Ecology of *Borrelia burgdorferi sensu lato* and epidemiology of borrelial infections in Cumbria

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Declaration

This thesis represents approximately three and a half years of research for the award of the degree of Doctor of Philosophy, commencing in October 2014 and funded by a Graduate Teaching Studentship from the University of Salford. Results within this thesis have been published in conference proceedings over the course of the PhD and are being utilised as part of papers that have been submitted to peer-reviewed journals.

Four months of longitudinal data (June 2013- September 2014) from Chapter 3 of this thesis has been used in part submission for a Master by research at Salford University obtained by the author. Chapter 3 data is a continuation of the longitudinal study and so it was deemed sensible to add in the previous data.

Although the author carried out the majority of experiments and analysis for the results of this thesis, part of the work is as a result of collaboration. Ms Kathrine Alpers (Erasmus student, University of Salford) identified and tested a subset of ticks in 2016 from Chapter 4. The data presented in Chapter 4 is the combined work of Ms Cathrine Whitemann (Sfam studentship), Mr Talinfu (PhD student, University of Salford) and the author. Squirrels were processed by Ms Whitemann in 2014, by the author in 2015 and this work was taken over by Mr Talinfu in 2016 due to the author developing an allergy to squirrels.

List of abbreviations

6FAM	6-carboxy-fluorescein
ACA	Acrodermatitis Chronica Atrophicans
bp	Base pairs
CDC	Centre for Disease Control
d.f	Degrees of freedom
DIN	Density of infected nymphs
DNA	Deoxyribonucleic acid
DOA	Density of adults
DON	Density of nymphs
ECDC	European centre for disease prevention and control
EDAC	1-Ethyl-3-(3-Dimethylaminopropyl) carbodiimide, Hydrochloride
EDTA	Ethylenediaminetetraacetic acid
ELC	Enhanced chemiluminescent
EM	Erythema migrans
FC	Forestry Commission
GLM	Generalised linear model
GP	General Practitioner
GPS	Global positioning system
Km	Kilometres
LB	Lyme borreliosis
MLST	Multi locus sequence typing
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium Hydroxide
ONS	Office of National statistics
OS	Ordinance Survey
PCA	Principal Component analysis
PCR	Polymerase chain reaction
PHE	Public Health England

qPCR quantitative polymerase chain reaction

RIVM Rijksinstituut voor Volksgezondheid en Milieu (Dutch: National Institute for Health and Environment)

RLB Reverse line blot

RLF Relapsing fever

RT-PCR Real time Polymerase chain reaction

s.l Sensu lato

s.s. Sensu stricto

SDS Sodium dodecyl sulphate

SE Standard Error

SSPE Saline sodium phosphate ETDA

TAMARA 6- carboxyl-tetramethyl-rhomadine

TBD Tick borne disease

TBE Tris Borate EDTA

TBP Tick borne pathogen

UK United Kingdom

USA United Sates of America

VBORNET European Network for Arthropod Vector Surveillance for Human Public Health

Abstract

Borrelia burgdorferi sensu lato (s.l.) is the causative agent of the tick-borne zoonotic disease Lyme borreliosis (LB), of which around 2-3000 cases are reported annually in the UK. This project took a "One Health" approach to studying *Borrelia burgdorferi* s.l. in southern Cumbria exploring its medical and veterinary importance and quantifying the environmental hazard it presents in the region.

Under a "One Health" umbrella, this project comprised of three discrete studies: (1) a longitudinal study of tick population dynamics, and *B. burgdorferi* s.l. transmission dynamics in these ticks, was carried out between June 2013 and September 2017 in three separate but closely located woodlands, (2) the role of grey squirrels (*Sciurus carolinensis*) in the natural maintenance of *B. burgdorferi* s.l. was assessed, and (3) a veterinarian-based survey of ticks infesting companion animals was completed.

The most noteworthy findings in these studies were: (1) the phenologies of the three questing tick populations studied were similar, but there was consistent and significant variation in tick abundance between the three populations. Overall tick abundance rose during the study. *B. burgdorferi* s.l. circulated in all three tick populations, but at significantly different prevalences. The annual density of infected ticks varied significantly at all three sites but this variation was asynchronous. Four *Borrelia* genospecies were encountered in the study, but the relative contribution of each to the borrelial community varied markedly between populations and but stayed constant through time. (2) Grey squirrels were susceptible to *B. burgdorferi* s.l. (prevalence 19.74%) and infection with all four *Borrelia* genospecies was encountered. The prevalence of infections in ticks feeding on squirrels was almost 11.57% significantly higher than that in local questing tick populations (3.69%). (3) Three *Ixodes* species parasitized companion animals, with *I. ricinus* being the most common. Parasitism peaked in July. Infection rate in companion animal-associated *I. ricinus* ticks was 1.29%, was significantly lower than that observed in questing ticks

This thesis provides an insight into tick phenology and what species of ticks bite companion animals in the area. Although the peak in tick bites on companion animals did not coincide with the peak in questing ticks from the longitudinal study this can be explained by

behaviour and species of ticks. Regarding *B. burgdorferi* s.l. infections in questing ticks tend to fluctuate throughout the year with cases of temporary absence at some sites. Infection prevalence in ticks from companion animals was lower than that found in questing ticks and grey squirrels had a high prevalence of infection particularly *B. afzelii* and *B. garinii.* Both of which dominated sites during the longitudinal study. This holistic approach to disease ecology shows that *B. burgdorferi* should not only be discussed in regard to human disease but to explore the wider concept to gain a better insight into the spirochetes place in the world.

Chapter 1 Introduction

1.1 Tick-borne disease

Ticks have the ability to transmit an abundance of viral, bacterial and protozoan pathogens (Table 1) and are second only to mosquitos as disease vectors (Parola & Raoult, 2001). Of the ~900 currently identified ticks world-wide, 10% of these are a threat to public or veterinary health (de la Fuente *et al.,* 2017). Tick borne diseases (TBDs) are seen as a major health problem in many European countries and have been on the rise in recent decades (Randolph, 2004a).

Table 1. Tick-borne diseases that have been detected in the most common ticks in the UK. Those in grey have only been detected in the vector and those in red have been confirmed to be transmitted by the vector.

Tick-borne diseases	Causative agent	Tick Species	Reference
Anaplasmosis	*Anaplasma phagocytophilum*	*I. ricinus* *I. hexagonus*	MacLeod & Gordan, 1933, Jahfari *et al.,* 2017
Lyme borreliosis	*Borrelia burgdorferi s.l.*	*I. ricinus, I. hexagonus*	Burgdorfer *et al*., 1983 Gern *et al.,* 1991
Relapsing fever	*Borrelia miyamotoi*	*I. ricinus* *I. hexagonus*	Hansford *et al.,*2015 Jahfari *et al.,* 2017
Spotted fever	*Rickettsia helvatica*	*I. ricinus* *I. hexagonus* *D. reticularis*	Beati *et al.,* 1993 Jahfari *et al.,* 2017 Dobec *et al.,* 2009
	Rickettsia raoultii	*D. reticularis*	Mediannikov *et al.,* 2008
Babesiosis	*Babesia divergens*	*I. ricinus*	Joyner *et al.,* 1963
	Babesia gibsoni	*I. ricinus*	Smith & Wall, 2013
	Babesia canis	*I. ricinus* *D. reticularis*	Cieniuch, *et al*., 2009 Hansford *et al.,* 2016

	Babesia venatorum	*I. canisuga*	Smith & Wall, 2013
Ehrlichiosis	*Ehrlichia canis*	*I. ricinus*	Wilson *et al.,* 2013
Q fever	*Coxiella burnetii*	*I. ricinus*	Řeháček, *et al.,* 1991
Cat scratch disease	*Bartonella henselae*	*I. ricinus*	Cotté *et al.,* 2008
Toxoplasmosis	*Toxoplasma gondii*	*I. ricinus*	Obsomer *et al.,* 2013
Febrile illness	*Candidatus Neoehrlichia mikurensis*	*I. ricinus,* *I. hexagonus*	Jahfari *et al.,* 2017
Tick borne encephalitis (TBE)	Tick borne encephalitis virus	*I. ricinus* *I. hexagonus*	Benda, 1958
Louping ill	Louping ill virus	*I. ricinus*	Jones *et al.,* 1997
Congo Crimean fever	Crimean-Congo haemorrhagic fever virus	*I. ricinus*	Obsomer *et al.,* 2013
West Nile fever	West Nile Virus	*I. ricinus*	Obsomer *et al.,* 2013

The TBD of greatest medical importance in temperate regions of the northern hemisphere is Lyme borreliosis (LB). Although LB has long been recognised, its aetiology was only clarified in 1982, when its causative agent, *Borrelia burgdorferi sensu lato* (s.l.) was discovered (Burgdorfer *et al.,* 1982). Today in the USA, the Center for Disease Control estimates that there is an average of 8,344 reported cases per year (Schwartz, 2017) and in Europe there are thought to be more than 65,500 reported cases per year (Rizzoli *et al.,*2011). Although there are thought to be more unreported cases per year that go undocumented. For example, in Scotland it is thought only 20% of cases are reported (Evans *et al.,* 2014).

The epidemiologies of TBDs are continually changing and, globally, the number of reported cases of LB has risen dramatically since the 1980s. Although much of this can be attributed to improved recognition and reporting of the disease, case numbers continue to rise in some countries where public health surveillance of LB is well established. This increase has been associated with, change in land use, direct effects of climate change, which has enabled *Ixodes* ticks to broaden their distribution and abundance and indirect effects such as host increases in abundance and distribution (Medlock *et al.,*2013). For example, in

Canada at the beginning of the 1990s the only known population of *I. scapularis* was in Ontario (Barker *et al.,* 1992), since then it has been shown by using human records of LB and a passive surveillance system *I. scapularis* has been identified in five provinces (southern Ontario, Nova Scotia, south-eastern Manitoba, New Brunswick and Quebec), (Ogden *et al.,* 2006b; Ogden 2008).It is expected that *I. scapularis* will continue to expand northwards into Canada with the projected increases in temperature due to climate change (Ogden *et al.,* 2006a). This has also been seen in northern Europe, with the latitudinal expansion of ticks in Norway, Finland and Sweden (Jore *et al.,* 2011; Laarksonen *et al.,* 2017; Jaenson *et al.,* 2012).

B. burgdorferi s.l. has a complex life cycle between the tick vector and competent reservoir hosts, because of this there have been calls for a holistic approach to investigating TBDs. The "One Health" approach encompasses the veterinary, medical and environmental aspects of TBDs (Dantas-Torres *et al.,* 2012). Both the reservoir hosts and ticks are affected by environmental factors such as habitat and climate. How an area is used by humans and animals affects the incidence of human and animal TBDs and is relative to the number of infected ticks and reservoir host in an area. The goal of one health is to use a multidisciplinary approach to work collaboratively in advancing our knowledge in health and understanding that human health is linked with animal health and the environment. Increasing communication and sharing knowledge about the distribution of tick species and what pathogens they carry will help to inform both medical and veterinary professional and the wider community.

This thesis aims to look at *B. burgdorferi* s.l. in southern Cumbria using the One Health approach. *B. burgdorferi* s.l. circulates in the vector and reservoir hosts but can be transmitted to humans and some animals where it causes pathogenesis. Using the one health approach will enable exploration into the prevalence of this pathogen in ticks from the environment, to assess tick burden and pathogen prevalence in reservoir hosts, understand what percentage of ticks that bite animals are infected and assess the public health burden of this bacteria in the study area.

1.2 Ticks

Ticks belong to the Class Arachnida and the sub-class Acari. There are three families of tick the soft ticks (*Argasidae*), hard ticks *(Ixodidae*) and *Nuttalliellidae*, which contains only one species (Latif *et al.,* 2012). *Argasidae* contains 194 species, which are found predominantly in warmer climates and *Ixodidae* contains 703 species that are found worldwide (Service, 2012). Ticks are blood sucking arthropods that are found on all seven continents (Gressitt & Leech, 1961) and can transmit many pathogens (Parola & Raoult, 2001). Figure 1 shows the worldwide distribution of ticks that are vectors of pathogens which can infect both humans and animals.

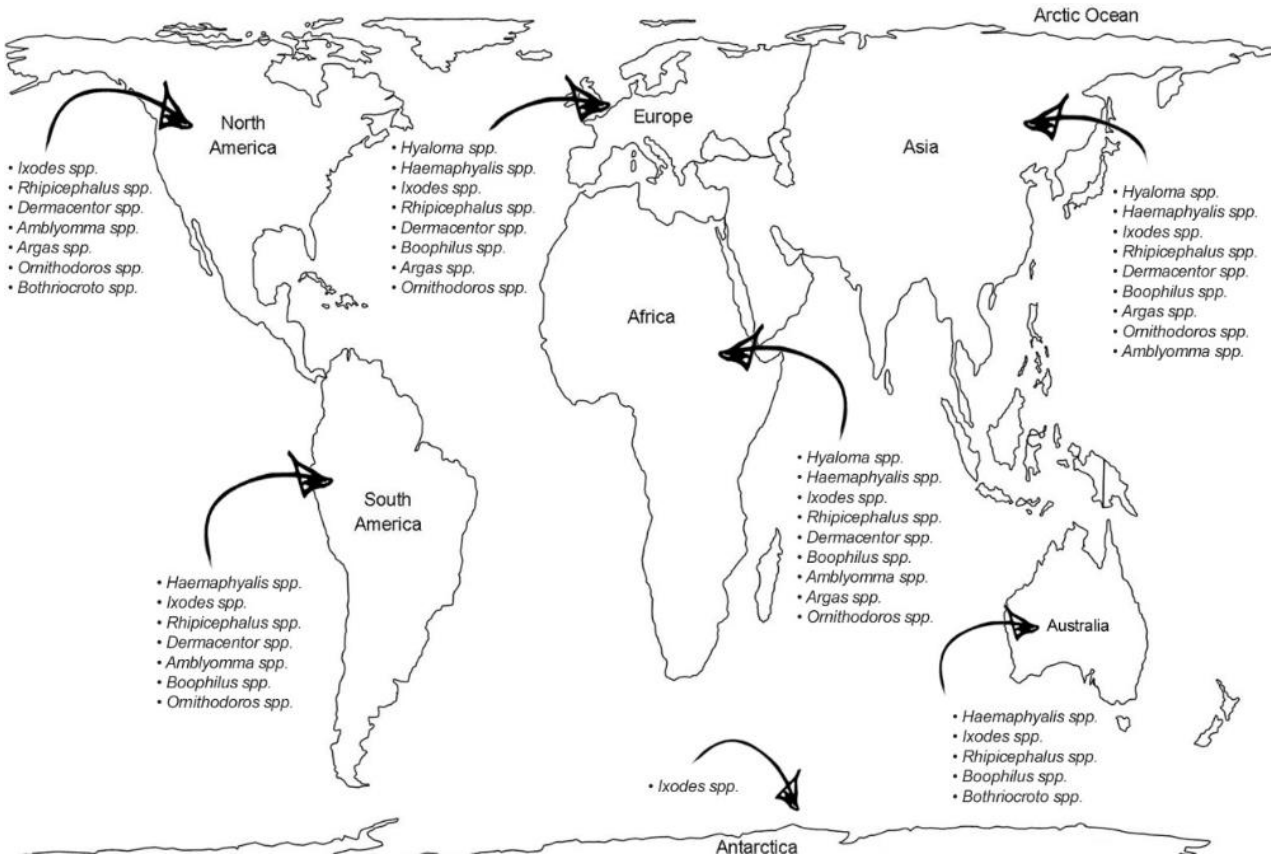

Figure 1. A map showing the known distribution of ticks that are vectors of human and animal pathogens created from de la Fuente *et al.,* 2008.

1.2.1 Hard ticks

Hard ticks are found all over the world but each species is restricted to specific ecological niches that suit their life cycle. Some ticks are adapted to the desert, some to humid or tropical climates and some to the cooler climate such as *Ixodes* (Beugnet, 2013). The *Ixodes* genus with 247 species it is the largest genus. *Ixodes* is the most wide spread tick. 59 species

are known to inhabit the Palaearctic Region (Estrada Pena *et al.,* 2017). *I. ricinus* is one of the most frequently encountered ticks in Europe because of its non-nidiculous life style and catholic feeding (feed once per stage) habits (Jameson & Medlock, 2011). This tick is free living and actively 'quests' for a host. Questing is where the tick ascends the vegetation and ambushes the host as it goes past (Crooks & Randolph, 2006). It is hypothesized that each stage of tick quests at different heights to target their preferred host. Larave quest at a lower heigh than nymphs and nymphs lower than adults. Therefore they can attach to different sized host for example larvae to small rodents that are lower to the ground and adults to deer. The height at which each stage quests is restricted by humidity and temperature (Mejlon & Jaenson, 1997).

1.2.2 *Ixodes ricinus* life cycle

There are four life stages; the egg, the larvae, the nymph, and the adult (Figure 2). The larvae hatch from the egg, quest to find a host and take a blood meal then return to the ground to moult into the nymphal stage. The nymph then quests and take a blood meal, returning to the ground to moult into an adult. Adults also quest for a host, on which they mate. During this process females blood feed and when fully engorged, drop to the ground to oviposition (lay eggs) after which she dies (MacLeod, 1932). The likelihood that an individual tick will complete all life stages is minimal; it is estimated that only around 5% of the eggs will hatch into larvae, of which 10% will moult into nymphs, of which about 20% will go on to develop into adults. Thus 1000 eggs yield a single adult (Randolph, 1998).

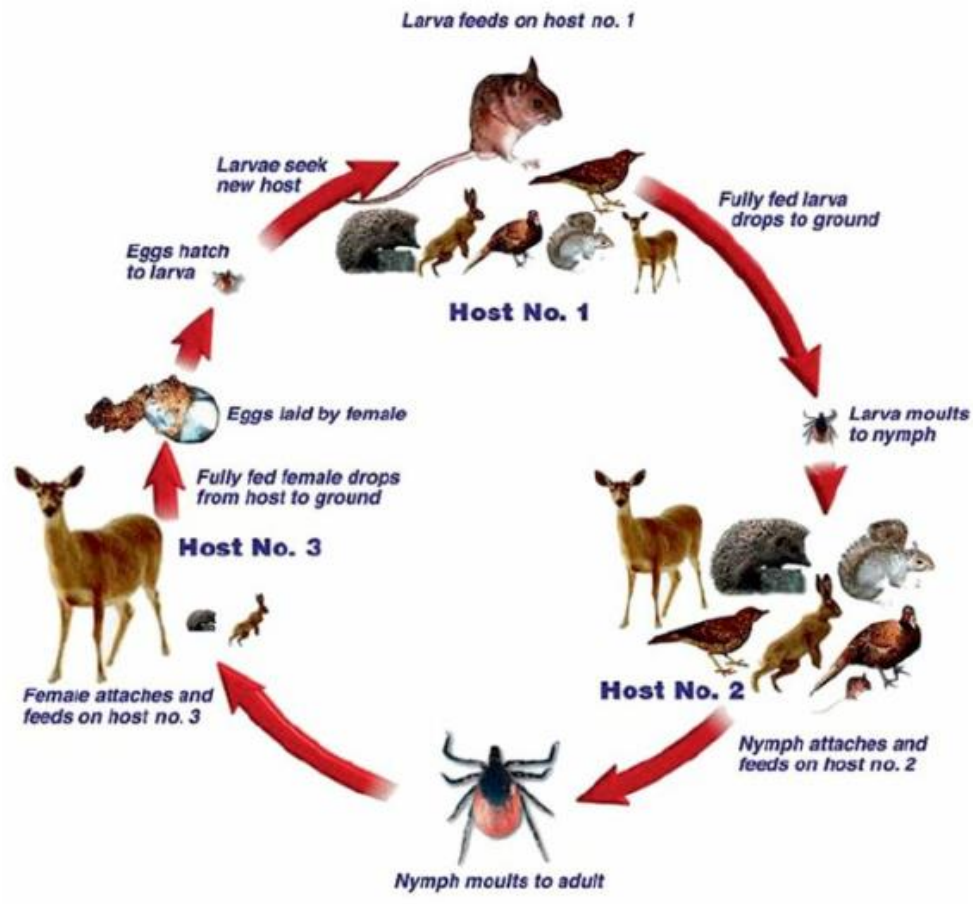

Figure 2. Life cycle of *I. ricinus* with each stage (egg, larvae, nymph and adult) and the preferred blood meal host for each stage.

The blood meal is an integral part of tick development. Bartosik and Buczek (2012) observed *I. ricinus* adult females and their feeding behaviours, they documented that on average *I. ricinus* blood meal lasted between 8-9 days, to begin with the tick would attach to the host for one hour and then the feeding period would begin. There are two stages of feeding the first is the slow/early phase where the tick ingests around ten times its own weight in blood over several days, the second phase is where the tick increases its weight by ten times again but in around 12-36 hours (Hillyard, 1996). This period of feeding is known as “the big sip”. (Sonenshine & Anderson, 2014).

Ticks attached to a host alternate between feeding and salivation. This occurs in cycles of five to twenty minutes. The blood meal is concentrated in the mid-gut and at the same time water and electrolytes are forced into the haemocoel and are then returned to the host in the tick’s saliva. This alternating pattern is needed to enable the tick to evade the host immune system as the saliva contains many different properties including anticoagulants

and anti-inflammatory compounds (Francischetti *et al.,* 2009). It is during the blood meal when the tick can become infected with pathogens or transmit the pathogen to the host. Some TBP's can be transovarial transmitted from the female to the eggs for examples *Babesia* species (Bonnet *et al.,* 2007).

1.2.3 *Ixodes ricinus* European distribution

Ixodes ricinus is one of the most commonly reported ticks in Europe. It can be found throughout much of Europe, including the UK (Rizzoli *et al.,* 2011; Martyn, 1988). The distribution of this tick (Figure 3) extends between longitudes of 30°N (Egypt) to 66°N (Norway) (Soleng *et al.,*2018) and latitudes of 45°E (European Russia) and 10°W (Ireland) (Pietzsch *et al.,*2005). To the east of its range, *I. ricinus* overlaps with its sister species *I. persulcatus* (Figure 4) in the Baltic regions and Russia (Korenberg, 1994) and Finland (Laaksonen *et al.,* 2017). These two species can mate and successfully produce hybrid larvae (Bugmyrin *et al.,* 2016). Studies exploring the altitudinal range of *I. ricinus* have found *I. ricinus* at high altitude in the Italian Alps at over 3000m, and 1000m in the Czech Republic (Daniel *et al*., 2003; Rizzoli *et al.,* 2002).

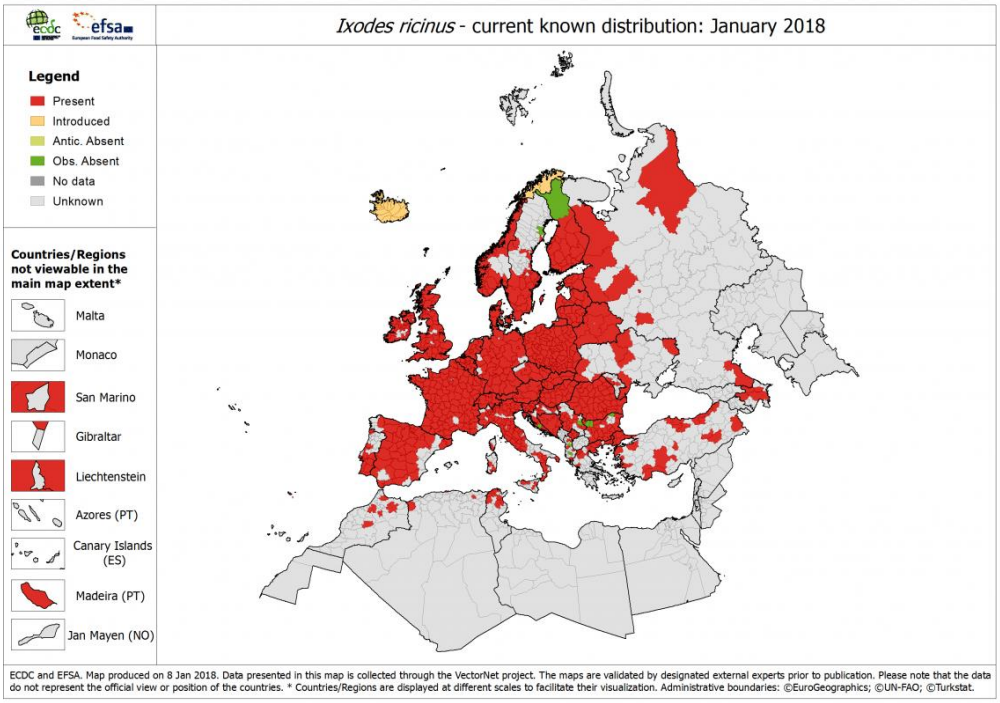

Figure 3. The distribution of *I. ricinus* assessed by the VBORNET project (ECDC, 2018a)

The distribution of *Ixodes* species has been shown to be changing in Russia *I. persulcatus* range had increased from over 60 years ago, whereas the range of *I. ricinus* has not changed but *I. ricinus* has become less abundant, meaning the sympatric zone for *I. persulcatus* and *I. ricinus* has widened (Bugmyrin *et al.,* 2013). Although elsewhere there have been reports that the distribution of *I. ricinus* has been expending its range in recent years. A recent crowd sourcing study in Finland reported that *I. ricinus* has expanded 200-300km northwards over 60 years. Samples of *I. ricinus* as northerly as 67°N were reported but these were sporadic and so the population here may not be fully established (Laaksonen *et al.,* 2017). Similar results were found in Sweden, from a questionnaire which asked the public about *I. ricinus* distribution. This study found that *I. ricinus* had expanded northward in Sweden over the last 30 years (Jaenson *et al.,* 2012). Although these questionnaires are useful to estimate the distribution of *I. ricinus*, they also come with their own limitations. Participants may have incorrect memories or could be biased by more awareness of ticks in recent years. To test for these biases a control questions should be implemented such as asking something which has been quantified to decrease or increase over time.

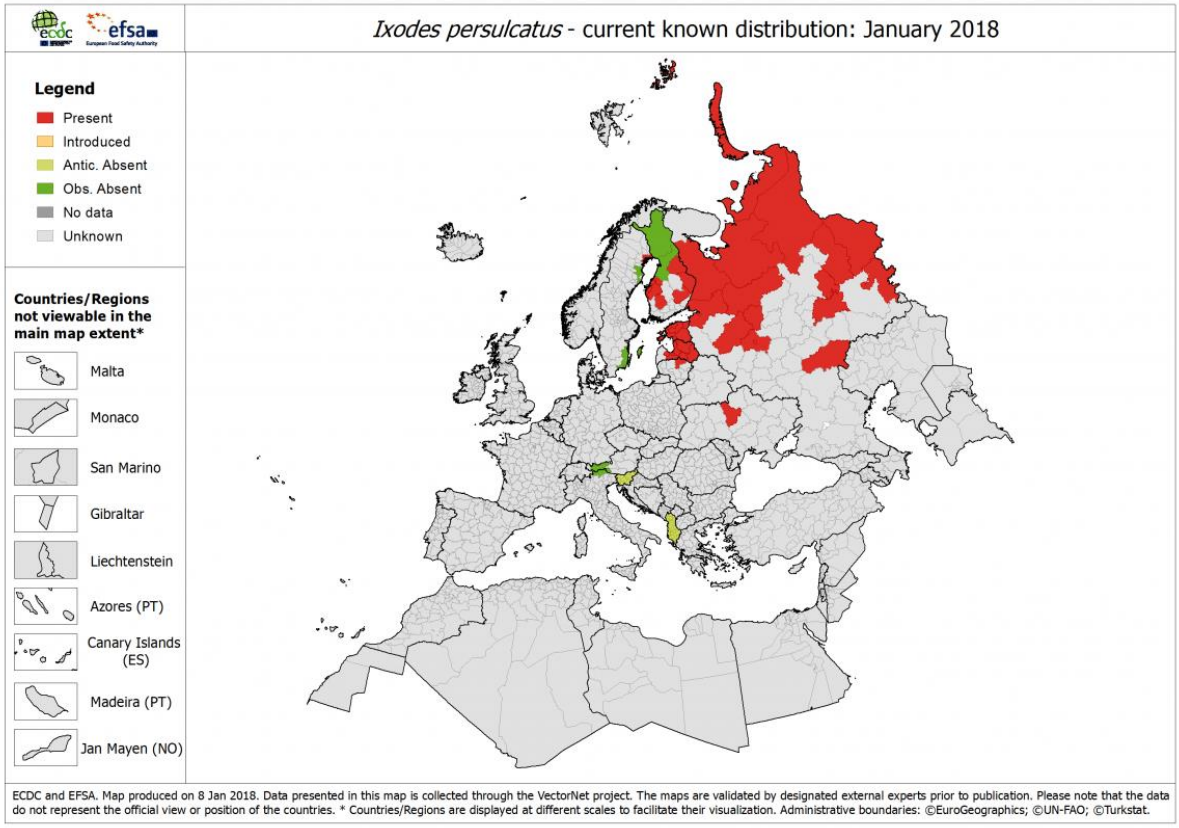

Figure 4. The distribution of *I. persulcatus* assessed by the VBORNET project (ECDC, 2018b)

I. ricinus from Europe, Asia and North Africa were tested for genetic differences in 2011, variability was low between European and Asian *I. ricinus* but a genetically distinct clade was found in North Africa (Noureddine *et al.,* 2011). Since then, a new species known as *I. inopinatus* has been reported in the Mediterranean areas of Spain and Portugal and in North Africa; Morocco, Tunisia and Algeria. The authors describe how this species could have been mistaken for *I. ricinus* in previous studies (Estrada Pena *et al.,* 2014). More recently a population of *I. inopinatus* has been identified in southern Germany, which is the most northerly report for this species. This article gives evidence that *I. inopinatus* and *I. ricinus* can occur sympatrically but it is yet to be discovered if the two can mate to produce hybrids (Chitimia-Dobler *et al.,* 2017).

Within Europe phylogenetic analysis on a local or continental scale has found no clear genetic differences within *I. ricinus* populations (Paulauskas *et al.,* 2016; Capri *et al.,* 2016). It has been suggested that the lack of correlation between genetic and geographic structure was due to tick geographic locations reflecting that of its host (Carpi *et al.,* 2016). This had been shown before by Kempf and colleagues (2011) who analysed ticks attached to local host species, from five countries in Europe, and found genetic differentiation between host-associated *I. ricinus* populations. However, in other studies geographically related genetic differences have been shown. For example, British *I. ricinus* were genetically distinct from European (Latvian) *I. ricinus* (Dinnis *et al.,* 2014). Then in 2016, a larger study which took *I. ricinus* from 22 sites in northern Europe, found that *I. ricinus* from Great Britain were genetically distinct but this study found three genetic lineages. *I. ricinus* from western Norway was genetically distinct from the rest of Europe, although it shared a more recent common ancestry than those from Great Britain (Røed *et al.,* 2016).

1.2.4 *Ixodes ricinus* in the UK

There are thought to be 20 species of tick indigenous to the UK, three are from *Argasidea* family and the remaining 17 species are from the *Ixodidae* (Jameson & Medlock, 2011; Martyn, 1988). Current surveillance of ticks in the UK is limited to Public Health England's (PHE) voluntary tick surveillance system, which relies primarily on members of the public submitting ticks they encounter (with the majority of submissions being collected from companion animals). (Jameson & Medlock, 2011). This surveillance system received over 14,000 ticks from 2010-2016. The majority (97.0%) of these were native ticks, although

some submissions (3.0%) were of imported ticks. Submission came from wildlife hosts, companion animals, and humans. *I. ricinus* was the most frequently found species accounting for 59.2% of all submissions (Cull *et al.,* 2018).

As in Europe, *I. ricinus* is not ubiquitous in the UK. Early attempts to map the distribution of *I. ricinus* in the UK was based on observations of infested sheep (MacLeod, 1932; Milne, 1946). In 1988, an effort was made to collate all previous data and create tick distribution maps. This included *I. ricinus* (Figure 5a), which showed that *I. ricinus* was widely distributed in the UK. As mentioned above PHE set up a voluntary tick surveillance system, in 2005 an article was published with an updated map of *I. ricinus* distribution which included records from 1878-2003 (Figure 5b), which shows how *I. ricinus* distribution has expanded (Pietzsch *et al.,* 2005).

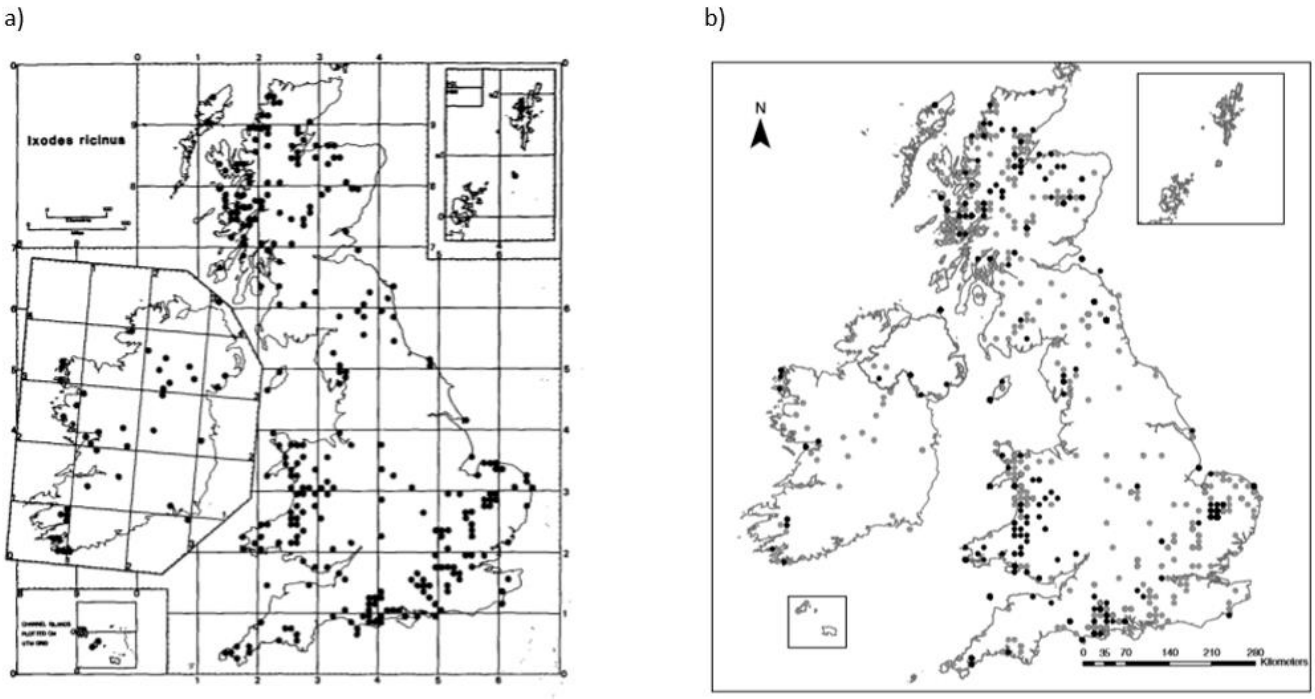

Figure 5. *I. ricinus* distribution maps. a) From Martyn, 1988 showing tick records up to 1988, b) from Pietzscht *et al.*2005 showing the records from 1878-2003, black dots are established sites and grey spots are reported sites for *I. ricinus*.

A survey by Scharlemann *et al*. (2008) used questionnaires to assess the public's perception on tick abundance. Responses were derived from 159 different locations. 97%of the responses agreed that ticks were present in their area, of which 61% were from locations

where ticks have previously been perceived as being absent. Interestingly some reports were over 10km away from any site where *I. ricinus* had been formally documented, the majority of these were in Scotland and northern England. However, as mentioned before these results should be used with caution as memories could be biased. Support for these observations is derived from the quantification of tick burden on red grouse (*Lagopus lagopus scoticus*) on 13 Scottish moorland estates from 1985 to 2003, these data showed that tick burden of red grouse chicks increased significantly over the 19 year period, from 2.6 ticks per chick in 1985 to 12.7 ticks per chick in 2003. Furthermore, the proportion of chicks parasitized increased dramatically to from 4% in 1985 to 92% in 2003 (Kirby *et al*., 2004). Collecting ticks from host has been used to map the distribution of ticks in the UK, most recently, collecting ticks from companion animals. In 2000, in a collaboration with veterinarians throughout the UK Ogden *et al.* mapped the distribution of ticks on cats and dogs. *I. ricinus* was reported but also *I. hexagonus, I. canisuga, Dermacentor reticulatus* and *Haemaphyalis puntctata*. This study highlighted that urban areas were able to support ticks especially (*I. hexagonus*). In 2011, another nationwide study was conducted in collaboration with veterinarians. This study showed *I. ricinus* was the most common tick found attached to dogs and that it was widely distributed in the UK, although there were significantly more *I. ricinus* ticks collected in rural areas (Smith *et al.,* 2011). Most recently this study was repeated and again demonstrated that *I. ricinus* was the most commonly found tick, with the widest distribution in the UK. This study went on to create a risk map of the UK, these maps represented the likelihood of being bitten by a tick (Abdullah *et al.,* 2016). Although this risk map gives an idea of the threat of a tick bite, it groups large areas of the country together which have been shown to have different densities of ticks. For example, the whole of Scotland has been categorised as high risk, when studies have shown local difference in tick abundance (James *et al.,* 2012; Millins *et al.,* 2016).

The abundance of *I. ricinus* is not static in time but had peaks and trough. In the UK the phenology of ticks has classically been described as bimodal, with peaks in late spring and early autumn (Randolph *et al.,* 2002). Initially, phenology was monitored by using data of ticks feeding on sheep, it was thought that ticks first fed in the spring and again in autumn, with the ticks developing to the next stage in summer and winter (Wheler, 1899 in Milne 1944). This theory was referred to as the "Two Broods" theory by both MacLeod

(1932,1939) and Milne (1949), who both explore the theory using different densities of sheep to assess if a change in the peak abundance of feeding ticks occurred. From these studies, MacLeod concluded that the two brood theory was not the whole explanation for the bimodal pattern of tick phenology, but that tick behaviour was temperature driven (MacLeod, 1939).

It is now thought that the two peaks are due to one cohort which starts in the autumn. If the ticks are successful in feeding in the spring they moult without diapause (without delay) and those that feed in the autumn moult after a diapause and so development is delayed until the next autumn. (Randolph *et al.,* 2002). A unimodal pattern of tick phenology has been recorded in England and Wales (Bown *et al*., 2008; Steele & Randolph, 1985). In areas of long cold winters and short summers, diapause can occur earlier slowing development as tick questing begins later in spring and ends earlier in autumn, causing a unimodal phenology (Steele & Randolph, 1985). A mix of bimodal and unimodal phenology has been recorded in the UK as early as 1947 (Edwards & Arthur, 1947).

The tick's life cycle can take from 1-6 years depending the environment conditions (Hillyard, 1996). If conditions such as climate, host availability and humidity are favourable, then they can pass through more than one moult in a year (Gray, 1982). When conditions are too hot or too cold ticks can go into behavioural diapause. According to Gray *et al.* (2016), diapause is a strategic response to conditions that adversely affect the water balance or deplete the energy of the tick. Diapause can affect the phenology of tick species as it delays development. Photoperiod, mating behaviours, and temperature change have been shown to cause or disrupt diapause (Gray, 2008; Gray, 1987; Campbell, 1948).

1.2.5 Abiotic and Biotic drivers of *I. ricinus*

Many studies have assessed why *I. ricinus* distribution is not ubiquitous in Europe and the UK. *I. ricinus* life cycle has specific requirements to meet or the tick will die. It has been shown that different abiotic and biotic factors affect tick phenology and abundance.

1.2.5.1 Climate

I. ricinus has proven to be hardy in an extensive range of weather conditions. *I. ricinus* spends the majority of its life "off host" and so it is essential that they can adapt to changing conditions in order to survive. In Britain, Macleod (1936) reported that the temperature must reach 7°C before questing behaviour begins and questing halts at around 16 °C.

Although more recently this has been shown to be geographically regulated. A larger proportion of ticks taken from cooler climates (Scotland and England) quested at lower temperatures than those from warmer climates (France), showing that *I. ricinus* can adapt to local climates and that there is phenotypic plasticity to questing behaviours (Gilbert *et al.,* 2014).

Ticks have been recorded questing at temperatures as low as 1.9°C and up to 35°C, which shows the extensive range of temperatures they can endure (Perret *et la.,* 2000; MacLeod, 1935). Extreme heat or cold can disrupt the life cycle of ticks. High temperatures can cause a drop in humidity and this can be very detrimental to the tick it has been shown that mortality increases, as ticks are at risk of desiccation, and questing periods decrease when humidity is low (Perret *et al.,*2000). Perret *et al.* (2003) found that the duration of questing for nymphs was limited by saturation deficit. The saturation deficit is used to measure the drying out power of the atmosphere by using a combination of the temperature and humidity. Whereas, snow cover can protect the tick from extreme temperatures and freeze thawing. It has been hypothesised that ticks are affected by altitude as tick density decreases with increasing altitude (Jouda *et al.,* 2004). In Scotland the effect of altitude on tick densities was evaluated. As the altitude increased the tick density decreased but analysis of the data using mathematical models found that this association is most likely due to a decrease in temperature as the altitude increased (Gilbert, 2010).

As mentioned previously, studies have shown that *I. ricinus* distribution is increasing (Laaksonen *et al.,* 2017; Jaenson *et al.,* 2012; Jore *et al.,*2011). Climate change has been suggested as one explanation for this increasing distribution. In Sweden a study suggested that the northward expansion of *I. ricinus* was in part due to warmer climate which is more favourable to the ticks life cycle (Jaenson *et al.,* 2012). Another study from Sweden found that tick abundance was associated with areas that had over 180 days a year over 5°C and areas which had less than 125 days per year of snow cover (Jaenson *et al.,* 2009). Projections have been made for *I. ricinus* distribution in Sweden using climate change predictions. This shows that *I. ricinus* will expand northwards over time. The authors state that this expansion is due to the climate being more favourable for ticks, but also because of indirect effects from climate change such as the expansion of the range of host for ticks (Jaenson & Lindgren, 2011).

1.2.5.2 Habitat

Ticks have been recorded in woodlands, bracken, sand dunes, heather moorland, short grass, floodplain forest, even in urban parks, and private gardens (Dobson *et al.,* 2011; Welinga *et al.,* 2006; Hubálek *et al.,* 2003; Jameson & Medlock, 2011; Hansford *et al.,* 2017a). Many studies, including a large study assessing 105 habitats in 16 countries across Europe, have tried to understand which habitats have the greatest abundance of ticks. Many found that mixed forests have the highest densities of ticks (Gray, 1991; Dobson *et al.,* 2011; James *et al.,* 2013; Lindstrom and Jaenson, 2003; Tack *et al.,* 2012b). This is thought to be due to higher levels of humidity in woodlands and because the habitat provides food (seed, berries) and shelter for an abundance of hosts thus increasing the ticks chance of coming across a blood meal (Gray, 1991). The tick must coordinate the need to stay hydrated and questing activities perfectly or risk desiccation and the loss of a potential blood meal. Therefore, living in a habitat that provides everything in one place is more profitable to the tick (Herrmann & Gern, 2015).

I. ricinus requires a relative humidity of at least 80% in the environment therefore the distribution of this tick is limited to areas with rainfall and vegetation which enables a high humidity (Macleod, 1935). For this reason, the understorey of a forest is an important part of the tick habitat. This is shown in a study from Belgium that cleared shrubs from the understory of a forest and found that the tick numbers significantly decreased for the next two years compared with a control plot (Tack *et al.,* 2013). Soil condition, leaf litter and microclimate were shown to be important drivers for larval abundance in a large European study which looked at many different habitat variable (Ehrmann *et al.,* 2017). This is because the hatching success and the larvae questing success is affected by the temperature and humidity at ground level.

Ticks are not only found in the forest but can also be found in urban environments (Rizzoli *et al.,* 2014; Gern *et al.,* 1997). A recent publication from the UK studied *I. ricinus* at 25 urban sites in southern England. At 14 of the sites *I. ricinus* was present. Ticks were found in grassland, hedge, parks, woodland and woodland edge, although ticks were significantly higher in woodland edge habitats and in spring and summer (Hansford *et al.,* 2017a).

Habitat configuration has shown to be important in the distribution of *I. ricinus.* In North America it has been shown the fragment forest has been associated with higher Lyme

borreliosis incidence (Tran & Waller, 2013). Across Europe and in the UK efforts are being made to reduce fragmented forest and increase connectivity through “Green Corridors” (Medlock *et al.,* 2013). Although greater connectivity has been associated with a higher abundance of ticks, it is thought that host movement pathways along with suitable climate variables are the drivers for this (Estrada Pena, 2002).

1.2.5.3 Hosts

I. ricinus are known to parasitize over 200 vertebrate hosts, including mammals, reptiles and birds (Anderson & Magnarelli, 1993). Small and medium hosts are the primary blood meal source for the larval and the nymphal stages of *I. ricinus*. Small animals such rodents have been shown to feed more larvae than nymphs (Matuschka *et al.,* 1991). Larger hosts are the main providers of a blood meal for the adult stage. In the UK ungulates such as sheep, cows and deer have been shown to carry ticks (Ogden *et al.,* 1997; Cull *et al.,* 2018). Although it has been demonstrated that deer can feed larvae and nymphs (Kiffner *et al.,* 2011), while adult ticks have been found to feed from small rodents (Bown *et al.,* 2003).

Deer are an important host for ticks as they are seen as the “reproductive host”. They feed more adults enabling them to mate and oviposition (Gray, 1998a) Recently a study found that it was presence or absence of deer that was more important in maintaining ticks and not deer abundance (Hoffmeester *et al.,* 2017).This paper used camera traps to assess deer abundance which is not as good of a measure as other deer indicators such as dung counts. Many other studies have found associations with deer density and tick abundance. In Scotland a “natural experiment” using fenced forested areas as deer exclusion zones found a strong positive relationship between tick density and deer abundance (Ruiz-Fons & Gilbert, 2010). As the reproductive host it makes sense that the more deer to feed that adults, the more likely ovipositioning is successful and therefore more eggs and consequently ticks in the environment. Managing deer using fenced areas has been explored by assessing the tick populations inside and outside of these enclosures. In the short term, tick numbers increased creating a higher health risk, this is thought to be due to more questing ticks that would have otherwise been attached to deer (Rand *et al*., 2004). Studies that looked at older fenced enclosures (3 years and above) found these fenced areas to have a lower density of questing ticks (Bown *et al.,* 2008; Gilbert *et al.,* 2012). A study from the USA using meta-analysis found tick abundance was associated with the size of the

exclusion zone. They found that a reduction in questing tick was found if the enclosure was larger than 2.5 ha but below this there was an increase in questing ticks and higher tick burdens on small animals, the authors suggested that in small areas small mammals were able to maintain the tick populations (Perkins *et al.,*2006). In contrast to these findings Gilbert *et al.* (2012) found that even small enclosures were effective at reducing tick abundance. Although small mammals are able to feed adult ticks they are not able to feed the large quantities of adult ticks that deer can and therefore ticks are vital in sustaining tick populations.

1.3 *Borrelia burgdorferi sensu lato*

1.3.1 Taxonomy

Borrelia is a genus of spirochete (of the order Spirochaetales) bacteria, characterized as Gram negative organisms that possess an unusual mode of motility by means of axial filaments (endoflagellae) (Schwan & Piesman, 2002). The genomes of *Borrelia* species are unusual in that they are comprised of a linear chromosome along with numerous linear and circular plasmids, which vary with species and strains (Casjens *et al.,* 2000; Wang & Scwartz, 2015). To date, 42 *Borrelia* species have been proposed. Phylogenetic analysis of these species indicates they form two divergent clades, informally referred to as the "relapsing fever" clade and the "Lyme disease" or "*Borrelia burgdorferi* sensu lato" clade. The extent of divergence between these two clades is such that it has been proposed that each would be better accommodated in its own genus (Adeolu & Gupta, 2014), with members of the relapsing fever clade remaining as *Borrelia* species, and members of *B. burgdorferi* s.l. clade being reclassified into *Borreliella*. However, the sense of this proposal continues to be hotly debated (Margos *et al*., 2017a; Barbour *et al*., 2017). Given this ongoing uncertainty, this thesis will refer to all 42 species as members of the genus *Borrelia*.

The genus currently contains 42 species of which over 20 are considered to belong to *B. burgdorferi* s.l. clade (http://www.bacterio.net/borrelia.html; Wang & Schwartz, 2015). This clade originated with the proposal of *B. burgdorferi* as the causative agent of LB in North-eastern USA in 1984 (Johnson *et al*., 1984) and has grown as close relatives to this species have been encountered either in LB patients or in surveys of potential reservoir hosts or vectors (and, with this expansion, the original clade member has been renamed *B.*

burgdorferi sensu stricto (s.s.)). Thus the clade now contains several *Borrelia* species that are associated with LB, and several that, as yet, have not. *B. burgdorferi* s.l. genome is unusual along with possessing a linear chromosome and multiple linear and circular plasmids, the 16S rRNA gene is singular whereas it contains of many tandem repeats of the 23S and 5S rRNA genes. It has been reported that a large proportion of plasmid DNA is in a state of evolutionary decay and that many of the plasmid genes have had recent DNA rearrangement. Alongside this it has a significantly higher frequency of lipoprotein-encoding genes that other prokaryotes (Wang & Schwartz, 2015).

1.3.2 Enzonotic cycles of *B. burgdorferi* s.l.

This spirochete is maintained in nature by alternating between the tick and the host, this means that in order to survive it must quickly adapt to its surroundings. In the tick the spirochete resides in the midgut, until the tick begins to feed (Francischetti *et al.,* 2009). Once the tick attaches to its host the spirochetes begins to double in numbers (Burgdorfer *et al*., 1989) and then move to the haemocoel and spirochetes enter the host via the saliva (Speilman *et al.,* 1987).

As mentioned above *I. ricinus* has been found to feed on a variety of different hosts. However, all host are not equal in the epizoonotic cycles of B*. burgdorferi* s.l. some are competent reservoir host (Table 2). A competent reservoir host is a species that when it is fed on by an infected tick becomes infected and the spirochetes persist for a considerable time enabling this host to pass the infection onto other feeding ticks. *I. ricinus* are infected with the spirochete, transstadially when they take a blood meal from an infected reservoir host they can become infected, this infection then stays in the tick throughout the developmental moults. Therefore, every blood meal that is taken is an opportunity to become infected and once the tick is infected, every blood meal is an opportunity to infect a competent reservoir host.

However, competent reservoir host are not always needed for the transmission of *B. burgdorferi* s.l. Co-feeding is another route of transmission for *B. burgdorferi* s.l. animals do not have to be systemically infected for a feeding tick to become infected. Co-feeding occurs on the host. Ticks that are feeding in close proximity to one another can pass the spirochete

to an uninfected tick. This was explored by feeding infected nymphs next to sterile larvae and using xenodiagnosis, to show infection can occur when the host remains uninfected (Gern & Rais, 1996). Transovarial transmission is another route of transmission (although rare), this is where the infection is passed from the adult female to the eggs. Evidence of this has been found in *I. persulcatus* (Nefedova *et al.,* 2004), *I. hexagonus* (Toutoungi & Gern, 1993) and in *I. ricinus* for *B. burgdorferi* s.l. and specifically with *B. afzelii,* it was also demonstrated that the larvae could transmit *B. afzelii* to rodents (van Duijvendijk *et al.,* 2016).

In Europe *I. ricinus* and in Eurasia *I. persulcatus* are the key vector of *B. burgdorferi* s.l. Although other ticks have been shown to be competent vectors, not all vectors are equal in *B. burgdorferi* s.l. transmission. Of the most common ticks in the UK, *B. burgdorferi* s.l. has been detected in *I. hexagonus, I. trianguliceps* and *I. canisuga,* (Bown *et al.,* 2008; Toutoungi & Gern, 1993; Burgdorfer *et al.,* 1983; Gorelova *et al.,* 1996) these are not regarded as important vectors in the transmission of *B. burgdorferi* s.l. (Barbour *et al.,* 1983). In the sympatric zone in Sweden, *I. persulcatus* was significantly more likely to be infected with *B. burgdorferi* s.l. than *I. ricinus* (Laarksonen *et al.,* 2017).

1.3.3 *B. burgdorferi* s.l. geographic distribution

The distribution of *B. burgdorferi* s.l. is limited to areas where there are competent vectors and reservoir hosts to create the enzoonotic cycles. Figure 6 shows that *B. burgdorferi* s.l. is encountered across all temperate regions of the northern hemisphere, each clade member has a far more restricted distribution (and some have only been encountered on one or a handful of occasions).

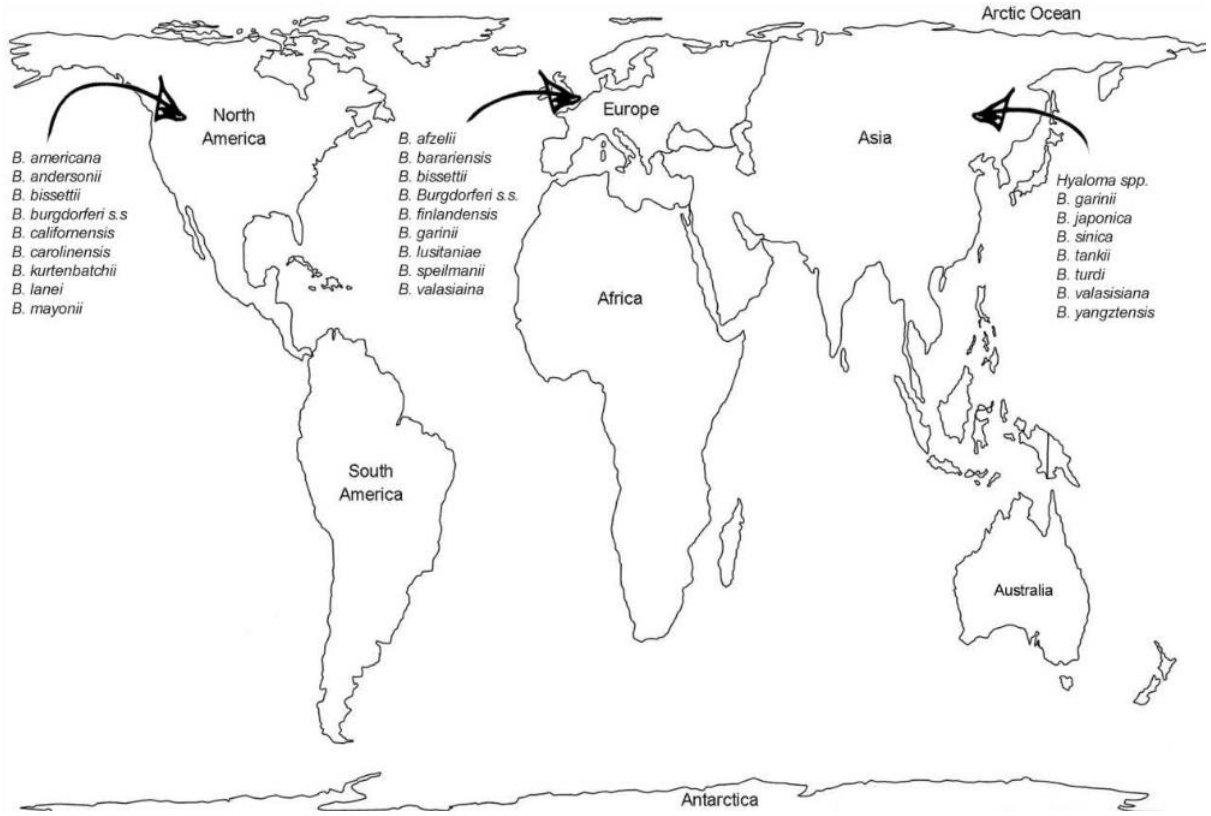

Figure 6. *B. burgdorferi* s.l. genospecies and the geographical locations that they are found.

A review of the literature in 2005 found that the infection prevalence of *B. burgdorferi* s.l. in European countries varied considerably from between 0.0% to 50.0%between and within countries, increasing from west to east. The meta-analysis showed that overall there was a difference in developmental stages, infection rates in nymphs was 10.1% and almost double that in adults with 18.9% (Rauter & Hartung, 2005). A more recent meta-analysis of European published data on the prevalence of *B. burgdorferi* s.l. in ticks between 2010-2016 found that the overall prevalence of *B. burgdorferi* s.l. has significantly increased in nymphs over the past 10 years. This study also reinforced the general opinion that *B. burgdorferi* s.l. is more abundant in eastern Europe than western Europe (Strnad *et al.,* 2017). The analysis revealed that *B. afzelii* is the most common genospecies, then *B. garinii, B. valaisiana* and *B. burgdorferi s.s.* (Strnad *et al.,* 2017). Both these studies compared literature that used different methods of detection and used studies which includes pooled samples, therefore the assessment may not be accurate but gives an estimation of the distribution of *B. burgdorferi* s.l. in Europe.

1.3.4 *B. burgdorferi* s.l. in the UK

The distribution of *B. burgdorferi* s.l. in the UK is not fully understood. However there have been many studies which look at the prevalence of *B. burgdorferi* s.l. in *I. ricinus* throughout the country. In 1994, Livesley *et al*. described the difficulties of isolating *B. burgdorferi* s.l., reporting no positive cultures from 108 attempted isolations. As the authors used the same protocol to successfully obtain an isolate from Swedish and Slovakian ticks they suggested that the culture medium they used, Barbour-Stoenner-Kelly (BSK) II, was not suitable for the isolation of British borrelial strains. In 1999, 11 isolates were established by culture from ticks in the Scottish Highlands using BSK (Davidson *et al*. 1999). A year later, 12 isolates cultured from ticks in the Scottish Highlands were characterised as *B. afzelii* (5 isolates) and *B. burgdorferi s.s.* (7 isolates) (Ling *et al*., 2000). In 1998, in Dorset 56.6% of ticks collected from pheasants were infected with *B. burgdorferi* s.l. (Kurtenbach *et al*., 1998a).

Within the UK *B. burgdorferi* s.l. prevalence in questing *I. ricinus* populations appears to vary spatially. In 2011, Vollmer *et al.* found no evidence of *B. burgdorferi* s.l. in *I. ricinus* from Wales but concurrent sampling in southern England found an infection prevalence of 12.4%. Then in 2013, a study of 25 sites in southern Scotland and northern England, found a range of 0-8.9% *B. burgdorferi* s.l. infection rate (Bettridge *et al.,* 2013), comparable results were found in Scotland with a range of 0.8- 13.9%(James *et al.,*2013). In 2015, nymphs from a large peri-urban park in London were reported to have an infection prevalence of 2.4% (Nelson *et al.,* 2015). In 2016, another large-scale study across Scotland, reported an infection prevalence range of 0-6.0% (Millins *et al.,* 2016). In the south of England four woodland sites were found to have an infection prevalence of 1.5-5.6% (Layzell *et al.,*2017). Most recently a small-scale study in an urban town in southern England survey 25 sites for *I. ricinus* of the 14 sites where *I. ricinus* was present, seven of these sites were found to have infected nymphs a rage of 8.2% -24.3% infection prevalence was found at these sites (Hansford *et al.,*2017a).

Within the UK four *Borrelia* genospecies have been found in questing ticks these are *B. afzelii, B. burgdorferi* s.s., *B. garinii* and *B. valaisiana* (Vollmer *et al.,* 2011; Bettridge *et al.,* 2013; James *et al.,* 2013; Millins *et al.,* 2016; Hansford *et al.,* 2017a; Layzell *et al.,* 2017). Very recently *B. spielmanii* was found from a tick attached to a dog in Yorkshire (Abdullah *et*

*al.,*2017) and *B. lusitaniae* was detected in a tick attached to a fox in south west England (Couper *et al.,*2010) but both species have yet to be found in questing ticks.

1.3.5 Abiotic and biotic drivers of *B. burgdorferi* s.l. prevalence

Many factors have been associated with *B. burgdorferi* s.l. infection prevalence, some directly and some have indirect effects.

1.3.5.1 Climate

Temperature has indirect effects on *B. burgdorferi* s.l. as it affects the tick and its life cycle, as explained in section 1.2.5.1. In Scotland, *B. burgdorferi* s.l. prevalence was positively associated with the number of growing degree days. Growing degree days is the sum of the number of degrees per day the mean temperature is higher than 4°C in the summer. The authors suggested that this association was due to warmer areas being more suitable for rodents which are competent hosts for *B. burgdorferi* s.l. (Millins *et al.,*2016). However, another Scottish study found growing degree days not to be a significant factor in *B. burgdorferi* s.l. prevalence but that a seasonal pattern was significant, this found that more ticks were infected later on in the season (James *et al.,* 2013).

In laboratory experiments infected nymphs were significantly more likely to survive heat stress than none infected nymphs. For adults this relationship with infection of *B. burgdorferi* s.l. was beneficial if the number of spirochete was low (Herrmann & Gern, 2010). *B. burgdorferi* s.l. infections in *I. ricinus* have been shown to modify the behaviour of the tick. *Borrelia* -infected nymphs have been shown to be less sensitive to a dry environment, in experimental conditions infected nymphs were less likely to move horizontally on a humidity gradient but rather stay in lower humidity areas (Herrmann & Gern, 2012). In the field, infected ticks were shown to be significantly associated with higher relative humidity (James *et al.,* 2013).

1.3.5.2Habitat

Many studies have tried to define the type of habitat where *B. burgdorferi* s.l. is most prevalent in *I. ricinus*. In the UK studies have shown that nymphs are more likely to be infected in mixed or deciduous woodland than other habitats (James *et al.,* 2013; Bettridge *et al.,*2013). This is most likely due to the host that live in this specific habitat. Millins *et al.* (2017) identify that deciduous woodland is the most planted in the EU and that this could cause an increase in both *I. ricinus* and *B. burgdorferi* s.l. prevalence.

1.3.5.3 Host

Many small mammals are competent reservoir host for *B. burgdorferi* s.l. (Table 2) this makes these animals very important in the enzoonotic cycle of the disease as they could potentially infect the larvae that feed off them.

Larger hosts are the main providers of a blood meal for the adult stage. Although deer are incompetent reservoir host for *B. burgdorferi* s.l., (Jaenson &Tälleklint, 1992; Telford., *et al* 1988) they are seen as "reproduction host" as they carry a high abundance of ticks especially adults (Gray, 1998). When a large number of deer are in one area, this can cause high densities of ticks, these ticks must feed to entre the next developmental stage, when most of the ticks are feeding from the deer the prevalence of *B. burgdorferi* s.l. is low. However, if there is a large number of deer in an ecosystem that also contains *B. burgdorferi* s.l. competent hosts then there would be a high density of ticks with a high *B. burgdorferi* s.l. prevalence. Equally if there are low deer numbers the population of ticks would be lower but if these ticks were feeding from competent reservoir host then the *B. burgdorferi* s.l. prevalence will be high. Evidence has shown that managing deer numbers would decrease tick numbers (Gilbert *et al.,* 2001) and so decrease infections such as Louping ill in wild life and LB in people. However, disrupting an ecosystem is not simple, as mentioned above decreasing the amount of deer can cause an increase in pathogen prevalence in ticks as they find other host that are competent reservoirs for these diseases to feed from (Gray *et al.,* 1999; Rand *et al.,* 2004).

Table 2. Competent reservoir hosts found in Europe adapted from Gern *et al.,* 1998.

Birds	Small mammals	Medium / large mammals
Alca torda (razorbill)	*Apodemus sylvaticus* (wood mouse)	*Lepus timidus* (varying hare)
Anthus trivialis (tree pipit)	*Apodemus flavicollis* (yellow-necked field mouse)	*Lepus europaeus* (brown hare)
Coccothraustes coccothraustes (hawfinch)	*Apodemus agrarius* (black striped mouse)	*Erinaceus europaeus* (hedgehog)
Erithacus rubecula (robin)	*Clethrionomys glareolus* (bank vole)	*Rattus norvegicus* (brown rat)
Fringilla coelebs	*Glis glis* (edible dormouse)	*Rattus rattus* (black rat)

(chaffinch)		
Luscinia luscinia (thrush nightingale)	*Microtus agrestis* (field vole)	*Sciurus carolinensis* (grey squirrel)
Luscinia svecica (bluethroat)	*Sorex minutus* (pigmy shrew)	*Sciurus vulgaris* (red squirrel)
Parus major (great tit)	*Sorex araneus* (common shrew)	*Vulpes vulpes* (red fox)
Phasianus colchicus (pheasant)	*Neomys fodiens* (water shrew)	*Ovis aries* (sheep)
Phoenicurus phoenicurus (redstart)		*Meles meles* (badger),
Psylloscopus collybita (chiffchaff)		
Sylvia atricapilla (blackcap)		
Sylvia communis (whitethroat)		
Troglodytes (wren)		
Turdus merula (blackbird)		
Turdus philomelos (song thrush)		

Each genospecies has been associated with a specific reservoir host. *B. afzelii* has been associated with rodents, *B. garinii* and *B. valaisiana* have been associated with birds and *B. burgdorferi s.s.* is more of a generalist (Kurtenbatch *et al.,* 2002a). An investigation using the serum of different animals confirmed that each genospecies showed resistance to complement to different animals (Kurtenbatch *et al.,* 2002a). *B. afzelii* was shown to infect wood mice and voles and was able to be transmitted to sterile larvae using xenodiagnosis, from both wood mice and voles (Hu *et al.,* 1997; Hanincova *et al.,* 2003). It was also shown that birds which were challenged with *B. afzelii* infected nymphs did not become infected, and so did not pass on the infection to larvae in xenodiagnostic test. The *B. afzelii* infected

nymphs that had fed on birds were tested two weeks after feeding and no evidence of *B. afzelii* as it was eliminated after the blood meal (Kurtenbatch *et al.,* 2002b). *B. burgdorferi s.s.* was shown to infect both rodents and birds. Xenodiagnosis showed that larvae that fed from these rodents and birds could be infected with *B. burgdorferi s.s.* (Kurtenbatch *et al.,* 1998 Kurtenbatch *et al.,* 2002b ;). *B. garinii* and *B. valaisiana* infected nymphs were shown to transmit the spirochete successfully to birds and for *B. garinii* it was shown that these newly infected birds could transmit *B. garinii* to sterile larvae via xenodiagnosis (Kurtenbatch *et al.,* 2002b). *B. garinii* was originally thought of as five outer surface protein A (OspA) serotypes, with one was specific to rodents and the other four to birds, but now this serotype has been classified as *B. bavariensis* as it was sufficiently different enough from the other four serotypes (Margos *et al.,* 2008). The host associations with *Borrelia* species are not as clear cut as they appear as evidence of expectations have been published. *B. afzelii* has been found in birds (Heylen *et al.,* 2010) and *B. garinii* in squirrels (Millins *et al.,* 2015).

1.4 Lyme borreliosis

LB is the clinical manifestation of *B. burgdorferi* s.l. infections in humans. Typically**,** symptoms of LB develop in the days following the bite of an infected tick. Most patients complain of fatigue, headaches, chills and fever. The most common acute-phase presentation and the most distinctive symptom of LB is erythema migrans (EM), is a red rash (Figure 7) with a central clearing which forms at the site of the tick bite (Nadelman & Wormser, 1995). A short course of antibiotic treatment such as doxycycline for 10 days has shown to be successful for treating early LB (Kowalski *et al.,* 2010). However, if left untreated more profound manifestations can develop.

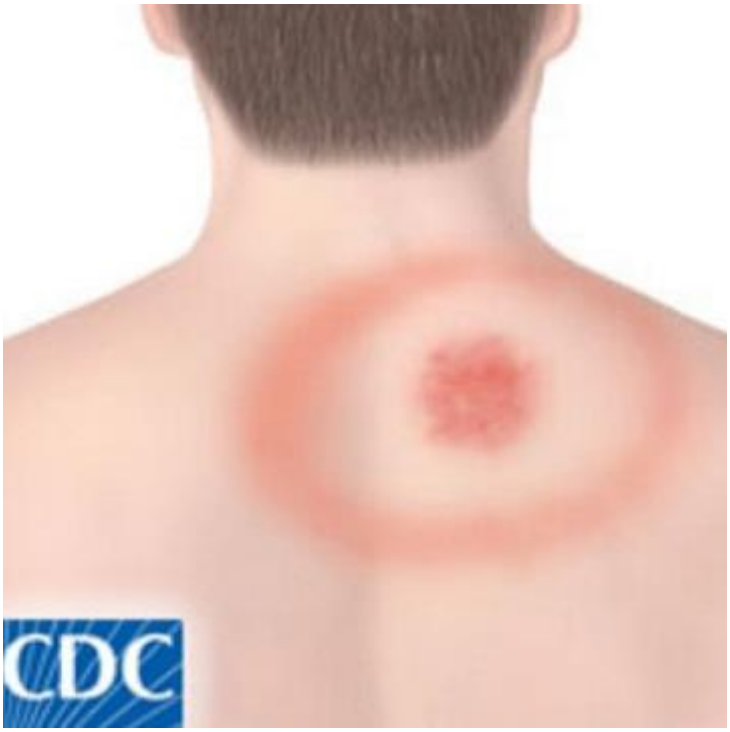

Figure 7. Erythema migrans (EM) rash also known as the Bulls eye rash

Different *Borrelia* species have been associated with different late symptoms of LB. *B. afzelii* has been detected in patients who present with acrodermatitis chronica atrophicans (a skin condition), *B. garinii* and *B. bavariensis* has been associated with neurological symptoms and *B. burgdorferi* s.s. with arthritis and neurological symptoms (Balmelli & Piffaretti, 1995; Coipan *et al.,*2016). *B. speilmanii* has been shown to be pathogenic in humans, with the presentation of Erythema migrans (EM) (Maraspin *et al.,*2006).

1.4.1 Chronic Lyme borreliosis

Chronic LB is a controversial subject, Feder *et al.,* (2007), defines chronic Lyme to encompass four categories; i) those who have symptoms of LB but no evidence of *B. burgdorferi* s.l. infection, ii) those who have a well-defined illness unrelated to *B. burgdorferi* s.l. (miss-diagnosed), iii) those with symptoms not consistent with LB, but with evidence of *B. burgdorferi* s.l. infection and iv) those with symptoms consistent with LB and evidence of *B. burgdorferi* s.l. (post-Lyme syndrome). Treatment for chronic LB is a hotly debated subject as many studies have shown that prolonged use of antibiotics have no effect and there are

ethical issue around antimicrobial stewardship, when prescribing long courses of antibiotics to patients without evidence of infection (Feder *et al.,*2017). There are many patient groups and some clinicians who believe that chronic LB can be cured with lengthy courses of antibiotics, however there is little evidence to back this up. Rather than focusing on potentially unreliable treatment it has been suggested that sero-negative cases previously described as chronic LB should now be described as chronic arthropod- borne neuropathy and patients should undergo further investigation of their illness (Drydent *et al.,* 2015).

1.5 LB epidemiology in the UK

PHE began recording cases of LB in 1986, reporting 15 confirmed cases in that year. The incidence of LB has continued to increase dramatically, such that in 2017, PHE reported 1,534 human cases of LB (https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/660591/hpr4117_zoos.pdf accessed on 8/1/2018). However, this number is widely considered to be a gross underestimation, and the estimated number of actual cases per annum is thought to be around 1,000-2000 cases higher (British Infection Society, 2011), with the majority of the cases reported from Exmoor, the New Forest, North Yorkshire Moors and the Lake District (PHE, 2018). In Scotland, epidemiological data are collated by Health Protection Scotland. Their reports indicate a rise from no cases in 1988 (Health Protection Scotland, 2006) to 200 cases in 2015 (http://www.hps.scot.nhs.uk/resourcedocument.aspx?id=5469) Again this has shown to be an underestimation as only 20% of suspected LB cases are referred for laboratory testing (Evans *et al.,* 2014). Reasons for this increase in the number of annual reports of LB in the UK are unclear but it has been suggested that a mix of; improved awareness of LB by the public and healthcare professionals and improved diagnostics, increasing numbers of people visiting tick-infested areas, and expanding tick populations and/or increased natural abundance of *B. burgdorferi* s.l. may also be influential (Medlock *et al.,* 2013). Of the four *Borrelia* genospecies that have been so far reported in questing ticks from the UK, three are considered of public health importance. These are *B. burgdorferi* s.s.*, B. afzelii,* and *B. garinii* (Balmelli & Piltaretti, 1995; Maraspin *et al.,* 2006).

1.6 *Borrelia miyamotoi*

As mentioned above, the *Borrelia* genus encompassed two clades that cause human disease the first is those species that cause Lyme borreliosis and the second is those that cause relapsing fever. The relapsing fever group contains over 20 species. The majority of these species are vectored by members of the argasid ticks, some hard ticks and one species (*B. recurrentis*) by lice (Wang & Schwartz*.,* 2015). The only species of this group to be vectored by the *Ixodes* genus is *B. miyamotoi*.

B. miyamotoi was fist isolated in Japan (Fukunaga, 1995) and is known to be pathogenic to humans (Platonov *et al.,* 2011). Symptoms are described as a high fever, with flu-like symptoms including malaise, myalgia/arthralgia and headaches, in serve cases (usually in immunocompromised patients) infections can result in meningoencephalitis (Hovius *et al*., 2013). Reports of patients presenting with EM have also been documented but it is suspected that these cases are due to co-infection with *B. burgdorferi* s.l. (Sato *et al.,* 2014; Platonov *et al.,* 2011; Molloy *et al.,* 2015). Cases of *B. miyamotoi* caused illness have been reported in the USA (Molloy *et al.,* 2015), Russia (Platonov *et al.,* 2011), Japan (Sato *et al.,* 2014), The Netherlands (Hovius *et al.,* 2013), and Germany (Boden *et al.,* 2016). Currently no cases of *B. miyamotoi* caused illness have been reported in the UK but this could be because it is circulating in very low numbers or due to a lack of awareness of the disease. Recently in the UK, *B. miyamotoi* has been confirmed to infect questing *I. ricinus* in the UK (Hansford *et al.,* 2015).

B. miyamotoi has been found in questing ticks in much of Europe although with varying rates of infection. In 2011, 55 questing ticks from France were tested for the presence of *B. miyamotoi* DNA and 3.6% of the ticks were found to be positive, *B. miyamotoi* was only found in adult females (Reis *et al.,* 2011). Larvae were tested for *B. miyamotoi* DNA using PCR questing larvae from two sites in the Czech Republic found between 0.0- 6.0% were positive for *B. miyamotoi.* This study also took engorged females from dogs in Germany and allowed them to oviposition, from these egg batches pools of larvae were tested and over 90% of them were positive for *B. miyamotoi* DNA (Richter *et al.,* 2012), which suggests transovarial transmission of *B. miyamotoi*, however as this contradicts with most of the

other literature perhaps the PCR assay used in this study was too sensitive or cross contamination occurred in these samples. In Norway in 2015, nymphs were found to contain *B. miyamotoi* DNA with 0.6% (10 /1789 adults and nymphs) reported as positive. Larvae were tested (n=210) but no evidence of infection was found (Kjelland *et al.,* 2015). From Belgium 1.1% (5/439) of questing ticks from seven locations were found to be positive for *B. miyamotoi* and in the Netherlands a much higher infection prevalence of 3.8% (20/520) of questing nymphs were positive for *B. miyamotoi* (Cochez *et al.,* 2015). In Serbia in 2016, 71 ticks taken from dogs and the vegetation were tested for *B. miyamotoi* and one (1.4%) positive questing nymph was found (Potkonjak *et al.,* 2016). In 2017, in Spain 357 ticks were tested by PCR for *B. miyamotoi* DNA 1.1% (4/357) were found to be positive these were two adult female ticks and two questing nymphs (Diaz *et al.,*2017). In the UK, the first report of *B. miyamotoi* in questing nymphs came from southern England with 0.3% (3/954) infection prevalence (Hansford *et al.,* 2015). Since then *B. miyamotoi* has been reported in other areas in southern England at 0.6% and 0.7% respectively (Hansford *et al.,* 2017b; Layzell *et al.,* 2017).

As noted above a large percentage of unfed larvae were infected with *B. miyamotoi*. This suggests that *B. miyamotoi* can be transmitted transovarially from adult female to the eggs. It has been shown that *I. ricinus* larvae can transmit *B. miyamotoi* to mice under laboratory conditions (van Duijvendeijk *et al.,* 2016). More studies need to be carried out to assess what role the larvae have in the transmission of *B. miyamotoi* in the wild. It has been established that small mammals are reservoir host for *B. miyamotoi* and that these host can often have co-infections with *B. burgdorferi* s.l. (Barbour *et al.,* 2009; Cosson *et al.,* 2014).

Aims and objectives

This project aims to better understand the medical and veterinary importance of LB in southern Cumbria. As part of a One Health initiative this project will attempt to explore key determinants of *B. burgdorferi* s.l. in a holistic manner by i) surveying the *I. ricinus* population dynamic and *Borrelia* spp. prevalence at sites across the study area, iii) assess the tick burden and infection prevalence of the grey squirrel, a reservoir host in the area, and iii) collaborate with veterinary clinics in the area to collect samples from companion animals.

To fulfil the first aim, the *I. ricinus* populations will be monitored every four weeks at three field sites. This should unravel any seasonal patterns that occur in the tick populations and will give an idea of when the environmental hazard of tick biting is at its highest in respect to human and animal health. These ticks will be tested using molecular methods to determine monthly infection prevalence for *B. burgdorferi* s.l. and *B. miyamotoi* which can then be analysed to see if infection prevalence remains stable thought the year. Using both these data sets together the density of infected nymphs (DIN) can be analysed to calculate the environmental hazard posed in the area. *B. burgdorferi* s.l. will be delineated to genospecies to see if any spatial of temporal patterns are revealed. Then using mathematical models the data will be used to determine what scale of sampling must be undertaken to accurately assess the tick population and DIN of a specific area.

The second aim of identifying the importance of vertebrate hosts will be tackled by exploring the role of an invasive host, the grey squirrel. To do this tick burden and infection prevalence will be assessed from squirrel carcasses. The ticks from the positive squirrels will also be tested to see if they share the same genospecies and larvae will be tested to assess transmission from squirrel to tick. These results will be compared with those from questing ticks in the area to understand if a specific *B. burgdorferi* s.l. genospecies is being filtered into the environment.

The third aim will be achieved as a collaborative effort with veterinarians from Cumbria. The vets will collect the tick samples from animals visiting the clinics, then they will be sent to the University of Salford to test them for the presence of *B. burgdorferi* s.l. This will reveal the species of ticks that bite companion animals and to explore the infection prevalence

compared with that from the questing ticks. Using data from the host animals of these ticks analysis can be performed to determine if any host characteristics are associated with tick burden.

Collating this data together will bring a One Health view of ticks and *B. burgdorferi* s.l. prevalence in Cumbria. This should help to inform policy makers when planning green space, public health practitioners by giving a more accurate view of the environmental hazard of LB to the public, and veterinarians as it should give and idea of what tick species are attaching to companion animals and when to apply acaracide to animals as a prevention method.

Chapter 2 Methods

2.1 Sample collection

Questing *I. ricinus* nymphs and adults were collected using a simple drag technique (Milne, 1943). Questing nymphs were collected from three longitudinal sites and from 13 sites close to where the grey squirrels were caught (Figure 8). In this study a woollen blanket of 1.6 m width x 2.0 m length was dragged across the woodland floor for 10m, then turned over and examined for ticks. All ticks observed on the blanket were removed into 70% ethanol. This process was repeated nine more times, after which the total number of nymphs and adults collected was recorded. If less than 200 nymphs were collected during these first 10 drags, further drags were completed until 200 nymphs were obtained or until 60 minutes had been spent at the field site. Any adults that were found were also collected and noted, larvae were difficult to quantify and collect, due to their size, so they were not included. The air temperature was recorded at each site once dragging had been completed, and a brief description of the weather made. Deer dung was counted on all the quantitative drags and a score given out of ten depending on how many times on these drags it was observed. Data were also collected from the weather station at Levens Hall, 11km away from the central study point.

I. ricinus were collected at approximately four-week intervals (dragging was only performed on dry days so the exact sampling schedule had to be amended to account for weather conditions) between June 2013 to September 2017. The data from June 2013 – October 2013 was collected as part of a separate research project (MSc by research, Jessica Hall, University of Salford).

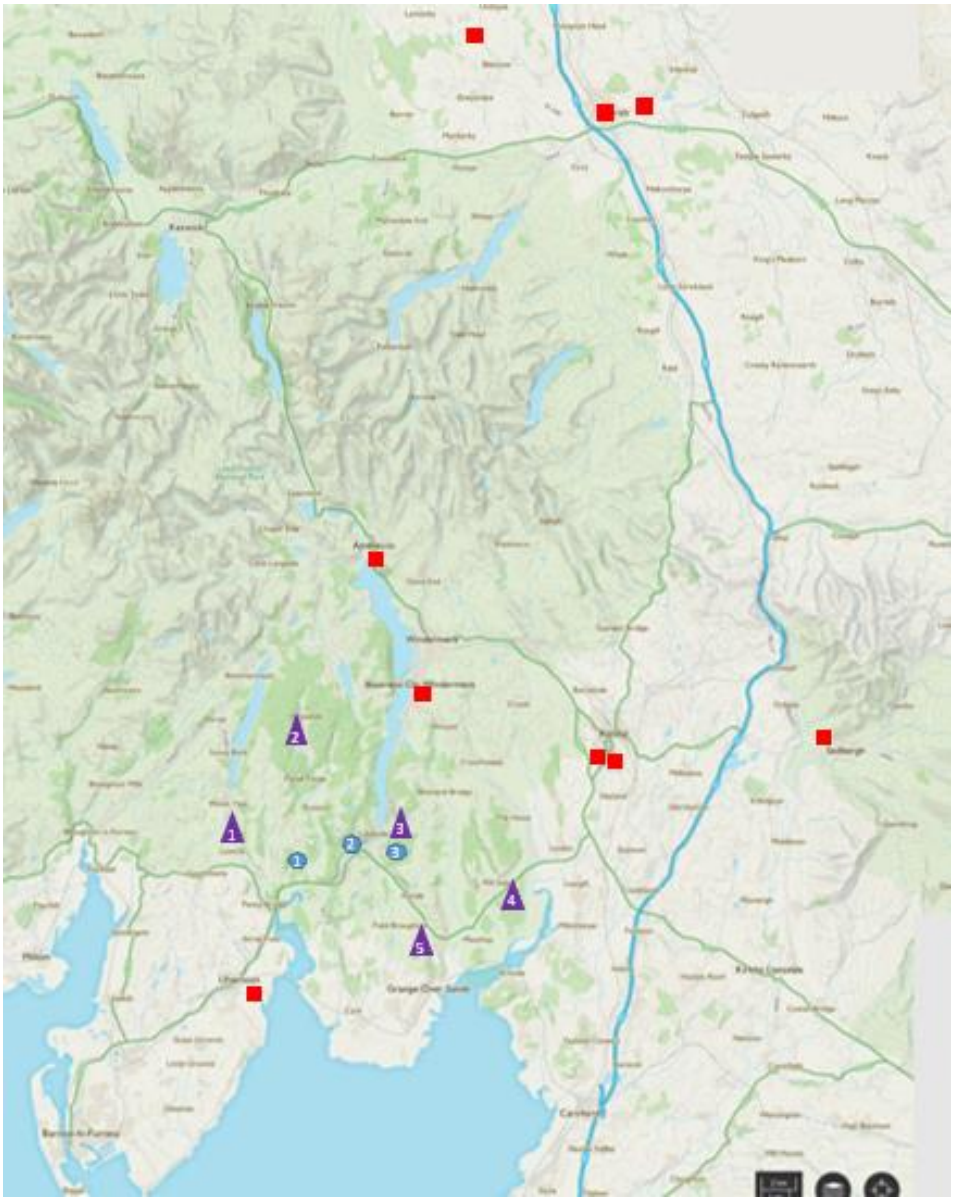

Figure 8. A map of Cumbria showing the location of the; longitudinal sites (Blue octagon- 1, Bouth; 2, Linsty Green; 3, Chapel House), participating veterinary practices (Red squares), and squirrel trapping areas (Purple triangle - 1, Arklid; 2, Grizdale; 3, Lakeside; 4, Foul Shaw Moss; 5, Eggerslack) (Ordinance Survey, 2010).

2.2 Tick identification

Nymph and adult tick samples from companion animals and squirrels were identified to species and stage using a dissection microscope and taxonomic keys (Arthur, 1963; Hillyard, 1996). Identification was made on the basis of at least three distinct morphological differences. Nymphs were difficult to identify to species level and were recorded as Pholeoixodes if ambiguous. Larvae were only identified to stage and adults were also sexed.

2.3 DNA extraction

2.3.1 Tick samples

For all nymph and adult tick samples DNA extraction was carried out as described by Gern *et al* (2010). Briefly, each tick was placed into an individual 1.5 ml Surelock microcentrifuge tube (Starlabs, Milton Keynes) containing 100µl (un-engorged nymph), 200µl (engorged nymphs or un-engorged adults), 500µl (engorged adults) of 1.25 % ammonium hydroxide (Sigma, Dorset). The tick was ground using a sterile pipette tip then the tube was closed, locked and placed onto a heating block at 100^{o}C for 20 min. Each tube was briefly centrifuged then returned to the heating block for a further 10 min, with the lid open, to evaporate off half of the solution. Finally, each tube was closed, relocked and stored at -20^{o}C until required. In order to control for cross-contamination between samples, cross-contamination controls were included every time DNA extractions were prepared. These comprised of tubes containing only 100µl, 200µl or 500µl of 1.25 % ammonium hydroxide that were co-processed with tubes containing ticks. One control was used for every four ticks processed. If the ticks were heavily engorged, one in ten dilutions of the DNA extracts was made for engorged nymphs and one in a hundred for engorged adults to prevent any inhibition during PCR. DNA was not quantified after each extraction due to the volume of samples that were processed

For larvae samples, DNA extraction was carried out using a DNeasy extraction kit (Qiagen, Hilden Germany), using the method for purification of total DNA from ticks (Qiagen, Hilden Germany). Up to 10 larvae were pooled for DNA extraction. Only larvae from the same squirrel carcass were pooled together, resulting in pools from 1 to 10 larvae. Up to 10 larvae were placed in a Sure lock microcentrifuge tube (Starlabs, Milton Keynes) with 180µl of buffer ATL. The larvae were homogenised using a sterile pipette tip. 20µl of proteinase K was added and then the lids closed, all samples were thoroughly vortexed and then placed into a water bath at 56^{o}C overnight. The samples were then vortexed for 15 seconds and 200µl of AL buffer was added to the sample and vortexed again. The samples were incubated at 70^{o}C for 10 mins. 1µl of carrier RNA was added to the samples and vortexed, after which 230µl of ethanol (96-100%) was added and then again vortexed.

This mixture was transferred from the microcentrifuge tube into a DNeasy mini spin column which was placed into a 2ml collection tube. This was centrifuged at 6,000 x g for 1 min and the flow through was discarded. 500µl of buffer AW1 was added to the spin column and it was centrifuged again for another min at 6,000 x g, the flow through was discarded. 500µl of buffer AW2 was added to the spin column and it was centrifuged for 3 mins at 20,000 x g, the flow through was discarded. The spin column was placed into a 1.5ml sure lock microcentrifuge tube (Starlabs, Milton Keynes), 35µl of AE buffer was added to the column, directly on top of the membrane and left to incubate at room temperature for 1 min before being centrifuged for 1 min at 6,000 x g to elute, another 30µl of AE buffer was then added to the spin column and the incubation and centrifugation step repeated to have a final eluate of 60µl. The eluent was analysed on the Nano drop 2000 Spectrophotometer (Thermoscientific, USA) to obtain the nucleic acid concentration and to assess if the extraction had worked. The eluent was kept at -20^{o} until testing.

2.3.2 Tissue samples

DNA extraction of squirrel ear biopsy punches was carried out using an Isolate II Genomic DNA kit (Bioline, London). For each squirrel five biopsy punches from each ear were combined. Extraction controls were made every ten samples. The 10 biopsy punches were place in a Surelock microcentrifuge tube (Starlabs, Milton Keynes) with 180 µl of Lysis buffer GL and 25µl of proteinase K, then were thoroughly vortexed and placed into a water bath at 56^{o}C overnight. The samples were vortexed for 15 seconds and 200µl of Lysis buffer G3 was added and vortexed. The samples were then incubated at 70^{o}C for 10 mins. Then 210µl of ethanol (96-100%) was added and vortexed.

This mixture was transferred from the microcentrifuge tube into an Isolate II spin column that was placed into a 2ml collection tube. This was centrifuged at 11, 000 x g for 1 min and the flow through was discarded. 500µl of buffer GW1 was added to the spin column and it was centrifuged for a further min at 11,000 x g. The flow through was again discarded. 600µl of buffer AW2 was added to the spin column and it was centrifuged for 1 min at 11,000 x g, the flow through was discarded. To dry the silica membrane the spin column was centrifuged at 11,000 x g for 1 min to remove any residual ethanol. The spin column was then placed into a 1.5ml Sure lock microcentrifuge tube (Starlabs, Milton Keynes), 100µl of

elution buffer which has been preheated to 70°C was added to the column, directly on top of the membrane and left to incubate at room temperature for 1 min and then centrifuged for 1 min at 11,000 x g to elute. The eluent was analysed on the Nano drop 2000 Spectrophotometer (Thermoscientific, USA) to obtain the nucleic acid concentration and to assess if the extraction had worked. The eluent was kept at -20° until testing.

2.4 Molecular diagnosis

Only the questing nymphs from June 2013 to November 2016 were tested for *B. burgdorferi* s.l. All the ticks taken from companion animals were tested for *B. burgdorferi* s.l. From Chapter 4 all the squirrels were tested for *B. burgdorferi* s.l. along with the questing nymphs and a subset of attached ticks from *B. burgdorferi* s.l. positive squirrels from 2015.

All samples were first tested using a RT-PCR (2.4.1) to determine if *Borrelia* DNA were present. Then any sample yielding an amplicon was tested using a nested PCR targeting the 23s-5s intergenic spacer region(2.4.2) any samples yielding an amplicon were sent off for sanger sequencing if the budget allowed or they were run on the reverse line blotting method (2.5). If no amplicon was produced, then these samples were tested for *B. miyamotoi* using a PCR assay targeting the glpQ gene (2.4.4).

A subset of RT-PCR positive samples from Chapel house (June 2013 – June 2014) and Bouth and Linsty Green (June 2014- June 2016) were taken to RIVM to be tested there as a cost saving exercise these sample were tested using a PCR targeting the 23s-5s intergenic spacer region (2.4.3) and all these samples were tested for *B. miyamotoi* using a PCR assay targeting the glpQ gene (2.4.4).

Table 3. Primer sequences used and their target gene with expected fragment size.

Primer / probe	Sequence (5'-3')	Target	Fragment size	Reference
Bb23Sf	CGAGTCTTAAAAGGGC GATTTAGT	23s rRNA gene *Borrelia spp*	75bp	Courtney *et al*., 2004
Bb23Sr	GCTTCAGCCTGGCCAT AAATAG	23s rRNA gene *Borrelia spp*	75bp	Courtney *et al*., 2004
Bb23S probe	FAM-GATGTGGTAGACCCG AAGCCGAG TG-TAMRA	23s rRNA gene *Borrelia spp*	75bp	Courtney *et al*., 2004
23SN1	AAGCTGACTAATACTA ATTACCC	5s23s intergenic spacer region *Borrelia spp*	~354bp	Rijpkema *et al*., 1995

23CN1	ACCATAGACTCTTATT ACTTTGAC	5s23s intergenic spacer region *Borrelia spp*	~354bp	Rijpkema *et al*., 1995
23SN2	ACCATAGACTCTTATT ACTTTGACCA	5s23s intergenic spacer region *Borrelia spp*	~202bp	Rijpkema *et al*., 1995
5SC	ACCATAGACTCTTATT ACTTTGACCA	5s23s intergenic spacer region *Borrelia spp*	~202bp	Rijpkema *et al*., 1995
B5sBorseq	GAGTTCGCGGGAGAG TAGGTTATTGCC	5s23s intergenic spacer region Borrelia spp	~410	Heylen et al., 2013
B23sBorseq	TCAGGGTACTTAGATG GTTCACTTAA	5s23s intergenic spacer region Borrelia spp	~410BP	Heylen et al., 2013
B-5SBor (RLB)	biotin-GAGTTCCGGGAGAGT AGGTTATT	5S rRNA gene	103bp	Alekseev *et al,* 2001
23SBor (RLB)	TCAGGGTACTTAGATG GTTCACTT	23S rRNA gene	103bp	Alekseev *et al,* 2001
B. burgdorferi s.l.	CTTTGACCATATTTTTA TCTTCCA	*B. burgdorferi s.l.*		Alekseev *et al,* 2001
B. afzelii	AACATTTAAAAAATAA ATTCAAGG	*B. afzelii*		Alekseev *et al,* 2001
B. burgdorferi s.s.	AACACCAATATTTAAA AAACATAA	*B. burgdorferi s.s.*		Alekseev *et al,* 2001
B. garinii	AACATGAACATCTAAA AACATAAA	*B. garinii*		Alekseev *et al,* 2001
B. valaisiana	CATTAAAAAAATATAA AAAATAAATTTAAGG	*B. valaisiana*		Alekseev *et al,* 2001
VSNE	TATATCTTTTGTTCAAT CCATGT	*B. valaisiana*		Poupon *et al.*, 2006
GANE	CAAAAACATAAATATC TAAAAACATAA	*B. garinii*		Poupon *et al*., 2006
GANE1	AAAATCAATGTTTAAA GTATAAAAT	*B. garinii*		Poupon *et al.,* 2006
LusiNE	TCAAGATTTGAAGTAT AAAATAAAA	*B. lusitaniae*		Poupon *et al.,* 2006
LusiNE1	CATTCAAAAAAATAAA CATTTAAAAACAT	*B. lusitaniae*		Gern *et al.,* 2010
LusiNE2	AAATCAAACATTCAAA AAAATAAAC	*B. lusitaniae*		Gern *et al.,* 2010
SpiNE2	GAATGGTTTATTCAAA TAACATA	*B. spielmanii*		Gern *et al.,* 2010
SpiNE3	GAATAAGCCATTTAAA TAACATA	*B. spielmanii*		Gern *et al.,* 2010
BisNE1	AAACACTAACATTTAA AAAACAT	*B. bissettii*		Gern *et al.,* 2010
BisNE2	AACTAACAAACATTTA AAAAACAT	*B. bissettii*		Gern *et al.,* 2010
RFLNE	CTATCCATTGATCAAT	Relapsing fever-like		Gern *et al.,*

	GC			2010
glpQ F	ATGGGT TCAAACAAA AAGTCACC	*B. miyamotoi* glpQ gene		Hansford *et al.,*2015
glpQ R	CCAGGGTCCAATTCC ATCAGAATATTGTGC AAC	*B. miyamotoi* glpQ gene		Hansford *et al.,*2015

2.4.1 Real time PCR

The presence of *B. burgdorferi* s.l. DNA in each sample was determined using a previously-described real-time PCR assay (Courtney *et al*., 2004). Each PCR mix (20µl) contained a 10pmol μl^{-1} solution of primer Bb23Sf and 10pmol μl^{-1} solution of primer Bb23Sr, 3.25pmol μl^{-1} solution ofBb23s probe, the dual labelled Taqman probe (Table 3), 5 µl of PCR grade water and 10µl of 2 x MyTaq Mix (Bioline Ltd, London). Finally, 2µl of DNA extract was added. Typically, PCRs were carried out on 96 well plates, exposed to the following thermal cycle: 95^{o}C for 5 min, then 40 cycles of 95^{o}C for 15 seconds and 57^{o}C for 1 min, on an MJ Research Opticon 2 machine (Bio-Rad, California). Each plate included a reagent (negative) control and a culture (positive) control, together with the cross-contamination controls described above. The fluorescence was recorded by the machine at the end of each extension step and all results were visualised via Opticon monitor software. Each samples was recorded as *B. burgdorferi* s.l. positive or negative.

2.4.2 23s-5s Intergenic spacer region nested PCR

Any *B. burgdorferi* s.l. positive samples were then subjected to a nested PCR which targeted the 23s-5s intergenic spacer region, this method was taken from Rijpkema *et al* (1995). This assay allows for identification of the genospecies when the amplicon is sent for Sanger sequencing. Each PCR mix (20µl) contained a 10pmol μl^{-1} solution of 23SN1 primer and 23CN1 primer (Table 3 for sequences), 6µl of PCR grade water and 10µl of 2 x working concentration MyTaq clear Mix (Bioline Ltd, London). Finally, 2µl of DNA extract was added. Typically, PCRs were carried out on 96 well plates, exposed to the following thermal cycle: for the first round conditions were 94°C for 1 min, then 25 cycles of 94°C for 30 s, annealing temperature of 52°C for 30s, and an extension step of 72°C for 1min. Followed by an extra extension step for 1 min, the samples were kept at 4°C. For the second round a PCR mix

(50µl) containing 10pmol μl^{-1} solution of 23SN2 primer and 5SC primer (Table 3), 19µl of PCR grade water and 25µl of 2 x working concentration MyTaq Red Mix (Bioline Ltd, London). Finally, 2µl of the first round product was added. For the second round conditions were 94°C for 1 min, then 40 cycles of 94°C for 30 s, annealing temperature of 52°C for 30 s, and an extension step of 72°C for 1min. Followed by an extra extension step for 1 min, the samples were kept at 4°C.

Samples were visualised on an agarose gel. The agarose gel was prepared by adding 1.5g of agarose powder (Bioline, London) with 100ml of 1 x TBE (Severn Biotech, Worcestershire) to a conical flask. This was heated and agitated until the powder has dissolved and the liquid was clear. This was left to cool on an agitator at room temperature for 5 mins, then 100µl of Gel red was added (Cambridge scientific, Cambridge), the mix was then poured into a mould and a comb added to create the well. This was allowed to solidify before it was added to an electrophoresis tank (Bio-rad, California) and covered with 1x TBE. 5ul of Hyper ladder 1KB (Bioline, London) was added to the first well and then 5µl of sample was added to subsequent well. The agarose gel was run for 75 mins at 100 volts. The gel was then taken from the tank and visualized using Genesnap (Syngene).

Any yielding an amplicon was subject to the Isolate II PCR & Gel clean up kit (Bioline Ltd, London). For each sample an Isolate II column was placed into a collection tube and labelled, then 45µl of the PCR product was pipetted directly onto the filter in the spin column along with 90µl of binding buffer CB. This was centrifuged at 11,000g for 30 secs, and the flow through was pipetted back into the spin column and again centrifuged at 11,000g for 30 secs, then the flow through was discarded. 700µl of CW wash buffer was added to the spin column and centrifuged at 11,000g for 30 seconds. The columns were then centrifuged at 11,000g for 1 min, the flow through discarded, and the spin column were left with the lids open at room temperature for 10 mins to evaporate off any residual ethanol. 15µl of elution buffer C was added to the membrane and incubated at room temperature for 1 min, then the spin columns were transferred into Surelock microcentrifuge tubes and centrifuged at 11,000g for 1 min.

The eluent was analysed on the Nano drop 2000 Spectrophotometer (Thermoscientific, USA) to obtain the nucleic acid concentration. Samples were then diluted to have an end

concentration between 2-20ng/µl. The samples were then sent off for Sanger sequencing by Biosource (Rochdale) in both directions.

2.4.3 23s-5s Intergenic spacer region conventional PCR

This assay targeted the 5s- 23s intergenic spacer region as described in Heylen *et al.,* 2013. Each PCR reaction (25µl) contained 10pmol μl^{-1} solution and of 5Sborseq and 23Sborseq (Table 3). 7.5µl PCR grade H_2O, 12.5µl Hotstart taq mastermix (Qiagen, Hilden), and 3µl of DNA under the following conditions: 94°C for 15 mins, then 94°C for 20 seconds, 70°C for 30 seconds and 72°C for 30 seconds, for 10 cycles, lowering the annealing step 1°C per cycle. Then 40 cycles of 94°C for 20 seconds, 60°C for 30 seconds and 72°C for 30 seconds, followed by an extension at 72°C for 7 mins. The product was imaged on an agarose gel (as above). 5µl of PCR product was taken from any sample that produced an amplicon and mixed with 2µl of ExoSAP-IT™ (Thermosfisher) on ice. These samples were then incubated at 37°C for 15 mins to degrade remaining primers and nucleotides and then incubated at 80°C for 15 mins to inactivate ExoSAP-IT™ reagent. The samples were sent off for Sanger sequencing in both directions (Baseclear, Leiden). This method was carried out at RIVM.

2.4.4 glpQ *B. miyamotoi* conventional PCR

This assay targeted the glpQ gene for B*. miyamotoi* as described in Hansford *et al.,* 2015. Each PCR reaction (25µl) contained 10pmol μl^{-1} solution and of glpQ F and glpQ R (Table 3). 7.5µl PCR grade H_2O, 12.5µl Hotstart taq mastermix (Qiagen, Hilden), and 3µl of DNA under the following conditions 95°C for 15 mins, then 94°C for 30 seconds, 62°C for 30 seconds and 72°C for 60 seconds, for 10 cycles, lowering the annealing step 1°C per cycle. Then 40 cycles of 94°C for 30 seconds, 53°C for 30 seconds and 72°C for 60 seconds, followed by an extension at 72°C for 10 mins. The product was imaged on an agarose gel (As above). 5µl of PCR product was taken from any sample that produced an amplicon and mixed with 2µl of ExoSAP-IT™ (Thermosfisher) on ice. These samples were then incubated at 37°C for 15 mins to degrade remaining primers and nucleotides and then incubated at 80°C for 15 mins to inactivate ExoSAP-IT™ reagent. The samples were then sent off for Sanger sequencing in both directions (Baseclear, Leiden). This was carried out at RIVM.

2.5 Reverse line blotting

The identity of the infecting *B. burgdorferi* s.l. genospecies in all ticks yielding a real-time PCR product was determined using reverse line blotting (RLB), when sequencing did not give a clear result or for samples after September 2016. This method was as described previously (Alekseev *et al,* 2001). For full recipes of solutions see appendix 2.

2.5.1 Activation of the membrane and linking of genospecies-specific oligonucleotides to the membrane.

The Biodyne C membrane was activated by incubation with 5ml of 16% (w/v) EDAC (Sigma, Dorset) solution for 15 min at room temperature. The membrane was rinsed with distilled H_2O for 2 min on a mechanized rocker then placed on the inverted top half of a 45 lane mini-blotter base (Immunetics, Boston). Once the position of the membrane had been verified, a blotting cushion, then the bottom half of the mini-blotter were placed on top of it, completing the "sandwich", which was held together by tightened screws.

Each oligonucleotide (Eurofins, Luxemburg) (Table 3) was diluted in 150µl of 0.5M $NaHCO_3$ (pH 8.4) to a concentration of 10pmol $150\mu l^{-1}$. To the mini-blotter 150µl of ink solution (1µ of ink in 100µl of 2X SSPE) was added to the first lane. Then into each subsequent lane 150µl of each oligonucleotide was added and in the last lane the ink solution. Once all the samples have been applied to the min-blotter it was incubated at room temperature for 1 min and then the oligonucleotides and ink were removed by aspiration in the same order they were applied.

Forceps were used to transfer the membrane from the mini-blotter into a tray containing 100ml of 0.1M NaOH, in which the membrane was washed for 9 min at room temperature on a mechanized rocker. The membrane was rinsed in distilled H_2O then incubated in 250ml of 2XSSPE/0.1% SDS at 50°C for 10 min in a rocking oven. Next, the membrane was incubated in 100ml of 20 mM EDTA at room temperature for 15 min on the mechanized rocker, after which it was wrapped in cling film and stored at 4°C until needed.

2.5.2 Generation of 23S-5S rDNA intergenic spacer region amplicons

Each sample was processed by PCR, each well contained a 10pmol μl^{-1} solution of biotin labelled primer B-5SBor primer 23SBor (Table 3), 7.5µl of PCR grade H_2O and 12.5µl of 2 x MyTaq Red Mix (Bioline Ltd, London). Finally, 2µl of DNA extract was added. Typically, PCRs were carried out on 96 well plates, exposed to the following thermal cycle: 94°C for 3 mins, 94°C for 20 sec, 60°C for 30 sec, 72°C for 30 sec. The annealing step then decreases by 1°C until it reaches 52°C and then it is repeated for 40 cycles. Then a 72°C extension step that lasted for 10 min. The product was immediately placed on ice. 10µl of each PCR product was then added to 150µl of 2X SSPE/0.1% SDS in a 1.5ml Surelock microcentrifuge tube (Starlabs Ltd). This was heat denatured by placing the sample on a heating block at 100°C for 10 min. Then the products were again immediately placed on ice to cool and left there for at least 5mins before centrifuging at 11,000 x g for 15 seconds, then returned to the ice.

2.5.3 Hybridisation of amplicons to oligonucleotides linked to the membrane and detection of bound amplicons

The membrane was washed for 5 min in 250ml of 2XSSPE/0.1% SDS at 50°C in the rocking hybridizing oven. The membrane was then once again applied to the mini-blotter so the holes are perpendicular to the position of the applied oligonucleotides. Any residual fluid was removed from the holes of the mini-blotter by aspiration. 150µl of the diluted PCR product was added to the blotter again taking care not to introduce bubbles. This was left to hybridize for 45 min at 50°C keeping perfectly horizontal, after which the mini-blotter was removed from the oven and the PCR products aspirated. The membrane was removed from the mini-blotter using forceps and was washed in 250ml of 2X SSPE/0.5% SDS for 10 min at 60°C and this wash was repeated.

The membrane was allowed to cool to prevent inactivation. 5 µl streptavidin POD conjugate (Roche, Basel Switzerland) was added to 25ml of 2X SSPE/0.5% SDS, which had been preheated to 42°C, and the membrane was incubated in this solution for 30 mins at 42°C in the rocking oven. Then the membrane was washed in 250ml of 2X SSPE/0.5% SDS for 10 min at 42°C in the rocking oven and this wash was repeated. The membrane was then washed in 250ml of 2 XSSPE (Sigma, Dorset) for 5 min at room temperature on the mechanized rocker

and this wash was repeated. 5ml of ECL 1 and 5ml of ELC 2 (GE Healthcare, Buckinghamshire) was added to the membrane in quick succession so the membrane was covered and incubated for 1 min with agitation. The membrane was then carefully wrapped in cling film and taped into a film cassette and taken to the dark room.

Working by red light only Amersham Hyperfilm, ECL (GE Healthcare, Buckinghamshire) was overlaid onto the blot in the cassette, which was sealed and left for 7 mins. The film was taken out of the cassette and placed in developer (Kodak, New York) for 1 min with constant movement, it was then placed in tap water for 1 min to rinse off the developer and then into fixative (Kodak, New York) for 1 min. The film was examined.

2.6 Statistical analysis

Univariate statistical analysis was performed using Minitab 18 (Minitab Ltd, Coventry) 18, Philadelphia). Generalized linear models (GLM) were performed in R Version 3.4 (R, Development core team, Vienna).

Chapter 3: Temporal and spatial monitoring of questing *I. ricinus* abundance and *Borrelia burgdorferi* s.l. prevalence

3.1 Introduction

The public health risk of Lyme borrelisosis (LB) can be described in two parts, (i) the hazard, which is the quantification of the potential danger in the environment and (ii) the risk, which is how likely the hazard will affect humans (Dobson *et al.*, 2011). The hazard for LB is the number of infected ticks in the environment specifically the nymphal stage (Gray, 1998). Therefore, the hazard of LB is linked with the density of nymphs (DON) in an area and has been described as directly linked with the density of infected nymphs (DIN) in an area (Vourc'h, *et al.,* 2016). DIN is calculated to understand the environmental hazard of *B. burgdorferi* s.l. and is the product of the DON and the infection prevalence of *B. burgdorferi* s.l. found in those nymphs (Ostfeld *et al.,* 2001). DIN is useful for assessing if an area is high or low risk of LB to public health and can be used to understand where and when preventative tools are best deployed (Coipan *et al.,* 2013). Consequently, the study of *I. ricinus* phenology is important in understanding the risk of LB.

The phenology of *I. ricinus* is shaped by the four life stages that are affected by many biotic and abiotic factors. In the UK a bimodal pattern of phenology is found in the south of England as the classical *I. ricinus* phenology, with peaks of abundance in late spring and early autumn (Randolph *et al.,* 2002). However, in other parts of the UK such as Wales and northern England a unimodal phenology has been described (Edwards & Arthur, 1947; Bown *et al*., 2008). It is thought that areas with short summers and long cold winters cause a unimodal pattern of phenology, by diapause occurring earlier in the winter and tick questing commencing later in spring both due to unfavourable conditions (Steele & Randolph, 1985). DON and DIN have shown to be associated with different abiotic and biotic factors such as temperature, photoperiod, saturation deficit, host availability, habitat structure and composition (Gray, 1982; Vourc'h, *et al.,* 2016; Sprong *et al*., 2012; TäLleklint & Jaenson, 2014)

Host abundance has been shown to affect DON. Deer are the host that have been most heavily studied as they are an important reproductive host for the adult stage ticks. Many

studies have found a positive correlation between deer density and DON (Sprong *et al.,* 2012; Gray *et al.,* 1992; Gilbert *et al.,* 2012; James *et al.,* 2013). High deer populations have been associated with an increase in *B. burgdorferi* s.l. prevalence as they are the reproductive host and so increase the tick population, however a high deer presence had been associated a dilution effect and a lower *B. burgdorferi* s.l. prevalence as deer are incompetent host for *B. burgdorferi* s.l. (Rosef *et al.,* 2009; Gray *et al.,* 1992). Gray *et al.* (1992) suggested that rodents rather than deer create higher *B. burgdorferi* s.l. prevalence in *I. ricinus* as they are competent reservoir hosts for *B. burgdorferi* s.l. A variety of hosts for all tick stages to feed from, some of which are competent host for *B. burgdorferi* s.l. and that are important for tick reproduction which will potentially create enzoonotic cycles of *Borrelia* species (Matuschka *et al.,* 1991). For TBEV (as is possibly the case for *B. burgdorferi* s.l.) when the number of deer increase the numbers of co-feeding ticks on mice also increased until this plateaued and after which at very high densities of deer this started to decrease (Cagnacci *et al.,* 2012). The more ticks co-feeeding on mice the more likely infection is to transmit to both the ticks and the mice but as the ticks begin to feed from the deer less ticks are likely to be infected with *B. burgdorferi* s.l. This shows that deer can cause a both amplification and dilution effects depending on their densities.

The dispersal of *I. ricinus* ticks is thought to be due to their host range therefore, habitat and host factors are closely linked (Carpi *et al.,* 2016). Prior studies exploring DON and DIN have found the structure of these habitats is important. DON has been associated with forest composition (Vourc'h, *et al.*, 2016; Tack *et al.,* 2012a; Tack *et al.,* 2012b; James *et al.,* 2013), soil composition (Greenfield, 2011; Medlock *et al.,* 2012), altitude (Gilbert, 2010), forest cover and the structure of the landscape (Vanwambeke *et al.,* 2016; Tack *et al.,* 2012b). Connectivity between fragmented forests has been associated with higher DON and tick dispersal, it is thought that host movement pathways along with suitable climate variables are the drivers for this (Estrada Pena, 2002). DON has been shown to be higher at the woodlands edge than in the woodland, park, hedge or grassland. There was no significant association between DIN and habitat type (Hansford *et al.,* 2017a).

Woodland areas with leaf litter and scrubs have been shown to create microclimates with high relative humidity and low saturation deficit. Saturation deficit is a measurement of the "drying out" power of the air (Perret *et al.,* 2000). Several studies have found that high

saturation deficit is detrimental to the unfed tick's ability to quest due to water loss (Randolph & Storey*.,* 1999; Perret *et al.,* 2000; Tagliapietra *et al.,* 2011). Saturation deficit is thought to influence DIN because ticks find a host to feed from by questing. Questing requires energy and can leave the tick dehydrated and so the environmental conditions must be adequate for the tick to quest (Lees & Milne, 1951). This is most likely to affect DIN as a lower DON is directly liked to DIN.

In the UK there are areas known as LB "hotspots" but these areas are not necessarily where the DON or DIN is the highest. These hotspots are identified as areas with a high tick density and where many human cases of LB are found, this is based on the public health data collected in the UK (Dubrey *et al.,* 2013). In England and Wales there were 1,534 human cases of LB reported in 2017 (https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/660591/hpr4117_zoos.pdf accessed on 8/1/2018), although as LB is not a notifiable disease this is likely to be an underestimation. This data is based on cases that have been submitted to the laboratory for testing, it is thought that there are more likely to be around 1,000-2,000 annual cases in the UK (British Infection Society, 2011). In Scotland it has been estimated that only 20% of suspected LB cases are referred for laboratory testing, thus reinforcing the view that annual cases are greatly underestimated (Evans *et al.,* 2014). Some areas of the UK have been identified as high areas of LB, with up to a five-fold higher incidence rate than the rest of the country, these are the LB "hotspot" areas (Drydent *et al.,* 2015; Mavin *et al.,* 2014). As mentioned above these "hotspots" may not have more ticks but they maybe riskier to public health. Thinking of the definition of risk by Dobson *et al.* (2011) these areas are perhaps where people are more likely to encounter ticks and so have a higher risk. LB "hotspots" are mainly rural areas that attract plenty of tourism. Recently our behaviour to outdoor space is changing with much emphasis being put on the health benefits of outdoor activities (Allen-Colinson, 2018).

Efforts have been made to quantify the DON throughout the UK. In 2008, nine locations in Wales were sampled and differing densities of questing ticks were found this ranged from 0 ticks per $10m^2$ to 21 ticks per $10m^2$ (Medlock *et al.,*2008). Then in 2011, 16 plots inside a park in London were sampled for ticks these plots were all within a $10km^2$ area. The DON differed at these plots even on this small scale (Greenfield, 2011). Another study in 2011

compared three sites across the south of England this found a range of DON from 0 nymphs per $10m^2$ to 30.9 nymphs per $10m^{2,}$ (Dobson *et al.,* 2011). In 2012, a large-scale survey across Scotland sampled at 25 sites and found an overall DON of 5.5 nymphs per $10m^2$ (James *et al.,* 2013). A large-scale study was undertaken at four parks in south London in 2015, of these parks two had no evidence of *I. ricinus* being present. At the two remaining parks the DON was only expressed for one this was 2.6 nymphs per $10m^2$ (Nelson *et al.,* 2015). In 2016, another large-scale study in Scotland which sampled 19 woodland sites reported a range of 0.6-11.5 nymphs per $10m^2$ (Millins *et al.,* 2016). These studies show that the DON differs spatially around the UK and have the potential to create "hotspots" for tick biting and LB. These studies reported that vegetation type, and deer density, have been associated with higher DON (Dobson *et al.,* 2011; Medlock *et al.,* 2008; James *et al.,*2013).

Many of these studies tested the nymphs for the presence of *B. burgdorferi* s.l. DNA and found differing rates of infection. In Wales and southern England, 2,737 ticks were tested by PCR for *B. burgdorferi* s.l., in Wales positive samples were not reported, but the ticks in southern England had an infection prevalence of 12.4% (Vollmer *et al.,* 2011). From a study across Scotland, 2,000 nymphs from 25 woodland sites were tested for *B. burgdorferi* s.l. and reported infection prevalence ranging between 0.8-13.9 % (James *et al.,* 2013). Another large-scale study which focused on southern Scotland and northern England tested 2,204 ticks from 11 sites by PCR for *B. burgdorferi* s.l. and a range of 0.0-8.9% *B. burgdorferi* s.l. infection prevalence was reported (Bettridge *et al.,* 2013). In London, nymphs from a large peri-urban park were tested for the presence of *B. burgdorferi* s.l. DNA and it was reported that an infection prevalence of 2.4% was found (Nelson *et al.,* 2015). In 2016 another large-scale study surveying 19 sites across Scotland tested 3,800 ticks reported an infection prevalence range of 0-6.0% (Millins *et al.,* 2016). Most recently a small-scale study in an urban town in southern England surveyed 25 sites for *I. ricinus* of the fourteen sites where *I. ricinus* was present, seven of these sites were found to have infected nymphs and infection prevalence's of between 8.2% -24.3% was recorded (Hansford *et al.,* 2017a). Similar to the DON data the infection prevalence data from around the UK differs showing that *I. ricinus* populations and *B. burgdorferi* s.l. infection prevalence are heterogeneous in the UK and subsequently so is LB risk. These studies also report conflicting results on associations with *B. burgdorferi* infection prevalence. For example, Millins *et al.* (2016) found a negative

correlation with nymphal abundance and *B. burgdorferi* infection prevalence, whereas no such correlation was supported by other work in Scotland. (James *et al.,* 2013). Similarly, James *et al.* found a positive correlation with Roe deer and *B. burgdorferi* s.l. infection prevalence, whereas no significant associations with deer were found by Millins *et al.* (2016).

It is clear from these studies that *I. ricinus* populations and *B. burgdorferi* s.l. infection prevalence differ geographically but what is not clear from these studies is if these patterns remain static overtime. To a lesser extent this has been studied in the UK. DON was shown to differ over time in Wales, from annual sampling tick density generally was increasing over a three-year period although not significantly (Medlock *et al.,* 2008). In southern England no annual increase in DON was found but this study did find a seasonal pattern, significantly more nymphs in spring than in summer or autumn (Hansford *et al.,* 2017a). Exploring infection prevalence over time, in Scotland, six sites were sampled in 2007, 2008, 2012 and 2013 and a difference in infection prevalence over time was reported but was not significant. However, when considering the genospecies proportions at each site significant differences were found over time (Millins *et al.,* 2016).

Four *Borrelia* genospecies have been reported in questing ticks in the UK; *B. afzelii, B. garinii, B. valaisiana* and *B. burgdorferi* s.s. Two other species, *B. spielmanii* and *B. lusitaniae,* have been reported in the UK in ticks taken from animals (Abdullah *et al.,* 2018; Couper *et al.,* 2010), but have yet to be encountered in questing ticks. Genospecies distribution in the UK has shown a different distribution from north to south (Figure 9). For example, *B. burgdorferi s.s.* has only been found in Scotland (James *et al.,* 2013; Millins *et al.,* 2016). *B. afzelii* is the most common genospecies in questing ticks in Scotland (James *et al.,* 2013; Millins *et al.,* 2016), this genospecies is found with less frequency further south in the UK (Vollmer *et al.,* 2011; Bettridge *et al.,* 2013; Hansford *et al.,* 2017a; Layzell *et al.,* 2017). In England, the more dominant species are *B. garinii* and *B. valaisiana* (Vollmer *et al.,* 2011; Bettridge *et al.,* 2013; Hansford *et al.,* 2017a; Layzell *et al.,* 2017).

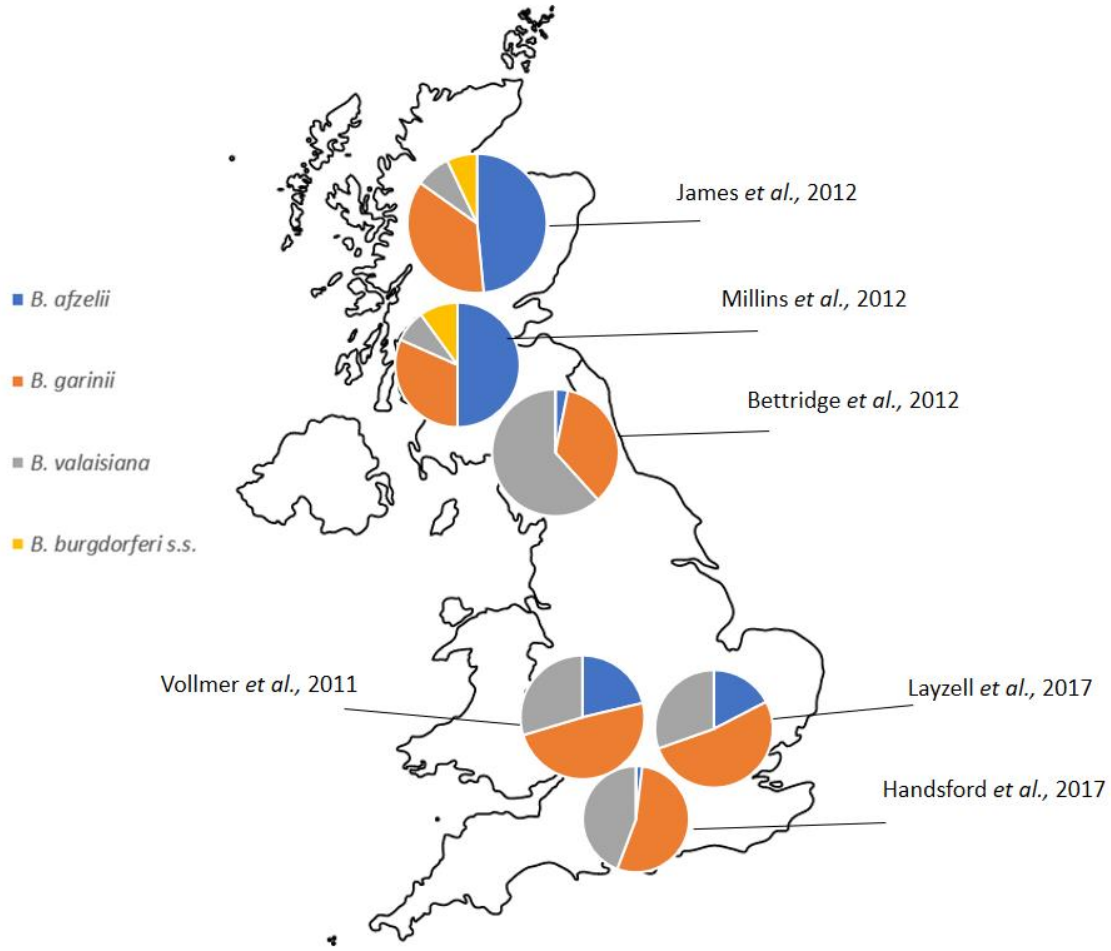

Figure 9. The genospecies distribution in the UK from previous findings in the UK.

In 2014, a relapsing fever group Borrelia, was detected for the first time in questing nymphs from the UK (Hansford *et al.,* 2015). *Borrelia miyamotoi* has been shown to cause disease in humans in Europe (Platonov *et al.,* 2011), but as of yet no cases have been reported in the UK. Since 2017 it has been confirmed in other parts of southern England (Layzell *et al.,* 2017; Hansford *et al.,* 2017a).

The most sought after tool in LB prevention is the ability to predict the risk of LB to humans. As described above the DIN gives a good indication of the environmental hazard to humans but efforts are being made to predict DON and DIN in the future. In the USA a link had been made between the acorn production from oak trees and the risk of LB to humans two years later. The acorn production increases the amount of food available to rodents, this creates a larger rodent population the following year, these rodents are (i) competent reservoir host for *B. burgdorferi* s.l. and (ii) are host for larvae. Therefore, a higher acorn yield in year zero can cause a higher rodent population in year one which feed more larvae who are more

likely to become infected as they are feeding from a competent reservoir host and then they moult to become infected nymphs in year two (Ostfeld *et al.,* 2001*).* Some critics, especially in Europe, have labelled this prediction system too simple as it does not take into account all variables that contribute to LB risk. In Europe there is more than one genospecies of *B. burgdorferi* s.l. which are found in more reservoir hosts and so the acorn system is not complex enough to account for this (Estrada-Pena, 2009; Randolph, 1998). The acorn system has been tested in Poland with the addition of using Internet search data. These results show that after a high acorn year google search terms for "mice" in the first year and "ticks" and "Lyme disease" in the second year increased, suggesting a link similar to that found in the USA (Bogdziewicz & Szymkowiak, 2016). This prediction system still requires sampling from the environment to quantify the acorns. Other computational systems have been shown to be good predictors for tick borne pathogens and are less laborious than field work. NDVI along with ground temperature was a good predictor of tick-borne encephalitis in central Europe and the Baltic regions (Randolph, 2001). As mentioned by Dobson (2013) many studies sample a site once per year and infer from this the abundance of ticks. The analysis from this paper revealed that to correctly predict nymphal abundance throughout a year, sampling must take place ideally at three-week intervals (Dobson, 2013).

The aim of this study is to track the phenology of *I. ricinus* in southern Cumbria and test them for *Borrelia* species. This study aims to identify any spatiotemporal effects on tick population dynamics and in *B. burgdorferi* s.l. and *B. miyamotoi* prevalence, at three different sites, which have different deer densities and differing habitats. This longitudinal monitoring aims to quantify any seasonal patterns in the *Borrelia* genospecies that are found with implications for risk awareness.

3.2 Methods

3.2.1 Sample collection

Three field sites were used to explore *I. ricinus* phenology, *B. burgdorferi* s.l. and *B. miyamotoi* infection dynamics. These sites, Bouth, Chapel House and Linsty Green, lay within 5km of one another in southern Cumbria, which is a known "tick hostspot" and

attracts many tourists. The sites were chosen as they were known to have ticks present. *I. ricinus* was collected at each site at four-week intervals as described in section 2.1.

Chapel House is a remote upland setting (elevation = 200 m) bordered by pine woodland to the north, east and west and a minor road to the south (Figure 10). The habitat can be summarized as open grassland with few trees, the dominant species of tree are Sitka spruce (*Picea sitchensis*), European larch (*Larix decidua*), and Japanese larch (*Larix kaempferi*). Running through the center of the site is a track that is lined with bracken (*Pteridium aquilinum*) and bramble (*Rubus fruticosus ag*.) which then opens out into grassland. Wildlife recorded at this site include; wood mice (*Apodemus sylvaticus*) bank voles (*Myodes glareolus*), badgers (*Meles meles*), foxes (*Vulpes vulpes*), stoats (*Mustela erminea),* grey squirrels (*Sciurus carolinensis*), red squirrels (*Sciurus vulgaris*), red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*). Qualitative data of deer impact was collected each year by the Forestry Commission (FC) it shows that the deer activity is low from 2013-2016.

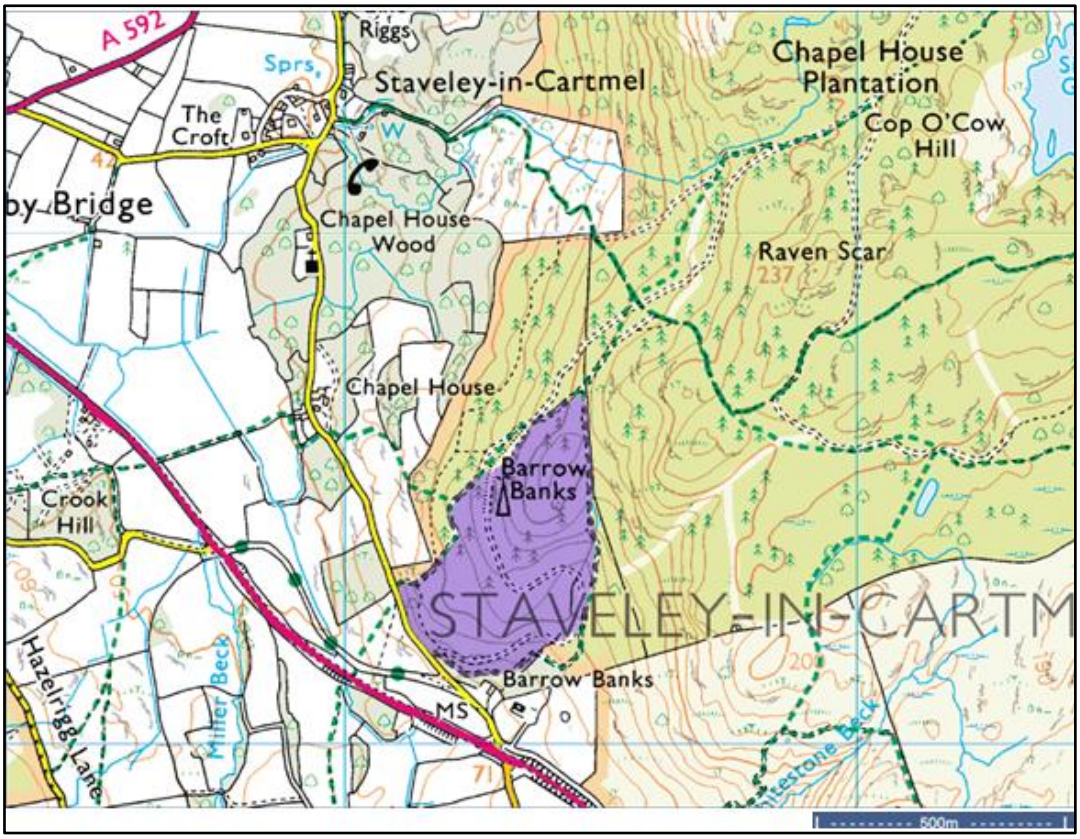

Figure 10. The location of the Chapel House study site (purple) OS grid reference SD 380860 (Ordinance Survey, 2008).

Linsty Green (elevation = 70 m) is denser lowland woodland, which is bordered by a minor road to the north and the east, a stream to the west and more deciduous woodland to the south (Figure 11). Consisting primarily of, grasses, beech (*Fagus sylvatica*), oak (*Quercus spp*), hybrid larch (*Larix eurolepis*), Scotch pine (*Pinus sylvestris*), hybrid poplar (*Populus serotina/ Populus trichocarpa*) and Norway spruce (*Picea abies).* Linsty Green has a dense understory which comprised of mainly bracken (*Pteridium aquilinum*) with bramble (*Rubus fruticosus ag*.) lining the west wall and sporadic bushes of holly (*Ulex europeaus).* Potential tick host that frequented this area included; doormice *(Muscardinus avellanarius),* pygmy shrew *(Sorex minutus),* wood mice (*Apodemus sylvaticus*) bank voles (*Myodes glareolus*), badgers (*Meles meles*), foxes (*Vulpes vulpes*), grey squirrels (*Sciurus carolinensis*), pheasants *(Phasianus colvhicus)* red deer (*Cervus elaphus*), roe deer (*Capreolus*) and many passerine birds. This land is privately owned and a hobby deer stalker controls the deer populations and the FC class the deer in this area to be high.

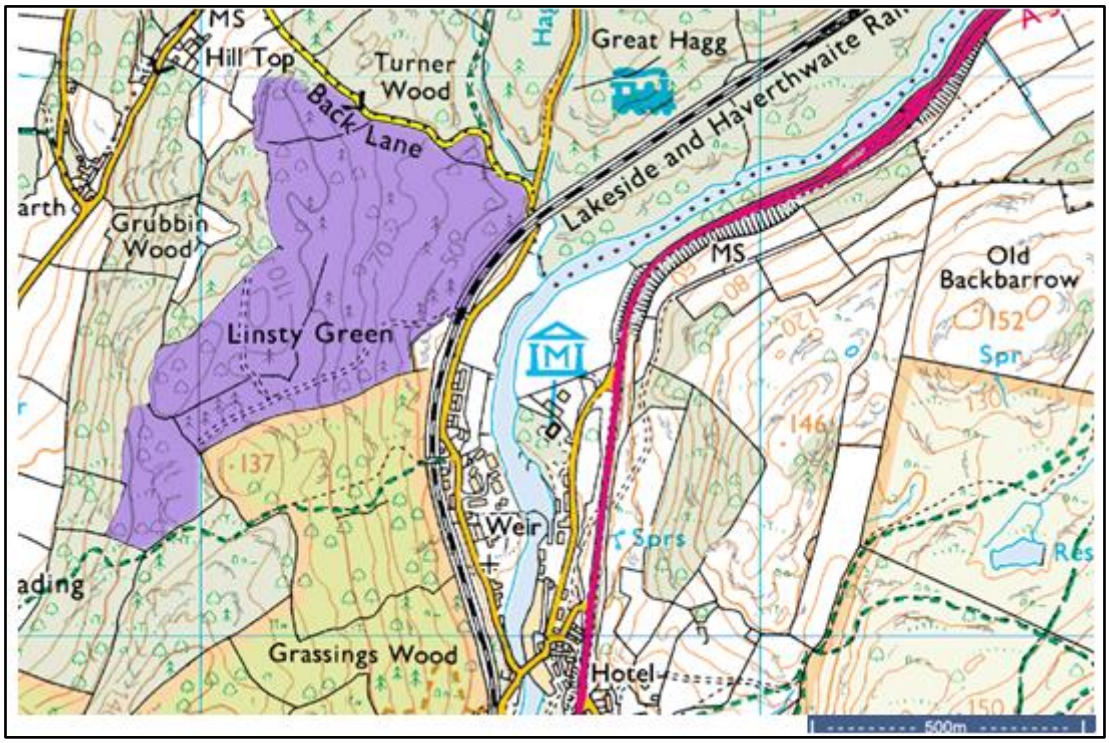

Figure 11. The location of the Linsty Green study site (purple) OS grid reference 353854 (Ordinance Survey, 2008).

The habitat at Bouth (elevation = 90 m) is similar to that at Linsty Green although the tree density is somewhat greater across the site (Figure 12). This site is bordered by a stream to

the north, pasture and a farm to the east, grassland for camping to the west and more deciduous woodland to the south. The area is dominated by European larch (*Larix decidua*), birch (*Betula pubescens/Betula pendula*), and oak (*Quercus spp*). The understory is mainly bilberry (*Vaccinium myrtillus*), with sporadic bushes of holly (*Ulex europeaus)* and patches of bracken (*Pteridium aquilinum)* and grasses. At this site there has been evidence of tawny owls (*Strix aluco)*, wood warblers *(Phylloscopus sibilatrix*), siskins *(Spinus spinus*) and other passerine birds, wood mice *(Apodemus sylvaticus*) foxes (*Vulpes vulpes*), grey squirrels (*Sciurus carolinensis*), red squirrels (*Sciurus vulgaris*), red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*). Qualitative data of deer impact showed that the deer activity is medium for Bouth 2013-2015 and classified as low in 2016.

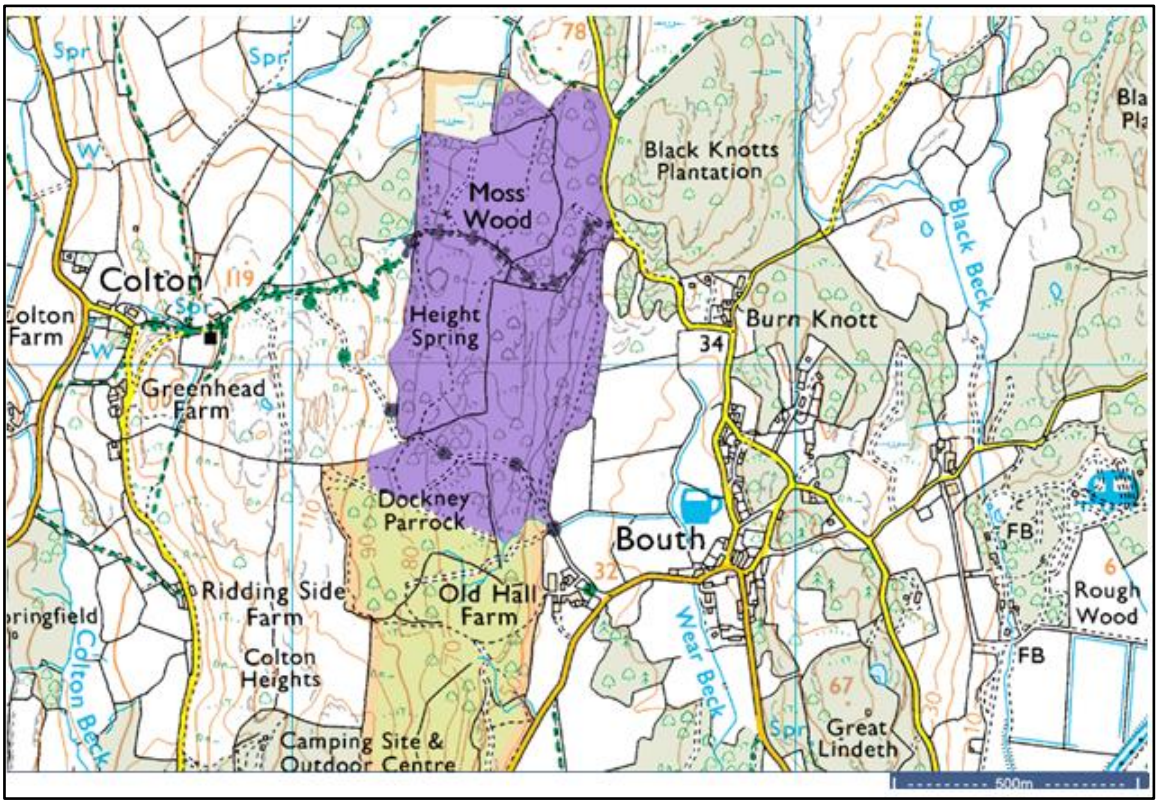

Figure 12. The location of the Bouth study site (purple) OS grid reference SD324863 (Ordinance Survey, 2008).

3.2.2 Sample processing

Nymphs collected from 2013-2016 were tested for *B. burgdorferi* s.l. A maximum of 200 samples per site per month were processed. Nymphs were individually extracted for DNA, using the ammonium hydroxide method as described in section 2.3.1, with cross-contamination controls of tick-less ammonium hydroxide solution included at a ratio of one

for every four tick samples. These extractions were then used as template in a real time PCR assay (section 2.4.1) specific for *B. burgdorferi* s.l. Extracts yielding an amplicon were incorporated into either (i) a nested PCR which targets the 5S-23S rDNA intergenic spacer region (section 2.4.2), (ii) a conventional PCR which also targets the 5S-23S rDNA intergenic spacer region (section 2.4.3), or (iii) a reverse line blot as described in section 2.5. To validate that these three methods of delineating genospecies were in agreement, five samples were processed using all three methods. Once we established that these methods were in 100 % agreement a sample from each genospecies which had been confirmed using sequencing was used as the positive controls of the Reverse line blots.

Samples were tested by method 2.4.3 if they were taken to RIVM for testing (as a cost saving exercise). Samples before 2016 were either taken to RIVM or tested using method 2.4.2. After 2016 the RLB (2.5) was fully optimized and it is cheaper to delineate samples to genospecies using this method.

To test for *B. miyamotoi,* positive samples from the RT-PCR assay for *B. burgdorferi* s.l. that did not yield an amplicon when subjected to the nested PCR targeting the 5S-23S rRNA gene for *B burgdorferi* s.l. were then subjected to a PCR assay that targets the glpQ gene for *B. miyamotoi* which is described in section 2.4.4.

3.2.3 Statistical analysis

Univariate statistical analysis was performed using Minitab 18 (Minitab Ltd, Coventry). The factors associated with the response variable tick abundance (nymphs per 100m) were investigated using generalized linear models (GLMs) which assumed a negative binomial error and a log link. The variables that were considered were maximum/ minimum monthly and daily temperatures, days of frost, hours of day light, all of which were recorded by the Met Office, also included was ground temperature, cloud coverage, site, month, season, time of sampling, deer presence which were recorded by myself on the day of sampling. Deer presence was monitored by how many drags at each site has evidence of deer dung. The factors associated with the response variable *B. burgdorferi* s.l. (positive or not for each

individual tick) were investigated using GLMs which assumed a binomial error and a log link. The variable that were considered were maximum/ minimum monthly and daily temperatures, days of frost, hours of day light, ground temperature, all of which were recorded by the Met Office, also included was cloud coverage, site, month, season, time of sampling, deer presence and tick abundance which were recorded by myself on the day of sampling (Table 4). All analyses were carried out using R 3.4 (R. Development Core Team, 2017) using either the glm.nb function for negative binomial GLMS, or .glm for the binomial GLMS. To test for associations of site with genospecies, multinomial generalized linear mixed model with a cumulative logit link, using multinom in the package nnet**.** Model selection was based on backward stepwise model selection with variables dropped according to p-value, with only those variables significant at the $p<0.05$ level being retained in the final model.

Table 4. Description of variables used in the GLM's

Variable	**Description**
DON	Nymphs per 100m per visit per site collected by operator on day of sampling
DIN	Infected Nymphs per 100m per visit per site collected by operator on day of sampling
Infection prevalence	Presence or absence of *B. burgdorferi* s.l. in each nymph per visit per site
Season	Spring (March, April, May), Summer (June, July, August), Autumn (September, October, November), Winter (December, January, February)
Ground temperature	Temperature taken at the time of sampling by operator on day of sampling
Cloud coverage	The percentage of sky with cloud at the site on day of sampling taken by the operator
Time of sampling	The time of day that site was visited recorded by the operator
Deer presence	Deer dung observed on quantitative drags (score out of 10) recorded by the operator on the day of sampling
Ground condition	Dry, damp, moist, frozen recorded by the operator on the day of sampling
Minimum day temperature	Minimum measurement from weather station on the day of sampling recorded by the Met Office.

Maximum day temperature	Maximum measurement from weather station on the day of sampling recorded by the Met Office
Average monthly minimum temperature	Mean average minimum measurement from weather station for the month of sampling recorded by the Met Office
Average monthly maximum temperature	Mean average maximum measurement from weather station for the month of sampling recorded by the Met Office
Hours of day light	Hours of day light recorded on the day of sampling recorded by the Met Office
Monthly average hours of day light	Mean monthly average of day light recorded for the month of sampling by the Met Office
Monthly average days of frost	Mean monthly average of days of frost for the month of the sampling recorded by the Met Office.

Minitab 18 was used to perform a principle component (PCA) analysis which was used to create an overarching climate variable. The PCA was then used as an explanatory value in the GLMs replace the climate variables provided by the Met office. PCA was performed with data on the day of sampling this included; maximum/ minimum daily temperatures, cm of rain, hours of day light, ground temperature, and cloud coverage. PCA was also performed for monthly data this included mean values for; mean maximum/ minimum monthly temperatures, hours of day light, days of frost, ground temperature, and cloud coverage. As sampling was every four weeks both day of sampling records and monthly records were used to create separate PCAs to examine any differences.

3.3 Results

3.3.1 Density of Nymphs (DON)

Ticks were encountered at all three study sites. Chapel House had the overall lowest DON with an average of 11 nymphs per 100m^2. Bouth had an intermediate DON with an average of 24 nymphs per 100m^2 and Linsty Green had the overall highest DON with an average of 56 nymphs per 100m^2.

At Chapel House the highest peak of activity was in June 2015 with 68 nymphs per 100m² (Figure 13). Nymphal activity peaked in late spring /early summer in all years at Chapel House. Thereafter, the DON gradually fell throughout the remaining months of the year. However, these dynamics varied between years. In 2014 the ticks decreased throughout autumn and in winter no ticks were recorded at Chapel House. In 2015, an increase in tick numbers occurred in early autumn with a decrease into late autumn, and again no ticks were recorded during the winter months. In 2016, the DON peaked in early summer and again in late summer with a third peak in early autumn, tick numbers decreased over autumn and no ticks were reported in the winter.

The peak DON at Bouth was in February 2015 with 78 nymphs per 100m² (Figure 14). Generally, at Bouth a peak in DON would occur in early spring, with another peak occurring in mid-summer, and a third smaller peak occurred in autumn in 2014 and 2015 but not 2016. Nymphs were reported at all sampling visits at Bouth.

At Linsty Green the peak of activity was in June 2016 with 146 nymphs per 100m² (Figure 15), over double the amount found at Chapel House. Linsty Green had a similar phenology for most years where the peak activity occurred late spring, with a dramatic decrease during the summer months and then an increase in activity in early autumn. Nymphs were found at every sampling visit for Linsty Green.

Adults were found in almost a 10-fold lower abundance than nymphs at all three sites. The average density of adults (DOA) at Chapel House was 1 adult per 100m^2. The average DOA at Bouth was 2 adults per 100m^2 and the average DOA at Linsty Green was 7 adults per 100m^2.

At Chapel House the highest DOA was in June 2015 with 12 adults per 100m². The adults showed two peaks, in spring and autumn each year at Chapel House. In 2014 the spring peak was smaller than the second peak in autumn. In 2015 and 2016 the spring/summer peak was larger than the autumn peak.

At Bouth the peak of adult activity was in May 2016 with 8 adults per 100m². In general at Bouth adults peak three times a year in spring, summer and autumn, with the largest peak in spring. Adults were frequently not reported in winter at Bouth and in April 2017.

Linsty Green had the highest DOA of all three sites. March 2016 was the peak of adult activity with 18 adult ticks per 100m². The adult ticks at Linsty Green also had two peaks of activity in each year in spring and autumn with both peaks being of similar quantities. Adults were found at all visits to Linsty Green with the exception of February 2016.

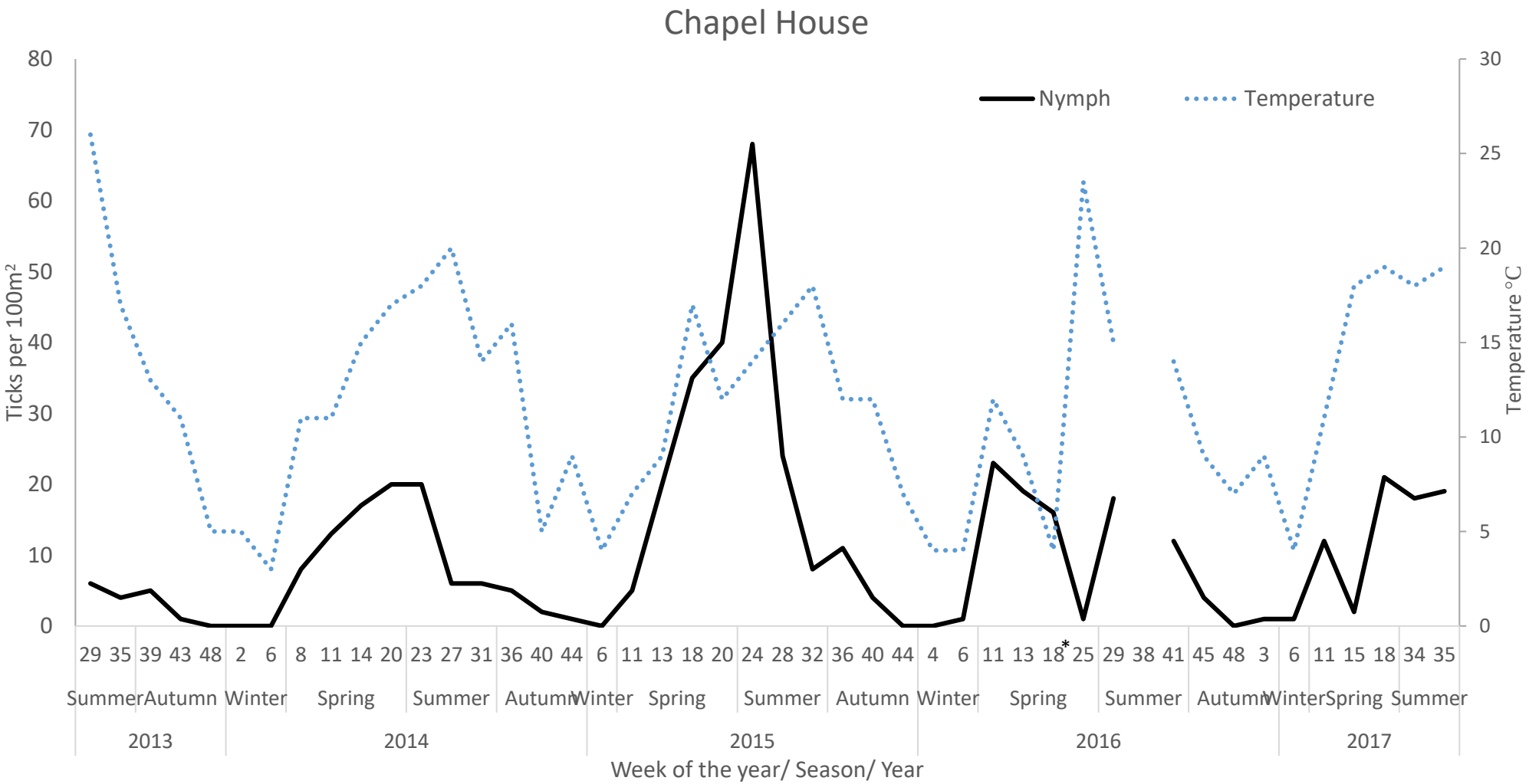

Figure 13. Density of nymphs (Black) per 100m^2 at Chapel House from July 2013 until September 2017 at approximately four-weekly intervals. The air temperature at the site on the day of sampling is indicated as a dashed line, * intermittent hail.

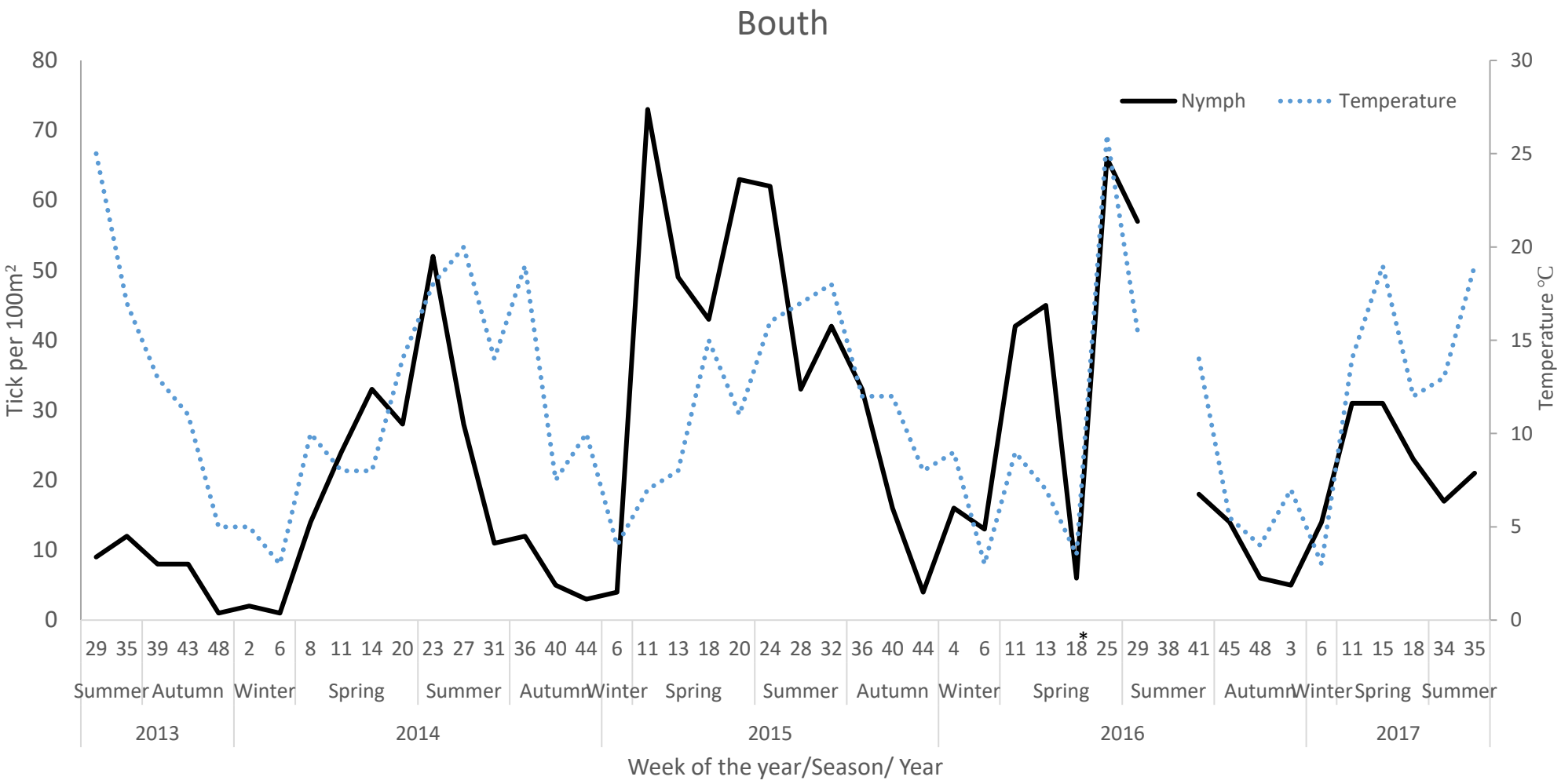

Figure 14. Density of nymphs (Black) per 100m² at Bouth from July 2013 until September 2017 at approximately four-weekly intervals. The air temperature at the site on the day of sampling is indicated in as a dashed line, * intermittent hail.

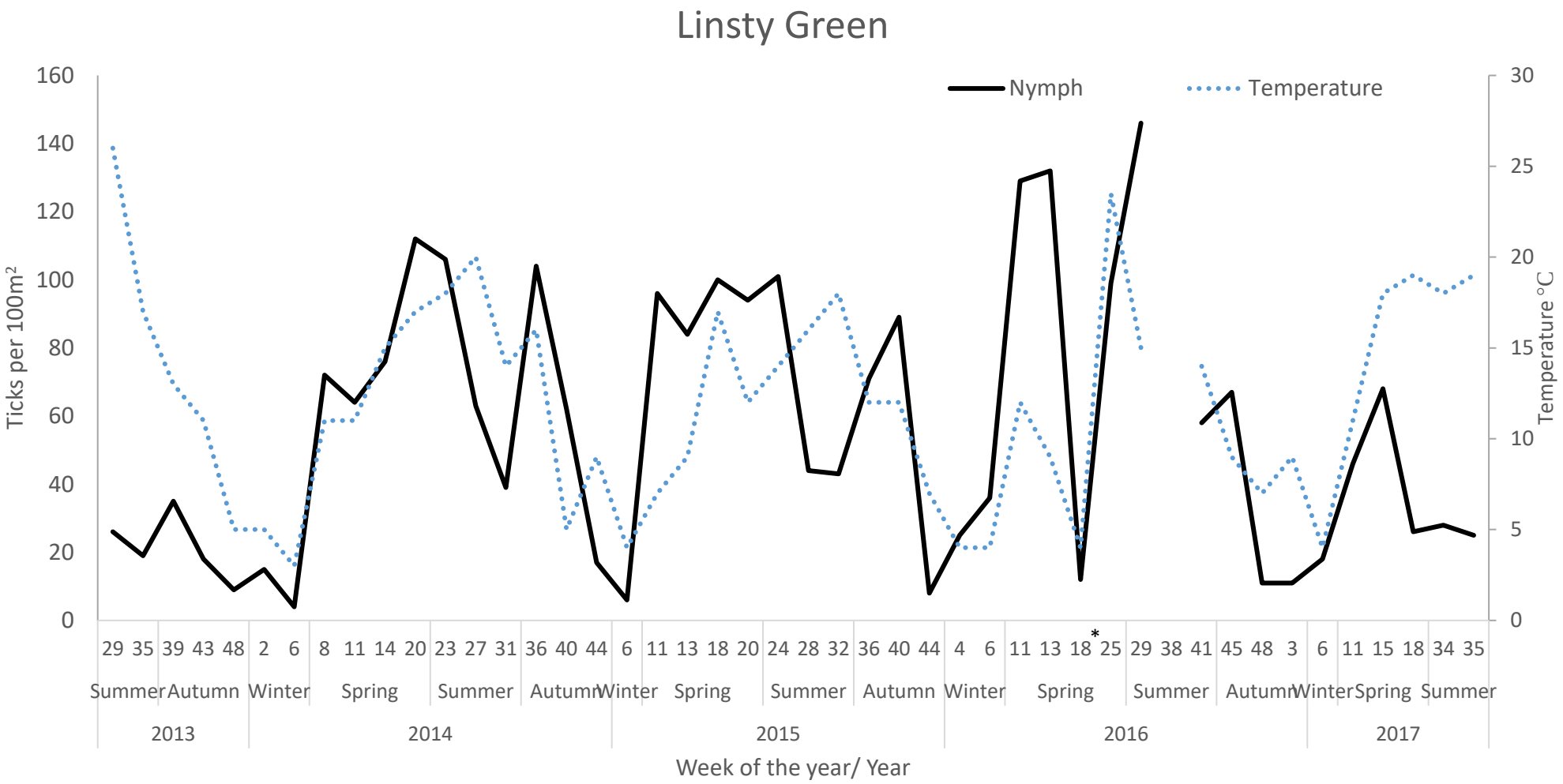

Figure 15. Density of nymphs (Black) per 100m² at Linsty Green from July 2013 until September 2017 at approximately four-weekly intervals. The air temperature at the site on the day of sampling is indicated as a dashed line, * intermittent hail.

3.3.1.1 Univariate analyses of DON

When analysing the overall DON, significant differences between sites were evident, tick density was highest at Linsty Green (Kruskal -Wallis; H value =45.08, d.f = 2, P- value = 0.0001). The overall DOA was also significantly different at all three sites (Kruskal -Wallis; H value = 41.64 d.f = 2, P- value = 0.0001), the highest was at Linsty Green. Overall DON was not significantly different between years (2014-2016) (Kruskal -Wallis; H value =3.04 d.f = 2, P- value = 0.218) nor the overall DOA (Kruskal -Wallis; H value =6.01 d.f = 2, P- value = 0.053). Overall the three sites the DOA was significantly lower than the DON (Wilcoxon = 8368, N = 129, P- value = 0.0001).

When analysed by site, at Chapel House (Kruskal -Wallis; H value =2.09 d.f = 2, P- value = 0.351), Bouth (Kruskal -Wallis; H value =5.02 d.f = 2, P- value = 0.081), and Linsty Green (Kruskal -Wallis; H value =0.07 d.f = 2, P- value = 0.968) no significant difference was found for DON at each year (2014-2016). There was no significant difference found between year and DOA at Chapel House (Kruskal -Wallis; H value =0.71 d.f = 2, P- value = 0.703), but at Bouth (Kruskal -Wallis; H value =10.17 d.f = 2, P- value = 0.006), and Linsty Green (Kruskal -Wallis; H value =8.52 d.f = 2, P- value = 0.014) there was a significant difference between DOA in each year with 2016 having the most adults at Bouth and Linsty Green.

3.3.1.2 Multivariate analyses of DON

Principle component analysis was performed on the climate data to create an overarching climate parameter. These were calculated for the specific day of sampling and then using the mean monthly values. The Eigen values were calculated for each model (Table 5). PCA1 Day and PCA2 Day explained 73.7% of the variation in that data. PCA1 month and PCA 2 month explained 85.8% of the variation in the data.

Table 5. Eigen values and proportion of each model generated using climate data.

	Principle component analysis			
	Day		**Monthly mean**	
Model	PCA1 Day	PCA2 Day	PCA1 Month	PCA2 Month
Eigen value	2.786	1.635	3.083	1.206
Proportion	0.464	0.273	0.617	0.241

Significantly more nymphs were found in spring and summer than autumn and significantly fewer in winter than were recorded in autumn. There were significantly more nymphs at Linsty Green than at Bouth and significantly less nymphs at Chapel House than Bouth. The PCA shows that a day with more hours of sunshine, a higher temperature and less rainfall was associated with more questing ticks. Year was positively associated with tick abundance (Table 6). There was no significant association of DON with deer dung presence, time of day the sampling took place, ground condition (Dry, damp, wet), or the month of the year.

Table 6. Parameter estimates and standard errors for the negative binomial model of DON. Baseline = 2014, Bouth, autumn.

Parameter	Coefficient (SE)	Z Value	Probability > Z value
Intercept	-6.773+02 (1.501e+02)	-4.512	6.42e-06
Year	3.377e-01 (7.449e-02)	4.534	5.80e-06
Spring	1.201e+00 (1.782e-01)	6.743	1.56e-11
Summer	8530e-01 (2.194e-01)	3.889	0.001
Winter	-6.728e-01 (3.013e-01)	-2.233	0.025
Chapel House	-8.343e-01 (1724e-01)	-4.839	1.30e-06
Linsty Green	1.078e+00 (1648e-01)	6.540	6.17e-11
PCA1 Day	3.211e-02 (1.114e-02)	2.884	0.003
PCA2 Day	-6.460e-03 (3.153e-03)	-2.049	0.040

3.3.1.3 Modality of the phenology

At Chapel House the phenology of nymphs was similar each year peaking in spring, declining through summer and then a small rise in autumn (Figure 16). The DON peaked earlier at Chapel House in 2016. Overall at Bouth the phenology peaked later in summer then starts to decline into the autumn (Figure 17). Each year was slightly different at Bouth, 2014 peaked in June rather than May as in 2015 and 2016. At Linsty Green the overall phenology peaked mid-summer with a second peak in early autumn (Figure 18). The second peak always occurred in September but with the first peak occurring in May in 2014 and 2015 and in June in 2016. In 2017, sampling was not undertaken in every month and so data is missing for this year.

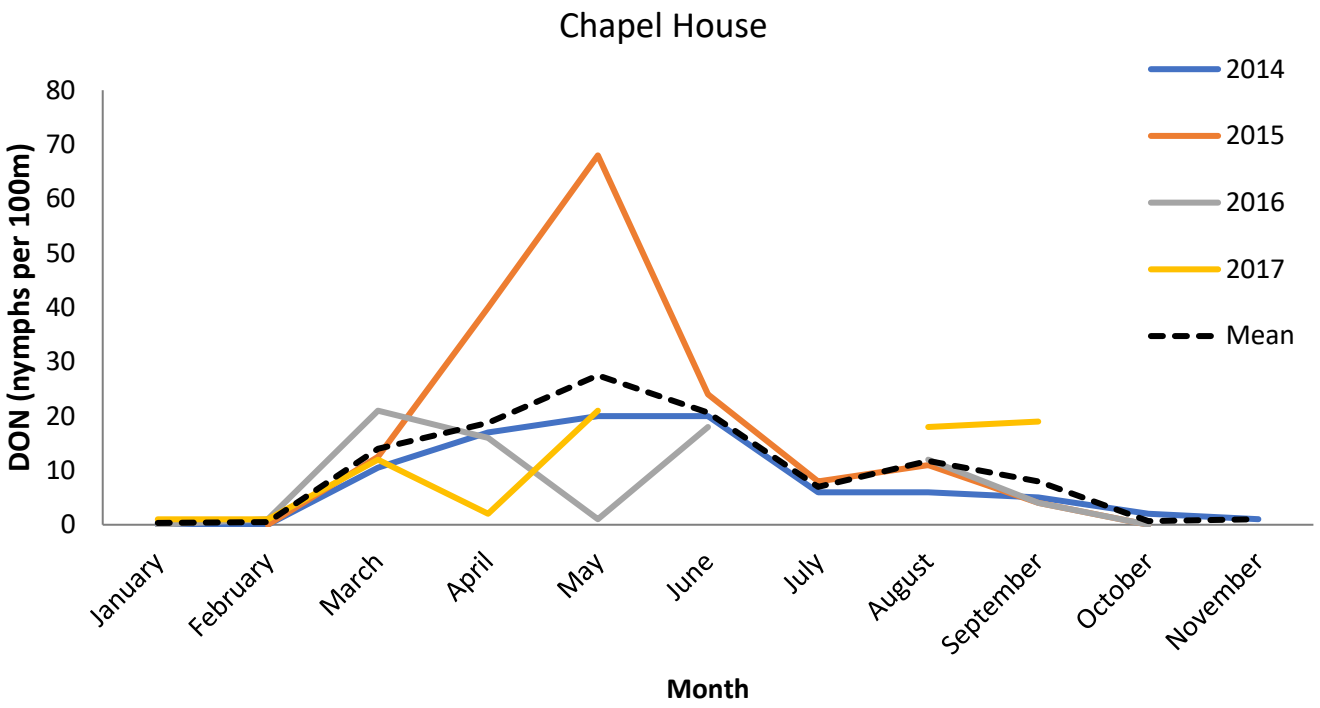

Figure 16. Density of nymphs per 100m² by year at Chapel House

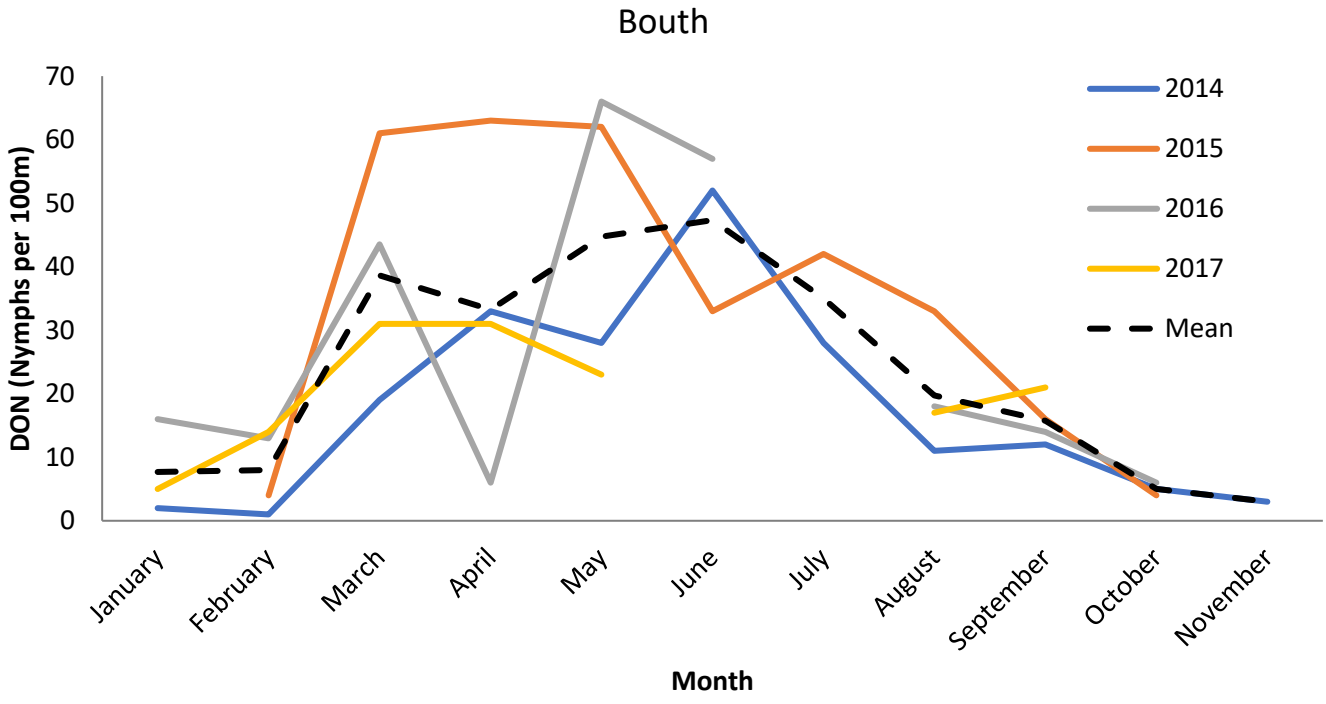

Figure 17. Density of nymphs per 100m² by year at Bouth

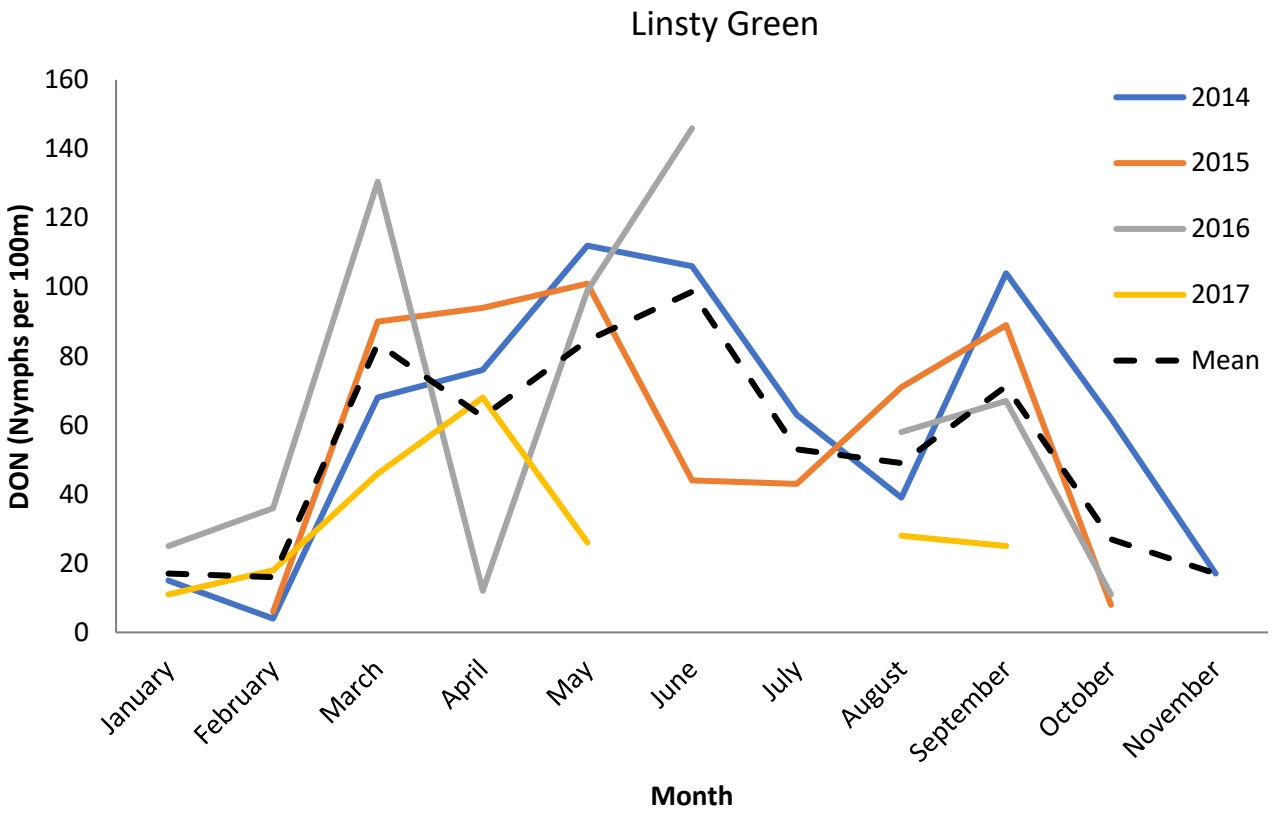

Figure 18. Density of nymphs per 100m^2 by year at Linsty Green

3.3.2 Infection prevalence

B. burgdorferi s.l. was found at all three sites, but not at every sampling occasion. Although samples sizes were sometimes low, even with high samples sizes *B. burgdorferi* s.l. was not found. Overall 561 of the 14,972 (3.75%) nymphs tested were found to contain *B. burgdorferi* s.l. DNA and thus were considered infected.

At Chapel House 255 nymphs of the 3,194 were positive for *B. burgdorferi* s.l. therefore infection prevalence at Chapel House was 7.98% (95% binomial confidence intervals = 7.07% to 8.98%), this was the highest infection prevalence of the three sites. Infection prevalence at Chapel House (Figure 19) fluctuates throughout the year. In general, at Chapel House the infection prevalence is higher in spring and then decreases throughout the rest of the year. In 2013, autumn has a very high infection prevalence along with large confidence intervals. In 2014 infection prevalence rose in spring, and again in autumn. In 2015, a similar pattern occurred although the increase in autumn was lower than that in spring. In 2016, a rise occurred in spring and in early summer. On 11 sampling occasions no infection prevalence was found at Chapel House, all of which occurred in either autumn or winter. For four of

these sampling occasions no ticks were found to be tested, for the remaining seven between 1-160 nymphs were tested (see Fig. 19).

At Bouth 148 nymphs out of 4,995 were positive for *B. burgdorferi* s.l. and so had an overall intermediate infection prevalence of 2.96% (95% binomial confidence intervals = 2.51% to 3.47%). Infection prevalence at Bouth remains stable in 2013 through summer and autumn, no infection prevalence was reported in winter, then increases again in spring 2014. In summer 2014, a rise occurred in infection prevalence with a rise again in autumn. Then again in winter no infection prevalence is found but in mid spring 2015 this increases throughout summer. Autumn and winter 2015 and 2016 the infection prevalence seems to be stable. 2013 and 2014 have higher infection prevalence and then this decreases in 2015 and 2016 (Figure 20). On nine sampling occasions nymphs were tested but no *B. burgdorferi* s.l. DNA was found and thus 0% infection prevalence was found at these sampling points. Samples sized ranged from 2-200 for these sampling occasions.

158 nymphs from Linsty Green were found to be positive out of 6,783. Linsty Green had the lowest infection prevalence of the three sites, with an overall infection prevalence of 2.33% (95% binomial confidence intervals = 1.98% to 2.72%). In 2013, infection prevalence increased in spring and again in autumn, then in the winter of 2014 no infection prevalence was found. Early spring 2014 infection prevalence increased and remained stable until late summer 2014 where no infection prevalence was found. A low infection prevalence was found in autumn but again no infection prevalence was found late autumn 2014. Winter 2015 infection prevalence rose then disappeared again in early spring 2015, then from mid spring 2015 until late autumn 2015 infection prevalence remained stable, a slight increase occurred in late autumn 2015 and the infection prevalence increased in winter 2016 to spring, with a peak infection prevalence occurring in late summer 2016 (Figure 21). No infection was found at five sampling points at Linsty Green where between 7 and 200 nymphs were tested.

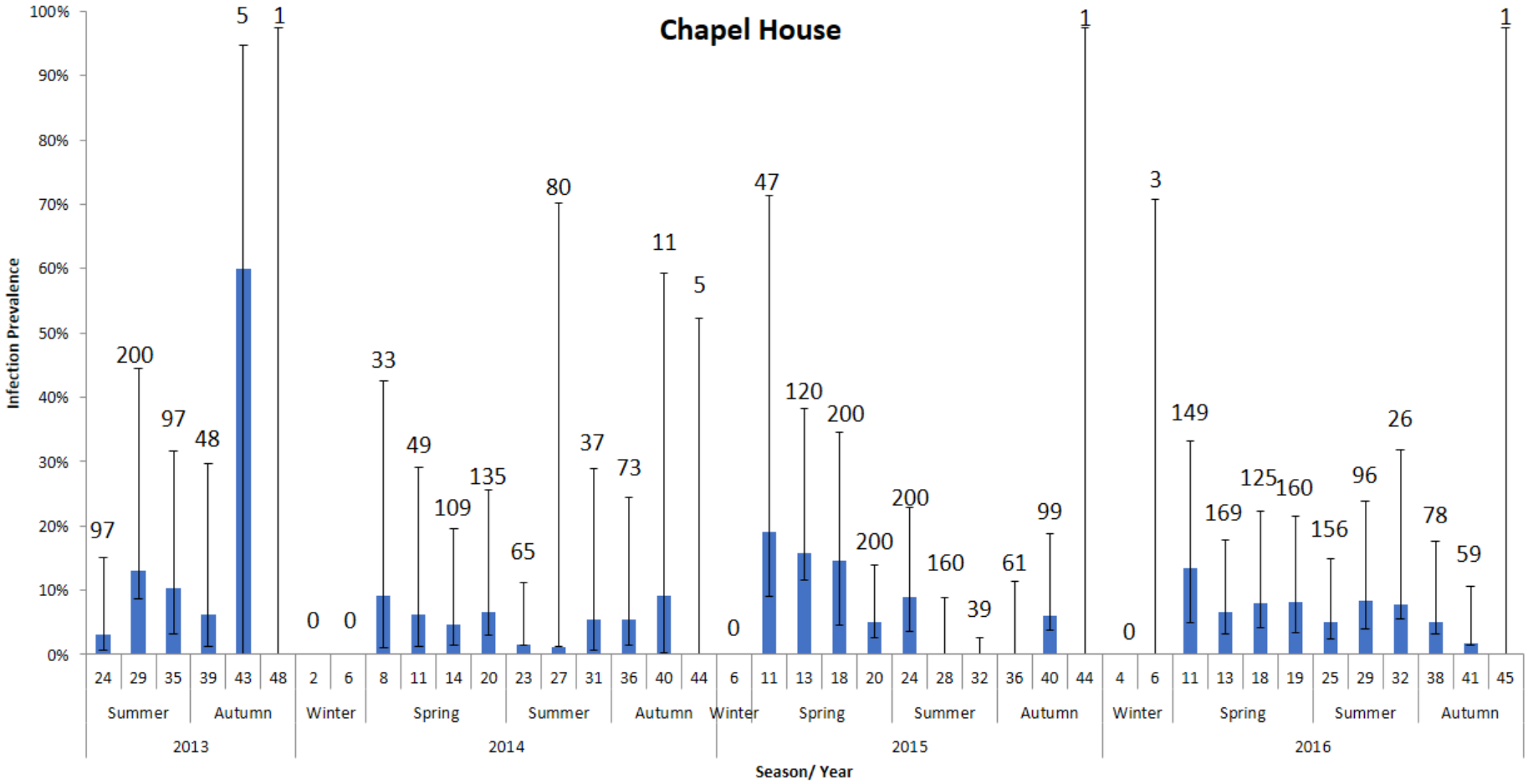

Figure 19. *B. burgdorferi* s.l. infection prevalence in nymphs and 95% exact binomial confidence intervals from Chapel House from July 2013 until November 2016. The number of ticks tested that month is displayed above the bar.

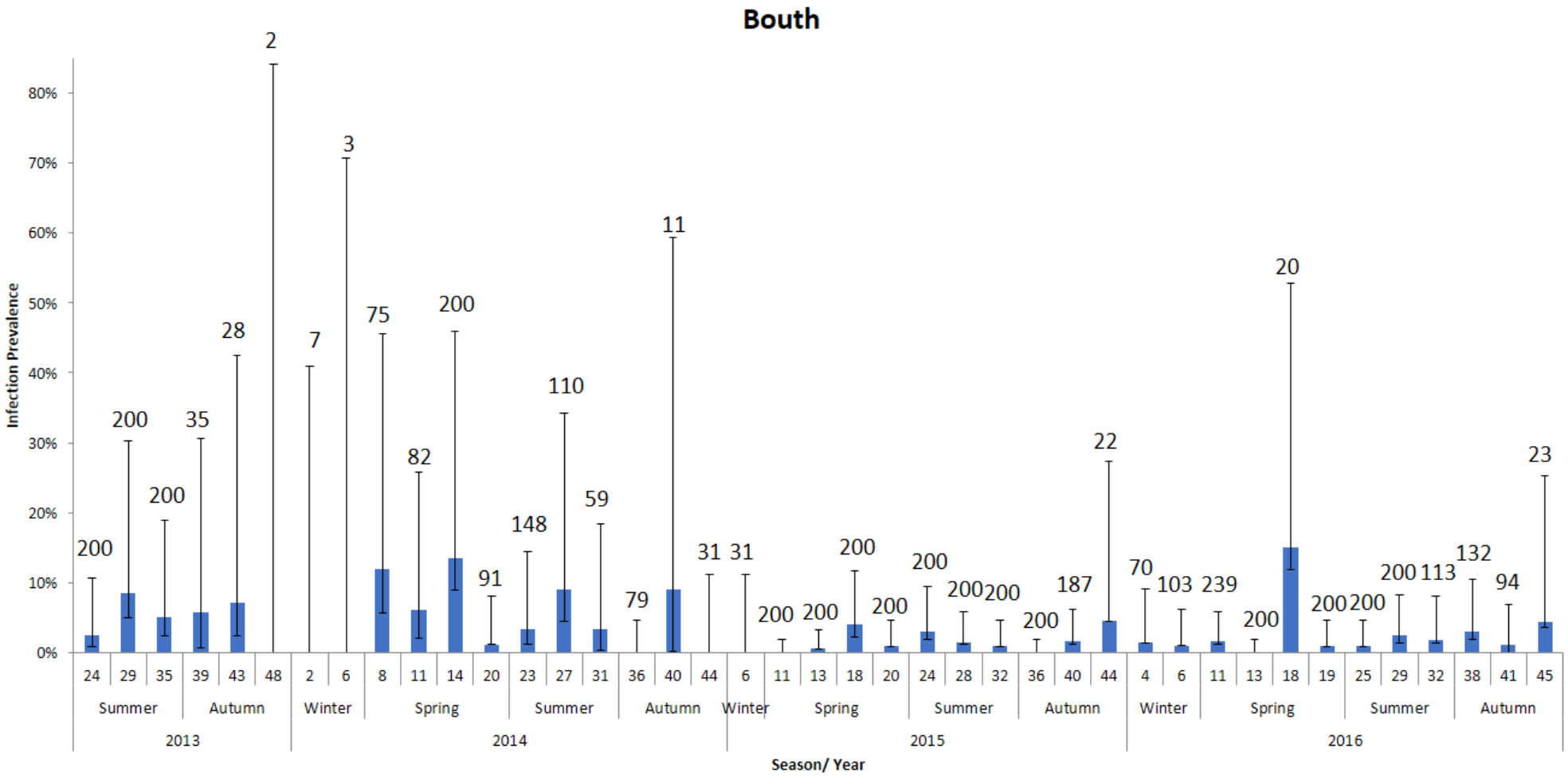

Figure 20. ***B. burgdorferi*** **s.l. infection prevalence in nymphs with 95% exact binomial confidence intervals from Bouth from July 2013 until November 2016. The number of ticks tested that month is displayed above the bar.**

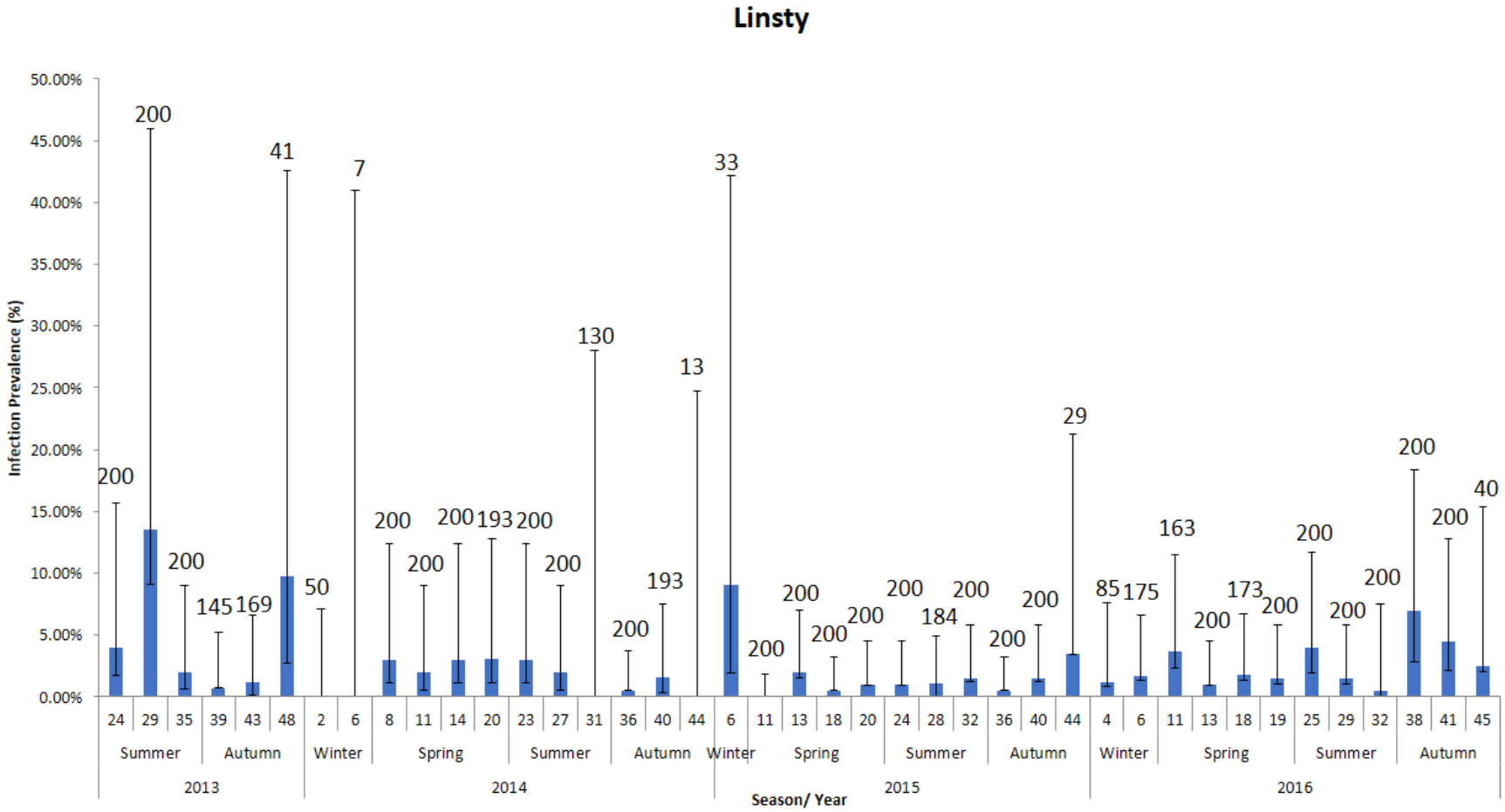

Figure 21. ***B. burgdorferi*** **s.l. Infection prevalence with 95% exact binomial confidence intervals in nymphs from Linsty Green from July 2013 until November 2016. The number of ticks tested that month is displayed above the bar.**

3.3.2.1 Univariate analyses of infection prevalence

When analysing overall infection prevalence there was a significant difference between the three sites (χ^2 = 200.35, d.f = 3, P value = 0.0001). Overall no significant difference was found for infection prevalence and year (χ^2 = 2.1, d.f =2, P value= 0.331) (excluding 2013 which is not representative of an entire year).

When the analysis was conducted by site for Chapel House, in 2015 nymphs were twice as likely to be infected than in 2014 (χ^2 = 11.37, d.f =2, P value= 0.003). At Linsty Green, nymphs from 2016 were significantly higher than those from 2015 but not those from 2014 (χ^2 = 11.70, d.f =1, P value= 0.001), and at Bouth 2014 was significantly higher than 2015 and 2016 (χ^2 = 60.19, d.f =2, P value= 0.0001).

3.3.2.2 Multivariate analyses of *B. burgdorferi* s.l. infection

Nymphs were significantly more likely to be infected at Chapel House, than Bouth (odds ratio (OR) = 2.56, 95% Binomial confidence intervals (CI) = 1.94-3.33) but there was no significant difference between infection prevalence at Linsty Green and Bouth. Nymphs were more likely to be infected in spring than in autumn (OR = 1.71, CI = 1.27-2.33) but no difference was found between summer and winter compared with autumn. Significant negative associations were found with *B. burgdorferi* s.l. infection prevalence and tick density and a negative association was found with *B. burgdorferi* s.l. infection prevalence and deer presence (Table 7). No significant associations between time of day sampling was undertaken, any climate variables, ground condition, or year were found with infection prevalence.

Table 7. Parameter estimates and standard errors for the binomial model of infection prevalence in questing ticks. Baseline = Bouth, autumn, No deer, 1pm.

Parameter	Coefficient (SE)	Z Value	Probability > Z value
Intercept	-3.333 (0.364)	-9.143	<2e-16
Chapel House	0.941 (0.133)	7.065	1.61e-12
Linsty Green	0.010 (0.162)	0.063	0.950
Spring	0.541 (0.155)	3.474	0.001
Summer	0.262 (0.169)	1.549	0.121
Winter	0.110 (0.360)	0.306	0.759
Tick density	-0.002 (0.001)	-2.654	0.007
Deer presence	-0.400 (0.198)	-2.014	0.043
Time of visit (hour)	-0.026 (0.026)	-1.004	0.315

3.3.3 Density of Infected Nymphs (DIN)

DIN at Chapel House was low throughout 2013 and 2014, then increased dramatically in 2015. DIN at Chapel House in 2016 was lower than 2015 but was higher than 2014 and 2015. In all years DIN is higher in spring at Chapel House (Figure 22).

DIN was low in 2013 at Bouth. This increased to its highest spring and summer in 2014, decreasing into autumn and winter. In 2015, the DIN was low throughout the year with the exception of two peaks, one in spring and one in summer. Similarly, in 2016 although the overall DIN was low, there was an increase in spring and summer (Figure 23).

The DIN at Linsty Green was high in July 2013 and then declined for the rest of the year. In 2014 the DIN increases in spring and summer and then decreases over the autumn and winter. The DIN stayed low throughout 2015 and then increased to its highest in 2016, with increases in spring summer and autumn (Figure 24).

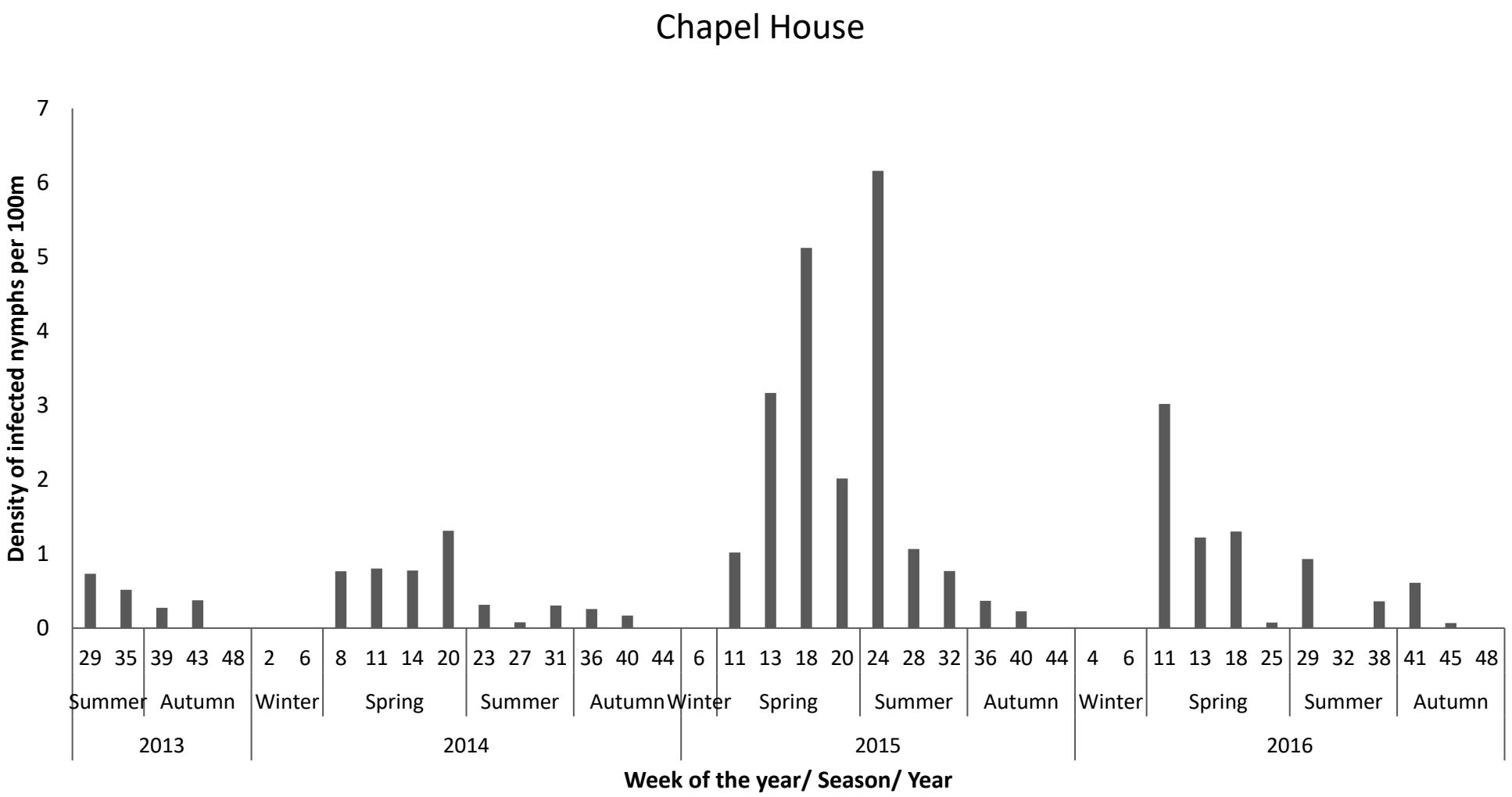

Figure 22. The density of infected nymphs per 100m^2 at Chapel House from 2013 to 2016.

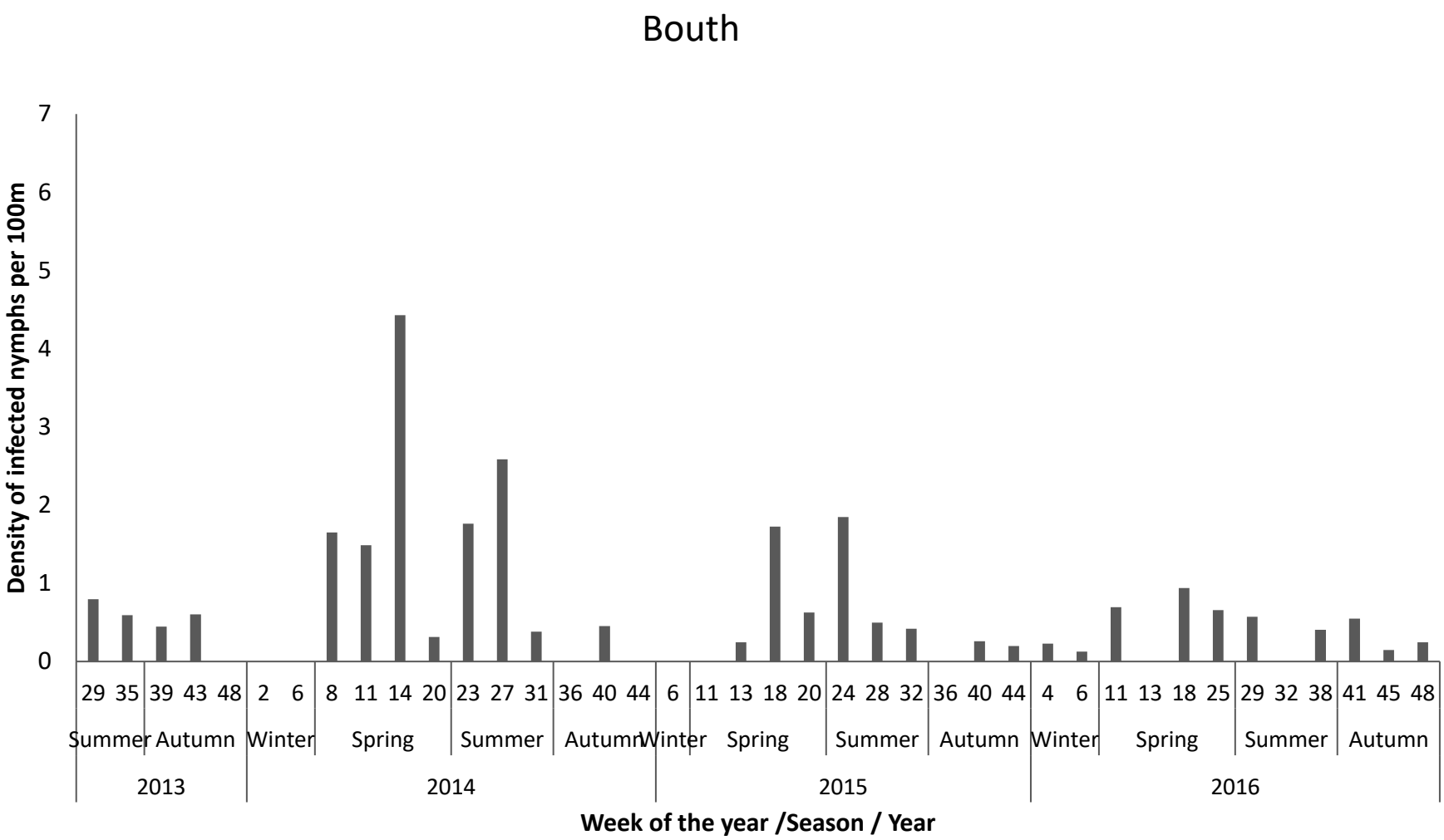

Figure 23. The density of infected nymphs per 100m^2 at Bouth from 2013 to 2016.

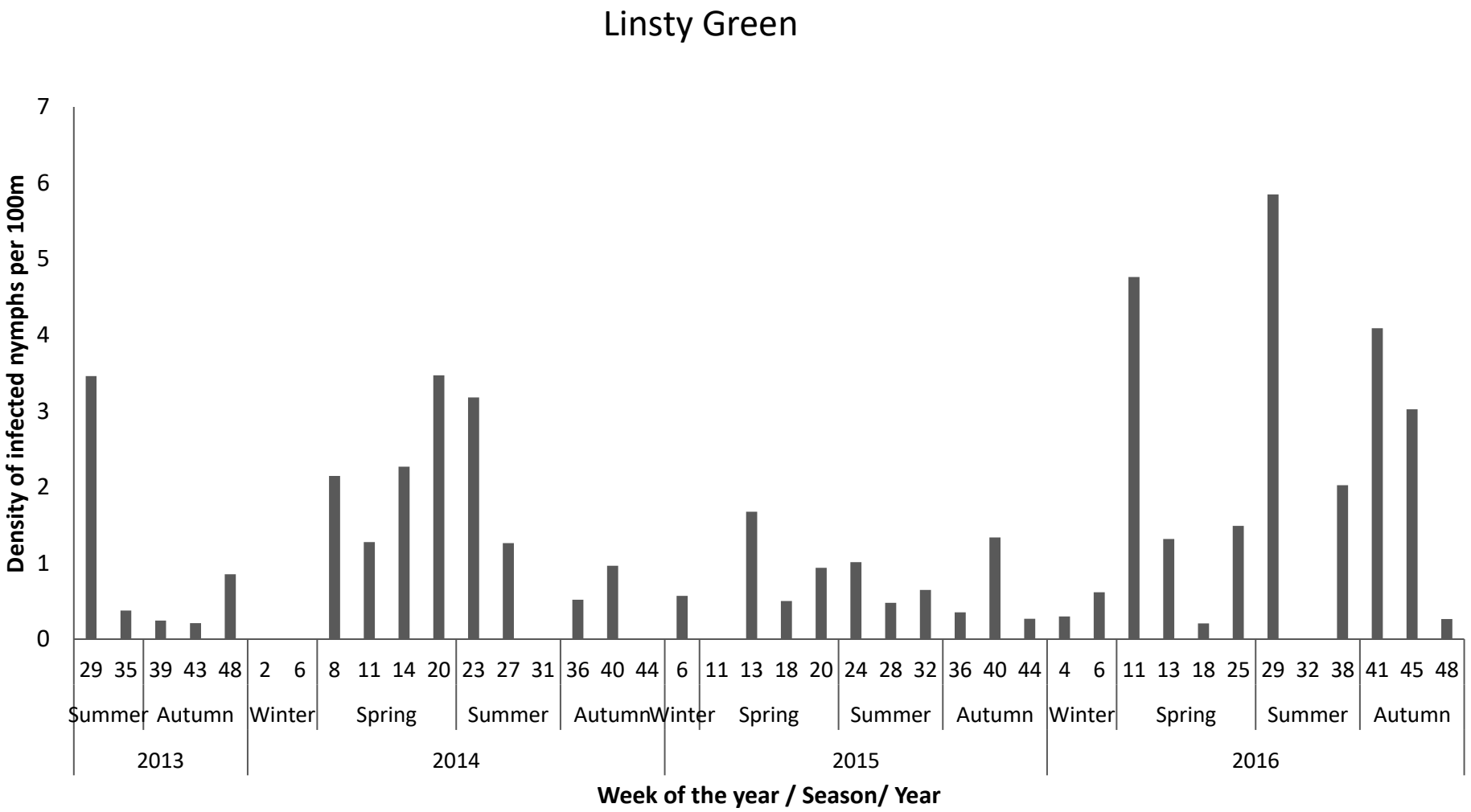

Figure 24. The density of infected nymphs per 100m^2 at Linsty Green from 2013 to 2016

3.3.3.1 Univariate analyses of DIN

When analysing the overall DIN, there was no significant difference between DIN and site (Kruskal -Wallis; H value =5.41, d.f = 2, P- value = 0.067). Overall DIN was not significantly different between years (2014-2016) (Kruskal -Wallis; H value =0.20 d.f = 2, P- value = 0.907).

When analysed by site, at Chapel House (Kruskal -Wallis; H value =3.9. d.f = 2, P- value = 0.140), Bouth (Kruskal -Wallis; H value =0.86 d.f = 2, P- value = 0.649), and Linsty Green (Kruskal -Wallis; H value =2.40 d.f = 2, P- value = 0.301) no significant difference was found for DIN at each year (2014-2016).

3.3.3.2 Multivariate analyses of DIN

DIN was significantly higher at Linsty Green, than Bouth but there was no significant difference between Chapel House and Bouth. DIN was significantly higher in spring and summer than in autumn but no difference was found between winter and autumn. (Table 8). No significant associations between time of visit, any climate variables, ground condition, deer, or year were found with DIN.

Table 8. Parameter estimates and standard errors for the negative binomial model of DIN. Baselines = autumn and Bouth.

Parameter	Coefficient (SE)	Z Value	Probability > Z value
Intercept	-1.338 (0.352)	-3.799	0.001
Spring	1.356 (0.335)	4.043	5.28e-05
Summer	1.210 (0.349)	3.467	0.0005
Winter	-1.063 (0.785)	-1.354	0.175
Chapel House	0.227 (0.310)	0.732	0.464
Linsty Green	0.739 (0.288)	2.565	0.010

3.3.4 Genospecies distribution

Only 236 of the 547 positives were successfully delineated to genospecies. Table 9 summarises the genospecies distribution at each site. At Chapel House all four genospecies were detected including some co-infections. *B. afzelii* dominates this site followed by *B. garinii, B. valaisiana* and *B. burgdorferi* s.s. Linsty Green was also dominated by *B. afzelii* then *B. garinii* and then *B. valaisiana*. *B. burgdorferi* s.s was absent at Linsty Green. Bouth

was dominated by *B. garinii*, then *B. valaisiana* and then *B. afzelii.* A co-infection of *B. afzelii* and *B. burgdorferi sensu stricto* was found at Bouth.

Table 9. Number of samples delineated to each genospecies *B. burgdorferi* s.l. and *B. miyamotoi* at each site (Percentage of total delineated ticks).

Site	*B.a*	*B. g*	*B. v*	*B. s*	*B. a/ B. s*	*B. a/ B. g*	*B. a/ B. m*	*B. m*	Total identified	unknown
Chapel House	106 (67.5)	24 (15.4)	14 (8.9)	5 (3.2)	0 (0.0)	1 (0.6)	4 (2.5)	3 (1.9)	157 (100.0)	58
Bouth	12 (18.6)	31 (48.5)	18 (28.1)	0 (0.0)	1 (1.6)	0 (0.0)	0 (0.0)	2 (3.2)	64 (100.0)	91
Linsty Green	12 (36.4)	9 (27.2)	5 (15.2)	0 (0.0)	0 (0.0)	1 (3.0)	1 (3.0)	5 (15.2)	33 (100.0)	87
Overall	130 (51.1)	64 (25.1)	37 (14.6)	5 (2.0)	1 (0.4)	2 (0.8)	5 (2.0)	10 (4.0)	254 (100.0)	236

B. a = B. afzelii, B. g= B. garinii, B. v = B. valaisiana, *B. s = B. burgdorferi sensu stricto, B. m = B. miyamotoi, B.a/B.s = B. afzelii* and *B. burgdorferi sensu stricto* co-infection, *B.a/B. g = B. afzelii* and *B. garinii* co-infection and *B.a/ B. m = B. afzelii* and *B. miyamotoi* co-infection

Over all three sites the genospecies the proportions remains similar (Figure 25) in proportion of genospecies. *B. afzelii* is the most dominant species in all four years, then, *B. garinii, B. valaisiana* and then *B. burgdorferi s.s.* In 2016 no positive samples for *B. burgdorferi s.s.* were found.

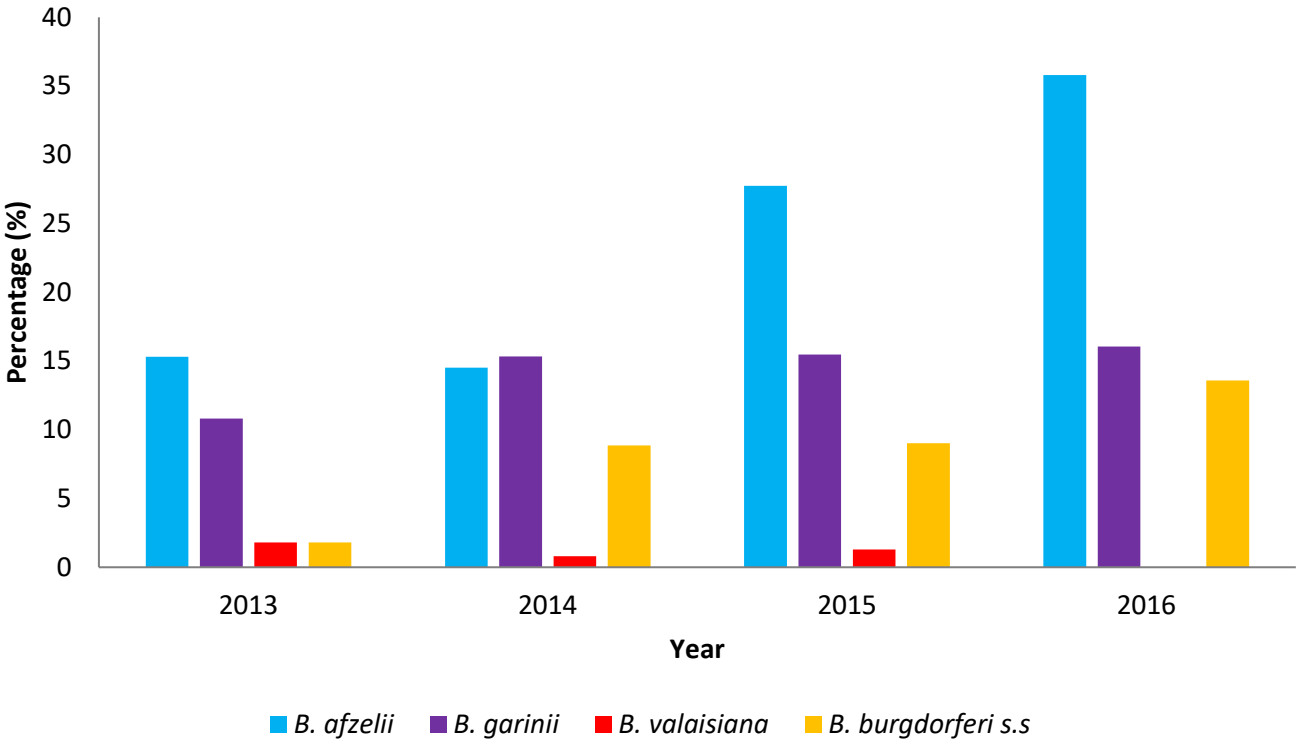

Figure 25. The relative percentage of positive samples comprising of each genospecies by year for all three sites.

Over all monthly distribution of genospecies (Figure 26) shows that from March to October the relative proportion of genospecies is fairly stable. *B. afzelii* is the most common genospecies in all months with the exception of February. Again with the exception of February *B. afzelii* and *B. garinii* were found in all months. *B. valaisiana* was found from February to October. *B. burgdorferi* s.s was only found in March, April, June, July and September.

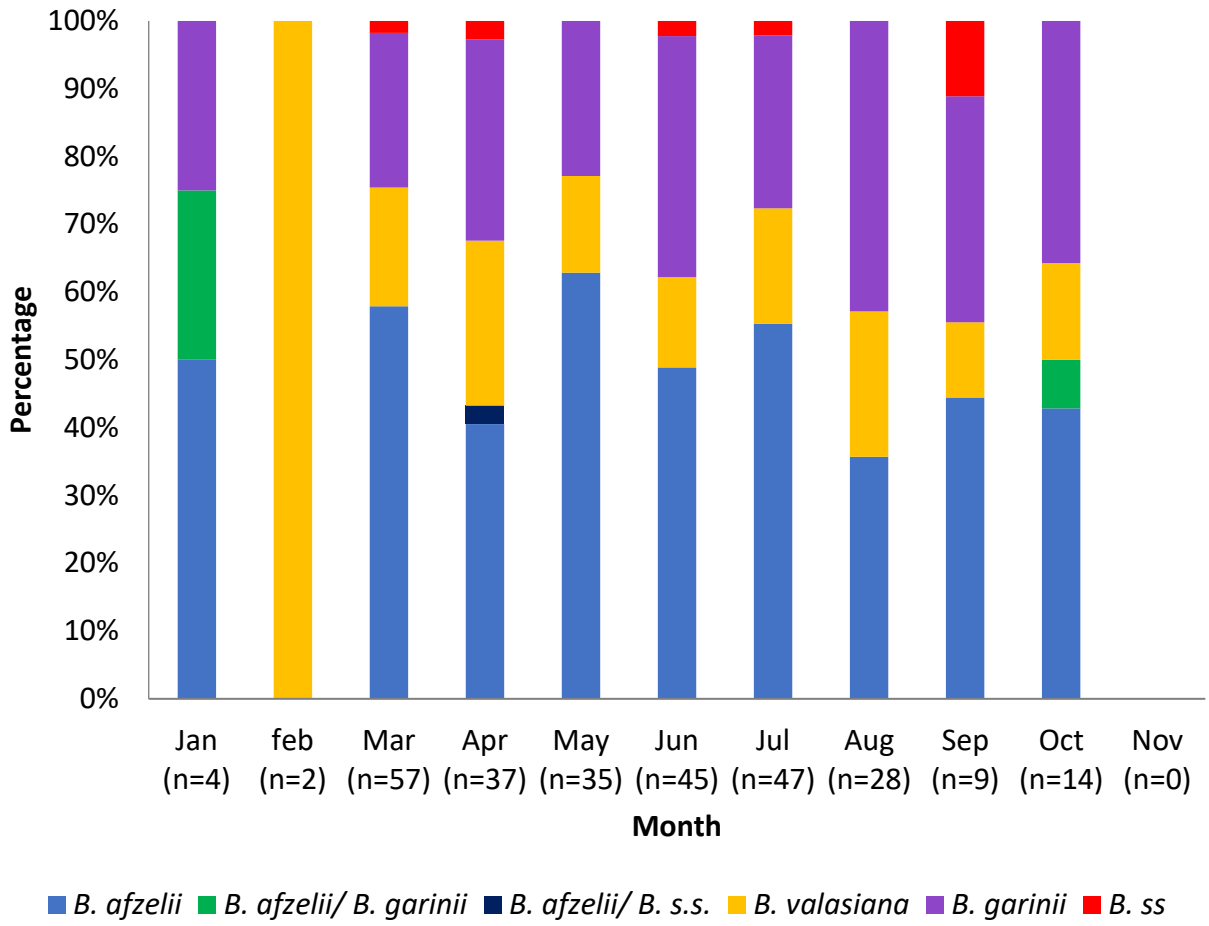

Figure 26. The relative proportion of genospecies at each month. Co-infections are shown as species 1/ species 2.

Table 10. Parameter estimates and P vales for the multinomial generalised linear model of *B. burgdorferi* s.l. genospecies and *B. miyamotoi* data in questing ticks. Baseline = *B. afzelii.*

Genospecies	Intercept			Chapel House			Linsty Green			Spring			Summer			Winter		
	Exp	Z Value	P > Z value	Exp	Z Value	P > Z value	Exp	Z Value	P > Z value	Exp	Z Value	P > Z value	Exp	Z Value	P > Z value	Exp	Z Value	P > Z value
B. garinii	5.568	2.611	0.009	7.355e-07	6.305	2.867e-10	0.240	-2.906	0.003	0.569	-0.950	0.341	0.615	-0.807	0.419	1.628e+27	8.036e+01	0
B. miyamotoi	8.006e-01	-0.192	0.847	1.152e-01	-2.165	3.031e-02	1.375	3.353e-01	0.737	0.339	-1.130	0.258	0.092	-2.120	0.033	1.705e+27	6.476e+01	0
B. burgdorferi s.s.	1.848e-07	-29.505	0.000	6.098e+05	25.350	0.000	0.301	-1.141e+06	0.000	0.298	-0.949	0.342	0.704	-0.289	0.772	8.615e-01	-3.946e+14	0
B. valaisiana	2.527	1.162	0.244	7.465e-07	-5.354	8.574e-08	0.348	-1.916	0.055	0.886	-0.165	0.868	0.886	-0.974	0.329	2.940ee+27	7.874e+01	0

It is significantly more likely for *B. garinii* to be present at Bouth than Chapel House and that *B. afzelii* is more likely to be at Chapel House than Bouth (Table 10). Linsty Green was more likely to have *B. afzelii* than *B. garinii* compared with Bouth. *B. burgdorferi* s.s was significantly more likely to be at Chapel House than Bouth or Linsty Green. *B. burgdorferi* s.s was significantly less likely to be present than *B. afzelii*. *B. valaisiana* was significantly more likely to be found at Bouth than Chapel House. *B. miyamotoi* was significantly more likely to be at Bouth than *B. afzelii* and was more likely to be found in summer. The number of samples that were successfully delineated to genospecies, and in winter, were too few for this model to analyse.

3.3.5 Prevalence of *B. miyamotoi*

The relapsing fever group *Borrelia miyamotoi* was found at all three sites at 1.44%, 1.29% and 4.17% of all positives found at Chapel House, Bouth and Linsty Green, respectively Table 9 shows the number of ticks positive for *B. miyamotoi* and the co-infections found at each site.

3.4 Discussion

3.4.1 Density of nymphs

I. ricinus nymphs and adults were present at all three field sites with nymphs approximately ten times higher in abundance than adults. Although nymphs were found at all three sites, at Chapel House no ticks were found at four sampling points in autumn or winter. At Bouth and Linsty Green ticks were found at all sampling points although all sites saw a decrease in tick density over winter. The phenology of ticks can be explained by a number of factors including; Temperature, hosts density, and day length. As the temperature begins to rise during spring so does the questing ticks if the temperature is too cold the ticks remain inactive, 7°C has been suggested as the temperature when *I. ricinus* begin to quest in the UK, (MacLeod, 1932) then as the temperature rises a larger proportion of the population will quest (Gilbert *et al.,* 2014), until the temperature causes the saturation deficit to increase and cause the ticks to abandon questing to prevent desiccation (Perret *et al.,* 2000). Once the nymphs are questing a percentage will die and others will find a host, therefore there will be less ticks questing hence the first decline if the year. Once the ticks that have fed moult they can quest again in the same year, if conditions are favourable and this is where the second peaks occurs (Milne, 1945). The winter diapause is dictated by

photoperiod so as the days shorten the ticks go into diapause and cease questing (Randolph, 2004b).

All three sites showed a bimodal pattern of tick phenology, similar to that of Randolph *et al* (2002) but differed slightly with the peak of abundance between sites. The air temperatures between each site did not differ significantly, so this is unlikely to cause this difference. Rather than air temperature, microclimate has been suggested to affect ticks as it is responsible for the risk of ticks desiccating (Milne, 1950). Attempts to track the microclimate at each site were unsuccessful as the temperature and humidity loggers were either lost or broken. Spring was significantly associated with a higher nymphal abundance and winter was significantly associated all a lower abundance, which agrees with many other studies (Randolph *et al.,* 2002; Reye *et al.,* 2010).

Tick abundance was consistently higher at Linsty Green; this site is mainly deciduous wood with bracken covering the forest floor. Tick abundance has been reported to be higher in mixed woodland compared to coniferous forest (James *et al.,*2013) bracken, grass, heather (Dobson *et al.,* 2011) pine forest and heath land (Lindstrom & Jaenson, 2003). Leaf litter has been recognized as a determinant in tick survival as it provides ground cover, along with high bracken and moss coverage they provide a refuge for ticks during unsuitable conditions (Greenfield, 2011; Gassner *et al.,* 2011). Chapel House, which consistently gave the lowest yield of ticks, is mainly coniferous wood and grassland where leaf litter is sparse. It is assumed that the influence of vegetation is most likely due to different types of vegetation providing different micro-climates and host associations (Gassner *et al.,* 2011). Different vegetation causes different micro-climates as they create differing relative humidity's therefore different saturation deficit. The tick descends to the ground to rehydrate and so tick survival is based on the ability of the tick to adsorb water in this environment. It should also be recognized that the blanket dragging technique does not work as well on tall vegetation as this does not come into contact with ticks questing further down the vegetation (Dobson *et al.,* 2011).

Different type's vegetation are associated with different host as mentioned above (Gassner *et al*., 2011). This is important for tick densities as host feed ticks and so aid survival by providing the blood meal they need to develop onto the next stage. Host also transport ticks

to a specific area. *I. ricinus* is mainly mobile vertically and although ticks have been recorded walking (moving horizontally), where they move to us usually dictated by where the host is when the tick has completed its feed and drops to the ground to molts (Hasle, 2013). Each host can also carry a differing amount of ticks to an area, for example bank voles have an average of 2 larvae (Bown *et al.,* 2008), whereas a deer can carry an average of 10 larvae (Kniffer *et al.,* 2010).

Climate variables are not the only factor to affect DON. Varying associations have been found between tick abundance and deer. In Scotland a linear relationship was found between deer abundance, measured by the presence of deer dung, and tick abundance (Gilbert *et al.,* 2012). No significant association was found with deer presence and tick abundance in the current study. Deer presence was indicated by deer dung being identified on the quantitative drags and then a score of 0-10 was given for that specific sampling point. There are many limitations to this method such as misidentifying other dung (e.g. sheep) as deer dung, missing dung while concentrating on the dragging technique, dung may have been found elsewhere on the site but this was not taken into account. Therefore, the methods may have affected the results as many other studies show that a higher deer density creates higher tick density (Gilbert *et al.,* 2012; Li *et al.,* 2014; Ruiz-Fons & Gilbert, 2010)

Although, no significant association was found with deer presence and tick abundance qualitative data of deer impact (proxy for deer populations) was collected each year by the Forestry Commission (FC) (personal communication). Interestingly a significant decrease in tick abundance at Bouth was found a year after the deer population was described as low, when it had been described as medium in previous years. This data is only qualitative and so has its limitations, but the results are consistent with the theory that there is an association between deer density and tick density in the following year (Sprong *et al.,* 2012). Chapel house was classified as low deer density and this had the lowest population of ticks and Linsty green was classed as high deer density and had the highest tick density. These results agree with other studies that found a linear relationship with deer abundance and ticks (Gilbert *et al.,* 2012; Li *et al.,* 2014; Ruiz-Fons & Gilbert, 2010).

The annual tick densities (DON) at all three sites significantly increased year on year. Although the currently study is very short compared with some other studies this is consistent with European data, for example in Russia over a 35-year period ticks increased from 0.5 per 1km up to 18.1 per km (Korotkov *et al.,* 2015). In northern Sweden a questionnaire of residents found that tick coverage had doubled over a 10-year period with ticks inhabiting more northerly parts of the country than in recent years (Jaenson *et al.,* 2012). In the Netherlands field studies found a rise in the mean abundance of overall questing ticks of 21.0% from two studies spanning 9 years. However, this was only a 4.0% increase in nymphs and adults, which shows that the majority of the increase was due to larvae (Sprong *et al.,* 2012). It would be interesting to continue monitoring these sites to see if this population increase continues over the years. Perhaps it is a temporary increase due to population cycles of rodents or increasing numbers of deer. More efforts should be put into quantifying the host at these sites alongside the tick numbers to see if there is an association.

The data collected from the questing nymphs at the three study sites gives an indication of when tick numbers are at their highest (April- June) and therefore are more of a hazard to public health. The data reported that although these three sites are no more than 5km apart, the abundance of ticks in one area can differ dramatically to the next. It has been found that sites separated by distances as short as 200m can vary greatly in terms of tick abundance and *B. burgdorferi* s.l. infection prevalence (Wielinga *et al.,* 2006). This shows that local drivers must be causing this affect rather than a variable such as climate that is likely to be the same at these distances.

3.4.2 Infection prevalence

B. burgdorferi s.l. was found at all sites but not consistently throughout the year. When investigating infection prevalence, it is important to have large samples sizes to be confident in the results. If sample sizes are too small this can cause results which cannot be trusted. At Chapel House small sample sizes caused inflated infections prevalence in autumn and winter. *B. burgdorferi* s.l. was present at every time point at Chapel House where ticks were available to test. However, at Bouth there were four time points that nymphs (n=130-200)

were tested but no *B. burgdorferi* s.l. DNA was detected. Similarly, at Linsty Green this happened on three occasions. From a public health point of view, it would be interesting to know what caused this apparent loss of *B. burgdorferi* s.l. at these sites for these small periods of time, as it could possibly be used as a method of prevention.

The overall *B. burgdorferi* s.l. infection prevalence was 3.8% in this study, this is similar to the 3.3% found in another study in northern England (Bettridge *et al.,* 2013). The three sites differed significantly in infection prevalence (1.8% -7.3%). In Scotland, Millins *et al.* (2016) reported an overall infection prevalence of 1.7%, whereas James *et al.* (2013) also in Scotland reported a much higher overall prevalence of 5.6%. Throughout the UK there are studies that found differing infection prevalence at different sites. Site was significantly found to affect the *B. burgdorferi* s.l. prevalence in this study. Chapel House had a significantly higher infection prevalence than Bouth and Linsty Green. Chapel House is a mainly coniferous woodland and so this finding differs with the findings by other studies that higher infection prevalence is associated with mixed woodland (James *et al.,* 2013; Bettridge *et al.,* 2013). Although more recently Millins *et al* (2016) found no significant association with woodland type.

Nymphs found in the spring were found to be significantly more likely to be infected with *B. burgdorferi* s.l. compared with those from autumn. According to Randolph *et al.* (2002) these nymphs would have fed as larvae in the previous autumn. A seasonal pattern of infection has been reported in other countries in Europe. A three-year study in Switzerland, sampling ticks monthly at a deciduous woodland between April and September, used phase contrast microscopy to detect *B. burgdorferi* s.l. spirochetes in 1,800 ticks. The authors observed a peak in infection prevalence of *B. burgdorferi* s.l. in these ticks, the peak occurred in late spring or early summer during all three years. An increase of up to 30.0% was observed. They hypothesized that this was because of higher populations of rodents (which are competent reservoir hosts for *B. burgdorferi* s.l.) in the previous autumn when these nymphs would have fed as larvae (TäLleklint & Jaenson, 1996). Further study should be undertaken at the sites in Cumbria to assess if the rise in *B. burgdorferi* s.l. in spring is associated with rodents.

A significant negative correlation between tick abundance and *B. burgdorferi* s.l. prevalence was found, which is consistent with studies in Ireland (Gray *et al* 1991) and Scotland (Millins *et al.,* 2016). Although another study from Scotland found no significant associations with tick density and *B. burgdorferi* s.l. infections (James *et al.,* 2013). In Switzerland a positive correlation was found with infected nymphs and nymphal abundance (TäLleklint & Jaenson, 1996). This could be due to more ticks feed from incompetent host such as deer which have shown to feed a large number of ticks. If the larval stages are feeding from none competent host then infection prevalence in the nymphs is likely to be lower. In this study deer presence had a significant negative association with *B. burgdorferi* s.l. infection prevalence. This is logical as deer are incompetent reservoir host and so cannot transmit the infections. (Telford *et al.,* 1988). It is thought that the deer population can increase *B. burgdorferi* s.l. infections as they are the reproductive host for adults, but as the deer population increases, more nymphs feed from the deer and so this can cause a dilution effect as less ticks are infected (Rosef *et al.,*2009). In Italy, it was shown that tick borne encephalitis virus (TBEV) occurrence had a negative association with deer but that the number of co-feeding ticks on rodents had a positive association with TBE occurrence. The number of co-feeding ticks on rodents was linked with the deer density as the deer density increased so did the number of co-feeding mice until this plateaued and after which at very high densities of deer this started to decrease (Cagnacci *et al.,* 2012). This shows although deer can cause a dilution effect in disease transmission the deer densities must reach a critical density before this happens.

3.4.3 Density of infected Nymphs

The highest recorded DIN in this study was 6 infected nymphs per 100m^2. This is similar to results found in southern Sweden (Mejlon &Jaenson, 1993, Tälleklint & Jaenson, 1996), France (Voutc'h *et al*., 2016) and the USA (Allen *et al.,*2003), but lower than that found in Switzerland, which ranged from 2-30 infected nymphs per 100m^2 (Jouda *et al.,* 2004). The risk of tick borne disease is defined as the environmental hazard plus human exposure (Dobson *et al.,* 2011). DIN enables areas to be assessed as high or low hazard areas in terms of the environmental hazard of LB. The DIN at the three sites changed temporarily over the three-year period. The data shows that the DIN at each site changed dramatically each year,

although when this was analyzed overall, year was not significant. Another study which found significant annual differences in DIN, attributed this variation to a high variability in nymph abundance (Ruyts *et al.,* 2017; Millins *et al.,* 2016), which was shown in this study. DIN was significantly higher in spring, as was nymphal abundance.

Spatially the DIN also varied, each site had a different level of risk that changed throughout the study. DIN was significantly higher at Linsty Green than at Chapel House or Bouth. A study from France found DIN to be positively associated with roe deer abundance and deciduous woodland (Vourc'h *et al.,* 2016), which agrees with my findings as Linsty green had the highest density of deer and is a deciduous woodland. Ruyts *et al.* (2017) who surveyed 22 forest stands, which consisted of four basic forest types, found consistently higher DIN at oak stands than pine. This again agrees with my findings as Linsty Green is a mix woodland whereas Chapel house is open grassland with pine.

3.4.4 Genospecies distribution

A greater variety of genospecies were found in Chapel House than Linsty Green or Bouth. Although a high proportion was *B. afzelii,* whereas Bouth and Linsty Green had a more even distribution of genospecies. A study from Belgium which tested 3,254 nymphs from 94 forest stands, found that pine stands had lower genospecies diversity compared with oak stands (Ruyts *et al.,* 2016). This disagrees with the results from the present study, where more diversity was found at Chapel House which consists of Pine and open grassland

B. afzelii was the dominant genospecies at Chapel House and Linsty Green but was found at all three sites. *B. afzelii* is associated with rodents (Kurtenbach *et al.,* 1998). This genospecies is the most dominant species in Scotland (James *et al.,* 2014; Millins *et al.,* 2016) and much of Europe along with *B. garinii* (Rauter & Hartung, 2005). In other studies from England and Wales *B. afzelii* is not the dominant genospecies but the least found, next to *B. burgdorferi* s.s (Vollmer *et al.,* 2011; Bettridge *et al.,* 2012; Layzell *et al.,* 2017; Hansford *et al.,* 2017). *B. garinii* is the dominant species at Bouth which has been found in previous studies from southern England (Vollmer *et al.,* 2011). *B. garinii* along with *B. valaisiana* are associated with avian hosts (Kurtenbach *et al.,* 1998; Hanincova *et al.,* 2003). *B. valaisiana* is the dominating *Borrelia* genospecies in central England (Bettridge *et al.,*

2013). The current study sites are situated close to some of those sampled from Bettridge *et al* (2013). These were dominated by a mixture of *B. garinii* and *B. valaisiana*. Although the current study found *B. valaisiana* it was not the dominant species. *B. burgdorferi* s.s. is the least abundant species as it was only found in Chapel House at low levels and in a co-infected tick with *B. afzelii* in Bouth.

Habitat has been associated with genospecies diversity, mostly by indirect associations with host. In Scotland, *B. afzelii* and *B. garinii* were more likely to be found in mixed woodland, whereas *B. burgdorferi s.s* was more likely to be found in coniferous woodland (James *et al.,* 2014), which agrees with my results. In contrast, in Belgium *B. afzelii* was mostly found in pine whereas *B. garinii* and *B. burgdorferi* s.s were more likely to be found in Oak stands (Ruyts *et al.,* 2016). *B. afzelii* was found in both mixed woodland and coniferous woodlands but *B. burgdorferi* s.s was found almost exclusively in coniferous woodland in this study, similar to James *et al.* (2013) Host distribution and composition is likely to be one of the drivers of genospecies distribution this can be connected to the habitat. In Scotland, *B. burgdorferi s.s.* is mainly found in coniferous woods. The authors suggested that this could be due to host distribution, such as red squirrels, although caution is advised when interpreting these results due to the small number of positives (James *et al.,* 2014). *B. burgdorferi* s.s was found to be the most common genospecies infecting red squirrels (Vourc'h *et al.,* 2014) which support this theory. Chapel House is a coniferous woodland, with a known presence of red squirrels and so gives more evidence to uphold this theory. In Scotland although grey squirrels were found to be infected with *B. burgdorferi s.s*, higher proportions of squirrels were infected with Grey squirrels were found to be infected with all *B. afzelii, B. garinii,* and *B. valaisiana* (Millins *et al.,* 2015)

Only 43.1% of the *B. burgdorferi* s.l. positive ticks were delineated to genospecies. This is a limitation to this study. Whilst this chapter has provided good spatial and temporal data for tick abundance and *B. burgdorferi* s.l. infection prevalence, the data on the genospecies is incomplete. Although each positive sample was tested by sequencing or by the RLB and was retested up to three times if no genospecies was assigned, there are gaps in the data. Chapel House is over represented in the genospecies data and so could give a biased view of the genospecies data. Although up to 600 ticks per month were tested this only produced a

small number of positive samples per month. Unfortunately, this means that the temporal data for genospecies distribution is inadequate to make any meaningful conclusions. Small sample sizes over autumn and winter at Chapel House gives a skewed view of the data. Although every effort was made to sample every four weeks the weather caused disruption to this schedule and so no data was collected for December in any year and missed in January in 2015, therefore, winter in 2015 is represented by one month.

3.4.5 *B. miyamotoi*

B. miyamotoi was first reported in the UK in 2014, using qPCR 954 ticks from seven regions in southern England were tested for *B. miyamotoi* and DNA was detected in three ticks from three different regions (Hansford *et al.,* 2015). Since then another report of *B. miyamotoi* in southern England in ticks from four different locations tested by qPCR for *B. miyamotoi* which was detected in ticks from three of these locations (Layzell *et al.,* 2017). This study is the first report of this pathogen in the north of England and was found at all three sites. In the south *B. miyamotoi* has been detected at 0.3% and 0.7% (Hansford *et al.,* 2015; Layzell *et al.,* 2017), which is significantly higher than the low prevalences (0.07%) found in this study. *B. miyamotoi* positive ticks were found in every month apart from August and November and were found in all years (2013-2016) substantiating claims of endemicity in the UK (Hansford *et al.,* 2015). In the current study *B. afzelii* and *B. miyamotoi* co-infections were frequently found. In Switzerland, it has been shown that *Apodemus sylvaticus a. flavicollis* and *Myodes glareolus* can serve as a reservoir host for both these spirochetes (Burri *et al.,* 2014). The levels of *B. miyamotoi* in *I. ricinus* from the UK is very low compared to other reports in Europe which have reported infection rates as high as 3.1% in the Netherlands (Fonville *et al.,* 2014).

3.4.7 Conclusion

In conclusion this study has found many spatial differences in DON, DIN and infection prevalence, were all significantly different at all three sites. These sites are just over 5km apart from each other and the difference is staggering. This means that what drives these differences is a local factor and unlikely to be a variable such as climate which is similar at all three sites. Deer density could be the cause of these differences as each site had a differing deer density with the lowest deer density site (Chapel house) having less ticks but a higher

B. burgdorferi s.l. infection prevalence and the highest deer density site (Linsty Green) having more ticks and lower infection prevalence's. Habitat could also explain the spatial differences, different vegetation has been shown to produce microclimates, tick survival depends on this microclimate as if the saturation deficit is too high then the ticks will desiccate and die. Chapel House was mainly Pine forest and open grasslands which has been shown to have less favourable microclimate conditions due to a lack of leaf litter creating high humidity levels, therefore less ticks survive, and the population is smaller. While Linsty Green and Bouth had a higher population of ticks and both these sites were mixed deciduous woodlands with bracken, which has been shown to produce high humidity which aids tick survival. Habitat and host go hand in hand as the type of habitat can dictate what host can live there. Other host such as rodents could cause the significant difference in *B. burgdorferi* s.l. and DIN as they are competent for *B. burgdorferi* s.l. but these were not quantified during this study.

Temporally, the tick phenologies showed to have a seasonal pattern that peaks in spring and autumn, similar to that already documented further south in England. The tick populations showed to be increasing year on year. To understand what drives this would be interesting it has been shown that deer numbers are increasing in the UK and so it could be due to more deer. Other host such as rodents have shown to have cyclic population increases depending on resource availability if more hosts are available for the ticks to feed on them more ticks will survive. The infection prevalence showed a seasonal pattern being significantly higher in the spring. This is thought to be due to rodent populations with more ticks feeding on rodents in the autumn when their numbers are higher. When looking at the DIN the environmental to human and animal health become apparent. Each site's DIN changed over time and peaked in different year. This could be driven by a number of factors including habitat such as seasonal variation in the vegetation, host associations of the habitat at different times of year and sward height can affect the blanket dragging method and therefore the results. Overall the DIN at Linsty Green which is a high deer site with a mix woodland habitat was significantly higher than Chapel House which has low deer densities and is a pine forest or Bouth which has medium deer densities and is a mixed woodland. Suggesting that deer densities are linked with DIN.

The genospecies was also different at the three sites, with *B. afzelii* dominating at Chapel House and Linsty Green, while *B. garinii* dominated at Bouth. As each genospecies are associated with a different host it is most likely that the ticks from Chapel House and Linsty Green mostly feed from rodents and that those from Bouth feed from Birds.

This study has given evidence to suggest that phonologies and the abundance of ticks are caused by local scale drivers such as hosts and habitat. It has also shown that there is a significantly different spatial and temporal risk to public health across this small area of southern Cumbria which is reflective of the literature reporting from the UK.

Chapter 4: Tick burden and *Borrelia burgdorferi* s.l. infection prevalence in grey squirrels

4.1 Introduction

A key determinant of *Borrelia burgdorferi sensu lato* (s.l.) epidemiology is the vertebrate reservoir host community (LoGiudice *et al*., 2008). The abundance of this community will influence the feeding success of questing ticks whereas the composition of this community will influence the transmission dynamics of *B. burgdorferi* with, in general, transmission being favoured when the relative abundance of competent reservoir hosts is higher. Furthermore, some *Borrelia* genospecies have been shown to exhibit host-adaptation, thus the relative abundance of specific competent reservoir hosts will influence transmission of these genospecies. *B. afzelii* is thought to be specifically adapted to rodents (Hanicova *et al*., 2003), whereas *B. garinii* and *B. valaisiana* are preferentially amplified in birds (Kurtenbach *et al*., 1998). Although there is evidence of rodent associated genospecies detected in ticks from birds (Heylen *et al*., 2010) and bird-associated genospecies in ticks from mammals (Millins *et al*., 2015; Pichon *et al*., 2005). Other genospecies such as *B. burgdorferi sensu stricto* (s.s.) apparently lack this specificity thus are considered as generalists (Kurtenbach *et al*., 2006), although recent evidence from the USA suggests that *B. burgdorferi* s.s. consists of sub-populations that are associated with specific host species (Mechai *et al*., 2016).

Invasive species can disrupt an ecosystem directly through competition of resources (Williamson, 1996) and indirectly by damaging the habitat or disrupting a food web (Thoresen *et al.,* 2017). The eastern grey squirrel (*Sciurus carolinensis*) was introduced from North America in the 1930s and has since has replaced the native red squirrel (*Sciurus vulgaris*) in England and Wales (Mayle & Broome, 2013). It has been hypothesised that the grey squirrel's invasion was so successful due to direct aggression between the species, habitat changes being more profitable to the gey squirrel (Reynolds, 1985) and that the grey squirrels introduced squirrel poxs which caused a rapid decline in red squirrels (Sainsbury *et al.,* 2008). Many examples of invading species bring disease with them have been recorded in the literature e.g. white tail deer (*Odocoileus virginianus*) invading westwards in the US taking with it the menigial worm (*Parelaphostrongylus tenuis*) (Jacques & Jenks 2004) and European settlers bringing small pox to North America (Patterson & Runge, 2002). In North America only *B. burgdorferi* s.s circulates unlike in the UK where four *Borrelia* genospecies

persist. A recent investigation of *B. burgdorferi* s.l. in grey squrrels suggested that these invasive species are sceptable to all four *Borrelia* genospecies (Millins *et al.,* 2015). This means that grey squirrels are not only reservouir host for the rodent associated *B. afzelii* but for *B. garinii, B. burgdorferi s.s.* and *B. valaisisana.* Therefore they could affect the distribution of genospecies within an ecosystem.

The distribution of *B. burgdorferi* s.l. is not only defined by competent host but also by non-competent hosts. For example, deer are a non-competent reservoir hosts for *B. burgdorferi* s.l. but are reproductive host for the main vector in the UK, *I. ricinus* (Kiffner *et al*., 2010). Deer management such as culling or fencing to keep deer from entering has been shown to significantly reduce tick numbers (Gilbert *et al*., 2012), however it has been shown that lower deer density can be correlated with a higher infection prevalence (Gray *et al*.,1999). As transovarial transmission *of B. burgdorferi* s.l. appears to be very limited (Richer *et al*., 2012) the ticks primarily acquire infection whilst taking a blood meal, provided this blood meal is taken from a competent, infected host. The spirochete is then trans-stadially sustained by the tick through the moult so that it is infectious in its next life stage and thus able to transmit infection to the next vertebrate it feeds on (providing the host is susceptible to the pathogen).

Many studies have looked at host competence. Some host have been categorised as dilution host and some as amplification hosts. Results have found that deer can eliminate *B. burgdorferi* s.l. from the tick possibly by borreliacidal antibodies in the deer blood (Lacombe *et al*., 1993), therefore deer can be considered a dilution host for *B. burgdorferi* s.l. Whereas other host are amplifying host which increase the amount of *Borrelia* spp in the environment through high tick burden and host competency (Matuschka *et al*., 1994; Heylen *et al.,* 2015). Deer have been shown to not only dilute *B. burgdorferi* s.l. in questing ticks but also to increase it as they feed adult ticks which in turn produce larvae and if there are more larvae in an area then there are more ticks to become infected after feeding from a competent host (Cagnacci *et al.,* 2012). Studies have shown positive associations between deer density and infection prevalence (Rizzoli *et al.,* 2002; James *et al.,* 2013) but also negative effects (Gray *et al.,* 1992; Mysterud *et al.,* 2013; Millins *et al.,* 2016). The studies

which recorded a negative effect may have had more deer and therefore the larvae had to feed from incompetent hosts rather than competent host.

Ticks are also able to acquire infection by "co-feeding", a process in which borreliae are passed directly from infected tick to uninfected tick when they are feeding simultaneously on the same host, even if the host itself is not systemically infected (Randolph *et al*., 1996; Ogden *et al*.,1997). This shows that the density of ticks carried by a host is also important. Larger mammals such as deer have been shown to carry up to 10 times more ticks than small mammals (Cull *et al*., 2017; Vor *et al*.,2010) and this is reflected in the blood meal source of questing ticks with the highest proportion of ticks feeding on artiodactyls (Cadenas *et al*., 2007).

Each site in the longitudinal study (Chapter 3) had a variety of animals living there. The combination of animals is dependent on natural influences such as availability of food and suitable habitat and by human intervention, forest management and introducing new species. When a new host species is introduced to a community it is argued this can have one of two effects on the host-pathogen interaction. On the one hand it is thought that the new host could cause an increase in the infection prevalence as it is an amplification host. On the other hand, it is thought that a new host could reduce the infection prevalence as it is a dilution host (Keesing, Holt & Ostfeld, 2006). Rodents have been shown to act as dilution host for *Bartonella* species in Ireland. In areas where invading bank voles were present a decrease was detected for the prevalence of two *Bartonella* species in wood mice in the same area. This was not observed in areas where the bank vole was absent (Telfer *et al*., 2005).

Eastern grey squirrels were first introduced into England in 1876 from north America. Between 1876 and 1929 it is thought that grey squirrels were first introduced from country estates who imported them as exotic pets. In England and Wales they were introduced from around 30 estates and three estates in Scotland (Mayle & Broome, 2013) In 1946, a questionnaire sent to park managers showed that grey squirrels were present in 29% of all parishes in England and Wales (Shorten, 1946). Since then grey squirrel have colonised

much of England, Wales and southern Scotland (Figure 27) (Okubo *et al*., 1989; https://species.nbnatlas.org/species/NHMSYS0000332764, accessed on 25/5/2018). The grey squirrel is viewed as a pest as it damages broadleaf woodland and has been linked to the decline of the red squirrels. Today the Forestry Commission estimate that there are around 15, 000 red squirrels in England and over 2 million grey squirrels (https://www.forestry.gov.uk/squirrel-damage, accessed on 25/05/2018).

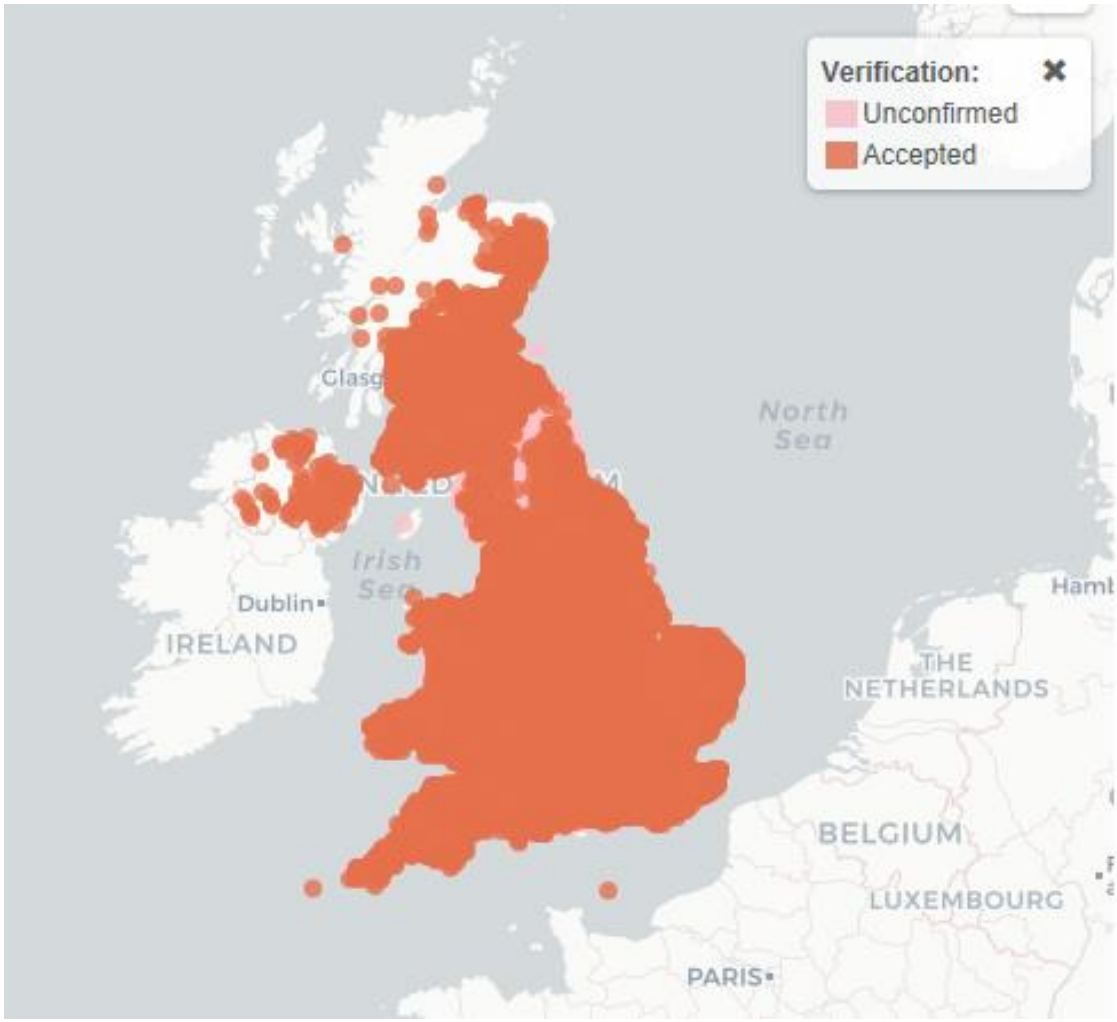

Figure 27. The current distribution of grey squirrels (https://species.nbnatlas.org/species/NHMSYS0000332764# accessed on 25/5/18)

Grey squirrels are competent reservoir host for *B. burgdorferi* s.l. Exploration of the impact of grey squirrel invasion on *B. burgdorferi* s.l. enzootic cycles in the UK has already begun. Craine *et al*. (1995) compared tick abundance from grey squirrels with wood mice (*Apodemus sylvaticus*). They found grey squirrels fed ten times more larvae than wood mice in the spring and summer months and carried a large number of nymphs. Squirrels are

thought to carry a high density of ticks because of their diurnal nature (which means they are more likely to come across questing ticks as ticks have been shown to quest in day light hours) and that they cover a greater area when foraging. Then in 1997, grey squirrels were trapped, and using xenodiagnosis, it was demonstrated that the grey squirrel is an amplifying and reservoir host *B. burgdorferi* s.l. From these squirrels a "wild" infection (a squirrel was already infected with *B. burgdorferi* from the environment) was found, which persisted for 11 weeks, and was characterised as *B. afzelii* (Craine *et al*,. 1997). More recently a study in Scotland detected *B. burgdorferi* s.l. DNA in 11.9% of the grey squirrels tested, this study found a range of genospecies in these squirrels (Millins *et al*., 2015). Feeding a large number of larval and nymphal ticks as well as being a competent reservoir host gives the grey squirrels potential to be an important reservoir host.

In Cumbria, as in other parts of Britain that have both red and grey squirrels present, grey squirrels are being culled to protect the native red squirrel and prevent further damage to woodlands. Small pockets of grey squirrels have been reported in Cumbria as early as 1930 (Lloyd, 1983). A recent study has shown that grey squirrels are infected with many different genospecies of *B. burgdorferi* s.l. (Millins *et al.,* 2015). This study will explore if grey squirrels from Cumbria are also infected with numerous *B. burgdorferi* s.l. genospecies and I aim to quantify the transmission of each genospecies to the feeding larvae to explore if grey squirrels are filter host for specific genospecies. Nymphs from the environment where the squirrels were caught will also be tested to determine if a specific genospecies is filtered in to the environment. Alongside this tick burden will be assessed for each animal to see if any host traits have an association with parasitism.

4.2 Methods

4.2.1 Collection of squirrels and ticks

Grey squirrel carcases were provided from five different areas of Southern Cumbria by the Forestry Commission as part of conservation efforts to reduce numbers of grey squirrels (Figure 28). The squirrels were trapped using humane squirrel traps and killed according to

UK legislation (shot). Immediately after death, individual animals were placed in sealed plastic bags and stored at -20°C until processing. Trapping was carried out between March to August each year from 2014-2017. Questing ticks were collected at 12 sites in the vicinity (within a 2-mile radius) of where the squirrels were trapped (Figure 28). Questing nymphs were collected by using a standard blanket dragging technique as described in section 2.1. Unfortunately, no questing ticks could be collected at Foulshaw Moss, due to site restrictions. Two sites represent the squirrels from Eggerslack wood, these ticks were collected in 2017. Squirrels from Lakeside were from a large area and so eight sites were used to represent these squirrels, these were collected in 2015. Arklid and Grizdale are represented by one site each and were collected in 2014 (Table 11). A maximum of 210 nymphs from each site.

Table 11. Details which questing ticks represent each subset of squirrels.

Squirrel batch (on map)	**Corresponding questing ticks**	**Year**
Grizdale (GRIZ)	Griz	2014
Arklid (ARK)	Ark	2014
Lakeside	RL, Ford, Ent, HD, TH, FIN, GH and CH	2015
Eggerslack wood (Egg)	Egg and HF	2016
Foulshaw Moss (FSM)	None	2016

Figure 28. The locations where the squirrels were trapped. Questing ticks were collected from woodlands where the squirrels were trapped and in neighboring woodlands.

4.2.2 Survey of squirrel carcasses

Each squirrel was processed individually. After removal from the freezer the carcass was placed in a large white plastic tray and allowed to thaw overnight. The bag from which the carcass was removed was inspected for the presence of ectoparasites. Each squirrel was then inspected systematically for the presence of ectoparasites attached to the skin or in the fur by eye and using a dissection microscope for a minimum of five minutes paying special attention to the eyes, ears, under arms and groin. A fine comb was used to brush the entire of the animal and forceps used to detach any ectoparasites still attached. Any

ectoparasites found on the animal or in the bag were identified to species/life stage by reference to taxonomic keys (Arthur, 1963; Hillyard, 1996) as described in section 2.2 and then stored in 70% ethanol until DNA extraction. Each squirrel was measured (nose tip to anus), weighed and sexed. Finally, each ear was cut off using scissors and ten 2mm biopsy punches were made in each ear using sterile biopsy puncher (Kai, Japan). These punches were then stored at -20°C until DNA extraction.

4.2.3 DNA extraction and molecular diagnosis of *B. burgdorferi* s.l.

DNA was extracted from both questing and attached nymphal and adult ticks as outlined in section 2.3.1 DNA from the squirrel ear biopsies was extracted using Isolate II Genomic DNA kit (Bioline, London) with slight modifications which are described in section 2.3.2. All DNA extracts were then used as a template in the RT-PCR assay to detect *B. burgdorferi* s.l. as specified in section 2.4.1. Firstly, the squirrel samples were tested to detect *B. burgdorferi* s.l. DNA, and then only the ticks from the positive squirrels from 2015 were tested. Any samples that yielded an amplicon were subjected to the nested PCR targeting the 5S-23S rDNA intergenic spacer region (section 2.4.2) and then sent for Sanger sequencing or subjected to the RLB as detailed in section 2.5.

4.2.4 Statistical analysis

Univariant analysis was performed using Minitab 18 (Minitab Ltd, Coventry) to determine any significant association with *Borrelia* spp infections and host associations including; age (grouped by weight), sex, length (with and without tail), location and total tick burden (all stages). Squirrel age was defined by weight, those squirrels that weighed 200g-500g were classed as sub adults and over 500g were classed as adults (Elton, 1951). The factors associated with total tick burden (number of ticks per squirrel, all stages) were investigated using generalized linear models (GLMs) which assumed a negative binomial error and a log link. The variables that were considered were age, sex, length and location. The factors associated with *B. burgdorferi* s.l. presence or absence in each squirrels were investigated using GLMs which assumed a binomial error and a logit link. The variable that were considered were age, sex, length, location and tick burden. Sex, length and weight were also tested as an interaction. All analyses were carried out using R 3.4 (R. Development Core Team, 2017) using either the glm. nb function for negative binomial GLMS, or. glm for the

binomial GLMS. Model selection was based on backward stepwise model selection with variables dropped according to p-value, with only those variables significant at the $p<0.05$ level being retained in the final model.

4.3 Results

314 squirrels were surveyed between 2014 -2017, 165 (52.6%) were male and 149 (47.4%) were female. Most squirrels were classed as adults (227, 72.3%), hence 87, 27.7% were subadults. The grey squirrels came from five different locations in southern Cumbria, 56 (17.8%) from Grizdale which is a coniferous woodland, 50 (15.9%) from Arklid, 65 (20.7%) from Lakeside which are both mixed woodlands and 60 (19.1%) from Foulshaw Moss which is a mixed woodland surrounded by a peatbog and 83 (26.4%) from Eggerslack which is a broad leaf woodland.

4.3.1 Tick burden

In total 8,783 ticks were found on all the squirrels all of which were *I. ricinus* (Figure 29), 269 (85.7%) squirrels were infested with at least one tick and 244 (77.7%) were infested with more than one tick. The median number of ticks per squirrel was 11. Some squirrels were very heavily infested with ticks, with some individuals carrying over 200 ticks. Ticks tended to be around the ears, eyes and mouth or in the armpits and groin areas.

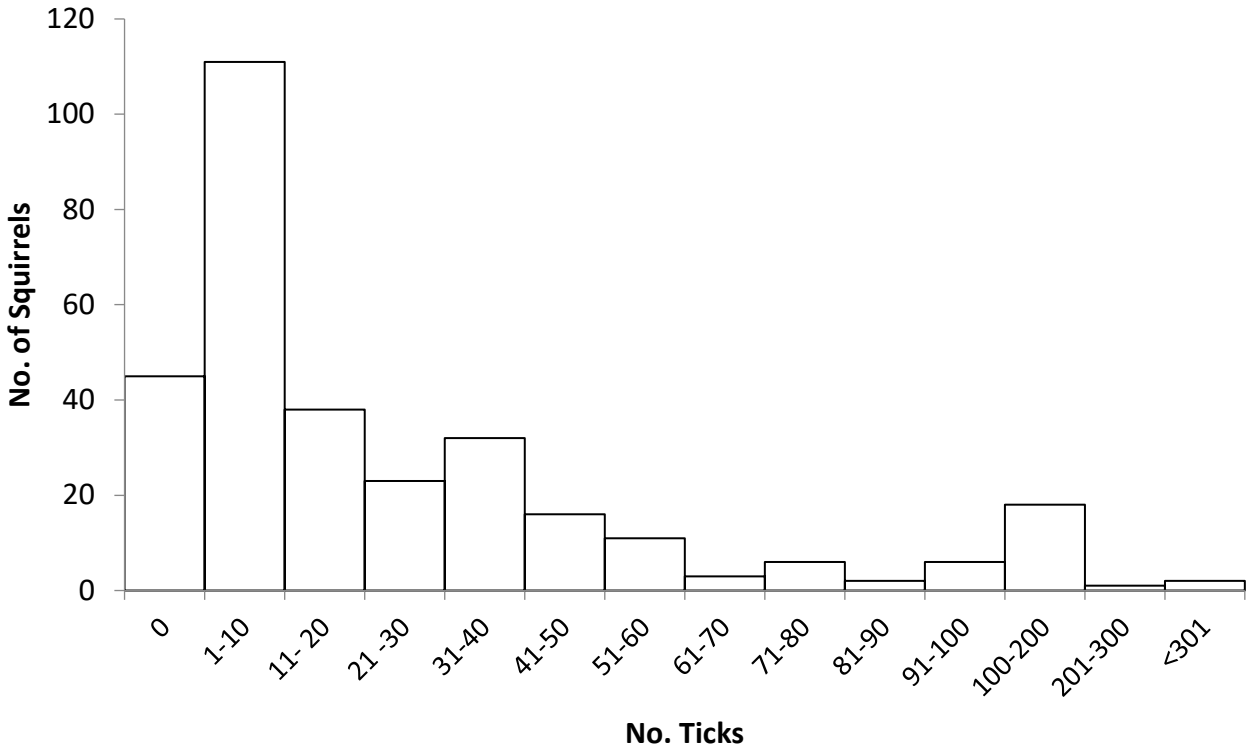

Figure 29. A histogram showing the distribution of tick burden on the squirrels.

A total of 4613 ticks collected from 208 squirrels trapped between 2015 and 2017 were identified to life-stage. Of these, only 10 (0.3%) were adults, 1,017 (22.0%) were nymphs and 3,586 (77.7%) were larvae. Whereas some squirrels were infested with only a single life stage, many were simultaneously infested with different I. ricinus life stages. One animal carried only a single adult tick, three (1.4%) were found to have an adult and at least one nymph, 27 (13.0%) squirrels had only nymphs, 103 (49.52%) squirrels had both nymph and larvae, 27 (12.98%) squirrels had just larvae and six (2.88%)squirrels had all three life stages of tick (Figure 30). Overall, 112 (53.84%) of squirrels were carrying more than one stage of tick. From squirrels carrying one or more ticks at any life stage, the median number of larvae was 4 (range, 0 to 300), and the median number of nymphs was 2 (range, 0 to 60). All the squirrels that harbored adult stage ticks came from Foulshaw Moss or Eggerslack Wood.

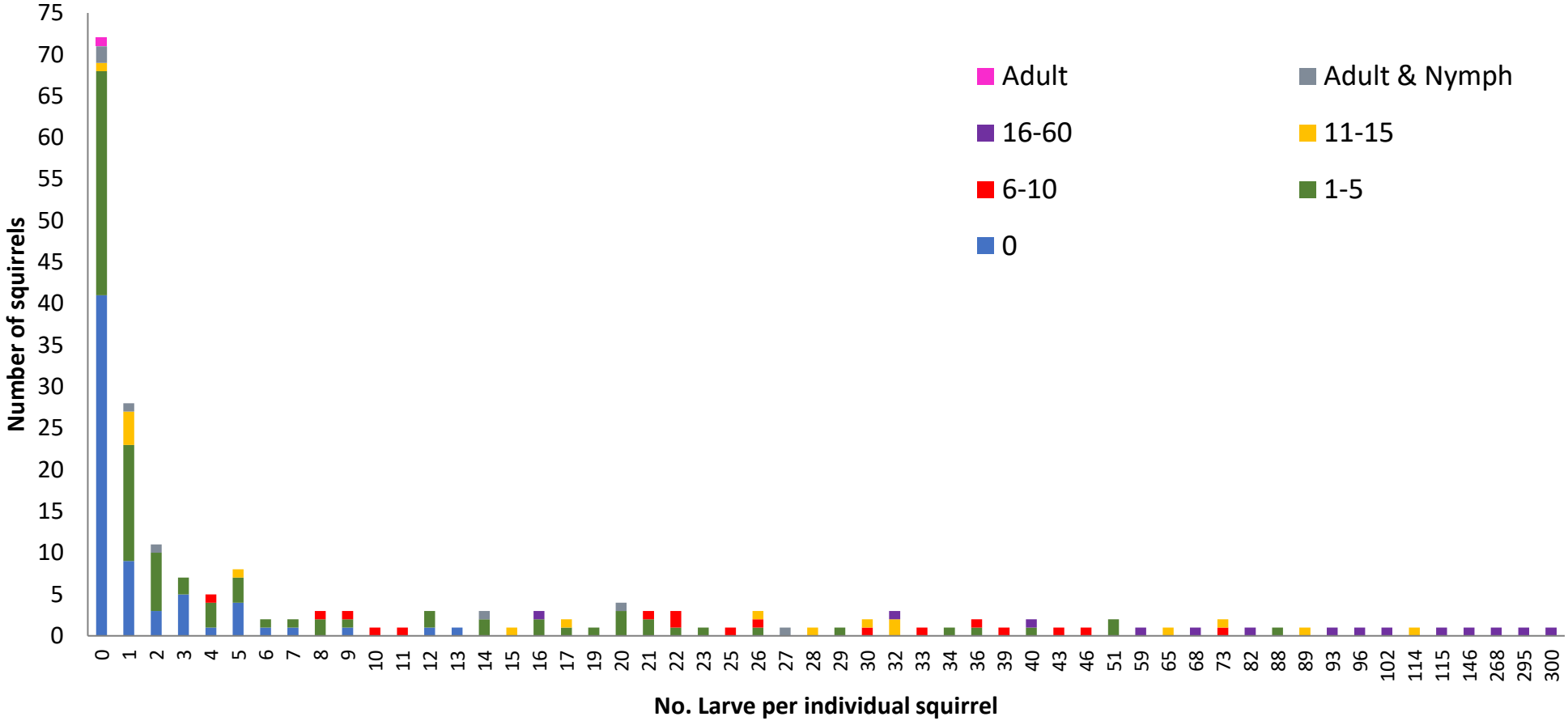

Figure 30. The frequency distribution of larvae on grey squirrels. At each intensity of larval infestation, the numbers of host also feeding; 0 nymphs (blue), 1-5 nymphs(green), 6-10 nymphs (red), 11-15 nymphs (yellow), 16-60 nymphs (purple), adults and multiple nymphs (grey), and a single adult (pink).

No significant association between squirrel sex and tick burden was found ($\chi 2$= 0.043, d.f = 2, P value =0.834), or between sub adults and adult squirrels ($\chi 2$= 2.003, d.f = 2, P-value =0.157). A spearmans rank correlation was used to test for correlation between weight and length (r = 0.45) and no strong correlation was found, therefore both variables were used in the GLM. Squirrels from Foulshaw Moss were found to have lowest infestations than those from other sites ($\chi 2$= 30.70, d.f = 5, P-value =0.001). The model which best fit this data included sex, weight and location. This revealed that that sex, and weight were not significant but squirrels from Foulshaw Moss were significantly less likely to be infested with ticks (Table 12).

Table 12. Summary table of results from a negative binomial generalised linear model testing for host and environmental factors for tick burden on grey squirrels. Baseline = Arklid wood and Females.

Parameters	Estimate (S.E)	T value	Probability
Intercept	37.846 (16.113)	2.349	0.019
Sex M	0.760 (5.068)	0.150	0.880
Weight	0.002 (0.027)	0.099	0.921
Eggerslack	-7.515 (7.950)	-0.945	0.345
Foulshaw Moss	-33.369 (8.622)	-3.870	0.001
Grizdale	-0.764 (8.649)	-0.088	0.929
Lakeside	-15.624 (8.432)	-1.853	0.064

4.3.2 Infection prevalence in squirrels

A total of 314 squirrels were tested for the presence of *B. burgdorferi* s.l. DNA. Of these, 62 (20.1%) were found to be positive. The infection prevalence ranged from 16.0% at Arklid to 24.6% at Lakeside. *Borrelia* genospecies could be delineated for 39 of these squirrels. 19 were infected with *B. afzelii*, 18 were infected with *B. garinii,* 1 with *B. valaisiana* and 1 with *B. burgdorferi* s.s. One mixed infection was detected (*B. afzelii and B. garinii*) (Table 13).

Table 13. The number of squirrels, attached ticks and questing ticks, how many were positive for *B. burgdorferi* s.l. and what genospecies were detected.

Area		Squirrels							Attached								Questing							
		No. tested	*B. burgdorferi* +ve (%)	*B.afzelii*	*B. garinii*	*B. valaisiana*	*B. burgdorferi s.s*	Unknown	No. tested	*B. burgdorferi s.l.* +ve (%)	*B.afzelii*	*B. garinii*	*B. valaisiana*	*B. burgdorferi s.s*	*B. garinii/ B. valaisiana*	Unknown	No. tested	*B. burgdorferi* +ve	*B.afzelii*	*B. garinii*	*B. valaisiana*	*B. burgdorferi s.s*	Unknown
Grizdale		56	9 (16.1)	2	4	1	0	2	NA		NA	NA	NA	NA	NA	NA	210	6 (2.9)	0	2	2	0	2
Arklid		50	8 (16.0)	4	3	0	0	1	NA		NA	NA	NA	NA	NA	NA	200	0 (0.0)	0	0	0	0	0
Lakeside	RL	65	16 (24.6)	10	4	0	0	2	95	11 (11.6)	1	5	3	0	1	1	200	8 (4.0)	0	4	3	0	1
	Ford																200	3 (1.5)	0	1	0	0	2
	ENT																200	5 (2.5)	0	4	1	0	0
	HD																200	10 (5.0)	0	5	3	0	2
	TH																200	14 (7.0)	0	6	6	0	2
	FIN																200	2 (1.0)	0	1	1	0	0
	GH																100	1 (1.0)	0	0	0	1	0
	CH																200	18 (9)	12	1	1	1	3
Eggerslack	Egg	83	16 (19.3)	1	7	0	0	7	NA	NA	NA	NA	NA	NA	NA	NA	200	4 (2.0)	1	0	0	0	3
	HF																108	12 (11.1)	0	1	4	0	7
Foulshaw Moss		60	14 (23.3)	2	0	0	1	11	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

No significant difference between males and females (X2= 0.163, d.f = 2, P value =0.686), or sub adults and adults (X2= 3.151, d.f = 2, P-value =0.075), or location (X2= 2.471, d.f = 5, P value =0.116) was found with *B. burgdorferi* s.l. infections. The best fit model included sex, weight, and length (excluding tail) as an interaction (Table 14). When analysing sex alone males are less likely to be infected than females, but when an interaction with weight is added heavier (older) males are more likely to be infected. When an interaction with sex, weight and length is analysed smaller males are more likely to be infected.

Table 14. Summary table of results from a binomial generalised linear model testing for *B. burgdorferi* s.l. infection in grey squirrels. Baseline = Females.

Parameter	Coefficient (Standard deviation)	Z value	Probability
Intercept	1.103e+01 (8.703e+00)	1.267	0.205
Sex (Male)	-3.776e+01(1.720e+01)	-2.195	0.028
Weight	-1.645e-02(1.781e-02)	-0.924	0.355
Length	-5.931e-01(3.927e-01)	-1.510	0.131
Male*Weight	7.744e-02(3.426e-02)	2.260	0.023
Male*Length	1.641e+00(7.361e-01)	2.229	0.025
Weight*Length	8.107e-04(7.675e-04)	1.056	0.291
Male*Weight*Length	-3.304e-03(1.437e-03)	-2.299	0.021

4.3.3 Infection prevalence in attached ticks to infected animals

Among the 65 *B. burgdorferi*-infected squirrels trapped in 2015, 16 (24.6%) were found have *B. burgdorferi* s.l. DNA present. A Borrelia genospecies was delineated for 13. A total of 95 ticks were collected off these 16 animals and tested for the presence of borrelial DNA. 74 nymphs were tested and four (5.41%) were positive; one *B. garinii*, one *B. valaisiana*, *one B. afzelii* and one was unable to be delineated. 21 larvae were tested and seven (33.33%) were positive; two *B. valaisiana*, four *B. garinii* and one coinfection with *B. garinii* and *B. valaisiana*. Two squirrels which were infected with *B. garinii* had larvae that were also infected with *B. garinii* and one squirrel which was infected with *B. afzelii* was infested with a nymph that was also positive for B. *afzelii*. Five larvae and two nymphs were infected with

genospecies different to the genospecies that was detected in the squirrel. For squirrel 13 the nymph and larvae had the same *Borrelia* spp. infections, but different to the infected squirrel (Table 15).

Table 15. Genospecies of the positive squirrels and the nymph and larvae that fed on them.

Squirrel	Squirrel genospecies	Nymph genospecies	Larvae genospecies
6	*B. garinii*		
12	*B. afzelii*		
13	*B. afzelii*	*B. garinii* + *B. valaisiana*	*B. garinii* + *B. valaisiana*
14	*B. garinii*		*B. garinii*
15	*B. afzelii*		*B. garinii*
16	*B. afzelii*		
25	?		
27	*B. garinii*		
28	*B. garinii*		
29	?		*B. garinii* + *B. valaisiana*
35	?		
37	?		
38	*B. afzelii*		
50	*B. afzelii*		
52	*B. afzelii*		
63	*B. afzelii*	*B. afzelii*	

4.3.4 Infection prevalence in questing ticks

Questing nymphs were collected at 13 sites in proximity to the squirrel trapping sites. 200 nymphs were tested at all sites with the exception of Grizdale (n = 210), WS (n=189), GH (n=100) and HF (n=108). The infection rates of *B. burgdorferi* s.l. at these sites ranged from 0.0% to 11.1%. HF had the highest infection prevalence and Arklid was the lowest as *B. burgdorferi* DNA was not detected in any ticks collected at this site it had no *B. burgdorferi* s.l. present (Table 13) Genospecies data revealed that *B. garinii* was the most abundant species (30.1%) and most widely distributed as it was present at nine out of the 12 sites. *B. valaisiana* was present at eight out of the 12 sites and was the second highest prevalence with 25.3%. *B. afzelii* (15.7%) and *B. burgdorferi s.s*. (2.4%) were only found at two single sites. 22 (26.5%) out of the 83 positives were undistinguished.

Overall a high percentage of the squirrels were carrying at least one tick (85.7%). 20.1% of all squirrels were positive for *B. burgdorferi s.l*. When the attached ticks were tested for B. burgdorferi s.l. had a significantly higher prevalence than the questing ticks ($\chi 2 = 11.69$, d.f =

2, P-value =0.0006). All four genospecies were found in both the squirrels and the questing ticks but not all genospecies were found in the attached ticks.

4.4 Discussion

4.4.1 Tick burden

Grey squirrels surveyed were primarily parasitized with *I. ricinus* larvae and nymphs, in agreement with previous studies in the UK (Craine *et al*., 1995; Millins *et al*., 2015). Cumbrian squirrels were found to support a median of four larvae and two nymphs, figures that are twice those reported for squirrels in Scotland (Millins *et al.,*2015) but much lower than those reported for squirrels living in southern England (Craine *et al*.,1995). Furthermore, the proportion of squirrels parasitized ticks in Cumbria was 85.7% which is, significantly higher than results of a similar study in Scotland which found 41.50% of squirrels to carry at least one tick (Millins *et al*., 2015). In the USA 80% of grey squirrels were reported to carry *I. scapularis* although they had very low tick burdens (0-1 per squirrel) (Hammer *et al*., 2005).

In the current study 0.3% of ticks from squirrels were adult ticks. These low levels are consistent with other studies from the UK which found no adult ticks (Craine *et al*., 1995) and more recent a study which found 0.1% ticks on squirrels were adults (Millins *et al*., 2015). Nine of the 10 squirrels harbouring adult ticks also carried nymphs (n=3) or both nymphs and larvae (n=6). Co-infestation were found on 15.9% of the squirrels, which is higher than that previously found on mice or voles (3.6%) in the south of England (Cull *et al.,*2017). Deer have been shown to carry much higher burdens of ticks with co-infestations of all three instars (Alberdi *et al*., 1993; Kiffner *et al*., 2010). A host such as the squirrel that is concurrently infested with all stages of the ticks and is a competent reservoir is potentially important in the transmission of *B. burgdorferi* s.l. because they may be infected by nymphs and/or adults and subsequently transmit the infection to the larvae. Co-infestations also facilitate co-feeding transmission of *B. burgdorferi* s.l. although with *I. scapularis* feeding from uninfected white footed mice, it was shown that an unrealistically high density of nymphs (~40) and larvae (~200) were needed to produce infection through co-feeding (Piesmann & Happ, 2001). However, in Europe transmission of *B. afzelii* by co-feeding

transmission was found to be between 1.6-55.3% when only one infected nymph was allowed to feed next to uninfected larvae (Richter *et al*., 2002). It has been shown that each genospecies shows different transmission rates via co-feeding (Voordouw, 2014).

Habitat was a significant factor in the tick burden of squirrels, those that came from Foulshaw Moss, a mixed woodland surrounded by peat bog, had significantly lower tick burdens. It has been previously shown that peat bogs have a very low abundance of ticks of all stages. This is thought to be partly due to the low density of deer that visit these sites (Gilbert, 2013). Most of the adult ticks attached to the squirrels came from Foulshaw Moss this could be because they cannot find any other suitable hosts to feed from.

4.4.2 Infection prevalence in squirrels

20.1% of Cumbrian squirrels were found to be infected with *B. burgdorferi* s.l. This prevalence is significantly higher ($\chi 2 = 7.12$, d.f = 2, P-value =0.007) than the 11.9% figure reported in Scotland (Millins *et al*., 2015) and the 0-11.1% range reported in the USA (Roy *et al*., 2017; Nieto *et al*.,2010).

Univariant analysis of the data indicated that males were significantly less likely to be infected than females. Although when analysed as an interaction, it appears that lighter, shorter males were more likely to be infected. Lighter and short males indicates that younger males are more likely to be infected. Other studies found sub adults more likely to be infected although not significantly and found no sex bias (Millins *et al*., 2015). In other rodent models males with higher testosterone levels fed more ticks (Hughes & Randolph, 2001) and had more fleas (Krasnov *et al.,* 2005), feeding more ectoparasites could lead to a higher chance of becoming infected with *B. burgdorferi* s.l. Along with immunosuppressing hormones causing males to be more susceptible to infection, it has also been suggested that energy trade-off may cause the squirrel to put more effort into self-maintenance and reproduction(by increasing their home range) rather than its immune system(Krasnov *et al*., 2005).Evidence from this has been found in rodents infected with *Bartonella.* Younger rodents were more likely to be infected, it was suggested this is because they are more mobile and therefore more likely to pick up more ectoparasites and that their immature immune system had yet to clear the infection (Fichet Calvet *et al*., 2000).

Millins *et al.* (2015) reported encountering four *Borrelia* genospecies (*B. afzelii, B. garinii, B. valaisiana* and *B. burgdorferi s.s.*), either alone or as co-infectors, in Scottish grey squirrels, and my findings are consistent with this. All the *Borrelia* species that were found in the squirrel tissue have been found before in questing ticks from Northern England (Bettridge *et al*., 2013; Chapter 3 of this thesis). The most prevalent genospecies in Scottish squirrels was *B. garinii* followed by *B. afzelii* then *B. valaisiana* and *B. burgdorferi s.s*. (Millins *et al*., 2015). This is similar to the current study the most dominant species were *B. afzelii* and *B. garinii. B. garinii* is associated with birds (Kurtenbach *et al*., 1998) but many reports of divergence from this host-associations have been published. *B. afzelii* has been found in bird associated larvae (Heylen *et al*., 2017) and *B. garinii* has been detected in larvae from both red and grey squirrels (Pisanu *et al*., 2014; Millins *et al*., 2015).

Unfortunately, the present study could not quantify seasonal tick burden or seasonal infection prevalence in squirrels as it was not practical to record the precise date on which each squirrel was trapped. Other studies have shown that there is a peak in *B. burgdorferi* s.l. infections in squirrels in June the authors suggested this was due to the infection only being short lived in the squirrels (Millins *et al*., 2015). The squirrels in the present study were trapped from March to August, and so is not representative of the entire year.

4.4.3 Infection prevalence in attached ticks vs questing ticks

The infection prevalence of feeding ticks was assessed from a subset of the squirrels. Overall 11.6% of attached ticks were infected with *B. burgdorferi* s.l. compared with an overall 4.1% of infected questing ticks. The attached ticks were significantly more likely to be infected than the questing ticks. As the questing ticks were nymphs no comparison can be made as to the status of the squirrel as an amplification host as it is important to explore the larval stage when considering transmission as nymphs could have been infected during their first blood meal.

38.1% of larvae were infected with *B. burgdorferi* s.l. this maybe an underestimation of transmission as not all the larvae had fully fed at the time of processing. A study in North America tested grey squirrels for *B. burgdorferi* s.l. and using xenodiagnosis with *I. pacificus* larvae found an average of 59.4% become infected with *B. burgdorferi* s.l. (Salkeld

et al., 2008). 37.5% of infected larvae in my study were found to have the same genospecies as the squirrel from which it was attached to, suggesting transmission from the host to the larvae, which has been demonstrated before in squirrels by xenodiagnosis (Craine *et al*., 1997). These were all *B. garinii* and not *B. afzelii* which was also found in Millins *et al.,* (2015). Interestingly it has been found that red squirrels in Europe do transmit both *B. afzelii* and *B. burgdorferi* s.s (Humair & Gern, 1998) which is anticipated as squirrels are rodents and *B. afzelii* the rodent associated genospecies.

However, a substantial proportion of the larvae (62.5%) did not share the same genospecies with the host. In one case the squirrel host was infected with *B. afzelii* but the associated larvae were with infected with either *B. valaisiana* or *B. garinii*, which was also detected in feeding nymphs from this squirrel, suggesting evidence of co-feeding, which has been shown before for *B. burgdorferi* s.l. from infected nymphs to uninfected larvae (Gern & Rais, 1996). However further studies with much larger sample sizes are needed to prove this hypothesis. These larvae could also have been infected by transovarial transmission a study from Scotland found that 0.7% of questing larvae were infected with *B. burgdorferi* s.l. (Hall *et al*., 2017). Similar results were found in the Netherlands with 0.6% of questing larvae being infected with *B. burgdorferi* s.l. Transovarial transmission of *B. afzelii* in larvae was shown to be transmitted to rodent host and so contribute to the cycles of *B. afzelii* (van Duijvendijk *et al*., 2016). A third option is that the larvae have partially fed on another host been interrupted and then fed on the squirrel. There has been evidence of partial feeds in ticks (Richter *et al.,* 2012).

B. garinii and *B. valaisiana* were the only genospecies to be found in larvae attached to squirrels. This could point towards the squirrel being a filter host for *B. garinii,* which then could go onto explain the high levels of this species found in questing ticks. The sample size in this study is too small to reach any solid conclusion and the results should be interpreted with caution but this result warrants further study into how efficiently squirrels transmit each *Borrelia* genospecies to larvae and what effect this has on the genospecies distribution in the questing nymph populations.

A limitation of this chapter is the incomplete genospecies picture for the squirrels, which leaves a gap in the data set. Due to time and budget restraints only, a subset of ticks from the positive squirrels were analysed to detected B. *burgdorferi* s.l. genospecies and it would have been a better to have used MLST on these samples to compare the *Borrelia* isolates from the squirrels and those in the infected feeding ticks and eventually to compare those with the questing nymphs that were positive for *B. burgdorferi* s.l. This could be taken on a future work.

This study has shown that a high percentage of squirrels were carrying ticks and over half of these were carrying more than one stage of tick. Hosting more than one stage is important for *B. burgdorferi* s.l. as it increases the chances of the larvae feeding from an infected squirrel. Overall a high *B. burgdorferi* s.l. infection prevalence was reported in the squirrels, with significantly more males with a smaller body condition being infected. Over a third of the feeding larvae were infected indicating that grey squirrels could be amplification host. All four *Borrelia* genospecies found in the UK questing ticks were present in the squirrels. The transmission of different *Borrelia* genospecies from squirrels to larvae should be further assessed with the investigations including co-feeding as a viable transmission route. This further study should try to understand if squirrels are a filter host for *B. garinii* rather than *B. afzelii* as there is evidence from this study and previous studies that squirrels do not transmit *B. afzelii* to larvae (Millins *et al.,* 2015), as was previously assumed, due to squirrels being rodents. More studies should examine the assumed host associtons within the *B. burgdorferi* s.l. genospecies to see if they hold true.

Chapter 5: Prevalence of *Borrelia burgdorferi* s.l. in Ixodid ticks from companion animals in Cumbria.

5.1 Introduction

It is estimated that there are about 8.5 million pet dogs and 8 million pet cats in the UK (https://www.pfma.org.uk/pet-population-2017). The health of these animals is potentially threatened by tick borne pathogens (TBPs); a handful of studies in the UK have shown that companion animals (that frequent the outdoors) are likely to come into contact with ticks (Jeanett *et al.,* 2013) and these ticks are capable of carrying TBPs of veterinary concern (Smith *et al.,* 2012; Abdullah *et al.,* 2017; Davies *et al.,* 2017).

The most common species of tick in the UK is *Ixodes ricinus* that has been shown to be a competent vector for pathogens such as *Borrelia* species, *Anaplasma phagocytophilum*, and *Babesia* species (Barbour *et al*., 1983*;* Grzeszczuk *et al*., 2002; Gorenflot *et al*., 1998). Case reports of dogs with no history of travel outside the UK have been published for Anaplasmosis, which can present as a hemolytic anemia and thrombocytopenia, leading to severe blood loss (Bexfeild *et al*., 2005), and Babesiosis which can present as thrombocytopenia, azotemia and jaundice leading to renal failure and can be fatal (Holm *et al*., 2006). Cases of tick-borne disease caused by pathogens generally considered exotic have also recently been reported in UK dogs. In 2016, *Babesia canis* was detected in four sick dogs with no travel history (Swainsbury *et al*., 2016; Phipps *et al*., 2016) living in Essex. Investigation of these cases resulted in the discovery of a local focus *of Dermacentor reticulatus* ticks, 17 questing adults were tested by PCR and 14 (82.4%) were positive for *B. carnis* (Hansford *et al*., 2016).

There is a void in the literature of case reports for tick-borne diseases (TBDs) in cats in the UK. However, elsewhere in the world, there have been numerous reports of TBDs in domestic cats. These include Feline Babesiosis, which is most common in South Africa (Jacobson *et al*., 2000) than in other parts of the world including Europe. Common symptoms of Feline Babesiosis are hemolytic anemia and an inflammatory response that can lead to organ failure (Solano-Gallego & Baneth, 2011). Anaplasmosis, which is

associated with a febrile illness that can lead to immunosuppression and subsequent secondary infections, have been reported in cats from Finland and other European countries (Heikkilä *et al.,* 2010). These observations suggest that cats are susceptible to TBPs where the enzoonotic cycles exist to sustain the pathogen. Other TBPs have been found in the blood of cats including; *Babesia* spp. *Anaplasma/Ehrlichia* spp, and *Borrelia* spp albeit at low prevalence. These pathogens were found in healthy and symptomatic cats (Maia *et al*., 2014).

In the UK the most prevalent TBP found in ticks attached to dogs is *Borrelia burgdorferi* s.l. (Abdullah *et al.,* 2017). *B. burgdorferi* s.l. is an umbrella term used to describe a group of phylogenetically clustered *Borrelia* species, some of which have been associated with Lyme borreliosis (LB) (Margos *et al.,* 2013). Dogs have been found to be infected by with all the genospecies of *B. burgdorferi* s.l. found in the UK (*B. afzelii, B. garinii, B. valaisiana* and *B. burgdorferi* s.s) (Hovius *et al.,* 1999), similar to squirrels which have also been found to be infected with these four genospecies (Millins *et al.,* 2015). This is thought to be due to their lifestyle and because their immune system is not able to destroy the bacteria (Skotarczak, 2014). In dogs LB has been attributed to infections of different organs but the most common symptom is migratory arthritis (Magnarelli *et al.,* 1987). *Borrelia* spp. have been detected in the liver, cerebral spinal fluid, bladder wall, skin, heart and bone marrow of dogs (Hovius *et al.,* 1999). Apparently healthy dogs have been found to be seropositive for *B. burgdorferi* s.l. which shows that the presence of *B. burgdorferi* s.l. antibodies is not always associated with pathology (Alho *et al.,* 2016). It has been suggested that some breeds of dog are predisposed to *B. burgdorferi* s.l. infections. A study of Burmese mountain dogs (n=106) showed that these dogs were significantly more likely to be seropositive for *B. burgdorferi* s.l. than the control group (n=62) (Gerber *et al.,* 2007).

Experimental infection of cats demonstrated that *B. burgdorferi* s.l. can infect cats when inoculated artificially, but none of the infected individuals developed clinical symptoms (Burgess, 1992). In 2005, naturally occurring *B. burgdorferi* s.l. infections in cats were reported for the first time, but these could not be significantly associated with clinical disease (Shaw *et al.,* 2005). However, a study in the USA found that *B. burgdorferi* s.l. seropositivity in cats was significantly associated with limb or joint problems either with or

without fever fatigue and anorexia (Magnarelli *et al.,* 1990). It is clear that, for both cats and dogs, more studies are needed to investigate their susceptibility to *B. burgdorferi* s.l. and the pathological consequences of infection.

In the UK, three studies have been carried out to quantify *B. burgdorferi* s.l. prevalence in companion animals. The first study was a serosurvey of 198 dogs. The dogs were categorised by tick bite history (bitten/not bitten) and location (rural/urban). The results found that dogs with a history of tick bite were significantly more likely to be seropositive than those without a history of a tick bite. Dogs from rural areas were also significantly more likely to be seropositive than dogs from urban areas (May *et al.,* 1991). The second study was a serosurvey of 477 cats, in which a seroprevalance of 4.8% was found. The authors concluded that *B. burgdorferi* s.l. causes a lower pathogenicity in cats compared with dogs and hypothesised that this is due to an early humoral response to *Borrelial* outer surface proteins in cats (May *et al.,* 1994). The third study used molecular methods to test for the presence of *B. burgdorferi* s.l. in the blood of systematically ill cats and dogs and found 3.33% and 4.16% infection prevalence respectively for *B. burgdorferi* s.l. They concluded that there were no statistically significant associations between the *B. burgdorferi* s.l. infections and the clinical signs shown by cats or dogs. (Shaw *et al.,* 2005).

Many serosurveys in Europe found *B. burgdorferi* s.l. antibodies in healthy controls as well as in symptomatic dogs (Cardoso *et al.,* 2012). Some of these studies found a higher prevalence of *B. burgdorferi* s.l. antibodies in the healthy controls than in the non-symptomatic dogs (Pantchev *et al.,* 2009; Amusategui *et al.,* 2008). Although these studies use different methods of detection and report a range of prevalence overall they indicate that, in dogs, possessing antibodies for *B. burgdorferi* s.l. does not correlate with any specific clinical symptoms. A European serosurvey of 271 cats which were suspected of feline LB, were tested and six cats were seropositive for *B. burgdorferi* s.l., this shows low levels of seropositivity in cats (Pantchev *et al.,* 2016).

An alternative approach to quantifying the threat of TBP is to consider rates of exposure to ticks rather than to the pathogens themselves. This data can be combined with information about local infection prevalence in ticks to formulate an estimate of the public or veterinary health threat posed. Public Health England manages voluntary national surveillance of host-

attached ticks. Collation of this data allow not only the generation of tick bite risk maps but also insight into the relative frequency with which ticks are encountered on humans and companion animals, and has shown that at least twice as many ticks are submitted after collection from dogs and cats than from humans (Jameson& Medlock, 2011). An alternative approach to quantifying the frequency with which cats and dogs are exposed to ticks is to monitor the electronic health records of veterinary practices. This method has been used across the UK to reveal that "tick removal" appeared at a rate of 18.3 per 100,000 electronic records per week for cats and 14.8 per 100,000 electronic records per week for dogs (Tulloch *et al.,* 2017). On a more local scale, one study attempted to correlate rate of tick attachment in dogs with their behaviour and the habitat in which they exercised (Jennett *et al.,* 2013). Using GPS collars, the paths taken by dogs through a large peri-urban park were monitored as were the number of ticks attached to dogs after their walks. The study found that all dogs appeared to be at equal risk of tick bite regardless of the path taken, with, on average, one tick attachment per dog per year being observed.

Recently, concerted efforts have been made to quantify the tick burden of dogs nationally (in the UK) by examining randomly selected dogs attending veterinary practices across the country. In the first of these studies (Smith *et al*., 2011), 22.9% of 3,534 dogs examined were parasitized by at least one tick, with the vast majority (90.4%) of ticks observed being adults. Identification of ticks revealed most to be *I. ricinus. I. canisuga, I. hexagonus* and *Dermacentor reticulatus* were also encountered. The infestations peaked in May and July (Smith *et al.,* 2011). A proportion of ticks collected (739) were tested for the presence of *Borrelia* spp. DNA, and 2.3% were found to be positive. These 17 ticks were collected from dogs in numerous locations across the UK (Smith *et al.,* 2012). A very recent extended study (Abdullah *et al*., 2016) employing similar methods, reported 30.7% of 12,096 dogs surveyed were found to have at least one tick, although as the authors point out, this number could be an over-estimate as the collaborating vet practices may have only sent positive samples rather than randomising their survey as dictated in the study design (Abdullah *et al.,* 2016). Pathogen detection using PCR tested 4,737 ticks collected in this survey, 2.0% were infected with *Borrelia* spp, and genotyping of infecting strains revealed them to belong to *B. afzelii, B. garinii, B. valaisiana*, *B. burgdorferi* s.s. and, for the first time in the UK, *B. spielmanii* was found in Yorkshire(Abdullah *et al.,* 2017).

Very few studies have attempted to explore tick parasitism of cats in the UK. Ogden *et al*. (2000) surveyed dogs and cats finding cats were significantly more likely to be infested with *I. hexagonus*, an observation possibly linked to cats' behaviour (Ogden *et al.,* 2000). More recently, a study similar to Abdullah *et al.* (2016) collaborated with veterinarians to randomly check cats for ticks. Of the 1,855 cats that were checked 6.6% of cats were infested with at least one tick. This study found more *I. ricinus* than *I. hexagonus* in contrast with the results of Ogden *et al.* (2000). The 541 ticks from this study were tested for TBPs and 1.8% of the ticks were infected with *B. burgdorferi* s.l. (Davies *et al.,* 2017).

In showing that a not-insignificant proportion of companion animals in the UK are parasitized by ticks, these studies justify the use of acaricides (impregnated collars, spot on, spray, tablets). However even if these measures are taken, dogs can still be exposed to tick borne diseases. In Portugal a study of 100 police dogs that had a very strict parasite control regime found 8.5% of these dogs were seropositive for *B. burgdorferi* s.l. (Alho *et al .,* 2016). In comparison another study of 557 dogs in Portugal found only 0.2% of healthy dogs to be seropositive (Cardoso *et al.,* 2012). To prevent companion animals from being infected with *B. burgdorferi* s.l. and other TBPs, a three-pronged approach targeting ticks has been proposed; 1) daily checks for ticks and swift removal, 2) up to date acaracide treatment, and 3) vaccination. Currently, there is a *B. burgdorferi* s.l. vaccine licenced for use in dogs in the UK but no vaccine for cats worldwide (Wright, 2013).

Pet owners wishing to move their animals in and out of the UK are required to have a "pet passport". Although acaracide treatment was originally required for the award of a passport, this criterion was removed in 2012. Unsurprisingly, the number of encounters with exotic ticks appears to be increasing. These ticks being imported on pets could introduce new pathogens to the UK (Featherstone *et al.,* 2012). A recent review of the tick surveillance system used by PHE has shown that *Rhipicephalus sanguineus* had been recorded on 27 occasions imported mainly from Cyprus and Spain on rehomed dogs. The presence of this tick in the UK is of particular concern as it is the vector of *Babesia* species associated with canine babesiosis and *Ehrlichia canis*, another well-established pathogen of dogs. Furthermore, it has the ability to live indoors and cause overwhelming infestations of homes as it reproduces very quickly (Hansford *et al.,* 2017b).

Previous studies that surveyed UK companion animals for ticks have encountered rare species such as *Haemaphysalis puntctata, D. reticulatus, D. variabilis* and *R. sanguineus*, showing that this is an effective way of assessing the tick species that are present in an area. (Ogden *et al.,* 2000; Abdullah *et al.,* 2016). There is limited information on TBPs in the UK and more so in the north of the UK although this region is represented in the country wide studies (Abdullah *et al.,* 2016; Smith *et al.*, 2011; Ogden *et al*., 2000) there has been no intense studies in the region as have occurred further south (Jeanett *et al.,*2013). This project aimed to build on this knowledge by collaborating with vets in the county of Cumbria that is a well-known tick "hot spot" (Medlock *et al.,* 2009). This study will It will use passive surveillance, therefore we cannot explore the tick infestation rate in dogs as in Abdullah *et al.* (2016) but we can explore, what species of ticks occur in this area and what factors such as breed, sex, age and location of the animal influence the probability of attachment of different species of ticks. Given the limited number of studies that have included cats to date (Ogden *et al.,* 2000; Davies *et al.,* 2017), this project will include ticks from both cats and dogs in the area. Any ticks collected will be tested for *B. burgdorferi* s.l. to understand what percentage of ticks attaching to companion animals are carrying *B. burgdorferi* s.l. and so estimating the risk of an infected tick attaching to a dog and by proxy people in Cumbria. Finally, any ticks found to be positive will be delineated to genospecies to understand which genospecies are circulating in the area.

5.2 Methods

The study population consisted of companion animals attending a veterinary consultation specifically for tick removal or where a tick was observed during a consultation by the attending veterinarian or veterinary nurse. After the first tick was observed, a further 2-minute check was performed on the animal to check for any additional ticks, paying special attention to the ears, underarms, and groin area. All ticks collected from an individual were removed intact, pooled and stored in 70% ethanol. Similar to a previous study (Ogden *et al.* 2000) it was deemed too time consuming to check that an animal was truly free of ticks and so no "tick negative" animals were used as controls in this study.

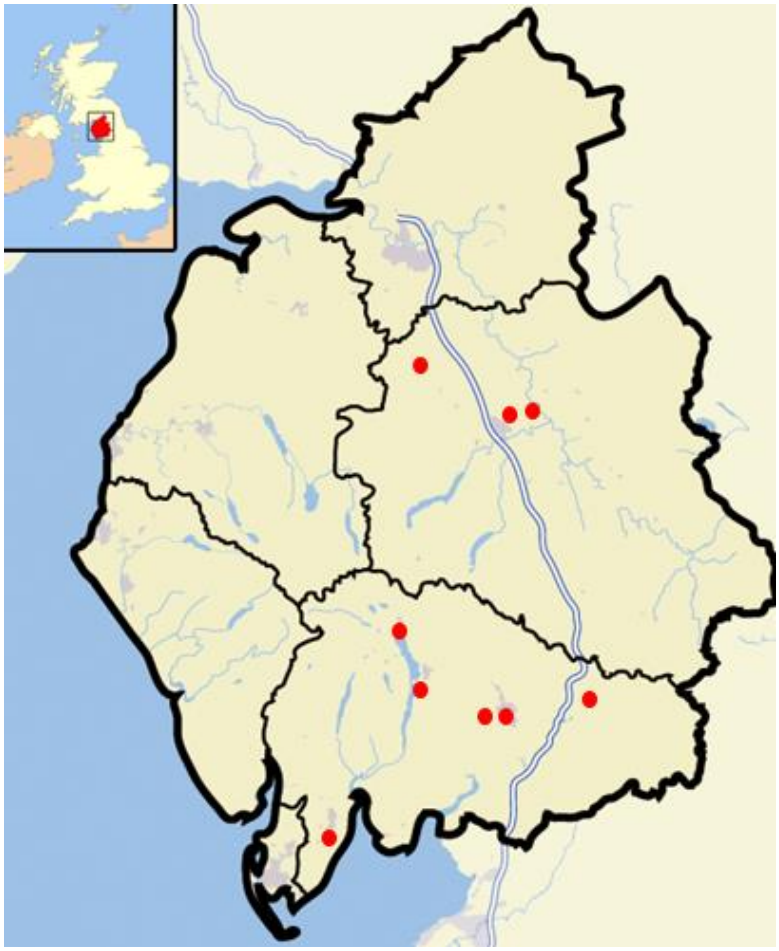

Figure 31. A map of Cumbria showing the locations of the nine collaborating veterinary clinics.

Recruitment to the study was carried out by Ian Wright, a veterinary parasitologist acting as a consultant for Merial, who sponsored this work. Nine Cumbrian veterinary practices were recruited into the study; the locations of these practices are shown on Figure 31. These practices were initially approached by telephone or email. After they agreed to participate, I visited each one to explain the aims and design of the study. These were further clarified in writing and were emailed to practices after my meeting with them. This written information was used to assist the lead veterinarian to disseminate information about the study to other relevant members of staff at the practice. Each practice was given 50 1.5ml microcentrifuge tubes (Starlabs, Milton Keynes) containing 70% ethanol for tick collection and data collection sheets (Appendix 1). The vets were also provided with a contact email and telephone number in case of any questions or if additional supplies were needed.

The data collection sheets that accompanied the tick samples included mainly questions relating to the individual from which ticks were collected, including species, breed, sex and age. A postcode of residence was used for geographical location, and it was noted if the animal had been in kennels or on holiday within or outside the UK within the previous two weeks. The data sheet also included a question about which (if any) acaracide treatment had been applied to the animal and the timing of these treatments. Questions on the ticks themselves included the date first noticed, where on the animal the tick was observed and the quantity of ticks collected. I visited practices at least twice a year to collect tick samples and completed data collection sheets. These visits also served to remind veterinarians about the survey and encourage their continued participation.

At Salford University, the species, life stage and sex of all ticks were determined by reference to an anatomical key (Section 2.2). DNA was extracted from individual ticks using the protocol in section 2.3.1, then DNA extracts were diluted either one in ten for fully engorged nymphs and semi-engorged adults or one in one hundred for fully engorged adults. Each extract was then used as template in the *B. burgdorferi* s.l.-specific RT-PCR described in section 2.4.1. All extracts that yielded an amplicon in this assay were analysed using reverse line blotting (section 2.5) to determine which genospecies of *B. burgdorferi* s.l. were present.

Univariate statistical analysis was performed using Minitab 18 (Minitab Ltd, Coventry). Relationships between aspects of the host animal and tick burden were assessed using generalized linear mixed models (GLMM) in R Version 3.4 (R, Development core team, Vienna). Non-significant factors were removed in turn until a minimal model remained. The practice the samples were submitted from was used a a random effect and variables including age, sex, location, breed, if the animal had been on holiday or in kennels two weeks previous, and month tick sample was collected were used as fixed effects. Dog breed was later categorized as one of the seven breed types listed by the Kennel Club (U.K.), namely: Gundog, Hound, Pastoral, Terrier, Toy, Utility or Working, cats were classified into domestic, breed or feral for clarity and postcodes were converted into north, south, east and west Cumbria. Missing values were replaced with NA before analysis.

5.3 Results

From March 2014 to June 2017, 608 tick samples were collected by nine collaborating veterinary practices. These ticks were collected from 356 animals including 210 dogs, 91 cats, 5 hedgehogs, 2 ferrets, 1 rabbit, 1 sheep, and 1 horse. 43 submissions had no information about the host species, these along with two submissions were excluded from this study as the first was from a human and the second was from a garden. Only the submissions from cats and dogs were used in the analysis.

5.3.1 Cats and Dogs

464 ticks were taken from cats and dogs, 316 (68.10%) were *I. ricinus,* 113 (24.35%) were *I. hexagonus,* three (0.65%) were *I. canisuga,* 26 (5.60%) were Pholeoixodes, and six (1.30%) were unidentifiable due to missing mouth parts. 373 (80.39%) of the ticks were adults, 80 (17.24%) were nymphs and five (1.08%) were larvae (Figure 32). All 3 (100%) *I. canisuga* were adults. 76 (67.25%) *I. hexagonus* were adults, 32 (28.32%) were nymphs. For *I. ricinus* 294 (94.04%) were adults, 22 (6.96%) were nymphs. The remaining 31 nymphs could not be identified to species level. All 464 ticks were tested for *B. burgdorferi* s.l. and six (1.29%) were positive. Of these, a *Borrelia* genospecies could be determined for four; three were *B. garinii,* and, one *B. burgdorferi* s.s. All positive ticks were *I. ricinus.*

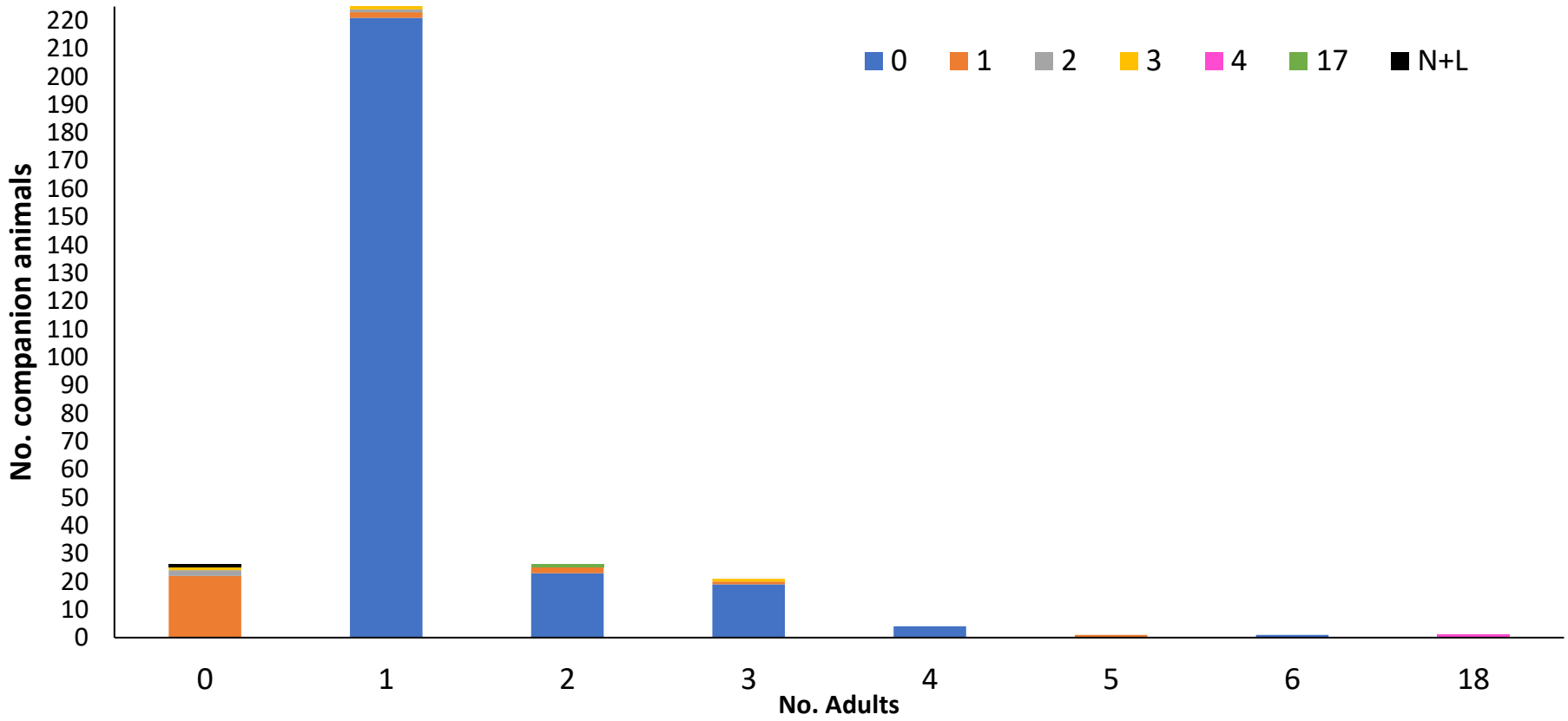

Figure 32. The frequency distribution of larval, nymphal and adult ticks on companion animals. At each intensity of adult infestation, the numbers of host also feeding; 0 nymphs (blue), 1 nymph (red), 2 nymphs (green), 3 nymphs (purple), 4 nymphs (pink), 17 nymphs (yellow) and multiple nymphs and larvae (black) is shown.

Analysis revealed that dogs were significantly more likely to have *I. ricinus* (82.41%) than *I. hexagonus* (9.31%). Cats were significantly more likely to have *I. hexagonus* (49.43%) than *I. ricinus* (42.25%) (X^2 13.18, d.f. 2, P value 0.001). A negative binomial pattern of infestation was found in both cats and dogs (Figure 33). When exploring infestation intensity, dogs were significantly more likely to have one tick than multiple ticks, whereas cats were significantly more likely to have 3 or more ticks rather than 1 or 2 (X^2 32.34, d.f. 3, P value 0.001). The most ticks submitted from a dog was 22, and from a cat 19.

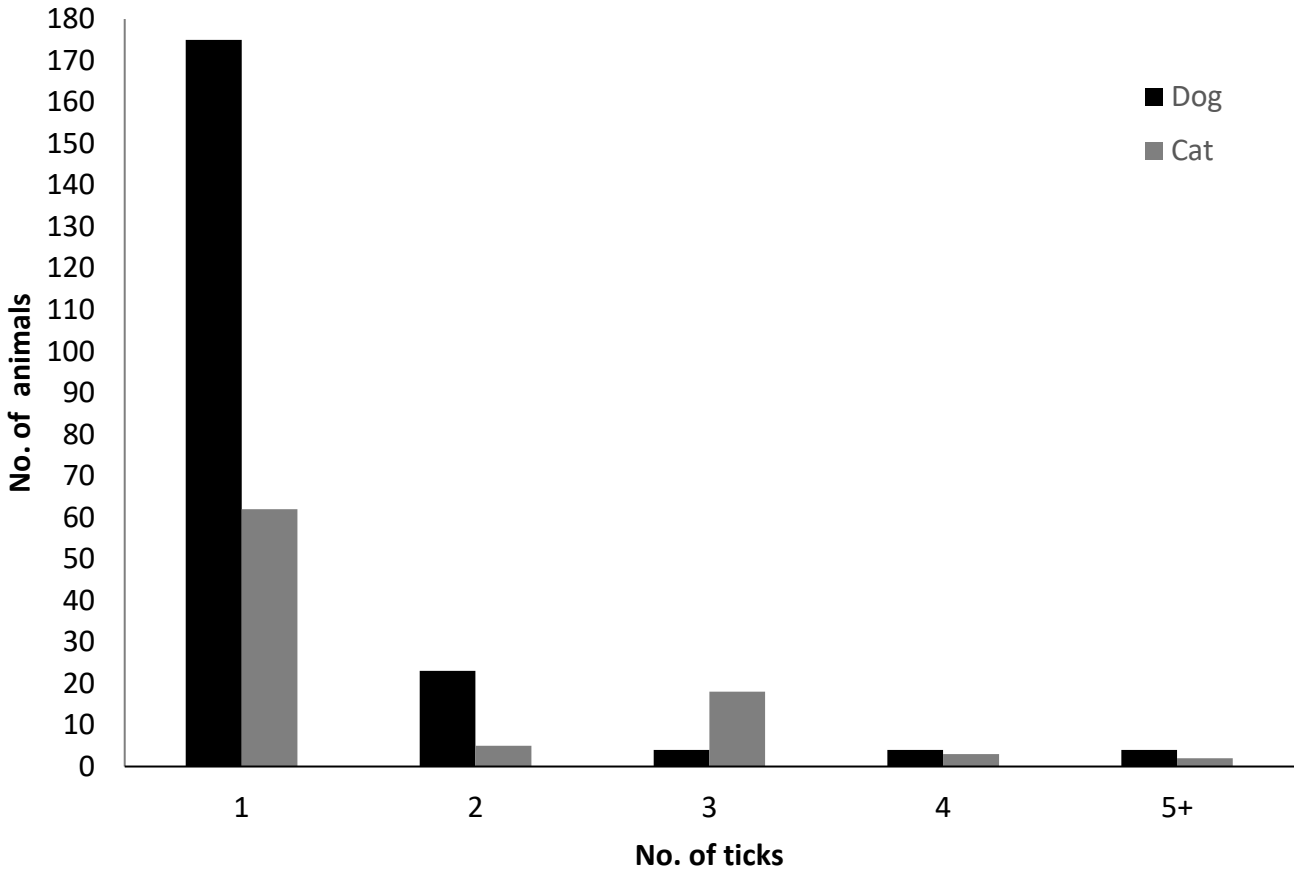

Figure 33. Numbers of ticks found on each cat or dog.

I. ricinus was identified in all months that samples were submitted, the most *I. ricinus* was found in July. *I. canisuga* was only found in April and May, *I. hexagonus* was found in April, May, June, July, and September. *I. hexagonus* sample submissions peaked in June (Figure 34).

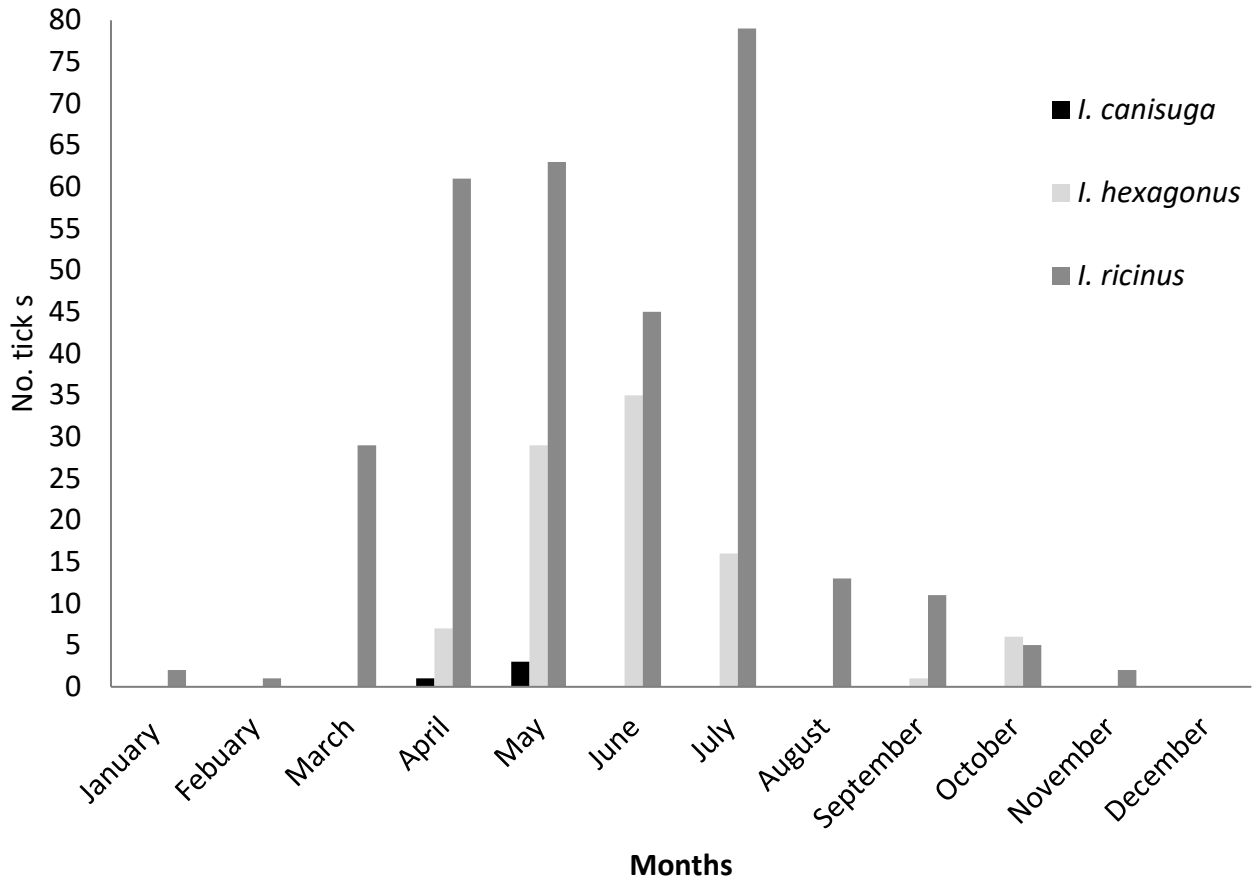

Figure 34. The monthly distribution of each species of tick found on companion animals in the study over the three years.

Ticks were encountered on cats and dogs throughout the year except for December (Figure 35). May was the month with the most dogs presenting with ticks, these samples showed a bimodal pattern of submission with a smaller peak occurring August / September. There were fewer submissions from cats, who also had a bimodal pattern of submission. The two peaks submissions from cats were in April and September.

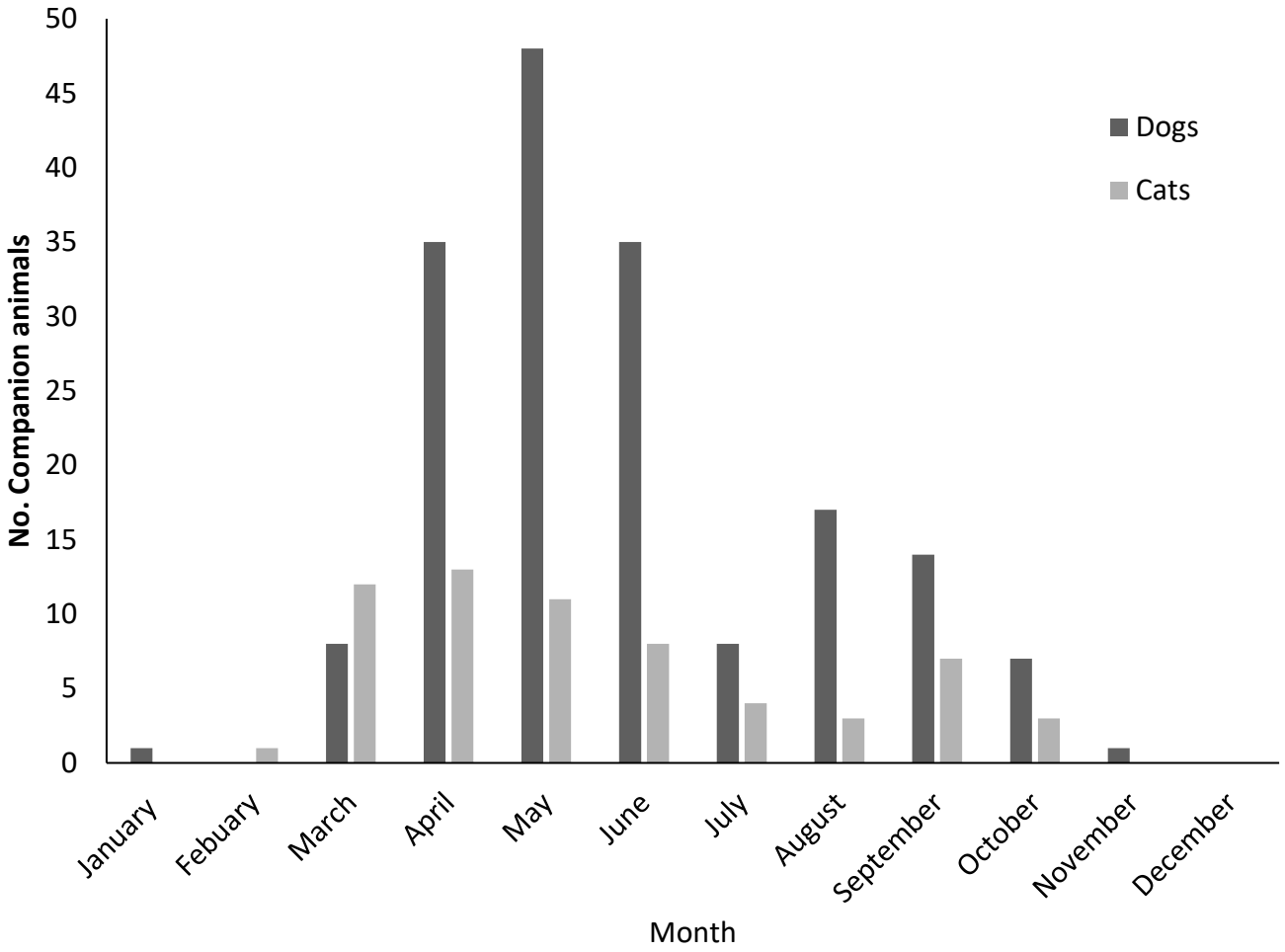

Figure 35. The monthly distribution of submissions from dogs and cats

Most ticks (72.96%) were observed on the head of the animals, with very few being found on the limbs or torso. *I. canisuga* was only found on the head (Table 16).

Table 16. The number (percentage) of each tick species on different anatomical sites of companion animals.

	I. ricinus	***I. hexagonus***	***I. canisuga***
Head	115 (68.5%)	17 (56.7%)	3 (100.0%)
Torso	42 (25.0%)	7 (23.3%)	0 (0.0%)
Limb	11 (6.5%)	6 (20.0%)	0 (0.0%)

For dogs, the GLMM showed that practice was not significant as a randome effect (varience= 0.062, Standard deviation =0.25). Male dogs were more likely to have a higher

tick burden than female dogs (Table 17). Significantly more ticks were found on dogs found on dogs in July.

Table 17. Summary table of results from a negative binomial generalised linear mixed model testing for host and environmental factors on the tick burden of dogs. Baseline = Female and April.

Parameter	Estimate (S.E)	Z value	Probability
Intercept	0.690 (0.283)	2.44	0.015
Sex M	0.390(0.174)	2.24	0.025
January	-1.412 (1.045)	-1.35	0.177
March	0.314 (0.411)	0.76	0.445
May	0.043 (0.243)	0.18	0.860
June	0.159 (0.264)	0.60	0.547
July	0.713 (0.264)	2.70	0.007
August	-0.279 (0.501)	-0.56	0.578
September	-0.166 (0.382)	-0.44	0.663
October	-0.259 (0.456)	-0.57	0.571
November	0.247 (1.036)	0.24	0.812

For cats, practice was not found to be significant as a random effect (Varience = 5.1e-07, standard deviation = 0.0007). There was no significant association between type of cat or the month the tick sample was submitted (Table 18).

Table 18. Summary table of results from a negative binomial generalised linear mixed model testing for host and environmental factors on the tick burden of cats. Baseline = Feral and April.

Parameter	Estimate (S.E)	Z value	Probability
Intercept	1.022 (0.462)	2.21	0.027
Domestic	-0.570 (0.350)	-1.63	0.103
March	-0.300 (0.455)	-0.66	0.510
May	-0.627 (0.482)	-1.30	0.194
June	-0.125 (0.477)	-0.26	0.793
July	-0.627 (0.598)	-1.05	0.294

August	-0.372 (0.689)	-0.54	0.590
September	-0.452 (0.482)	-0.93	0.350
October	-0.452 (0.651)	-0.69	0.488

5.3.2 Other animals

52 ticks from five hedgehogs were submitted to the study. These were all identified as *I. hexagonus* with the exception of four nymphs that could not be identified to species. The highest number of ticks a single hedgehog carried was 30 and the least was one. All these ticks were also tested for *B. burgdorferi* s.l. but none were positive.

Ticks were submitted from two ferrets; one ferret had one *I. hexagonus* the other had 21 ticks; 15 *I. hexagonus,* one *I. ricinus,* one *I. canisuga* and 4 nymphs which could not be identified to species. Three ticks from this second ferret were positive for *B. burgdorferi* s.l. two *I. hexagonus* and one *I. ricinus*. One *I. ricinus* each was submitted from a sheep, a horse and a rabbit, none were positive for *B. burgdorferi* s.l.

5.4 Discussion

5.4.1 Cats and Dogs

In the present study three tick species were found; *I. ricinus, I. hexagonus* and *I. canisuga*. *I. ricinus* was the most abundant species of tick encountered, making up 68.1% of those submitted, which agrees with other studies which found between 31.3% - 89.2%(Ogden *et al*., 2000; Smith *et al*., 2011; Abdullah *et al*., 2016). *I. hexagonus* was the second most frequently identified tick with 24.4% which is also consistent with other studies which found between 9.8% and 59.7%. (Ogden *et al*., 2000; Smith *et al*., 2011; Abdullah *et al*., 2016). The higher finding of 59.7% of *I. hexagonus* was explained by a high percentage of *I. hexagonus* being supported by urban parks (Ogden *et al*., 2000). The least common species of tick found in the present study was *I. canisuga*, 0.7%, this was similar to Abdullah *et al*. (2016) who found 0.8% but was significantly lower (X^2 value 490.99, d.f.3, P-value 0.0001) than in other studies (5.6%-11.9%) (Ogden *et al*., 2000; Smith *et al*., 2011).

Other studies found a wider range of species such as *H. punctata, D. reticulatus* and *D. variabilis* not encountered in the present study. This is possibly due to the study being

restricted to Cumbria, which is in northern England. *H. punctata* was found only in the south east of England, *D. reticulatus* was found sporadically in southern England, Wales and once in Yorkshire (northern England), whereas *D. variabilis* was found on a dog that had been imported from the USA (Ogden *et al.,* 2000; Smith *et al*., 2011; Abdullah *et al*., 2016). *R. sanguineus* is another tick of veterinary importance but was not found in the present study although it was found in previous similar studies and was only found on dogs that have travelled outside the UK (Abdullah *et al*., 2016). In 2014, in Essex a report of a house infestation, was cause *R. sanguineus* being imported from Spain on a rescue dog (Hansford *et al*., 2017b). Only one dog was recorded to have travelled abroad within two weeks of noticing the tick, this dog had *I. ricinus*, no exotic ticks were found in this study.

I. ricinus was found to attach to companion animals all year round and peaked in July, which is consistent with previous findings (Smith *et al.,* 2011). The present study found *I. canisuga* in April and May, this is not consistent with a previous study which found *I. canisuga* attached to dogs in the UK from March to September (Smith *et al.,* 2011). *I. hexagonus* was shown to attach to dogs from March to October in a previous national study (Smith *et al.,* 2011), this species of tick was found in a smaller window of time in the current study. *I. hexagonus* was collected from April until July and again in September. This is consistent with records of *I. hexagonus* on hedgehogs where two peaks; the first during April to June and then the second in September to October were recorded (Arthur, 1953).

I. hexagonus represents 24.4% of the ticks infesting companion animals. *I. hexagonus* was more prevalent on urban cats than rural cats in a study in 2000 which suggested that urban parks which can support animals such as badgers, foxes and hedgehogs can also support high densities of *I. hexagonus* (Ogden *et al.,* 2000). The focus of the present study was Cumbria which is predominantly rural, and so no comparison could be made between urban and rural environments in this study.

The life stage of ticks has been linked with different attachment sites on the bodies of humans. This is thought to be due to the optimal questing height for each tick, as the tick progresses through the life stage it is able to quest higher and for longer (Wilhelmsson *et al.,* 2013). In this study too few juvenile ticks were available to look into stage dependant

attachment. The majority of the submissions were found on the head and neck region. It is thought that the preferred attachment sites of ticks are the head, neck and ears, because these are areas where the animals groom less frequently so ticks can feed here for longer period's undisturbed (Koch, 1982; Dantas- Torres & Otranto, 2011). There is evidence to suggest that different genra of ticks prefer different locations on the host body for attachment (Koch, 1982). The present study found only I*xodes spp.* Of which the majority were found on the head or neck, which is in agreement with a study in Hungary that found most *Ixodid* ticks attach to the head and neck (Földvári & Farkas, 2005). When thinking of the host size dependant strategy hypothesised in Mejlon & Jeanson (1997) most adult ticks were seen to quest between 40-100 cm and the average height for a dog is 46.8cm (McGreevy *et al.,* 2013), which suggests that adult *I. ricinus* quest at an adequate height range to use dogs as host. This could be why most of the submissions were adults. However, it must also be taken into consideration that adults are larger and so easier to spot than nymph and larvae, especially in the fur of an animal, and so there is most likely some bias towards this stage. There is a void in the literature on observer bias of ticks but molecular evidence of blood meals from nymphs shows that the larval stage does feed on dogs and are perhaps unnoticed due to their size (Collini *et al.,* 2016).

Significantly more dogs carried only one tick rather than multiple, this was also the case in previous studies (Smith *et al.,* 2011*;* Ogden *et al.,* 2000; Abdullah *et al*., 2016). Ogden *et al.* (2000) found that cats were significantly more likely to carry *I. hexagonus* and dogs are significantly more likely to carry *I. ricinus.* This agrees with the results from the present study. *I. ricinus* actively quests for a host in woodlands, parks, moorlands and even gardens, all of which are environments frequented by dogs during exercising (Ginsburg *et al.,* 2008). It has been hypothesised that cats are more likely to have *I. hexagonus* due to their inquisitive nature, entering nest or burrows. Hedgehog nests have been shown to be infested by numerous (1 to 48) ticks of all stages (Arthur, 1953). The same study reported aggregated tick burden on a dog which was exercised on a patch of land where four hedgehog nests were located. This experiment was repeated over five times and the dog had a median of 8 ticks. Although this study is very basic it gives tantalising evidence that *I. hexagonus* is aggregated around nests in the environment (Arthur, 1953). Further work

should look at the distribution of *I. hexagonus* and build evidence to assess if this aggregation of attachment is true.

Male dogs were significantly more likely to be infested with ticks than females. Male-biased parasitism has been documented in many different vertebrate species. This is thought to be due to both hormonal and behavioural factors. Studies in rodents have shown males with higher testosterone levels feed more ticks that survive to the next developmental stage. It is thought that the males must make a trade-off between producing testosterone and putting energy into their immune system. Therefore, they do not generate the acquired or innate immune response to a tick bite (Hughes & Randolph, 2001). Alternatively, behavioural differences between the sexes have been suggested males generally cover a larger area and so have a greater probability of encountering a tick (Randolph, 1975; Ostfeld *et al.,* 1996).

No association was found with age, but in other studies dogs over a year old were found to be significantly more likely to have a tick (Abdullah *et al.,* 2016). No significant associations were found with a stay in the kennels but this could be due to the small sample size (n=4) of dogs that attended the kennels. Previously, dogs have been reported to be significantly more likely to carry *I. canisuga* after period in kennels (Ogden *et al.,* 2000).

Dogs but not cats were significantly more likely to be bitten by more ticks in July. Questing ticks are found in high populations in the summer months and so this could be why more ticks are feeding in July. These results agree with numerous other studies including a study that used health records to monitor tick bites. They found the highest biting levels between May to July (Tulloch *et al.,* 2017), a national study which collected ticks from dogs also found the highest biting prevalence to be May to July (Smith *et al.,* 2011) and a study of human health records that found arthropod biting peaked from July to September (Newitt *et al.,* 2016).

I. ricinus is the tick of most veterinary importance as it has the highest biting risk in the UK according to the tick surveillance scheme (Jameson & Medlock., 2011) and is a competent vector for many pathogens including *B. burgdorferi* s.l. (Barbour *et al.,* 1983; Grzeszczuk *et al.,* 2002*;* Gorenflot *et al.,* 1998*)* LB is suspected to cause pathogenesis in dogs (Littman *et*

al., 2006) and it has been suggested that LB can cause symptoms in cats (Magnarelli *et al.,* 1990). The samples submitted to this study were tested for *B. burgdorferi* s.l. and infections were only encountered in *I. ricinus*, at a prevalence of 1.3% (6/464). Two of these six ticks were collected from cats and four from dogs. *I. ricinus* was the only species of tick from companion animals to be positive for *B. burgdorferi* s.l. From other animals in this study *I. hexagonus* was found to be positive for *B. burgdorferi* s.l. This has been documented before (Smith *et al.,* 2011; Abdullah *et al.,* 2018; Davies *et al.,* 2017; Jahfari *et al.,* 2017). *B. burgdorferi* s.l. has also previously been detected in *I. canisuga* taken from samples from three badgers and one fox, this study reported eight out of 16 *I. canisuga* were positive for *B. burgdorferi* s.l. (Couper *et al.,* 2010). Only 3 *I. canisuga* were found in this study and none were positive for *B. burgdorferi* s.l. DNA using PCR detection.

Although the prevalence of *B. burgdorferi* s.l. in this study was lower than previous studies this difference was not significant (X^2 = 3.493, d.f = 3, P-value = 0.326). 1.4% of ticks from dogs in the current study were positive *for B. burgdorferi* s.l. Smith *et al*. (2012) found 2.3% of ticks from dog's positive for *B. burgdorferi* s.l. this study enlisted the help of vets nationwide and found that infected ticks were found throughout the UK. Another national study of ticks from dogs was conducted in 2015 which found a 2.0% infection prevalence and again this showed infected ticks throughout the UK (Abdullah *et al*., 2017). For cats, in this study 1.1% of ticks were positive for *B. burgdorferi* s.l. A national study found 1.8% *B. burgdorferi s.l.* infection prevalence from ticks taken from cats. Although samples were taken from all regions *B. burgdorferi* s.l. infected ticks from cats were only found in Scotland, the west of England and Wales. Although one infected tick was found in London (Davies *et al.,* 2017).

Comparing these results to European studies the UK prevalence of *B. burgdorferi* s.l. in ticks from companion animals is very low. In 1996 ticks taken from dogs in Germany were tested using PCR for *B. burgdorferi* s.l. and found 22.0% of adult ticks were positive for *Borrelial* DNA (Beichel *et al.,* 1996). Similarly in The Netherlands in 1998, ticks from dogs were tested using PCR and 14.5% of ticks were found to be positive, these samples were sequenced and *B. afzelii, B. garinii, B. burgdorferi* s.s. and *B. valaisiana* were identified (Hovius *et al.,* 1998). *B. afzelii* and *B. garinii* was also found in tick from dogs in Hungary in 2007, where 5.6% of

pooled ticks were found to be positive for *B. burgdorferi* s.l. (Foldvari *et al.,* 2007). In Poland only *B. afzelii* was identified from ticks taken from dogs with an overall infection prevalence of 6.2% (Zygner *et al.,* 2008). Most recently in Belgium ticks from cats and dogs were found to have an overall infection prevalence of 10.1%, when these samples were sequenced they were identified as *B. afzelii, B.garinii, B. burgdorferi* s.s*., B. valaisiana, B. lusitaniae* and *B. spielmanii* (Clareabout *et al.,* 2013). Differences in these results could be due to differences in assay specificity or could be genuine differences in infection prevalence.

The genospecies of *B. burgdorferi* s.l. infecting companion animal associated ticks in this study could only be determined from 3 of the 6 infected specimens. *B. garinii* and B. *burgdorferi s.s*, were both detected and have been found in questing ticks in the north of England (see Chapter 3 and Bettridge *et al*., 2013). Other studies in the UK have also found a mix of genospecies in ticks collected from dogs; *B. garinii, B. burgdorferi* s.s and *B. afzelii* were all found in *I. ricinus*. This study also reported an *I. ricinus* infected with *B. spielmanii*, which is the first time this genospecies has been detected in the UK (Abdullah *et al*., 2017). *I. ricinus* removed from cats were found to be infected with *B. afzelii* and *B. garinii,* one *I. hexagonus* was found to be infected with *B. afzelii* (Davies et al., 2017). *B. burgdorferi* s.s is the only genospecies that circulates in the USA. Significant associations were found with seroprevalance of *B. burgdorferi* s.s and symptoms in dogs, perhaps this *Borrelia* genospecies causes pathogenesis in dogs (Magnarelli *et al.,* 1990). This would be interesting to study in more detail.

5.4.2 Other animals

Collecting ticks from animals such as rabbits and ferrets that are usually kept in gardens, gives evidence to suggest that ticks are encroaching into gardens. This has been documented before (Jameson & Medlock, 2011). Ticks from livestock such as sheep and horses has been well documented and shows that these animals can act as a host for *I. ricinus* (Ogden *et al.,* 1997; Cull *et al.,* 2018).

The tick burden on each hedgehog and ferret varied considerably, this aggregation of ticks on hedgehogs has been recorded before (Jahfari *et al.,* 2017). The tick burden of hedgehogs

has been investigated with respects to the hedgehog's health and the study found that unhealthy hedgehogs were more likely to carry ticks than healthy hedgehogs (Bunnell *et al.,* 2011). All the hedgehogs in the current study carried *I. hexagonus* which has been shown to be a specialised tick that lives in the nest of small mammals especially hedgehogs (Jameson & Medlock*.,* 2011) but it has also been shown that hedgehogs can carry other species of tick such as *I. ricinus* (Jahfari *et al.,* 2017). The ferrets in this study carried a mixture of *I. ricinus, I. hexagonus* and *I. canisuga.* Ferrets have previously been shown to carry *I. hexagonus* (Beichel *et al.,* 1996) and *I. ricinus* (Macleod, 1934), *I. canisuga* has been reported to feed on foxes and badgers in the UK (Macleod, 1934).

I. hexagonus is a competent vector for *B. burgdorferi* s.l. under laboratory conditions, with bacteria being transmitted from infected ticks to naïve mice (Gern *et al.,* 1991). It has also been shown that the hedgehog is a competent reservoir host for *B. burgdorferi* s.l. (Gray *et al.,* 1994). This shows that the hedgehog and *I. hexagonus* have the capacity to create an infection cycle. However, the small number of ticks collected from the present study were not infected with *B. burgdorferi* s.l. A recent paper from the Netherlands found *B. burgdorferi* s.l. infections were highly prevalent in hedgehogs, with a diversity of genospecies encountered. The authors concluded that hedgehogs can sustain these spirochetes in natural cycles of infection (Jahfari *et al.,* 2017).

Two *I. hexagonus* were positive for *B. burgdorferi* s.l. these came from a ferret. One *I. ricinus* from the same ferret was also positive for *B. burgdorferi* s.l., unfortunately the *Borrelia* genospecies could not be delineated. Historic tick samples held at the natural history museum found *I. hexagonus* taken from a ferret. These were tested by PCR for *B. burgdorferi* s.l. of the 28 samples 4 (14.3%) were positive for *B. burgdorferi* DNA (Hubbard *et al.,* 1998).

Although this project has produced some useful data it is not without its limitations, the samples submitted are only part of the picture. The data from this study is limited to those who seek veterinary help to remove ticks, as are previous studies which use veterinary collaborators of information systems (Ogden *et al.,* 2000; Abdullah *et al.,* 2016; Smith *et al.*, 2011; Tulloch *et al.,* 2017). Even the tick surveillance system run by PHE, although it

provides good data on tick species and where they are located in Britain, it does not give the full picture of tick bite incidence (Cull *et al.,* 2018; Jameson & Medlock, 2011). To gain a more representative idea of how many companion animals or people in the UK are bitten by ticks, the information should be gathered from the general public. A study similar to Jaenson *et al.* (2012) or an extension of Scharlesmann *et al.* (2008) that uses public perception, would give a better idea of tick biting incidence nationally, although these also have their limitations.

Overall, this study found that *I. ricinus* was the most common tick to attach to companion animals and the infection prevalence in these ticks was low. Three species of ticks were found in this study area *I. ricinus, I. hexagonus* and *I. canisuga.* Dogs were significantly more likely to have *I. ricinus* than *I. hexagonus* and cats were significantly more likely to have *I. hexagonus* than *I. ricinus*. Host associations were found for dogs with tick burden. Males were significantly more likely to have a tick than females. This study suggests that in the Cumbrian area companion animals are at a low risk of being bitten by an infected tick. Therefore, the veterinary threat of LB to companion animals is very low.

Chapter 6 General discussion

6.1 Chapter summary

Lyme borrelisosis (LB) is the most common infection in the temperate regions of the northern hemisphere (Rizzoli *et al.,* 2011). In the USA and Europe many studies have been carried out on the infection prevalence of *B. burgdorferi* s.l. in humans, reservoir host, companion animals and questing ticks, by comparison only a handful of studies have explored this zoonotic pathogen in the UK.

This thesis aimed to explore tick abundance along with *B. burgdorferi* s.l. prevalence to add to the knowledge of this pathogens ecology in the UK, using as many parts of the one health philosophy to do so. Firstly Chapter 3 explored the environmental hazard, monitoring the population dynamics of ticks and the *B. burgdorferi* s.l. prevalence within them, calculating the DIN and estimating the environmental hazard over space and time. Then Chapter 4 assessed the tick burden of the grey squirrel, investigated the infection prevalence in these squirrels and the ticks that fed upon them. Infection rates from feeding ticks and questing ticks were compared to understand if squirrels amplified any specific *Borrelia* species in the area. Finally, Chapter 5 explored the veterinary and public health risk of being bitten by a tick, using companion animals. This Chapter not only quantified the tick burden and infection prevalence on these animals but also reported on what species of tick were present in the study area.

6.2 Major findings

6.2.1 Tick density

DON was significantly different at each site. According to Schwartz *et al*. (2009) Chapel House and Bouth were both categorised as medium abundance, whereas Linsty Green was categorised as very high tick abundance. The significant difference in DON at all three sites was constant throughout the three years. Significantly different DON at sites has been recorded before in other studies from Europe, although these studies were on a much larger scale (Takken *et al*., 2017; Jouda *et al*., 2004). Identify high and low risk areas for tick bites will help the public make an informed decision on firstly if they want to spend time in that area but secondly on if they are to use preventative measure such as wearing long clothing or treating their pets with acaracide. The results from Chapter 4 the squirrels showed

peatland areas are significantly less likely to have ticks. Which was hypothesised because less deer frequent these areas, the environmental data in Chapter 3 gives evidence to back up this theory as the sites with less deer (Chapel house) has less ticks than those with more deer (Linsty Green) had consistently more ticks. Areas with high deer populations could be used as a proxy for assuming a high tick population and so could be sign posted to warn humans about a high risk area for tick bites. Quantifying the hazard in the environment can help to understand the risk to animal and human health, showing that approaching disease ecology using a One Health approach is useful. Forestry workers and public health professionals could work together to try to build/manage forest in a manner that would create low risk areas, for example culling or fencing deer from areas which are known to have a high visitor rate or clearing the leaf litter from deciduous forests (Tack *et al.,* 2013).

Tick abundance was shown to significantly vary over time, an overall increase in tick abundance was found annually. If ticks are increasing in abundance, then more tick awareness campaigns should be set up. Many of the current tick awareness campaigns are run by pharmaceutical companies and are aimed at selling acaracide for companion animal and not on prevention methods for humans (e.g. http://www.bigtickproject.co.uk). As ticks become more common in the UK, the public should be educated on preventative measures. Although an annual increase in tick burden was not found in either companion animals or in squirrels, which suggest that the risk of a tick bite to humans or animals has not increased. The one health approach enables us to look at not only the environmental hazard but also the public / veterinary risk.

A strong seasonal variation in tick abundance was found. Spring had significantly higher tick abundance that any other season. This has been reported before as a product of bimodal phenology (Takken *et al*., 2016; Randolph *et al*., 2002). However, this peak is not reflected in the data collected from the companion animals, which suggests that the risk to human and animal health is a combination of tick density and behaviour to the environment as stated by Dobson *et al*. (2011).

Dogs were significantly more likely to be bitten in July. Although these results are consistent with previous studies which all found a peak in May-July (Wright *et al*., 2018; Tulloch *et al*., 2017; Smith *et al*., 2011), this does not coincide with the peak of questing tick abundance found in Chapter 3 which was in spring (March, April, May). It has been proposed that

people are bitten more in the summer due to fair weather causing more contact with the environment (Garcia-Marti *et al*., 2017). Dogs that are generally walked by an owner, which could explain why they showed a summer bias towards having more ticks, cats are less restrained in their movements and showed not significant associations between number of ticks with month or season. Ticks were collected from companion animals, all year round which agrees with the results of the questing ticks in Chapter 3. This shows that ticks are not just a seasonal threat to public and veterinary health. This information could be used to time tick awareness campaigns before the peak (July) which could help to lower the risk of a tick bite in humans and animals.

6.2.2 *B. burgdorferi* s.l. prevalence

To my knowledge this was the first study to sample *I. ricinus* monthly to explore the *B. burgdorferi* s.l. infection prevalence in the UK. Although studies exploring *B. burgdorferi* s.l. have sampled areas annually or seasonally (Hansford *et al*., 2017a; Millins *et al*., 2016) and tick population dynamics have been explored at three-week intervals (Dobson *et al*., 2011).

From this study, a seasonal pattern of *B. burgdorferi* s.l. infection prevalence was found, with a peak in the spring. This has been previously reported in Sweden, where the rise was attributed to larvae feeding from infected shrews and voles in the previous autumn (TäLleklint & Jaenson, 1996). In the UK mice have been linked to higher rates of *B. burgdorferi* s.l. in questing nymphs (Gray *et al*., 1995). This spring increase in infection rates and *B. afzelii* (which is the rodent-associated genospecies) being the most commonly found genospecies suggests that rodents are probably an important driver in *B. burgdorferi* s.l. prevalence in the study area. It would be interesting to investigate the rodents in the area to accurately assess how they contribute to the cycle of *B. burgdorferi* s.l. Although this thesis did assess a rodent reservoir host *B. afzelii* did not appear to be transmitted to feeding larvae as no *B. afzelii* was detected in the feeding larvae, but *B. garinii* was found in larvae from *B. afzelii* infected squirrels. Previous studies, show comparable results (Millins *et al.,* 2015). Possible explanations for this include co-feeding, transovarial transmission, or partial feeding on another host before feeding on the grey squirrel. The evidence is mounting that *B. afzelii* although able to infect grey squirrels is not transmitted to larvae from grey squirrels (Millins *et al.,* 2015) unlike the red squirrels (Humair & Gern, 1998) and more studies should look at transmission of *B. burgdorferi* s.l. between grey squirrels and larvae.

Overall annual infection prevalence in questing ticks did not significantly differ each year, however on a site level infection prevalence was significantly different each year. This has been documented in the south of the UK (Hansford *et al*., 2017a), which asks the question on what geographical scale should *B. burgdorferi* s.l. infection prevalence be investigated. In the UK significant differences in *B. burgdorferi* s.l. infection prevalence was found at different sites in numerous studies (Vollmer *et al*., 2011; James *et al*., 2012; Bettridge *et al*., 2013; Hansford *et al*.,2 017a; Millins *et al*., 2016). The infection prevalence at Chapel House was significantly higher than Linsty Green and Bouth. The current study was performed on a small geographical scale (5km) and significant differences were found for infection prevalence which is similar to other local (Hansford *et al*., 2017a), and larger scale studies (Bettridge *et al*., 2013; James *et al*.,2012; Millins *et al*.,2016). It is yet to be established on what scale *B. burgdorferi* s.l. infection prevalence should be monitored but the results from this study suggest that it must be smaller than 5km. The questing ticks taken from Chapter 4 were from sites with as little as 3km between them and these also had significant differences in infection prevalence. A study Wielinga *et al*. (2006) found that plots as little as 200m from each other had differing *B. burgdorferi* s.l. prevalence.

B. burgdorferi s.l. infection prevalence in ticks attached to companion animals had an overall infection prevalence of 1.29%. This gives an estimation of the risk of being bitten by an infected tick to animals, and by proxy, the risk to humans. This infection prevalence compared with the overall infection prevalence (3.75%) in the questing ticks from Chapter 3 and Chapter 4 (3.69%) is significantly lower. This may better reflect the risk of *B. burgdorferi* s.l. to human and animal health as it considers both the environmental hazard and risk (Dobson *et al*., 2011). As dogs tend to frequent the same environments as their owners, it can be assumed that the risk to humans is similar. A study using human participants assessed the infection prevalence from the biting ticks and questing ticks from the environment, there was no significant difference. The infection prevalence from the biting ticks was 12.4% which is much higher than that found from the companion animals (Hall *et al*., 2017). However, these human participants were exposed to moorland whereas the companion animals will have acquired ticks from various locations and the questing ticks from this study were from areas of know *B. burgdorferi* s.l. presence. Companion animals

were more likely to have adult ticks, which should have a higher infection prevalence as they have had more opportunity to be infected. Whereas the questing ticks were nymphs. Fully fed adults can inhibit PCR and although these samples are diluted to prevent inhibition it could be possible that inhibition still occurred giving a lower infection prevalence.

6.2.3 *B. burgdorferi* s.l. genospecies

In Chapter 4 only ticks from positive squirrels were tested for the presence of *B. burgdorferi* s.l. DNA. So, these cannot be compared with the ticks from companion animals which came from animals with an unknown infection status. However, the genospecies found in these ticks can be compared. In ticks from grey squirrels *B. garinii, B. afzelii* and *B. valaisiana* were found. Ticks from companion animal's *B. garinii* and *B. burgdorferi* s.s. were found. From the environment all four genospecies were found *B. garinii, B. afzelii, B. burgdorferi* s.s and *B. valaisiana* were identified from questing nymphs. The ticks attached to a host are more representative of the ticks that could potentially bite humans. These results show that people and animals in Cumbria could be exposed to all the pathogenic genospecies (*B. afzelii, B. garinii* and *B. burgdorferi* s.s).

Interestingly in Chapter 4 grey squirrels were shown not to transmit *B. afzelii* to larvae but were transmitting *B. garinii*. This was also found in a previous study in Scotland (Millins *et al.,* 2015). *B. garinii* was originally thought of as five outer surface protein A (OspA) serotypes, with one was specific to rodents and the other four to birds, but now this serotype has been classified as *B. bavariensis* as it was sufficiently different enough from the other four serotypes (Margos *et al.,* 2008). Millins *et al.* (2015) used phylogenetic analysis to determine if the *B. garinii* found in the grey squirrels were one genetic cluster. However, the results show that this was not true and many different strains of *B. garinii* were found in the squirrels. Unlike the native red squirrels which have been shown to transmit the rodent associated *B. afzelii* (Humair & Gern, 1998) my results show that the grey squirrels are infected with many genospecies of *B. burgdorferi* s.l. and only transmit *B. garinii* and *B. valaisiana.* These squirrels were imported from the US where the only *B. burgdorferi* s.s. is present. These squirrels may not have the immunity acquired by the native squirrels to fight against the *B. burgdorferi* s.l. infections and so this could be a reason why they are infected with so many genotypes.

Borrelia genospecies were monitored over space and time. *B. afzelii* was the prevailing genospecies at Chapel House and Linsty Green, whereas *B. garinii* was the most common genospecies at Bouth. Previous studies have shown associations with woodland type and genospecies. For example, In Scotland, *B. afzelii* and *B. garinii* were more likely to be found in mixed woodland, (James *et al.,* 2014) in contrast, in Belgium *B. afzelii* was mostly found in pine whereas *B. garinii* and *B. burgdorferi* s.s were more likely to be found in Oak stands (Ruyts *et al.,* 2016). In the current study *B. afzelii* was the dominant genospecies at both a mixed woodland site and a Pine site. Perhaps it is not the woodland type which defines these associations but the host that live within the woodlands. James *et al.* (2014) suggested that high levels of *B. burgdorferi* s.s. in coniferous woodland was due to red squirrels inhabiting these areas, although caution is advised when interpreting these results due to the small number of positives (James *et al.,* 2014). Reports from the forestry commission have confirmed that red squirrels can be found in Chapel House which is dominated by *B. afzelii* and red squirrels have been shown to be infected with *B. afzelii* (Pisanu *et al.,*2014). In Chapter 4 the grey squirrels were shown to transmit *B. garinii* and Bouth could have a high population of grey squirrels causing this to be the dominant genospecies in the questing ticks. However, more studies would need to be carried out to confirm this, they should look at a site from an ecosystem point of view and try to work out what contribution each host makes to the cycles of *B. burgdorferi* s.l. within that ecosystem.

In the current study the genospecies picture differed overtime, this was not significant. This does not agree with results reported in Scotland where a significant difference was found in the genospecies make up of some sites when they were resampled (Millins *et al*., 2016), although this study was over a longer timescale. A better picture of genospecies distribution could have been created if more success in delineating the genospecies had been achieved. It shows the public and veterinary health risk that the two dominating species are pathogenic to humans and potentially to some animals.

6.2.4 Biting risk

The risk of tick bite to humans and animals is often categorised using DIN. The results in this study show the DIN was significantly higher at Linsty Green and in spring. This study is one of only a few studies in the UK that looks at tick populations over time to understand the environmental hazard of LB. It is clear from the results that DIN is not static overtime. The

results show that the environmental hazard in the study area varies both temporally and spatially, which agrees with many studies across Europe (Takken *et al*., 2017; Richter *et al*., 2012; Tveten, 2013). Educating medical and veterinary professional on when peaks of infection occur can help them to give advice on preventative measures and a better understand of the disease. However, when you compare the DIN data with the tick burden data from companion animals. It suggests that animals are more likely to be bitten in summer but this could be due to human/ animal activity at this time of the year. Alternatively, the vets/ owners maybe more aware of ticks in summer and pay more attention to removing ticks at this time of the year.

6.2.5 *B. miyamotoi*

Alongside *B. burgdorferi* s.l., *B. miyamotoi* was found at all three sites and in all years. These results add to the geographical distribution of *B. miyamotoi* as it has only previously been reported in the south of England (Hansford *et al*., 2014; Layzell *et al*., 2017; Hansford *et al*.,2017) and Scotland (Hall *et al*., 2017). To date no confirmed cases of *B. miyamotoi* illness have been recorded in the UK but this evidence of the pathogen residing in the UK, should be motivation to train health professionals about the signs and symptoms of relapsing fever caused by *B. miyamotoi*. No evidence of *B. miyamotoi* was found in ticks from companion animals but this sample size was only small to detect a pathogen at such low levels. As this pathogen is circulating in such a low prevalence in the environment then this suggests that the risk to humans is low. Squirrels and squirrel ticks were not tested for *B. miyamotoi* but it would be interesting to see if this pathogen circulates in squirrels as the reservoir host is thought to be rodents. However, our results show that *B. afzelii* which is the rodent associated strain of *B. burgdorferi* s.l. does not transmit from grey squirrels so it would be interesting to see the if *B. miyamotoi* is specific to wood mice or all rodents including grey squirrels.

6.2 Further work

It would be interesting to understand on what spatial scale to monitor *B. burgdorferi* s.l. infection prevalence. The results presented in this thesis show that sites only 5km apart have significant differences but perhaps if the study design had included sites of varying differences from each other the concept of distance could be explored more thoroughly, this could be an extension of this study. To attempt to better quantify the risk to human

health it would be useful to quantify the human and companion animal "traffic" that passed through the longitudinal sites. This would have enabled us to establish which site is most risky to human and animal health. Further work could be undertaken by inviting people who regularly visit these sites to send in ticks they find attached to themselves or their companion animals along with data of where they have been and how long they spent in the area. This would be a great use of citizen science to explore the human risk of LB and perhaps could be designed as a mobile application that could track people via GPS, similar to Jennett *et al*., 2013 who used GPS to track dogs around a park to assess tick biting incidence in dogs. This could then be updated by the individual if they are diagnosed with LB.

To complement the longitudinal study, it would have been useful to have assessed the host population using methods such as mist netting, to quantify the bird population and small mammal traps for the rodent populations. Further work surveying the tick populations while simultaneously assessing the host diversity and abundance at each site, would hopefully shed some light on which hosts are important.

In Chapter 4 other ectoparasites were found on the grey squirrels such as fleas. It would be interesting to test for other infections vectored by these ectoparasites such as *Bartonella* species. With regards to the suspected co-feeding transmission and amplification of *B. garinii* occurring on/in the grey squirrels it would be interesting to follow this up in more detail.

To further develop the work carried out in Chapter 5, the samples collected could be tested for other TBP that cause disease in companion animals, such as *Babesia* spp and *Anaplasma spp* which have both been shown to be present in ticks collected from cats and dogs (Davies *et al*., 2017; Abdullah *et al*., 2017; Smith *et al*., 2013).

6.3 Concluding remarks

Southern Cumbria is a known tick "hot spot", where many animals, local people and tourists may come into contact with ticks. This project gave evidence that *I. ricinus* populations and the *B. burgdorferi* s.l. infection prevalence is heterogeneous over space and time in the environment. This means that predicting LB risk to humans and animals is difficult as the environmental hazard is not static but ever changing. Using a One health approach to *B. burgdorferi* s.l. ecology has enable me to explore the environmental hazard of *B. burgdorferi*

s.l. but also the risk of tick bite and the risk of an infected tick bite. Temporal patterns of tick population increased as well as *B. burgdorferi* s.l. infection prevalence increases have come to light and to educate vets and health professionals about this rise in spring could mean that they are extra vigilant to LB cases and removing ticks from pets or giving advice on when to use preventative measure such as treating animals with acaracide.

Understanding the importance of each host species in a woodland can help manage the woodland in a way that could reduce LB. This study showed the grey squirrels are an amplification host for *B. burgdorferi* s.l. and possibly filter *B. garinii* rather than *B. afzelii.* Forestry workers could use this information to better manage woodlands. For example, culling squirrels or fencing deer enclosures in areas with high tick populations and high visitor rates to decrease the risk of a tick bite. From surveying the ticks on companion animals, three tick species were found to reside in the area, this information would have been missed if environmental sampling alone had been carried out. This is useful to veterinary and public health professionals as it makes them aware that these ticks which could carry other pathogens than *B. burgdorferi* s.l. are in the area and so potentially could help to diagnose animals and humans with TBDs. The screening of environmental questing ticks and ticks feeding on animals showed that *B. afzelii, B. garinii, and B. burgdorferi* s.s. and *B. valaisiana* were present in the study area, all these genospecies except *B. valaisiana* are pathogenic to humans and possibly to companion animals. Knowing what *Borrelia* genospecies are in an area can help to inform health professionals about the signs and symptoms they should be aware of in late LB as each genospecies has been linked to different symptoms. This One Health approach to investigating LB in Cumbria has generated useful information on the ecology of LB over space and time.

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Appendix 1: Data collection sheet from veterinary clinics

Patient Details

Patient samples -

Post code of residence -

Date of presentation to clinic –

Species –

Breed –

Age –

Sex –

If tick treatment/preventative used:
Date of last treatment –
Product –

If the patient has left the country in the last 6 months:
Countries visited –

If the patient has left the county in the last 6 months
Counties visited –

Has the patient been in kennels in the past 2 weeks?

Tick Details

Number –

Location on body –

Date first noticed –

Appendix 2: Solution recipes

Final solutions	**Recipes**
20ml 1.25% Ammonium Hydroxide solutions	Add 1ml of Ammonium hydroxide solution (sigma) to 19ml of dH_2O
500 mL 2x SSPE	Add 50 mL of 20x SSPE(Sigma) to 450 mL of dH_2O
500 mL 1% SDS	Add 50 mL of 10% SDS(Sigma) + 450 mL of dH_2O
500 mL 2x SSPE/0.1% SDS	Add 50 mL of 20x SSPE(Sigma) and 5 mL of 10% SDS(Sigma) to 455 mL of dH_2O
500 mL 2x SSPE/0.1% SDS	Add 50 mL of 20x SSPE (Sigma) and 25 Ml of 10% SDS (Sigma) to 425 mL of dH_2O
100 mL of 20 mM EDTA	Add 20 mL of 100mM EDTA (Invitrogen) to 80 mL of dH_2O. Check pH is 8.0.