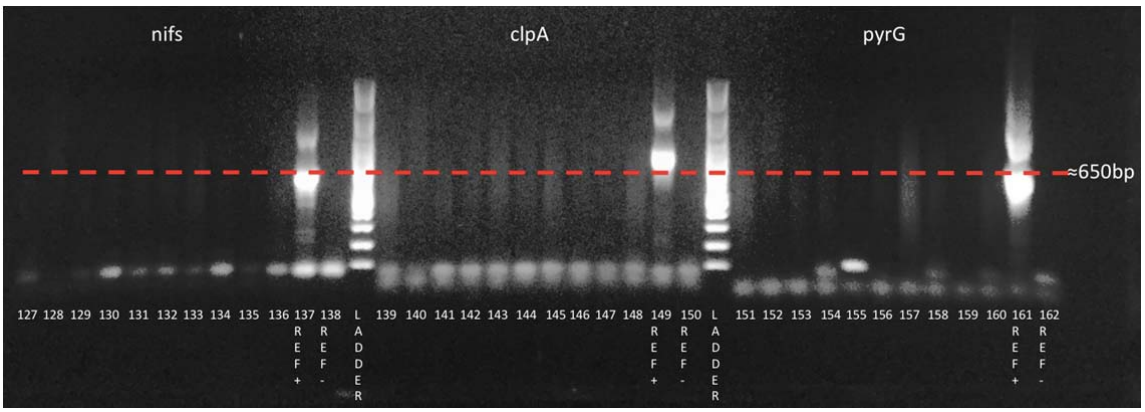

Figure 12. Agarose gel electrophoresis analyse round 5 (127-162), using nifS, clpA and pyrG primers. Positive and negative reference template (Ref+ and Ref§) were detected as seen in the Figure. The blurry grey clusters in the bottom are primer dimmers. None of the templates were tested positive in round 1-5 (N=44). Red dotted line show the threshold for 650bp. All 44 samples that were run did not show any positive results.

RT-qPCR analysis (Screening 2)

The RT-qPCR analysis of the screening samples showed 10 positive templates of N=44 (10/44) which give a *B. burgdorferi* s.l prevalence of 23%. The requirement for a positive

sample is that it is spatially distributed within the seventh (10^{6}-10^{-1}) standard curve range (Figure 13). All samples that exceeds C_t 33.0 are considered as negative results (34/44).

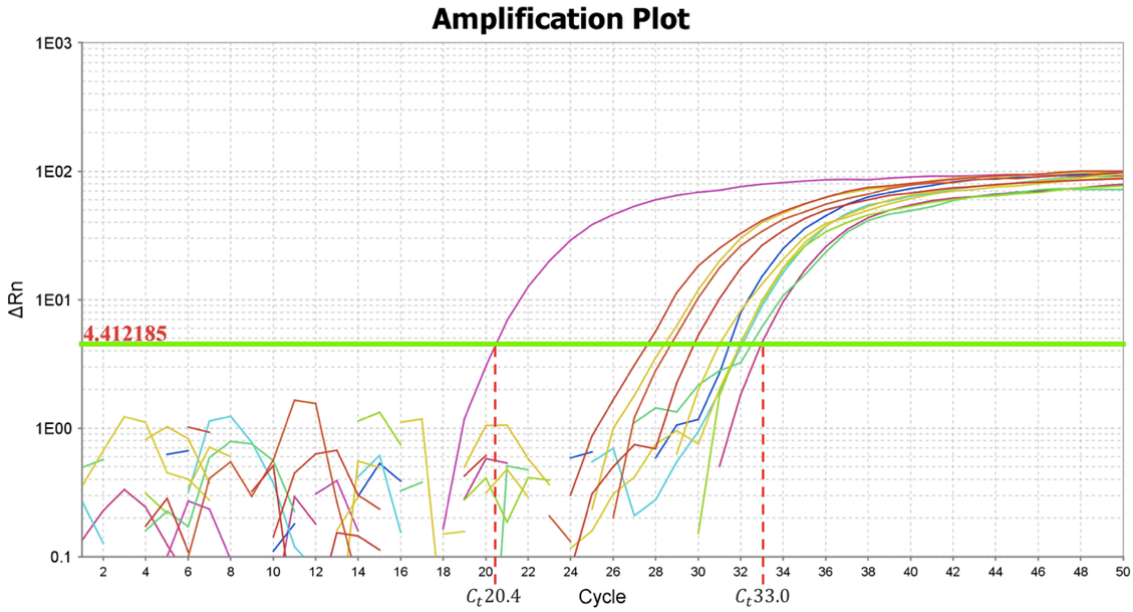

Figure 13. All samples spatially distributed within the 10^{6}-10^{-1} standard curve range (S_{max} and S_{min}) are considered *B. burgdorferi* s.l. positive. The purple curves show the S_{max} and the S_{min} curves and the other coloured curves represents the positive samples. The red dotted lines show the area in between positive samples should be.

RT-qPCR results

Since the usage of Synthesized External OligoNucleotide (SEON) failed, all RT-qPCR assays were C_t analysed with the first standard curve references, based on counted (Petroff-Houser chamber method) spirochete dilution (10^{6}-10^{-1}), giving a range of C_t20-C_t33. Based on that, 76 individuals of 350 tested rodents (*M. glareolus & Apodemus* spp.*),* was tested positive for *B. burgdorferi* s.l. spirochetes. Totally, in G/N, 25 *M. glareolus* and 16 *Apodemus* spp. were tested positive and in V/V, 30 *M. glareolus* and 5 *Apodemus spp*. were tested positive. *B. burgdorferi* s.l. prevalence comparisons for region, season, species and sex factors have been performed and range between 11-30%, depending on which factor that are analysed, which are shown in the table below (Table 9).

Prevalence hypothesis 1) The *B. burgdorferi* s.l. prevalence in G/N and V/V does not differ when including both spring and fall in the same prevalence calculation. But, within fall, V/V had higher prevalence compared to G/N, but not significantly so.

Prevalence Hypothesis 2) *M. Glareolus* had a significantly higher prevalence than *Apodemus* when including both regions and seasons. But, when analysing the prevalence in each region, the same result is shown in V/V, but not in G/N, where the prevalence is higher in *Apodemus.*

Prevalence hypothesis 3) The *B. burgdorferi* s.l. prevalence between males and females are almost equal, comprising region and season in the same analysis. In G/N, males have higher

B. burgdorferi s.l. prevalence compared to females, but not significant. On the contrary, in V/V the prevalence is significantly higher in females.

Prevalence hypothesis 4) There are no significant difference in *B. burgdorferi* s.l. prevalence between spring and fall (all rodent species).

Table 9. Prevalence calculations on rodents in region G/N and V/V (2012-2015). Prevalence differences within region, season, species and sex has been calculated in a prevalence comparison. P-values with bold text are significant.

Region / species / sex	**N tested**	**N pos.**	**Prev.**	**LCL**	**UCL**	**p-value**
GN & VV total	350	76	22%	N/A	N/A	N/A
GN & VV spring all	44	10	23%	0,1723	0,2672	1
GN & VV fall all	287	62	22%			
GN fall all	143	33	23%	0,2026	0,4096	0,2048
VV fall all	144	29	30%			
GN & VV *Apodemus*	124	19	15%	0,1994	0,3396	<0,00
GN & VV *Myodes*	163	43	26%			
GN fall *Apodemus*	61	14	23%	0,1518	0,3362	1
GN fall *Myodes*	82	19	23%			
VV fall *Apodemus*	63	5	8%	0,2026	0,4096	<0,00
VV fall *Myodes*	81	24	30%			
GN fall Males	68	18	26%	0,1198	0,3115	0,2546
GN fall Females	75	15	20%			
VV fall Males	72	8	11%	0,1935	0,4123	<0,00
VV fall Females	71	21	29%			
GN & VV males	175	37	21%	0,1660	0,2948	0,7507
GN & VV females	174	39	22%			

Calculated sample size for prevalence determination

Calculated least sample size for 34% expected *B. burgdorferi* s.l. prevalence (Humair *et al.*, 1993; Tschirren *et al.*, 2013) results in N=344 (±10). Prevalence studies within functional groups and species will fall short of the calculated sample size needed, due to insufficient rodent trapping sample size.

Generalised linear mixed model

To test my specific hypothesises, the relevant data was run in a Generalized linear mixed model fit by maximum likelihood (Laplace Approximation). The large GLMER analysed rodent fall data only (N=287), since the spring data had too few data (Table 10). A smaller GLMER model was run on the smaller spring data (N=63) from rodents (Table 11). Addressing hypothesis in chronological order:

Hypothesis 1) In the rodent fall data, there was no significant difference in *B. burgdorferi* infection probability between region G/N and V/V, although the tendency points to higher infection probability in V/V. The data from the spring population showed a significant difference between G/N & V/V, with higher infection probability among rodents in V/V.

Hypothesis 2) *B. burgdorferi* s.l. infection probability was significantly higher within *M. glareolus* compared to *Apodemus spp.* within the fall data. Spring data showed no significant difference in infection probability within the rodent species, but the same tendency as in fall with higher infection probability among males.

Hypothesis 3) There was no significant difference in *B. burgdorferi* infection probability between males and females (*M. glareolus and Apodemus spp.),* although the tendency was higher infection probability among males. No significant differences within the spring data regarding *B. burgdorferi* infection probability in males and females, but with the same tendency as fall, with higher infection probability within males.

Hypothesis 4) There was a significant difference of *B. burgdorferi* prevalence between rodents with low respectively high mass, showing that heavier rodents had higher prevalence compared to lighter rodents. Spring data did not show any significant differences within rodent mass, but the same tendency as fall population, with a higher prevalence among individuals with higher mass.

Hypothesis 5) There was no significant difference in *B. burgdorferi* infection probability among rodents in relation to rodent density. Although there was a tendency towards reduced infection probability in relation to increased rodent density. Spring data showed no significant difference in infection probability in relation to rodent density, but in contrast to the fall population, infection probability seemed to have the tendency to increase in relation to increased rodent density.

Hypothesis 6) There was no significant difference in *B. burgdorferi* infection probability within rodents with increased ungulate densities, neither for roe deer nor fallow deer. This hypothesis was not tested in relation to the spring population, because there was not enough data.

Table 10. Generalized linear mixed model for *B. burgdorferi* s.l. infection probability at individual level within rodents (fall 2012-2015) in relation to region, species, rodent density, rodent sex, rodent mass, roe deer and fallow deer. N=287.

Fall 2012-2015			
Infection prob.~	**Estimate**	**Std. Error**	**P-value**
Region (V/V)	0,413	0,714	0,56
Species (bank voles)	1,226	0,578	0,03
Density (all rodents)	-0,016	0,058	0,78
Sex (males)	0,297	0,451	0,51
Mass (all rodents)	0,087	0,033	0,01
Roe deer	-0,056	0,187	0,76
Fallow deer	-0,015	0,181	0,94

Table 11. Generalized linear mixed model for *B. burgdorferi* s.l. infection probability in rodents (spring 2013-2015) in relation to region, species, rodent density, rodent sex, rodent mass. Ungulate analysis was not performed due to weak amount of data points. N=63.

Spring 2013-2015

Infection prob.~	Estimate	Std. Error	P-value
Region (V/V)	-2,124	0,903	0,02
Species (bank voles)	2,607	1,361	0,06
Density (all rodents)	0,751	0,498	0,13
Sex (males)	1,547	0,889	0,08
Mass (all rodents)	0,146	0,093	0,12

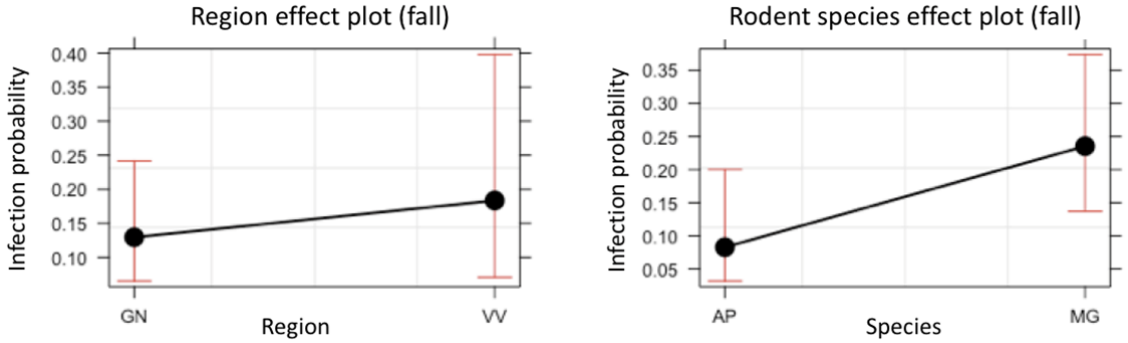

Figure 14-15. Effect plot for infection probability in fall, with region and rodent species as factors.

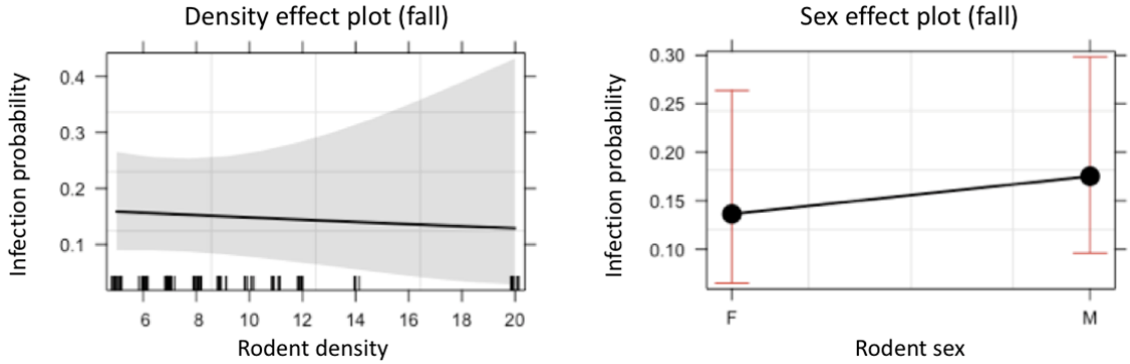

Figure 16-17. Effect plot for infection probability in fall, with rodent density and rodent sex as factors.

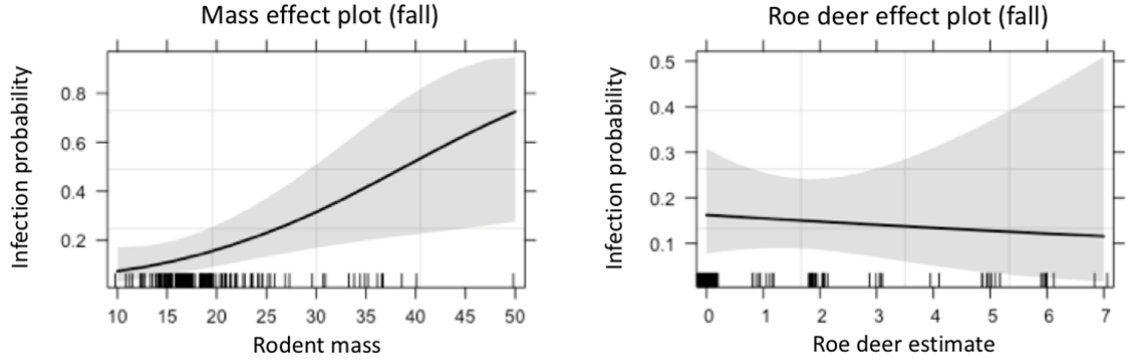

Figure 18-19. Effect plot for infection probability in fall, with rodent mass and rodent roe deer estimate as factors.

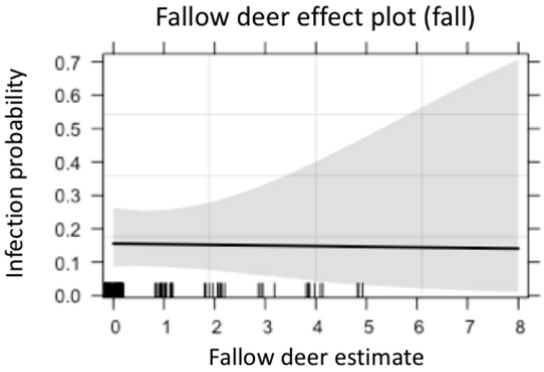

Figure 20. Effect plot for infection probability in fall, with fallow deer estimate as factor.

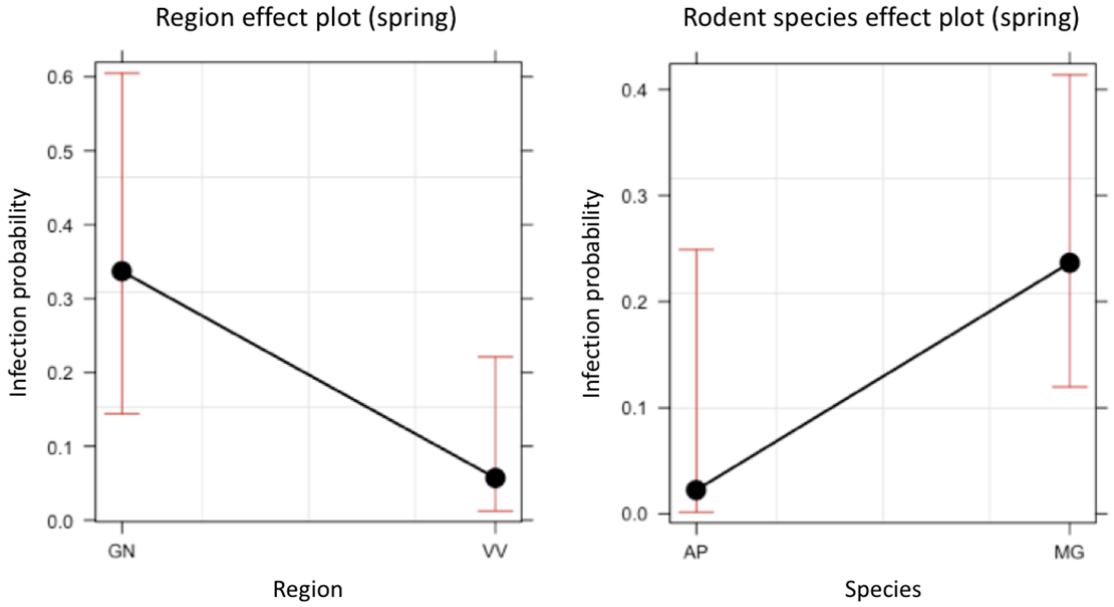

Figure 21-22. Effect plot for infection probability in spring, with rodent region and rodent species as factors.

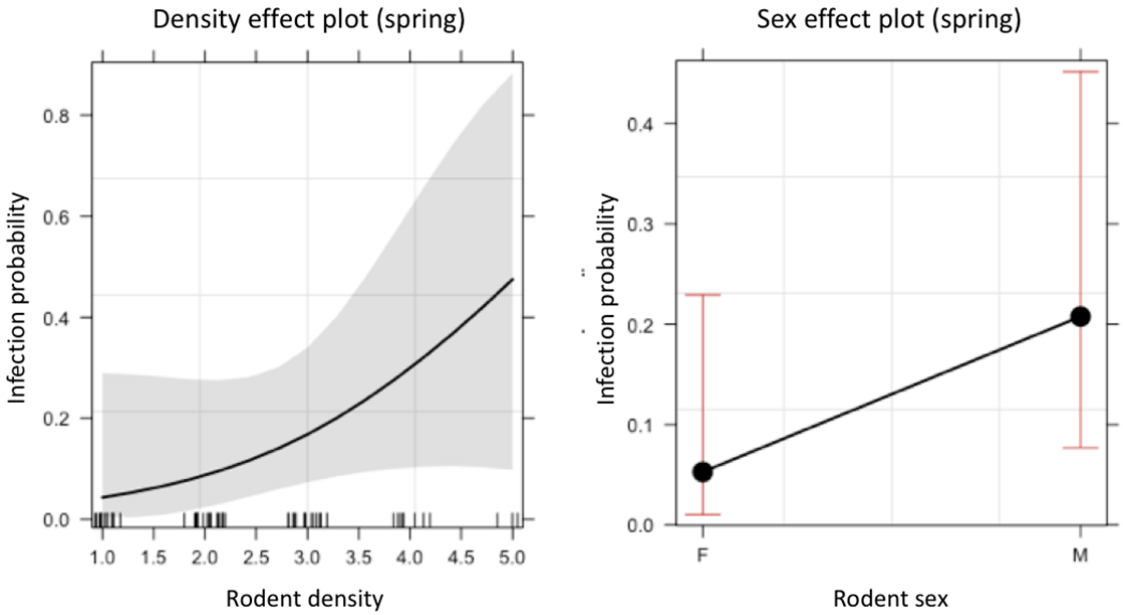

Figure 23-24. Effect plot for infection probability in spring, with rodent density and rodent sex as factors.

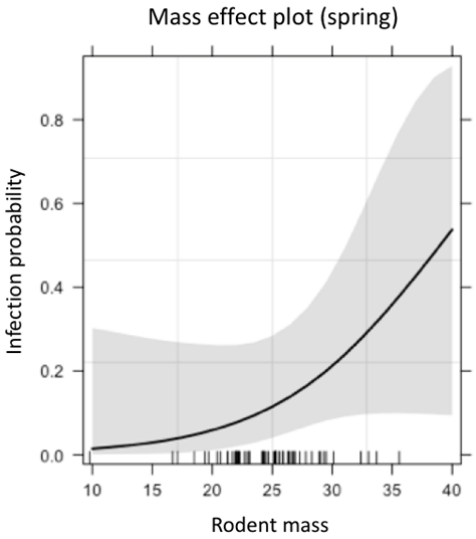

Figure 25. Effect plot for infection probability in spring, with rodent mass as factor.

Discussion

At a global scale, zoonotic vector-borne diseases are increasing and pose a serious threat to human health (REF). With estimations of 85 000 humans infected annually by pathogens within the *B. burgdorferi* s.l. complex, it is the most common tick-borne disease in Europe (Pritt *et al.*, 2016). According to an epidemiological study in Southern Sweden the annual incidence of *B. burgdorferi* infections in humans range between 5000-10000, with large variations between different Counties (Berglund *et al.*, 1995). However, an unpublished follow-up by public health authorities suggest it may be up to 30 000-35000 human

infections per year today (Olsson pers. comm.). Dahl *et al.*, (2015), emphasise the *B. burgdorferi* s.l. pathogen as a prioritized pathogen where the need of a deeper understanding is urgent. Following discussion will try to implement the results in the understanding of the *Borrelia* spirochete transmission in the wild.

Relative densities of rodents and ungulates

The results herein show differences in rodent abundances between the two regions 2012-2015, with higher rodent abundance in V/V compared to G/N. *M. glareolus* was more abundant than *Apodemus* spp. in both regions. In G/N, *Apodemus* spp. abundance was much lower in relation to *M. glareolus* compared to V/V. *M. glareolus* appears to be more a generalist than *Apodemus* and may adapt to less favourable habitats better than *Apodemus*, and due to *M. glareolus* dominance over *Apodemus*, *Apodemus* can be driven out of their habitat (Heyman *et al.*, 2009). In the current study, the higher ungulate density in G/N, may contribute to the lower *Apodemus* spp. abundances in this region, where food, e.g. acorns, is one of the resources competed for. There was also differences in ungulate abundances observed between the two regions, with higher ungulate abundance in G/N.

Regional effects on B. burgdorferi prevalence and infection probability

The overall *B. burgdorferi* s.l. prevalence comprising both regions and both rodent species studied was 22%. Even though the two regions showed large differences in relative rodent and ungulate densities, there were no significant differences in *B. burgdorferi* s.l. prevalence (G/N=23%, V/V=30% (LCL=0,2026, UCL=0,4096)). The infection probability analysis did not show any significant differences in *B. burgdorferi* s.l. infection probability between the two regions in fall. However, the small data-set from spring showed a significantly higher infection probability in rodents within G/N (std. err. 0,903, p=0,02). One explanation to this could be that the rodents in the spring population, probably mainly consists of older over-wintered specimens with longer exposure to ticks and therefore increased infection probability. Despite this, the *B. burgdorferi* s.l. prevalence was not significantly higher in the spring population compared to the fall population, which might be a true observation or biased due to small data set.

Rodent species specific prevalence and infection probability

Analysing at species specified level comprising both regions, B. *burgdorferi* s.l. prevalence was significantly higher among *M. glareolus* as compared to *Apodemus spp. M. glareolus* (M. *glareolus*=26%, *Apodemus* spp.=15%, LCL=0,1994, UCL=0,3396, p=<0,00). *M. glareolus* also showed a significant infection probability of *B. burgdorferi* s.l. compared to *Apodemus* spp. (std. err. 0,578, p=0,03) in fall. Same tendency was shown in spring, but the result was non-significant. This result is supported in literature, and are suggested to be caused by higher prevalence of antibodies in *Apodemus spp*., explained by higher tick burden compared to *M. glareolus* which subsequently leads to higher immune responses (Kybicová *et al.*, 2008). Since *M. glareolus* have higher prevalence of *B. burgdorferi* s.l. and are suggested to have higher infectivity (Humair *et al.*, 1999), *M. glareolus* might be the main contributor to increased infectivity among tick larva, nymphs and, subsequently, maybe also adult ticks. Further on, *Apodemus spp*. have a lower *B. burgdorferi* s.l. prevalence, higher tick infestation rate and contribute to higher moulting success among ticks (Humair *et al.*, 1999), might lead to that *Apodemus spp.* act as the contributor to tick abundances but not circulation of *B. burgdorferi* s.l..

In V/V, *M. glareolus* had a significantly higher *B. burgdorferi* s.l. prevalence compared to *Apodemus* spp. (M. glareolus=24%, Apodemus spp.=8%, LCL=0,2026, UCL=0,4096, p=<0,00). On the contrary, there were no prevalence difference between *Apodemus* spp. and *M. glareolus* in G/N (*Apodemus* spp.=23%, *M. glareolus*=23%, LCL=0,1518, UCL=0,3362, p=1). This leads to that the *B. burgdorferi* s.l. prevalence is higher in *Apodemus* spp. from G/N compared to V/V. This result could either be explained with uncertainties due to few data points, or due to ecological aspects as differences in rodent and ungulate densities, habitat or other environmental factors.

Rodent density, mass, and gender factors

In fall, the rodent density factor showed a non-significant tendency towards lowered probability of *B. burgdorferi* s.l. infection in relation to increased rodent densities. In spring the *B. burgdorferi* s.l. probability increased with rodent density. The higher densities in fall is due to the large proportion of young of the year, not yet tick bitten and exposed to *B. burgdorferi* s.l. infection, whereas in spring, the rodent population consist mainly of adult individuals. Adult individuals might have higher chance of being infected due to life-long infections (Tschirren *et al.*, 2013) and the probability that a tick bites an infected rodent should therefore be higher, which are shown in the density effect model from spring data. Increased mass in rodents, a proxy for age, increased infection probability, both in spring and fall, where, conclusively, younger individuals had lower infection probability compared to adults. Mass and gender as a factor for increased parasitism and pathogens is supported by Harrison *et al.*, (2010). However, the question remains why the current analysis of *B. burgdorferi* s.l. prevalence between rodent genders did not show a more consistent result. Possible biases could be that pregnant female rodents, and specimens with wet fur (from rain during sampling) when weighed (wet/dry factor noted but not taken in account for in the infection probability model), increased the measured weight. None of these two factors have been taken in consideration in prevalence and infection probability analyses.

Deer abundance

Densities of roe deer or fallow deer did not show any significant impact on *B. burgdorferi* s.l. infection probability within rodents, according to the model analysis. If this is correct, the conclusion could be that wild ungulates do not directly affect the pathogen dynamics in the wild within the studied system, since deer species are suggested to be *B. burgdorferi* s.l. reservoir incompetent and seems mostly to act as reproduction host for the tick (Jaenson & Tälleklint, 1992; Pacilly *et al.*, 2014; van Duijvendijk *et al.*, 2015). Deer species could, however, be the regulator for tick abundances, which also should have impact on the pathogen transmission between rodents. Differences in deer abundance might to some extent be the regulator for adult female ticks to obtain last blood meal and to fulfil the egg laying phase due to their contribution as reproduction hosts for female adult ticks (REF) However, since rodents, especially *Apodemus* spp., are main blood meal hosts for larvae and nymphs, a bottom-up regulation caused by *Apodemus* abundances might also lead to that the regulation of tick abundances also are regulated before the adult female tick enters feed on a large mammal, e.g. ungulate, reproduction hosts. Due to that nymphal ticks are suggested to be one the main vectors of the pathogen between rodents, they could also be the key actor on *B. burgdorferi* s.l. prevalence within rodents. In the current study, it was observed that rodent abundances were lower (especially *Apodemus* spp.) in G/N as compared to V/V, but the tick abundances were not estimated in the field. Out of the results, my opirnion is that it would have been exceedingly helpful to have access to tick data within both regions, both at spatial and temporal

Indirect ungulate abundance may locally have an impact on the *Borrelia* pathogen due to its impact on rodent communities, but I suggest is that the main factors in pathogen transmission are tick- and rodent abundances (tick lifecycle factors) and prevalence- and infectivity in rodents. Then, of course, climate and environmental factors may have effects on tick and rodent populations and are therefore also an important factor in *B. burgdorferi* s.l. prevalence, both in ticks and rodents.

Finally, I want to emphasize that the usage of Synthesized External OligoNucleotides (SEON) failed due because the SEON needed 8-10 more cycles to climb over the C_t threshold compared to the biogenetic standards, which skewed the C_t analysis with high C_t values as a result. The reason for this failure, might be either that the small single-stranded SEON was hard to detect by the primers due to its smaller size (88b) compared to the biological target chromosome (1mb), or that the single stranded SEON randomly fold and attach to itself with hydrogen bonds, which probably complicates the primer attachment and makes it harder for primers to locate unfolded SEON´s. Therefore, all RT-qPCR C_t analysis were based on the standard range that were established in the very first assay with Petroff-Houser chamber counted spirochetes. Also, note that one out of five RT-qPCR batches had a deviating prevalence compared with the other batches. The other batches had a prevalence range between 20-30%, whilst the deviating batch had a prevalence of 6%. It could either be a failed RT-qPCR assay, or the fact that the prevalence is ok in that batch. Also, the spring data-set contains few data points and therefore my focus was mainly on fall data, although I include the spring data in the discussion, one should be cautious about the conclusions from the spring data. Furthermore, I must emphasize that the most important actor in *B burgdorferi* s.l. transmission, the tick, is missing in my data, which leads to a huge gap in the understanding of *Borrelia* pathogen transmission between different vertebrate hosts. It would have been valuable to know the abundances and *B. burgdorferi* s.l. prevalence in the tick population within every region. The *B. burgdorferi* s.l. spirochete is a very complex pathogen due to multi species interaction to be able to maintain transmission in nature.

Conclusions

My major conclusions are that the sample size for DNA analysis was probably too small to yield accurate analysis results in prevalence analyses. Furthermore, tick abundance and *B. burgdorferi* s.l. prevalence in ticks would have greatly added to the strength of the data and added an important link to a broader understanding in the *Borrelia* pathogen dynamics.

Future research

If this type of study was to be replicated, the following suggestions would contribute to a more complete study: Initially, to obtain more accurate data, the sample size in rodent ear tissue should have been larger. Furthermore, future studies should perform tick data collection (flagging) at same spatial and temporal extent as rodent and ungulate samples to be able to sample ass phases in the circle of pathogen transmission. Also, an approved method to obtain local wild boar (*Sus scrofa*) density would contribute to the understanding of wild ungulates in relation to their environment and rodent communities. Especially

interesting are the piglets, which in size and behaviour aught be a proper blood meal host for ticks of any instar, and which could have an impact on tick abundances and subsequent pathogen transmission. Also, investigating the lagomorphs influence on pathogen transmission, would add an important piece in the puzzle. Finally, the usage of SEON´s instead of cultivated and chamber counted spirochetes as standard curves in Borrelia DNA analysis, is a method that has not been properly evaluated. A fully functional synthetic external oligonucleotide might save, both time and money in larger-scale studies on the *Borrelia* pathogen.

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