Exploration of the spatial epidemiology of tick borne pathogens of livestock in Southern Cumbria

Lauren Danielle Perrin

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Where to start?

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List of abbreviations

6FAM- 6-carboxy-fluorescein

AONB- Area of Outstanding Natural Beauty

APHA- Animal Plant and Health Agency

BB- Bovine Babesiosis

bp- base pairs

CDF- Cumulative Distribution Function

DTM- Digital Terrain Model

EDAC- Ethyl-Dimethylaminopropyl-carbodiimide, Hydrochloride

ELISA- Enzyme Linked Immunosorbent Assay

ESA- European Space Agency

FC- Forestry Commission

GIS- Geographical Information Systems

GLM- General Linear Model

GPS- Global Positioning System

HCl- Hydrochloric acid

HGA- Human Granulytic Anaplasmosis

Lat- Latitude

LiDAR- Light Detection and Ranging

LIV- Louping Ill Virus

Lon-Longitude

NaOH- sodium hydroxide

NDVI- Normalised Digital Vegetation Index

NIR- near infrared

OS- Ordnance Survey

PCR- Polymerase Chain Reaction

q-PCR- real-time Polymerase Chain Reaction

RGB- Red Blue Green

RLB- Reverse Line Blot

RS- Remote Sensing

RWF- Red Water Fever

SDS- sodium dodecyl sulphate

TAMRA- 6-carboxyl-tetramethyl-rhomadine

TBE- Tris-Borate-EDTA

TBEV- Tick Borne Encephalitis Virus

TBF- Tick Borne Fever

Abstract

Changes to farm production subsidies are provoking the maintenance of far less stock. Given that wildlife is more abundant in sustainable uplands, pathogens able to exploit both wild-living and domesticated hosts are of particular concern. Tick-borne pathogens are not only a case in point; but also their threat is now augmented by increasing tick abundance, changing climate, and the extraordinary nationwide increase in the abundance of deer (that serve as a key host species).

The major objective of this thesis was to further understand the spatial distribution of questing *Ixodes ricinus* ticks across farms in Southern Cumbria; as well as to attempt to understand the epidemiological and ecological factors that have a significant influence on the patterns of infections in livestock. This project integrated field work, GIS, molecular methods and citizen science in an effort to understand these complex epidemiologies.

Results demonstrated that: *I. ricinus* exhibit a patchy distribution across all study sites, with proximity to woodland indicated as the main driver behind this. The causal agent of tick borne fever, *Anaplasma phagocytophilum* was observed across the sites, with sheep being implicated as the main drivers behind this, rather than deer.

Conflicting to anecdotal evidence, *Babesia divergens* (the agent of red water fever in cattle) was not found at any of the sites. However, the discovery of *B. venatorum* in a new area of the UK is of potential medical importance. In addition to the confirmation of *B.* OO-2012, and a *B. odocoilei*- like species; this, to the best of my knowledge, is the first recording of these pathogens in the UK.

Contrary to scientific studies, *Borrelia afzelli* was observed in adult *I ricinus* females feeding on cattle; suggesting that the results from in vitro assays do not seamlessly translate into the field, and that further work is needed to quantify the role cattle may play in the circulation of Borrelia, which is of public health importance. Louping Ill Virus was sampled for, but was not confirmed.

The truly multidisciplinary nature of this project, has demonstrated the need for a holistic approach when considering the ecology and epidemiology of tick borne disease

Chapter 1: Introduction

1.1 General overview

Ticks have been recognised for their ability to transmit disease for hundreds of years and are currently considered to be second, only to mosquitoes, as vectors of human infectious diseases in the world. There are currently over 800 species of tick identified (Merino et al., 2005). Which have evolved to parasitize all classes of terrestrial vertebrate. From these blood-feeding habits, they are involved in the transmission of an impressive range of pathogens including viruses, nematodes, protozoa, bacteria and rickettsial infections (de la Fuente et al., 2008). Vector-borne pathogens are coming under increasing scrutiny in the UK, with changes in the environment, including global warming, changes in land use, growing wildlife populations and farming practice having a growing impact on the behaviour and ecology of both host and tick (Süss et al., 2008).

This is ultimately altering their geographical distributions, leading to new, previously uninfected areas, overlapping of infected areas which were once separate and emerging disease, including disease strains (Randolph, 2008). These changes could potentially have great effects on tick populations and the diseases they carry, of both human and veterinary importance.

With 20 different tick species carrying over 27 known pathogens in the UK alone, it is a very real and pressing issue. The farming industry in particular record massive losses due to tick borne disease, though there is great disparity between anecdotal evidence and reported data. Tick populations are patchy in the UK, with several geographical locations identified as 'hot-spots', including Gloucester, upland Scotland and Cumbria. In areas where ticks are found, disease can cause huge economic losses and, as of yet, is not always fully understood. A holistic, approach is really required to discover, understand and resolve much of the 'unknown' that is tick borne disease.

Changes to farm production subsidies have had a significant impact on livestock farming in the British uplands, provoking the maintenance of far less stock. The resulting decrease in grazing pressure potentially alters upland ecology in many ways, one of which is a shifting of the “infectious disease landscape” in which pathogens with transmission cycles favoured by a changing upland ecology will become of greater veterinary importance. Given that the consensus view of sustainable uplands is one in which wildlife is more abundant, pathogens able to exploit both wild-living and domesticated hosts are of particular concern.

Tick-borne pathogens are not only a case in point, but also their threat is now augmented by increasing tick abundance, changing climate, and the extraordinary nationwide increase in the abundance of deer (that serve as a key host species).

The three tick-borne diseases that pose the greatest threat to livestock in the UK uplands are (i) bovine babesiosis (red water fever) caused by *Babesia divergens*, (ii) tick-borne fever caused by *Anaplasma phagocytophilum*, and (iii) louping ill caused by the louping ill virus. All three are transmitted by the catholic-feeding tick *Ixodes ricinus*, and have established wildlife reservoirs.

The true impact of ticks and tick borne disease to cattle and livestock remains a relative unknown, with diseases being non-reportable, infection not necessarily resulting in overt disease and potential mis-diagnosis. A strong link between Southern Cumbria and the group at the University of Salford via previous projects studying *Borrelia burgdorferi,* the causal agent of Lyme disease, gave the group a presence there. From this, anecdotal evidence from the farmers was shared, regarding perceived 'ticky' farms, and almost cyclical incidences of disease. These farms and livestock are often in areas which are difficult to sample and require time consuming logistics. In an attempt to mitigate that; information from the farmers, access to their land and livestock, and their participation in sample collecting, embracing the Citizen Science ethos.

Recent studies have utilized this increase in interest to begin address its usefulness as a tool for public health and vector borne disease; collecting large numbers of samples without the need for complex field work (Curtis-Robels et al, 2015; Hall et al, 2017). This study explored the feasibility of expanding this method and idea to the farming industry; another aspect of the one health sphere. Followed by coupling Citizen Science with scientific research methods to take a holistic approach to considering the threat posed by ticks and the pathogens that they carry, across commonly grazed upland and a farm in Southern Cumbria.

This three year PhD project aimed to quantify tick populations and the diseases they carry at farms in the British uplands in Cumbria. These farms were identified by the Forestry Commission (FC) as having large tick populations, a history of disease, and a willingness to participate in the project.

The desired outcomes of this project were:

i) To determine the presence of ticks on a common grazed land and one upland farm in southern Cumbria and to map their distribution and discuss with farmers as to whether they matched the anecdotal reports of distribution

iii) To compare tick heat maps to other ecological data, such as vegetation and wildlife abundance, to explore if this can be used to identify drivers of tick distribution at these sites?

(iv) To explore the role of wild animal reservoirs as sources of infection to livestock by comparing sequence data from strains observed in ticks taken from the environment, ticks taken off sheep, and culled deer bloods.

(v) Sampling sheep for ticks; can 'super-spreaders'- ticky cohorts of sheep- be identified by sampling sheep. If so, can GPS tracking be used to see if ticky sheep were spending more time in the ticky parts of the Moor, identified by the heat maps.

(vi) Explore the feasibility of using Citizen Science within a farming capacity- can a mutually beneficial relationship be established via knowledge exchange, access, and sample collection

(vii) Considering all data collected, can recommendations be made to each of the participants in this study regarding the exposure of their livestock to ticks and the pathogens that they carry

1.2 Ticks

Ticks are small arachnids, belonging to the order Ixodida. They are ectoparasites that have developed a specialist mechanism for survival known as hematophagy, feeding on the blood of their hosts. Ticks are classified into three families, two of which are of medical importance; Ixodidae (hard ticks) and *Argasidae* (soft ticks). Anatomically, the two families are differentiated on the basis of the presence or absence of a hard plate, or scutum, respectively. Ticks are also known to carry an impressive range of disease; this incredible ability of these arthropod vectors is as a result of many of their traits that are seen through the order. Perhaps the most studied are their '3-host life cycles' and often catholic feeding habits. Also, once a pathogen is acquired, it can be maintained transstadially as the tick moults into its next life stage or even transovarially from the infected mother to the eggs as she lays.

Twenty different species of tick that have been recorded in the UK (www.britishticks.org.uk), this project will focus on arguably the most economically, medically and veterinary important: *Ixodes ricinus. I.ricinus* belongs to the hard tick family, and as such, exhibits a 3-stage lifecycle. In this lifecycle, newly hatched six-legged larvae encounter then attach to a host and take their first blood meal, which may last over a day. Once replete, the larvae drop off their hosts and remain free in the environment as they prepare to, then complete their moult into nymphs. They require specific conditions of high humidity provided by favourable vegetation for this to occur (Macleoad, 1936), the process takes at least a few weeks and, depending on climate, sometime much longer.

During the moult to nymph, the tick grows two legs, and the emergent, eight-legged nymphs encounter then attach to a host so that the tick takes its second blood meal. When fully engorged, the nymph drops off its host once more to moult into the adult stage. Adult ticks then encounter and attach to a third host. Only the female takes a blood meal on this host, but during this feed, she will be sought out by a male and copulation will take place.

This final feed may take up to seven days to complete after which the replete, gravid female drops off the host to lay her eggs, which number in the thousands. In optimal climate conditions, these eggs hatch within about 6 weeks, completing the lifecycle. The tick may be infected with a pathogen during a blood meal taken at each of its life stages. Once infection is acquired, the tick may then transmit the pathogen to a host when taking its next blood meal; resulting in the infection of the host with the pathogen. Dependant on the pathogen and immune status, this may cause overt disease (Figure 1).

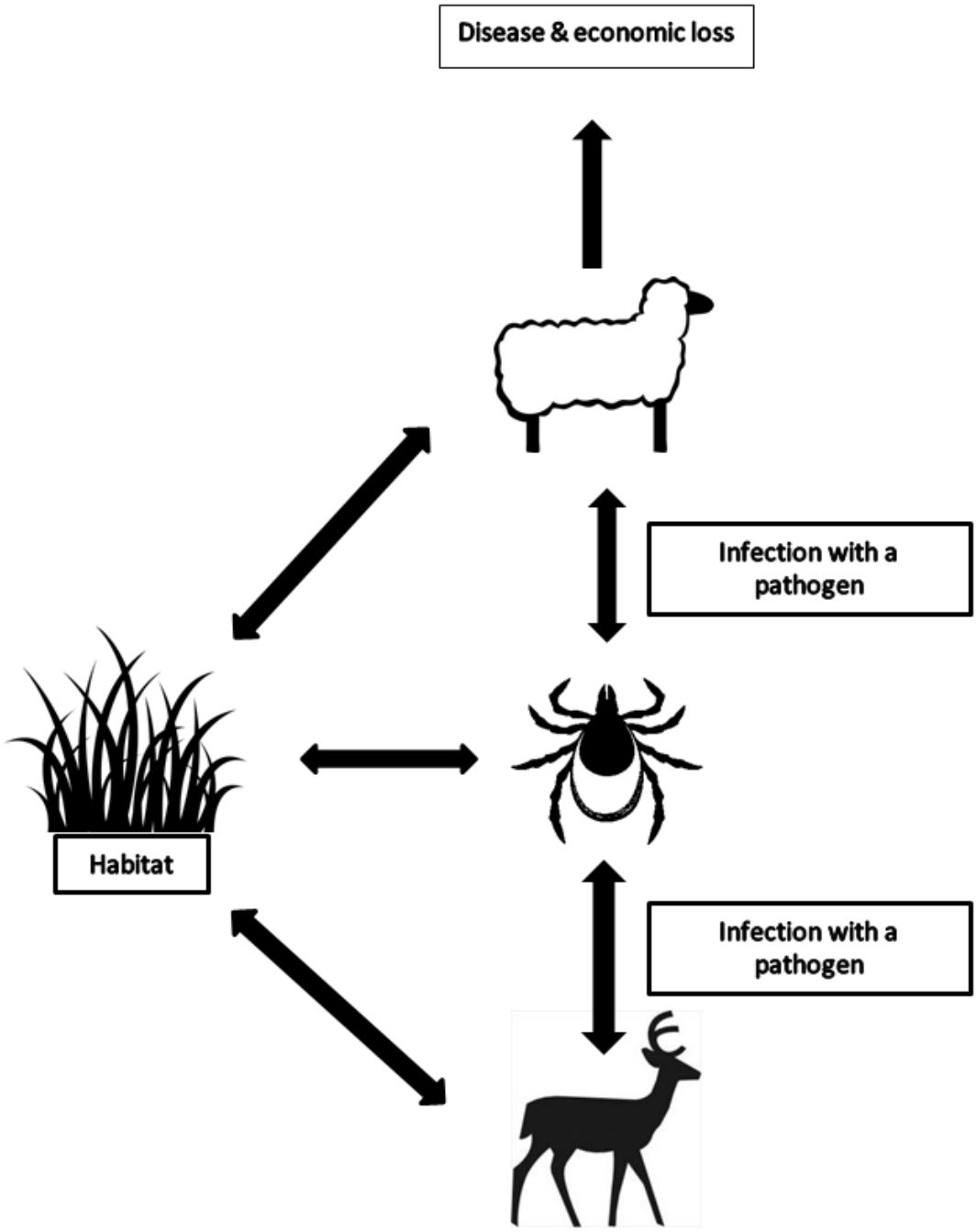

Figure 1 overview of interactions between the pathogen carrying tick, habitat, wildlife and domestic hosts.

I. ricinus is a generalist, feeding on a wide range of vertebrates, with each life stage of the tick preferring to feed on a different host animal (Keesing et al., 2010); which it locates by questing freely on vegetation. Thus, *I. ricinus* larvae tend to feed on rodents, shrews or birds, whereas nymphs feed on midsized mammals such as rabbits and squirrels, and birds, and adult females feed on large mammals such as deer or sheep.

Deer have long since been implicated as essential for maintaining tick populations (Wilson et al, 1988; Jaenson et al, 1992), and can carry hundreds of ticks at any one time (Carpi et al, 2008; Vor et al, 2010).

In the UK ticks are primarily active from mid-spring until mid-autumn. When the temperature drops below about 10^{o}C, ticks become inactive. Overwintering in the environment insulated in burrows, nests or deep in leave litter. All ixodid life stages are active throughout the warmer months, although in some habitats markedly different season patterns have been observed, with, for example, *I. ricinus* nymphs being most active during spring and early summer (Walker, 2001).

The tick life cycle coupled with the concurrent activity of the three life stages underlies the efficacy of ticks as vectors of mammalian and avian parasites. Indeed, an individual tick may be infected with several parasites simultaneously. Furthermore, the longevity of ticks, which may take several years to complete their lifecycle, enhances their contribution to the natural maintenance of the parasites they transmit, hence ticks are as much hosts in themselves as vectors for many parasites.

Currently, ticks are mainly controlled on an agricultural scale by acaricide based dips and pour ons. These typically only last around six weeks and so do not always prove helpful when sheep are turned out on to the fell for months at a time. Some farmers consider ticks in their husbandry and will move flocks around 'ticky' and 'non-ticky' ground in order to build up resistance to disease. Other areas for control involve the tick habitat; ticks are incredibly dependant on the micro climate and succumb easily to desiccation. Therefore they prefer dense vegetation matt that will limit their exposure to the elements; bracken is believed to play a role in supporting tick populations. In recent years, bans and restrictions have been put in place concerning the herbicide Asulox, which is used specifically against bracken. This ban could alter upland ecology and tick populations and is no longer a viable method of control.

1.3 Louping Ill Virus

Louping ill (LI) has been recognised as a disease of sheep in Scotland since the 18th century and associated with tick borne transmission for close to 100 years. The pathogen was not identified as a virus however until several decades later, when it was confirmed as the first arthropod-borne virus in Europe (Greig et al., 1931). Due to the pathogens association with farming, it is not often considered from a medical perspective. However, in recent years it has been considered as forgotten and re-emerging threat to human health (Jeffries et al, 2014)

Disease is caused by the enveloped, single-strand RNA louping ill virus. Between 40-50nm in length, the virus belongs to the *Flavivirus* genus, in the growing family *flaviridae.* The family also includes the closely related tick-borne encephalitis virus (TBEV), an emerging disease of increasing human importance in other areas of Europe. They are so similar antigenically that cross-reaction occurs in many serological tests.

This acute, viral pathogen primarily affects sheep and red grouse in upland areas of the UK; particularly Scotland, Cumbria, Wales and Ireland, and is often associated with upland and upland farming (Gilbert, 2015). The virus is acquired from the bite of an infected sheep tick, *I. ricinus,* and continues to cause severe clinical disease, even death, in up to 60% of lambs and naïve flocks that are moved to endemic areas (Hudson, 1992). LI is also recognised as a zoonotic agent and while human infection can occur, this is rare and unlikely to result in a fatality (Jeffries et al., 2014). Flu-like symptoms and neurological signs are more commonly associated with exposure.

1.3.1 Hosts & Geographic Distribution

In sheep, LI causes debilitating neurological symptoms and severe encephalitis, which in many cases proves to be fatal. The virus can display pathology in sheep of all ages, though is most frequently diagnosed in lambs and yearlings, or naïve flocks moved to an endemic area for the first time.

Prevalence varies depending on farming practices, seroprevalences of up to 90% can be found in untreated sheep (Hudson, 1992). In endemic areas, sheep mortality ranges from 5 to 10%, although it may be as high as 60% in newly introduced individuals. An extensive survey (Reid et al., 1984) of viraemias in a range of animals demonstrated that only sheep can support a sufficiently high viraemia to infect both larval and nymph tick stages.

Clinical cases have also been documented in other domestic species and wildlife; including cattle, goats, horses, pigs, dogs, deer and humans (Twomey et al., 2001, Gray et al., 1988, Hyde et al., 2007, Ross et al., 1994, MacKenzie et al., 1973, Reid et al., 1978a).

As none of these species develop a high-titre viraemia, are considered as unlikely to play a role in the maintenance of the disease, with humans being seen to be accidental hosts.

Fatal cases have been reported among experimentally infected red grouse (*Lagopus lagopus scoticus)* causing up to 80% mortality(Hudson et al., 1995) (Reid, 1975). The disease can also have a devastating effect on naturally infected red grouse populations in areas where estates are managed for commercial grouse shooting. The birds develop high viraemia with the disease and contribute to the persistence of the virus when uninfected ticks feed. Most recently, the mountain hare (*Lepus timidus)* has also been implicated in non-viraemic transmission. Infection persists via co-feeding (Jones et al., 1997), where uninfected ticks can become infected through feeding in close proximity to an infected tick. Additionally, one study suggests that horses may sometimes develop viraemia that is sufficient to amplify this virus(Hyde et al., 2007).

LI occurs mainly in the British Isles. This disease been reported throughout upland areas of Scotland, Ireland, northern England, and Wales wherever the tick vector *Ixodes ricinus* is found. A small number of cases have also been reported in Denmark, Norway, and more recently Spain (Skarpaas et al., 2006; Ruiz-Fons et al, 2014).

Across the continent, there are a number of closely related tick-borne viruses that cause neurological disease in sheep or goats, which have been described as louping ill-like viruses (Gao et al., 1997). These have included Turkish sheep encephalitis virus (Whitby et al., 1993), Spanish sheep encephalitis virus (Marin et al., 1995) and Greek goat encephalitis virus (Papa et al., 2008). Because of this, it is difficult to determine the true geographic distribution of LI. This is further exacerbated by the lack of a universally accepted, optimum classification of the different isolates of LIV and the LIV-like viruses within the tick-borne encephalitis complex.

Similar to the distribution of LIV in the UK, these pockets of LIV-like viruses are considered restricted and rarely seen. This is much unlike TBEV, which is increasing in both prevalence and distribution (Grard et al., 2007). It is currently unknown how or why these diseases with such genetic similarity are persisting in nature so differently.

1.3.2 Transmission

The natural vector of the disease is the hard sheep tick *I. ricinus* and the occurrence of LI can be closely correlated with its presence. All life stages of the tick can acquire infection while feeding and though transstadial transmission through the different life stages of the tick has been documented, transovarial transmission appears not to occur (Hudson et al., 1997, Gaunt et al., 1997).

The consistent high levels of viraemia adequate to infect ticks and amplify the virus have so far only been documented in the sheep and red grouse, making these hosts crucial to the epidemiology of this disease. Though grouse can only act as amplifying hosts for a short period, as they succumb to the disease very quickly (Hudson et al., 1995, Hudson, 1992). furthermore, high titres sufficient for virus amplification have also been reported in experimentally infected horses (Hyde et al., 2007). However, the viral titer was much lower than in sheep, and this is yet to be observed naturally.

LI is also known to be transmitted by other routes. Red grouse can be infected by eating ticks (Hudson et al., 1995), and one group of pigs became ill after ingesting raw meat from infected lambs (Bannatyne et al., 1980) Additionally, the virus is shed in the milk of goats, and sheep; a potential source of infection for lambs and kids when they nurse(Reid et al., 1984).

1.3.3 Incubation Period & Clinical Symptoms

In sheep, the disease is characterised by a biphasic fever. The first phase occurs after the incubation period of six- 18 days. This is the viraemic stage and may be accompanied by various non-specific symptoms, such as a fever which may reach 42° C, depression and anorexia. Following this, the pyrexia subsides and the second rise may occur around five days after the first appearance of clinical signs. This is as a result of virus activity in the brain. If it does not, the animal will recover quickly and develop a durable protective immunity to the disease.

In instances where the virus succeeds in invading the nervous system, encephalitis may develop. Clinical signs at this stage include muscle tremors, rigidity, incoordination and general dullness. Affected animals often develop the characteristic hopping gait, called a “louping gait,” during which they move both hind legs, then both forelegs, forward in unison. As the disease progresses, many appear blind as they are unable to avoid walking into objects put in their path. They may stand as if in a stupor displaying head pressing and, when disturbed, over react and appear startled. Sudden, loud noises have been known to cause them to fall and go into a fit. Gradually a paralysis develops and eventually recumbency. Recumbent animals frequently lie on one side and make cycling movements with their legs. Once neurological symptoms are present, prognosis is usually hopeless and death occurs 7-10 days after the onset of clinical signs. Co-infection with the tick borne

apicomplexan *A.phagocytophilum* is known to increase pathogenicity of the disease(Macleod and Gordon, 1932), most likely via immune suppression though the exact mechanism is yet to be described.

Similar clinical signs and fatalities can occur in other mammalian species, including cattle (Hyde et al., 2007, Gray et al., 1988, Twomey et al., 2001). The louping gait does not seem to have been reported in species other than sheep. However, incoordination and neurological symptoms are common, and exaggerated 'goose-stepping' of the hind limbs was documented in a llama(Macaldowie et al., 2005).

1.3.4 Morbidity & Mortality

The incidence of LI in an affected area correlates with the fluctuations in the surrounding tick population. Most cases are observed in spring, early summer and autumn, when tick activity is at its peak.

Morbidity and mortality in sheep varies with the animal's immune status, coexisting infections, severity of challenge and other factors, including farming practice. In endemic areas, the mortality rate is usually 5–10% (Reid, 1975, Hudson, 1992), and most cases occur in animals that are less than two years old. This is a result of protective maternal antibodies that are usually passed to lambs and provide cover for the first few months of life; alongside older animals have acquiring immunity over time. These passively protected lambs become susceptible to disease after 1 year, and infection rates of up to 60% have been observed. Despite the high prevalence seen in this group, mortality appears to remain lower than 15%.(Reid, 1975).

The incidence of LI in more mature members of the flock is usually low, though it is more common to see all ages affected in newly introduced flocks. The biggest losses are seen in 1 year old breeding replacement stock, with no previous exposure to the disease and mortality rates can reach 60% (Reid 2013). Once a sheep has developed encephalitis, the case fatality rate is approximately 50%. Both fatal cases and recovery have been described in other species of mammals (Bannatyne et al., 1980, Timoney et al., 1974).

Red grouse appear to be very susceptible to LI. It has been observed that in areas with a high population density of the vector *I. ricinus,* up to 84% of the adult birds may be seropositive, along with up to 80% mortality, in areas where *I. ricinus* is common (Reid et al., 1978b).

1.3.5 Diagnosis

Clinical

Due to the characteristic louping-gait, when displayed, LI can be easily recognised and the disease should be suspected. This should also be the case in sheep with fever and other neurological signs, especially when the flock has recently been introduced to tick–infested areas. It should also be considered when fatalities occur in grouse populations supporting ticks. Conversely however, due to the initial general symptoms of fever and malaise, LI may be confused clinically with a range of other infectious and non-infectious diseases. In any case, a diagnosis should remain speculative until corroboration and confirmation with further laboratory analysis.

Laboratory tests

LI can be diagnosed definitively by virus isolation, the detection of viral nucleic acids via a one-step reverse transcriptase polymerase chain reaction (TaqManRT-PCR), with confirmatory staining and serology (Marriott et al., 2006). LIV may be recovered from heparinised blood during the acute phase of the disease. However, virus isolation from the blood is not feasible after the onset of central nervous system signs. At this point, host immunological responses have inhibited the viraemia. In the majority of cases, virus isolation is attempted on the brain and spinal cord from animals that have died of suspected LI.

Serological tests used include serum neutralization, enzyme–linked immunosorbent assay (ELISA), complement fixation and haemagglutination inhibition, which indicates recent infection via the detection of virus-specific IgM. One downside to serological testing is the possibility of cross-reactions occurring with other flaviviruses due to their similarity (Klaus et al., 2014).

1.3.6 Treatment

There is no specific treatment available for advanced cases of LIV infection in humans or animals. Though, unlike sheep, cattle affected with LI may respond to good nursing and symptomatic treatment.

A formalin-inactivated vaccine against LI is commercially available within the UK and has previously been used with some success in endemic areas (Shaw and Reid, 1981). However, shortages in vaccine supply(Balsom, 2013), along with increases in cost, demonstrate the need for a more sustainable control method.

1.3.7 Current Approaches to Control & Surveillance

LI that is introduced into a new area may be eradicated by euthanasia of infected animals, quarantines, movement controls and other measures, combined with effective tick control. Conversely, knowledge of whether an area is harbouring ticks and disease is imperative before the movement of naive livestock, to prevent significant losses.

In endemic regions, sheep can be protected by vaccination, where available, or by preventing exposure to habitats where ticks are found (Gilbert, 2015). However, this requires an in-depth knowledge of tick ecology, resident reservoir host population, control and surveillance.

Lambs born to vaccinated or naturally infected ewes are usually protected by maternal antibodies for the first few months of life. Vaccines have also been used in cattle and goats. Acaricides can reduce tick populations, but it is difficult to protect animals by this method alone. There is no specific treatment for LI, but supportive therapy including good nursing may be helpful. Enveloped viruses such as LIV are generally susceptible to most common disinfectants.

As LI is not a reportable disease, the actual impact the virus is having remains largely unknown. The latest non-statutory zoonoses annual report by the Animal & Plant Health Agency (APHA) for 2016, diagnosed 26 cases (4 cattle, 20 sheep, 2 birds). 25 cases were diagnosed in 2015 and a total of 38 cases were confirmed in 2014 (29 sheep, 6 cattle), in comparison to the 24 reported in 2012 and 35 in 2013, this is as far back as these records go. Though these numbers are small, they are likely not capturing the whole picture. Additionally, between 5-10% of the flocks the submissions came from were reported affected, with a heavy tick burden frequently described. The APHA have also pointed out that this figure may also be misleading as they received far fewer samples in 2014 than normal and they also acknowledge that anecdotal evidence, particularly in Cumbria, suggests much higher incidences of disease (AHVLA, 2014),(2014)(APHA, 2017).

There is limited knowledge available on the ecology of the disease and how it is able to persist in nature. Despite European Health Schemes involving routine dipping and vaccination (Laurenson et al., 2007), a solid control approach has not, as of yet, been established in the UK. With vaccinations known to be subject to shortages, unrealistic increases in price and tick populations on the rise nationally, LI is likely to become an even bigger threat to the farming community in the next few years. In addition, its relatedness with the medically important European TBEV also gives cause for concern. With fears that the virus could spread to the UK, knowledge of how other viruses within the complex behave could be critical in our approach to controlling/eradicating TBEV.

1.4 Tick Borne Fever & Pasture Fever

Recent years have seen a growing scientific interest in bacteria of the genus *Anaplasma,* as a result of increased recognition of their pathogenic potential towards livestock, companion animals and humans. Members of the genus are widely distributed across the northern hemisphere, but *A. phagocytophilum* is the only pathogenic member of the genus that is endemic in the UK (Heyman et al., 2010).

A. phagocytophilum is an obligate erythrocytic bacterium, transmitted by several tick species, including *Ixodes ricinus* Endemic in the UK, this tick borne pathogen is of significant medical, veterinary and even economic importance. The rickettsia was first recognised as the causal agent of the potentially fatal tick borne fever (TBF) in sheep, in Scotland circa 1932 (Gordon et al., 1932). Though it was not until the 1990's that the human disease, human granulytic anaplasmosis (HGA) was identified and described in the USA, originally as human granulytic ehrlichiosis (Chen et al., 1994).

A. phagocytophilium is recognised as the agent of HGA, TBF and pasture fever in livestock. It also contributes to the polymicrobial syndrome tick pyaemia, which is a major cause of losses to the UK sheep farming industry. Surveys of livestock and wild animal populations have revealed that the pathogen is widespread across Europe and is able to infect a wide range of mammalian hosts (Woldehiwet, 2010; Stuen et al, 2013). The bacterium is coming under increasing scrutiny as recent studies have found that this pathogen can also exist within sub-populations that are adapted to different natural cycles (Massung et al., 2002, Bown et al., 2009).

1.4.1 Classification

All *Anaplasma* species are obligate intracellular haemotrophic bacteria, residing in either erythrocytes or different white blood cell types.

A. phagocytophilum is a Gram-negative coccus shaped bacteria, unusual in its affinity for neutrophils. The organism is small, generally between 0.2µl-1.0µl in size and enveloped by two membranes. The outer membrane of the bacterium is often ruffled, creating an irregular periplasmic space, with no capsule.

Unlike many gram-negative bacteria, *A. phagocytophilum* lacks lipopolysaccharide biosynthetic machinery, resulting in very fragile cells that are highly susceptible to stress (Lin and Rikihisa, 2003).

The bacteria reside and replicate in host endosomes found within the cytoplasm of eukaryotic host cells. Here they obtain the sufficient nutrients to carry out binary fission and form a cluster known as a morula (Dumler et al., 2005). This is a rather unique strategy within the order *Rickettsiaceae*, which ordinarily escape from phagosomes and replicate directly within the cytoplasm of eukaryotes (Rikihisa, 2011).

1.4.2 Hosts & Geographic Distribution

The detection of *A. phagocytophilum* DNA in a wide range of mammals and *Ixodes* species from across the globe suggest that it is a generalist parasite with the potential to exploit multiple hosts and vectors. However, there is increasing evidence that the species may, in fact, comprise of sub-populations adapted to specific hosts and/or vectors. In the USA, *A.phagocytophilum* genotypes solely associated with infections in deer have been reported (Massung et al., 2002, Massung et al., 2003). Whereas in the UK, *A.phagocytophilum* genotypes solely associated with the rodent-specific tick *Ixodes trianguliceps* have been described (Bown et al., 2009). These observations also carry some public health relevance as, for example, only sub-populations adapted to *I. ricinus* are likely to infect humans. The 'sheep tick' is also the vector of most importance in relation to the farming industry within Europe.

The role of sheep in the natural maintenance of *A. phagocytophilum* has been studied in extensively, and it is clear that in some circumstances they serve as its main reservoir. One such study in 2002 established that *A. phagocytophilum* was being maintained solely within flocks of fell-grazing sheep, in the absence of deer or rodents as other hosts (Ogden et al., 2002).

Although some theories and associations have been put forward, the real epidemiological implications of *A. phagocytophilum* diversity have yet to be fully elucidated (Massung et al., 2002). This has been further compounded by the discovery of multiple strains circulating simultaneously within flocks (Ladbury et al., 2008) and wildlife (Bown et al., 2009). Advances in molecular typing have demonstrated that geographic clustering of strains does not occur on an international level (Jahfari et al., 2014)

1.4.3 Transmission

Tick Borne Fever, caused by *A. phagocytophilum* is mainly transmitted by the hard tick *Ixodes ricinus,* though it has been documented in *I. persulcatus*, *I. trianguliceps* and *Haemophysalis punctata* (Stuen, 2013). An infected tick passes on the disease whilst taking a blood meal. Similarly, an uninfected tick feeding on an infected animal may also pick up the disease. The bacterium can be transmitted transstadially through the different life stages of the tick, but there is no evidence to suggest that it can be passed transovarially to the next tick generation (Macleod and Gordon, 1933).

1.4.4 Incubation Period & Clinical Symptoms

The bacterium exploits cattle and, particularly, sheep as reservoir hosts and, in general these infections have little clinical consequences, however disease can develop in animals exposed to the bacterium for the first time. The most characteristic symptom of the disease in domestic ruminants is high fever, which occurs between 3-7 days after a tick bite (Macleod and Gordon, 1933), though clinical signs may vary depending on host age, the strain of *A.phagocytophilum* involved, the host species and host immunity (Stuen et al., 2011).

In sheep, this disease is mainly seen in young lambs born in tick-infested areas, and in naive older sheep introduced to an endemic area. The characteristic, sudden fever that lasts for 4 to 10 days, reaching temperatures of 42.2°C (Macleod and Gordon, 1933). Other signs are more general and are usually mild, including; a loss in appetite, weight, listlessness, coughing and increased respiratory and pulse rates. Animals will usually recover from the disease themselves and no longer display symptoms. However, studies have shown that the bacterium may still be isolated from the blood months, even years after the initial infection and can still be infective (Foggie, 1951). Further studies explored this further and demonstrated that these low-level, persisting infections were often comprised of several strain types circulating within a flock (Ladbury et al., 2008). TBF has been known to induce abortions and stillbirths in pregnant ewes introduced onto infected pastures during the last stages of gestation, with abortions are usually observed 2 to 8 days after the onset of the fever (Stamp et al., 1950). While in rams, semen quality can be significantly reduced, even causing temporary infertility (Watson, 1964).

Bovine tick-borne fever, or pasture fever, usually occurs in dairy animals recently turned out to pasture. Again the clinical signs are variable in severity, dependant on age, previous exposure and immune status, but may include lethargy, anorexia, decreased milk production, coughing, respiratory distress, abortions, stillbirths and reduced semen quality (Tuomi, 1967, Hudson, 1950).

In humans, HGA is a multi-systemic disease that often difficult to diagnose due to its unspecific, 'flu-like' symptoms. Though HGA is rarely life-threatening in itself, the ability of *A. phagocytophilum* to infect and colonise neutrophils can lead to immunodeficiency and hence make a host more susceptible to more profound opportunistic infections (Dumler et al., 2005). The first recorded case of HGA in the UK was recently documented (Hagedorn et al., 2014).

1.4.5 Morbidity & Mortality

Infection prevalence decreases significantly with age and lambs or naive animals are far more susceptible, until immunity has developed.

In most cases, animals make an uneventful recovery within two weeks and deaths are uncommon with the exception of aborting ewes. However, *A. phagocytophilum* increases susceptibility to other illnesses, which may be more serious, or even fatal. Most commonly, *A. phagocytophilum*-induced immunodeficiency in lambs results in staphylococcal pyaemia, manifesting as irreversible arthritis ("stiffness") of the limbs that renders the animals lame and commercially worthless (Stuen et al., 2002, Rymaszewska, 2008, Foggie, 1957). A study on co-infection with the tick-borne LIV demonstrated a mortality rate of 60%, with the remaining 40% being euthanized 'in extremis' (Reid et al., 1986). While in cattle, previous exposure *A. phagocytophilum* appears to affect the severity of disease in *Babesia divergens* (Taylor et al., 1986, Zintl et al., 2003). Pasteurellosis and septicemic listeriosis are also common complications (Øverås et al., 1993, Gronstol and Ulvund, 1976).
Most problems occur in young lambs or naive sheep introduced to 'ticky' areas, though differences in susceptibility between breeds has also been reported (Stuen et al., 2011). In endemic areas, up to 90% of naive pregnant ewes can be affected by abortion storms when introduced to tick-infested pasture (Woldehiwet, 2006).

1.4.6 Diagnosis

Field

Many of the symptoms displayed in clinical cases of *A. phagocytophilum* are general and do not point towards a definitive diagnosis. However, TBF should be considered when sudden spikes of fever are observed in animals grazing on 'ticky' pastures. The history of the animal and area is also helpful.

Lab

Blood samples with Giemsa staining allow morulae to be observed via microscopy, though these may only be visible during the acute stage of the disease. After the acute stage, blood samples may be tested with sensitive q-PCR assays, targeting the highly repeated msp2 genes (Courtney et al., 2004).

1.4.7 Current Approaches to Control & Surveillance

There is no vaccine currently available and there are only a limited number of antibiotics commercially available that are effective for treating *A. phagocytophilum*. Tetracycline antibiotics, such as oxytetracycline in ruminants is commonly used and animals tend to respond well (Woldehiwet, 2007).

As such, TBF is usually managed by periodic treatment of animals with acaricides. One disadvantage of frequent exposure is that ticks may become resistant to these compounds. Alternatively, the risk of infection may be reduced by grazing animals on pastures with a low tick density, though this is not always feasible. Similarly, lambs could be kept in tick-free pastures until they have developed some immunity at around 6-7 weeks old.

As this pathogen is non-reportable, it is difficult to judge its true prevalence and the affect it may be having on the farming industry. In the APHA's 2014 annual report of small ruminant diseases, 7 abortions in a group of 70 ewes were confirmed to be as a result of *A. phagocytophilum.* Moreover, anecdotal evidence from Cumbria was again implicated in suggesting that there were far more unreported cases. In recent years, TBF caused considerable losses in the 2015 lambing season, with the quarterly report announcing a significant increase in confirmed cases, particularly in Wales, where lamb deaths due to TBF were reported 1-2 times a day over a 2 week period(2015). Though the true scale of the problem is difficult to quantify with any degree of certainty, as symptoms are often non-specific and can be attributed to TBF based on fever and the presence of ticks (personal communications with farmers). However, as the threat of *A. phagocytophilum* is not singular, but rather the level of immno-suppression it provokes (Grøva et al, 2013). This leads to an increased susceptibility to other pathogens, such as staphylococcal infections, louping ill, tick pyaemia and an increase in abortion rates (Stuen et al., 2011). As such, it is the indirect losses due to *A. phagocytophilum* that are much more important from a veterinary and economic perspective, which is difficult to quantify accurately.

1.5 Red Water Fever

1.5.1 Importance

The genus *Babesia* is comprised of several species of vector borne parasitic protozoa which affect various vertebrates, including humans. Historically, *Babesia* species have primarily been of veterinary importance(Zintl et al., 2003), while humans are only considered an accidental host. However, an increase in the number of human cases reported from North America and Europe have raised the profile of these pathogens. They are now coming under increasing scrutiny as to whether they may be emerging pathogens of human disease (Hildebrandt et al., 2013, Ismail and McBride, 2017)

Babesiosis, the disease caused by the *Babesia* parasite, is most common among dogs, cattle, horses, and rodents. Bovine babesiosis (BB) is a tick-borne disease of cattle caused by several *Babesia* species across the globe. The main agents of BB are: *Babesia bovis*, *B*. *bigemina* and *B. divergens*. Other *Babesia* that can infect cattle include *B. major, B. ovata, B. occultans* and *B. jakimovi* (Yabsley and Shock, 2013).Of these species, only two are found to infect cattle in Europe; *B. divergens* and *B. major,* the most common of which by far is *B. divergens* (Zintl et al., 2003). *B. divergens* is recognised as economically important to cattle, but was also responsible for the first ever documented case of human babesiosis, formally known as piroplasmosis (Skrabalo and Deanovic, 1957)

B. divergens is the main agent of red water fever (RWF) in cattle in Europe with common symptoms including fever, fatigue, chills, anaemia, haemoglobinuria and possibly eventual death. It can cause severe and potentially fatal disease in immunocompromised humans (Hildebrandt et al., 2013) and has also been documented in splenectomised sheep, where it produces transient parasitemias (Chauvin et al., 2002).

1.5.2 Classification

Babesiosis results from infection by haematoprotozoa in the genus *Babesia*, family *Babesiidae* and order Piroplasmida. With over 100 recorded species, affecting a wide range of hosts (Schnittger et al., 2012), *Babesia* species are second only to trypanosomes as haemoparasites (Yabsley and Shock, 2013).

RWF is of great economic importance within the European farming industry as an emerging, intraerythrocytic, zoonotic protozoan. *B. divergens* was first described in cattle just over 100 years ago and was given the generic name *Piroplasma divergens* (M'Fadyean and Stockman, 1911), due to the pear-shaped morphology of the multiplying stage found within the blood of the vertebrate host (Telford and Spielman, 1993).

B. divergens has been found to vary in size and position within the erythrocyte, depending on the host species (Zintl et al., 2003). In cattle, the pyriforms typically take a peripheral position within the red blood cell and are between 1.5µm-1.91µm in length, polyparasitism is infrequent, with usually just one parasite observed in a single erythrocyte (Zintl et al., 2003). When pairs of pyriforms do occur in the cell, the angle created between them is larger than in any other bovine *Babesia* species, giving rise to the divergent appearance to which the parasite owes its name (Zintl et al., 2003).

1.5.3 Hosts & Geographic Distribution

B. divergens is transmitted by *I. ricinus*, a member of the family of hard ticks (*Ixodidae*) and so is limited to its vectors range within Northern Europe.

Originally, *B. divergens* was thought of as a typical bovine babesia, with a narrow host range. This view was challenged when the parasite was found to be able to establish infections in a wide range of splenectomised animals. Including chimpanzees (*Pan troglodytes),* Rhesus monkeys (*Macaca mulatta)*, a wide range of cervids and domestic sheep (Garnham and Bray, 1959, Malandrin et al., 2010)

Infection may also have been detected in farmed reindeer in the UK (*Rangifer tarandus)*, though results were inconclusive and it may have been the deer related species *Babesia capreoli* (Malandrin et al., 2010). Much of the literature available disputes the role of non-splenectomised cervids in the maintenance of *B. divergens* (Zintl et al., 2011). However, recent research has demonstrated the presence of *B. divergens* in red deer (*Cervus elaphus)* (Michel et al., 2014) and could be of epidemiological importance.

The relationship between infection rates and clinical disease is yet to be fully understood, as there is a profound variation in the proportion of infected animals that go on to develop overt disease (Zintl et al., 2003).

1.5.4 Transmission & Life Cycle

B. divergens is observed across Northern Europe, along with its vector. However one report from Tunisia has suggests that the geographic range of the parasite may be further than initially thought and may extend beyond Europe into North Africa (Bouattour and Darghouth, 1996).

The incidence of disease is closely correlated with the behaviour of the tick vector, observed by a typical bimodal seasonal distribution, with an initial spring peak between April and June, followed by autumn peak from August to October (Zintl et al., 2003). Accounting for host seeking, attachment and incubation, the first cases and clinical signs of infection generally occur 2 weeks after nymphs and adult ticks become active in the spring peak (Donnelly, 1973).

All *Babesia* species exhibit two life cycles: one in the invertebrate tick host and one in the vertebrate host. The sexual cycle of *B. divergens* takes place in the tick and was not confirmed until 1990 (Mackenstedt et al., 1990). In summary, when an infected tick feeds on a vertebrate host, the *Babesia* parasite enters the host in trophozoite ring form. Following inoculation, the trophozoites invade the host's red blood cells, where they multiply through binary fission. This results in the pyriform bodies, known as merozoites. It is at this stage that the merozoite form of *Babesia* causes the most damage within the host, as it destroys red blood cells leading to anaemia. The merozoites can then re-infect red blood cells and continue the process. Many continue to divide, though a few become non-dividing, spherical gamonts to be taken up during the next tick blood meal. Uninfected ticks ingest the infected blood when feeding, and the uninucleated gamonts settle in the midgut and form ray bodies, which fuse to form a zygote which differentiates into polypoid kinetes. These kinetes then disseminate through the tick tissue including malphigian tubules, musculature and ovaries. Sporongy occurs, resulting in huge numbers of kinetes, at this point eggs may be invaded, resulting in infected larvae. Once the tick begins to feed on a new host, kinetes that invaded the salivary glands develop into haploid sporozoites and infect a host once again during the second part of a blood meal (Zintl et al., 2003).

Though larvae instar occurs in large numbers and transovarial transmission of this parasite has been documented (Donnelly and Peirce, 1975), larvae tend to feed on smaller, non-bovine hosts. Consequently, this life cycle stage is probably of limited importance for the epidemiology of bovine babesiosis.

In addition, *B. divergens* has been shown to persist in *I. ricinus*, in the absence of susceptible hosts , for up to two generations (Donnelly and Peirce, 1975). Meaning infection may be retained in a tick population for at least four years, making the low prevalence in ticks quite unexpected. Transstadial transmission of *B. divergens,* through moults, has also been observed (Mackenstedt et al., 1990).

It has also been documented that the parasite can be maintained within the environment by cattle which have recovered from previous infections or carry mild subclinical infections (Davies et al., 1958).

These low level parasitemias, are often asymptomatic and go unnoticed allowing them to persist for several years following the initial infection (Joyner and Davies, 1967) and may serve as a source of the infection of new ticks (Joyner et al., 1963).

It has been suggested that *B. divergens* may be transmitted transplacentally from heifer to calf, similar to *B. bovis* and *B. bigemina* which have both been documented as causes of abortion, (Egeli, 1996). This comes after it was observed that calves up to 1 year of age, although fully susceptible to infection, were resistant to the disease (Joyner and Donnelly, 1979). Further studies concluded that this resistance continues, though somewhat dampened up to the age of 2.5 (Christensson and Thorburn, 1987).

1.5.5 Incubation Period & Clinical Symptoms

The course of *B. divergens* infections are dependent on several factors, such as host fitness, strain virulence and the number of infected ticks feeding at the time of infection (Purnell et al., 1976b). Asymptomatic infections are quite common and often overlooked by owners, as the low parasitaemia may only cause a mild fever and slight anorexia which the animal will overcome on their own (Gray et al., 1985).

Clinical cases of *B. divergens* present with a sudden fever of 41°C, anaemia, anorexia, depression, increased heart rate and diarrhoea (Gray et al., 1985, Collins et al., 1970). Haemoglobinuria is the most characteristic symptom of the disease and gives it the name 'red water fever', this is usually the first clinical sign that is noticed by the owner and is a result of a peak parasitaemia of 30-45% along with haemolysis (Gray et al., 1985, Zintl et al., 2003).

Typically, the animal becomes more depressed as the anaemia worsens; dehydration also becomes more severe, resulting in constipation. Occasionally, a rapid, loud heart beat may be heard within a few feet of the animal (Zintl et al., 2003). In animals that are able to recover, eventually the body temperature drops and the red water ceases. However erythrocyte count and haemoglobin level continue to decrease for a couple of days (Zintl et al., 2003). In fatal cases, an animal may develop toxaemic shock, a weak pulse and display behavioural changes as a result of brain anoxia (Gray et al., 1985). Death is usually credited to cardiac failure or hepatic and kidney damage (Collins et al., 1970).

There have been several reports of co-infections with *B. divergens* and the tick-borne agent of pasture fever *A. phagocytophilum,* though this interaction is not fully understood (Zintl et al., 2003, Purnell et al., 1976a). It has been put forward that these concurrent infections do not generally induce more sever clinical signs than on their own (Brun-Hansen et al., 1997) and there is even some evidence to suggest that *B. divergens* may actually be suppressed by *A. phagocytophilum* (Purnell et al., 1977).

However, animals that are left with compromised immunity due to neutropoenia after fighting pasture fever and subsequently contract *B. divergens* may suffer a more severe form of the disease (Taylor et al., 1986). This realisation may have important epidemiological implications in the future of management of tick borne diseases. The recorded prevalence of *A. phagocytophilum* infected ticks is much higher than the prevalence of *B. divergens,* with a greater host range. It is therefore likely that *B. divergens* infections will be superimposed upon *A. phagocytophilum,* possibly resulting in more severe cases of disease (Zintl et al., 2003).

1.5.6 Morbidity & Mortality

The morbidity and mortality rates are highly variable. Treatment and previous exposure, as well as the strain of parasite, can affect the outcome. Case fatality rates are mainly influenced by the speed of diagnosis and treatment, and were estimated at 10% in Ireland in 1983. Along with an overall prevalence of 1.7% and considerable economic losses (Gray and Murphy, 1985). This has been shown to be on the decline in recent years (Zintl et al., 2014).

Cattle can develop lifelong resistance to a species after infection. Calves under the age of 9-12 months are as susceptible as adult cattle to infection with *B. divergens ,*however they are less likely to display clinical symptoms (Zintl et al., 2003). This phenomenon has been demonstrated in previous epidemiological studies and is known as inverse age resistance (Adam and Blewett, 1978, Christensson and Moren, 1987, Christensson and Thorburn, 1987). In endemic areas where tick transmission is a threat year round, animals are usually exposed to disease and infected when they

are young. They do not become ill, but become immune. This endemic stability can be upset and outbreaks can occur if climate changes, acaricide treatment or other factors decrease tick numbers and animals do not become infected during the critical early period. In older animals, immunity is reinforced by repeated tick challenge, explaining the low rate of clinical cases in endemic areas.

1.5.7 Diagnosis

Field

Babesiosis should be suspected in cattle grazing in known 'ticky' areas that develop fever, anaemia, jaundice and haemoglobinuria. A history of area and individual helps too.

Lab

Clinical evidence for babesiosis is usually confirmed by identification of the parasites in blood or tissues via Giemsa staining, polymerase chain reaction assays (PCR), serology, or transmission experiments (Zintl et al., 2003).

The persisting nature of *B. divergens* antibodies, even after the disease has cleared, creates problems for serological tests as they give no insight as to when infection was acquired, leading to overestimates of disease prevalence (Joyner et al., 1972, Donnelly et al., 1972).

1.5.8 Current Approaches to Control & Surveillance

Mild cases may recover without treatment. For more severe cases, treatment is available in the form of the babesicide Imidocarb, though immunity can develop and there are also concerns regarding residues in milk and meat (Traynor et al., 2013).

RWF is often only noticed at the onset of haemoglobinuria, when the disease is far advanced. Although chemotherapy and transfusion will generally save a severely afflicted animal even at in the advanced stage of the disease, it may continue to be markedly incapacitated for several months after recovery (Lewis et al., 1981). Therefore, the most logical approach to tackling this disease lies in prevention, rather than cure, for both economic and animal welfare reasons.

It has been suggested that the use of sheep as 'tick-mops' would be effective in the prevention of *B. divergens* (Taylor et al., 1982). However, the vector *I. ricinus* also carries other severe sheep pathogens, such as LIV and *A. phagocytophilum* and would be putting them at unnecessary risk. Additionally, the cattle would have to be absent from the *Babesia* endemic area for quite some time, as *B. divergens* infection can persist in ticks for at least two generations. Even in the absence of a suitable host (Donnelly and Peirce, 1975). It seems, therefore that use of acaricides and pour-ons are the best method available at present.

It has been postulated that integrated control in endemic areas may help maintain enzootic stability, by maintaining constant interaction between host, vector and pathogen (Lawrence and de Vos, 1990). Though there are no guarantees with this particular method.

Vaccination would be the best option, however there is no commercial vaccination currently available, despite promising studies (Zintl et al., 2003).

Chapter 2: Spatial distribution of *Ixodes ricinus* across mixed pasture

2.1 Introduction

As discussed in Chapter 1, in Europe, the single most important vector of human and animal health and economics is the sheep (or deer) tick, *I. ricinus* (Stuen, 2007, Rizzoli et al., 2007). This tick transmits a number of medical and veterinary pathogens including *Anaplasma* spp, louping ill virus and *Babesia* spp to livestock, and *Borrelia* spp and tick-borne encephalitis virus to humans (Greig et al., 1931, Zintl et al., 2003).

These tick borne pathogens are a burden to the agriculture sector both financially and in terms of land management and animal husbandry, with an estimated 300,000 lambs developing Tick Borne Fever (TBF) annually (Brodie et al., 1986). In addition, *Babesia* species which have historically been considered of veterinary importance, are coming under increasing scrutiny as to whether they may be emerging pathogens of human disease (Hildebrandt et al., 2013)

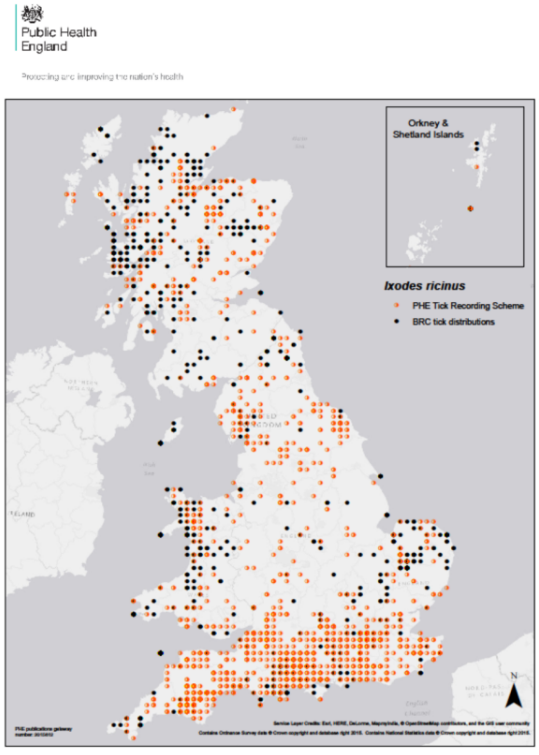

Figure 2 *I. ricinus* distribution map, from PHE

I. ricinus are widespread across the UK, but tick surveys have suggested patchiness in their distribution (Figure 2) (PHE 2016). Their geography is mainly determined by the availability of favourable habitat; deciduous and mixed forests, while being less abundant in coniferous forests and non-wooded areas (Milne, 1946, Randolph, 2000)(Milne, 1946, Randolph, 2000)(Milne, 1946; Randolph, 2000) .

There have been elegant and comprehensive studies on the spatial distribution of *I. ricinus* (Macleod, 1934, Milne, 1950b, Milne, 1946) across varying types of flora and habitat, often in relation to livestock.

These fundamental papers concluded that *I. ricinus* is sensitive to climatic conditions, and require a relative humidity of at least 80%, to avoid desiccation during its time spent off-host between life stages.

The tick is restricted to areas of moderate to high rainfall, in conjunction with vegetation that retains a high humidity (Medlock et al., 2013). These conditions more associated with rough, upland farming rather than grazed pasture. The need for favourable vegetation and sufficient numbers of appropriate animal hosts for all active stages are important factors in *I. ricinus* survival within a habitat, and for the completion of its life cycle (Milne, 1950a, Medlock et al., 2013).

Between host and habitat, the most important influence in determining I.*ricinus* distribution is not intuitively obvious, and has been discussed in literature ((Randolph, 2000, Estrada-Pena and Venzal, 2007). Studies have concluded that the presence of larger hosts, such as sheep and deer, are essential for the propagation of an *I. ricinus* population; providing a blood meal source for the reproductive adult tick life-stage and are the main determinants of tick abundance in tick-permissive habitats (Gray et al., 1992, Jaenson and Talleklint, 1992, Wilson, 1998).

However, tick permissive habitats are also largely determined by the availability of suitable vegetation, which plays a crucial role in the distribution and survival of *I. ricinus* (Medlock et al., 2013, Milne, 1946). This allows for the potential use of GIS and RS data in the mapping of habitat suitability and tick distribution. Cerny (1988) notably used this method in a study involving 20 years of tick collection from various types of habitat in the Czech Republic. These data where then correlated to Landsat-MSS imagery and used to establish that habitats comprised of favourable vegetation, and associated with higher tick infestation levels, could be identified remotely (Daniel and Cerny, 1990).

Ultimately, this proves that satellite imagery can be used to predict areas with high tick populations. This work was further built on in a study by James and colleagues (2012) which found a higher proportion of questing nymphs in mixed forest compared to coniferous, and an extension of this work by Gilbert and colleagues (2017) demonstrated a positive correlation between tick density and canopy cover, in addition to a negative correlation between percentage of grass cover and tick density. Though this study was conducted in western Norway, similar associations between questing *I. ricinus* and tree cover in heterogeneous landscapes in Scotland have also been observed (Ruiz-Fons and Gilbert, 2010) (Porter et al., 2013, Gilbert, 2016).

Since this study, GIS and RS methodology has progressed substantially, with significant progression from aerial photography to high resolution, multispectral scanners over the years (Mutanga et al., 2016). Their development and accessibility has been reflected in their increased use and accuracy within the sphere of vector borne disease research, particularly in mosquito vector monitoring and control (Eisen et al., 2009; Eisen et al., 2011; Adeola et al., 2015).

Studies at a large scale have also been carried out on mapping other tick species of public health importance across Spain, in relation to climate change (Estrada-Pena and Venzal, 2007).

High resolution satellite images are widely and freely available; particularly following the European Space Agency (ESA) launched Sentinel-2 satellite in June 2015. Sentinel-2 produces high resolution images for analysis, with an impressive 10m resolution in visual and NIR bands. Another advantage of Sentinel-2 over other satellites, such and Landsat-8, is the revisit frequency. The satellite makes a complete orbit every eight days, allowing for the potential capture of more relevant and frequent images, in comparison to Landsat-8's 16, which can be of great value when considering a highly seasonal system (Mandanici and Bitelli, 2016).

The production of tick 'heat-maps' from empirical ground data can provide a simple, extremely effective way of communicating exposure risk across a landscape. This would be of particular use within an agricultural setting, principally hill farmers, where there has been a long standing association with ticks and tick borne disease (Milne, 1945, Milne, 1946, Macleod, 1934) .This is due to the largely favourable habitat and abundance of hosts present in often encroaching woodland, with important wildlife hosts, such as deer, utilising the same habitat (Jones et al., 2010).

Rich species distribution maps of the vector are generated from field data. These can be coupled with other collated data sets, including knowledge on vegetation, abiotic factors, host abundance and movement. This can then be exploited by well-established statistical methods, providing the ability to infer correlations between various environmental factors and tick presence at a fine scale. Output will inform avoidance action and control by farmers at a fine scale. In an attempt to reduce risk to livestock by minimising the environmental hazards posed by the landscape and exposure rate, or contact between ticks and livestock.

The aim of this study was to explore the distribution of questing *I ricinus* ticks across the mixed pasture of Bowkerstead farm in Cumbria. Incorporating citizen science by discussing the perceived tick problem and distribution across the farm with the farmer and investigating this via by the production of tick 'heat-maps'.

With the intent to identify the ecological drivers behind the questing tick population, and to communicate the findings and any recommendations to the farmer in an appropriate way.

2.1.1 The Farm

Meetings with the Forestry Commission (FC) about the project and our aims allowed them to directly contact interested farmers. Bowkerstead Farm, Grizedale (lat/long: 54.3164, -3.0175) was identified and following discussions with the farmer (farmer 1), we were able to sample there. Bowkerstead Farm (Figure 3) was approximately 120ha, holding 500 breeding ewes and 12 rams.

The ewe flock was comprised of approximately even numbers of Swaledales and the North of England Mule, which is produced when a Swaledale ewe mates with a Blue Faced Leicester ram. There is a mixture of Swaledale, Blue Faced Leicester, Charollais Belltex, Texel Belltex and Suffolk rams resident at the farm.

The enduring Swaledale ewes graze and lamb on the higher fell, which was described by farmer 1 as the area of his farm where his flock is more likely to be infested with ticks (Area A, Figure 4). The Swaledale ewes are usually kept up on the higher ground (Area A). They are brought down on to the level fields, closer to the farm house (Area C) for three weeks at the end of October to mate with the Blue Faced Leicester male. After this three week period, the ewes are then sent back up to the higher ground with one of the other rams (Charollais Belltex, Texel Belltex & Suffolk) to ensure conception.

The ewes then stay on the higher ground all through the winter and lamb on the 'ticky' fell at the end of March. The lambs stay on this ground until July, when the young males are separated out and sent for fattening, prior to slaughter between November and January. All female lambs are kept for breeding; the pure Swaledales remain in Area A of the farm and become part of the farm's rotation.

The Mule ewes are kept on the other side of the farm (Area B), which was described by the farmer as the area where his flocks would be least likely to be infested with ticks. These ewes also breed in Area C in October, with the other rams (not the blue faced Leicester), to ensure lambing at the end of March. The ewes are brought in from the fields in the middle of March and lamb indoors. They are then sent back to Area B, until the male lambs are sent on for slaughter and females become farm's rotation.

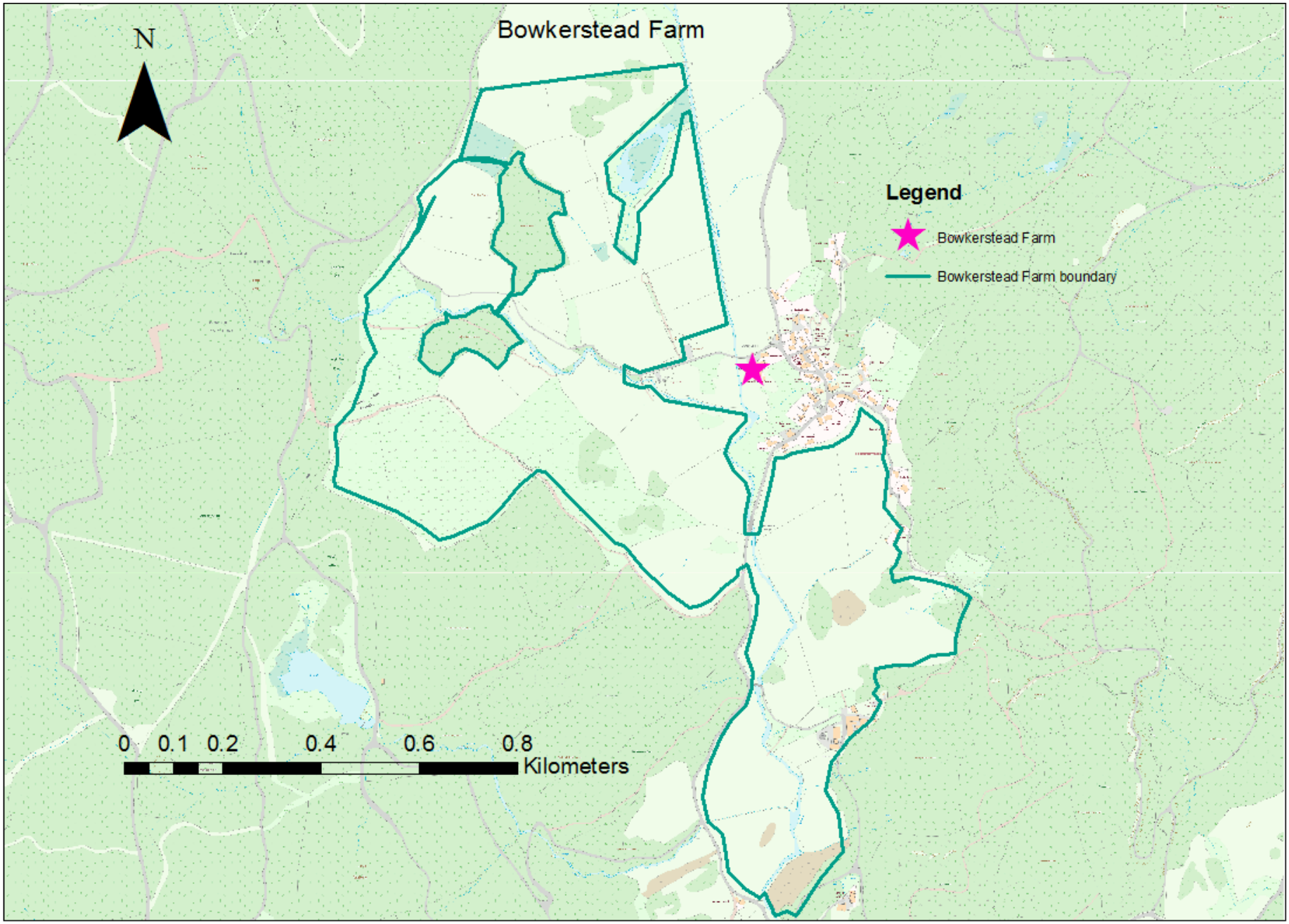

Figure 3 ArcGIS map showing Bowkerstead farm 1:9,000

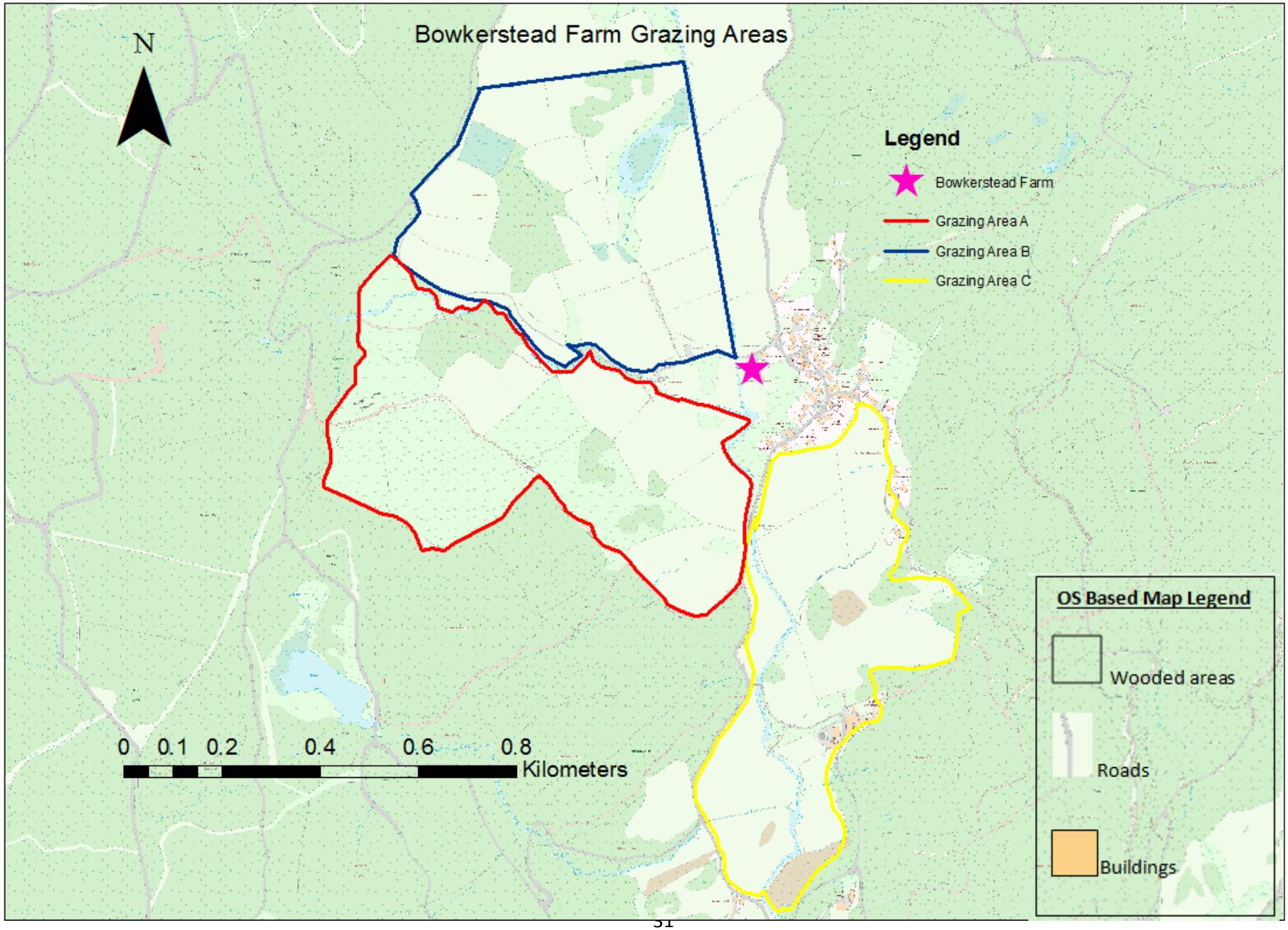

Figure 4 ArcGIS map showing different grazing and husbandry areas at Bowkerstead farm 1:9,000

2.1.2 Wildlife

There are frequent reports and sightings of deer on farmland or on land adjacent to farmland. The deer tend to take the same paths across the Bowkerstead farmland (trods), through the South of Area C, moving between the wooded areas.

2.2 Data collection & processing

2.2.1 Tick sampling

The collection of ticks from the environment was carried out via the well-established 'blanket dragging method (Macleod 1932; Milne 1943). Though there are known disadvantages of this method of tick collecting, it was deemed to be the most appropriate method at this site due to the low vegetation height. Blanket dragging is most effective when vegetation height is less than 30cm (Dobson 2013), the grazed land at Bowkerstead farm was well below this height. Drag efficiency decreases in wet weather (Dobson, 2013) due to ticks being unable to take suitable purchase on the blanket; to mitigate for this, drags were only conducted on dry days. Though these measures were put in place, dragging is still known to miss ticks and questing numbers can be highly variable at the same site dependant on when the site is sampled (Randolph et al, 2005; Gilbert et al, 2017). To ensure the most accurate picture of the farm was captured, multiple drags were conducted across the site (450). Dragging was conducted by myself and field volunteers, who I trained in dragging appropriately and in tick recognition. I then stayed with each volunteer while they carried out a few drags, until I was reassured of their competency and could be confident that ticks were not being missed on the blankets.

Preliminary dragging was conducted to confirm the presence of ticks. Once this was established, 450 drag points randomly distributed across the study site were generated, and visualised, using the 'create random points' tool in ArcMap 10.3 (Figure 5). The constraining extent was set to the boundary of Bowkerstead farm and no minimum distance between points was specified. The drag points were then uploaded to a Garmin GPS as waypoints, following manufacturer's guidelines, and software (MapSource) to allow for location of points on site.

After navigating to a waypoint via the GPS, drag direction was decided at random by following the second hand on analogue watch and recorded. At each waypoint, a 1.5m x 1.5m wool blanket was 'dragged' over the vegetation for 30m. Every 10m the blanket was checked for nymph and adult ticks, which were removed into 70% ethanol. All blanket-dragging took place on days when it was not raining between April-September, 2014-2015.

Tick confirmations

Identification of all ticks collected was verified by reference to taxonomic keys (Hillyard 2006).

2.2.2 Mapping

Mapping tick distribution

Distribution 'heat-maps' of questing *I. ricinus* ticks were produced using ArcMap in ArcGIS (version 10.3 ESRI Inc.) from tick count data derived from blanket dragging. Each drag point was assigned its tick count number in Excel, and then exported to ArcMap to undergo spatial analysis. The model used to create the maps was ordinary kriging, which was optimised by using the ArcMap optimisation tool. The resulting chloropleth maps were then clipped to farm boundaries using shapefiles drawn in QGIS or obtained from the Rural Land Registry.

Tick distribution over varying terrain was explored via 3D maps generated in ArcScene. Terrain and elevation data was compiled for the areas of interest from archived open access government data (http://environment.data.gov.uk/ds/survey/index.jsp#/survey?grid=SD39). The LIDAR Digital Terrain Models (DTM's) at 2m spatial resolution were collated and joined together in ArcScene to create a 3D mosaic of the area.

The relevant Ordnance Survey (OS) maps were obtained from Digimap (EDINA Digimap Ordnance Survey Service, downloaded May 2016.) mapping software at 5m spatial resolution. These maps were then draped over DTM's along with tick 'heat-maps', using the drape tool in ArcScene.

Mapping Distance to Woodland

Distance from each drag point to the closest woodland was also calculated in ArcMap. Polygons of adjacent and contained woodland of interest were drawn freehand using the draw and convert to polygon tools available in ArcMap (Figure 5). Once these were created, the ‘generate near table’ tool was used to produce all of the distances from each drag point to each of the woodland sites of interest. This was then fed in to a larger dataset, collating all potential explanatory variables, to be analysed as potential predictors of tick density.

2.2.3 Environmental Data Collection

Remote Sensing & NDVI

Remote sensing images of Bowkerstead Farm were obtained via freeware from the European Science Agency (ESA) and their Sentiel2 satellite. The satellite takes 10 days to make a complete orbit and collects data continuously. Images from the sampling date were downloaded from the

Scientific Data Copernicus Open Access Hub (https://scihub.copernicus.eu/). Standard Red Green Blue (RGB) colour images were visualised in ESA SNAPtoolbox software and cropped to contain the area of interest. Following this, Normalised Difference Vegetation Index (NDVI) images were generated using the relevant bands of the satellite. NDVI is a standardized index, which can be used to generate an image displaying greenness. Outputs are numerical values between -1.0 and 1.0; where any negative values are mainly generated from water. Very low values (< 0.1) of NDVI correspond to barren areas of rock, soil or sand. Moderate values (0.2 to 0.3) represent shrub and grassland, while high values (0.6 to 0.8) are indicative of forested areas.

The index takes advantage of the contrast of two bands from a multispectral raster dataset, namely the chlorophyll pigment absorptions in the red band and the high reflectivity of plant material in the near-infrared (NIR) band. NDVI images are created by following the standard NDVI equation: (NIR-RED)/ (NIR+RED). Bands 4 & 8 were used for NIR and red respectively. Generated Images were exported to ArcGIS as a map layer, where individual pixel values could be extracted, using the extract values to points tool. These values were then fed in to a larger dataset as a variable exploring tick density.

2.2.4 Statistical Analyses

All statistical analyses were carried out using R Studio (version 1.0.136, RStudio, Inc., Boston, MA); initial analysis and distribution fits were inferred using the fitdist function from the fitdistrplus library. Following this, all potential explanatory variables were explored in a Generalized Linear Model (GLM), using either the glm.nb function from the MASS library. 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. Variables considered in analysis were: Drag area, slope, NDVI, and distance to woodland.

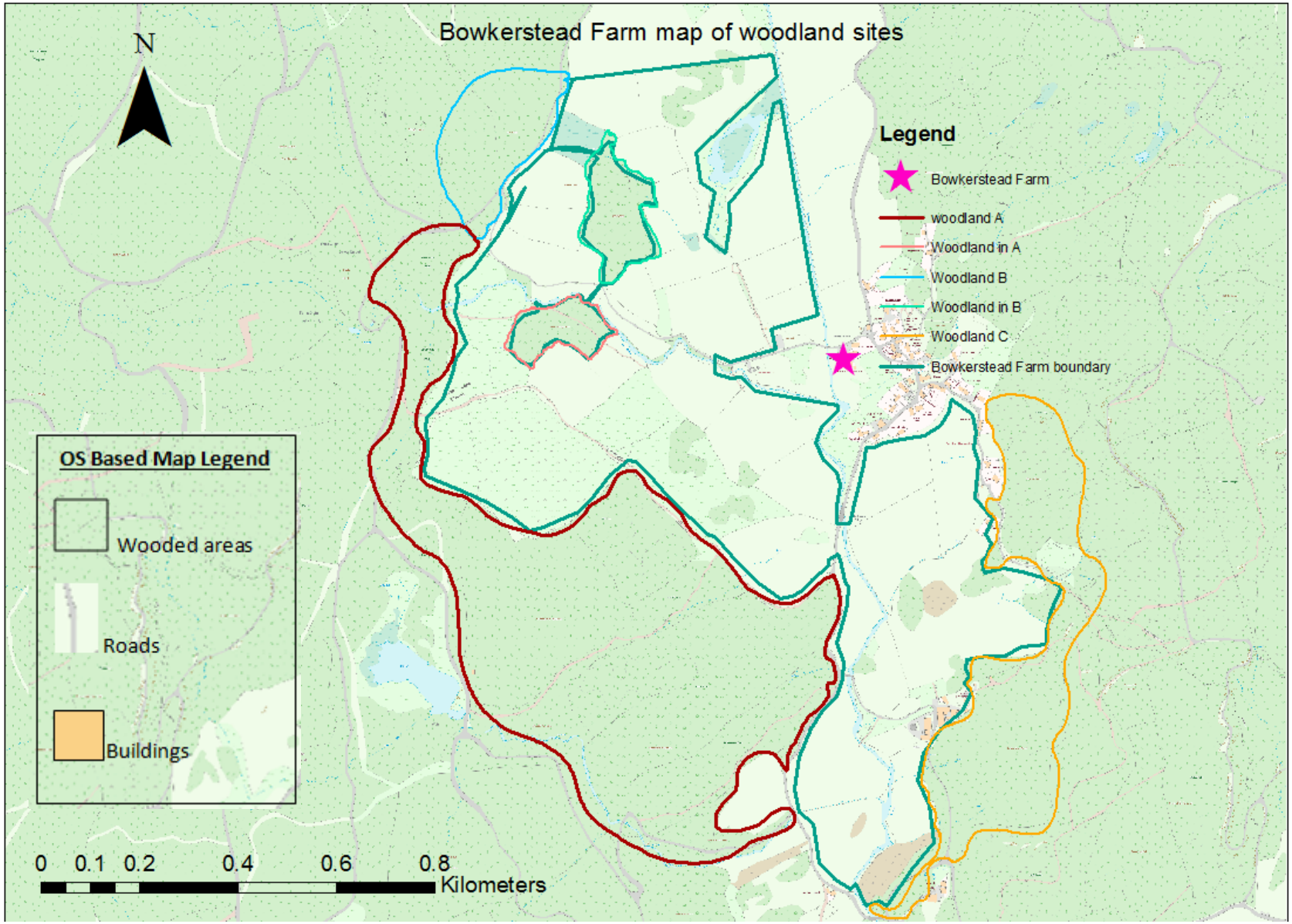

Figure 5 ArcMap map showing woodland surrounding and enclosed by Bowkerstead farm, 1:9,000

2.3 Results

2.3.1 Dragging

Six spatial surveys of questing tick abundance were completed on Bowkerstead Farm during the 2014 and 2015 tick seasons (April- September). In total, 450 X 30m drags yielded 329 ticks (Table 1). Of these, 307 were nymphs, 16 were adult males and 6 were adult females.

Table 1 showing all results from dragging across Bowkerstead Farm

	Drags			Number of ticks		
Survey Date	With ticks	Without ticks	**Total**	Nymphs	Adults	**Total**
04/04/2014	17	13	30	63	9	72
06/06/2014	1	10	11	27	1	28
17/07/2014	18	17	33	103	3	106
24/07/2014	3	12	15	15	1	16
28/07/2014	12	52	64	21	2	23
13/05/2015	9	24	33	20	0	20
14/05/2015	9	35	44	43	4	47
15/05/2015	1	22	23	1	0	1
08/08/2015	1	99	100	0	1	1
10/09/2015	10	87	97	14	1	15
Total	**81**	**371**	**450**	**307**	**22**	**329**

To begin to explore the spatial variation in tick distribution, tick count data from dragging at the separate areas of Bowkerstead Farm was compared to ascertain if there was a significant variation in tick density between them.

The drag data from the three areas of the farm (Figure 7) were compared and no significant difference in tick density between the three sites was found (Kruskal-Wallis test, $\chi^2 = 1.3654$, df=2, P=0.5053).

Dragging results were split into three different habitat categories: woodland, rough pasture (area A), and low pasture (areas B and C). Kruskal Wallis tests followed by Pairwise Wilcox test, state that there were significantly more ticks observed on drags conducted in woodland than there were on rough pasture and lower pasture; but that there was no significant difference between the number of ticks observed on drags conducted on rough pasture and lower pasture ($\chi^2 = 61.497$, df=2, P<0.001).

Table 2 demonstrating ticks observed on drags conducted in different habitat across Bowkerstead Farm

	No. Drags				
Habitat	With ticks	Without ticks	Total	% of drags infested	CI (95%)
Woodland	22	15	37	59.46	42-75
Rough pasture	22	115	137	16.06	10-23
Low pasture	37	236	273	13.55	9-18

2.3.2 Tick distribution

A histogram of tick count per drag was plotted in Rstudio (Figure 6). This showed that there was a large skew in the data, due to lots of drags with zero ticks. The data was plotted and compared against theoretical Poisson and Negative Binomial distribution vales, to determine which distribution model fitted the data best. Analysis confirmed that the tick count data was displaying a negative binomial distribution, with the theoretical negative binomial values in green, aligning much more closely with the empirical values in black, than theoretical Poisson figures in red (Figure 8).

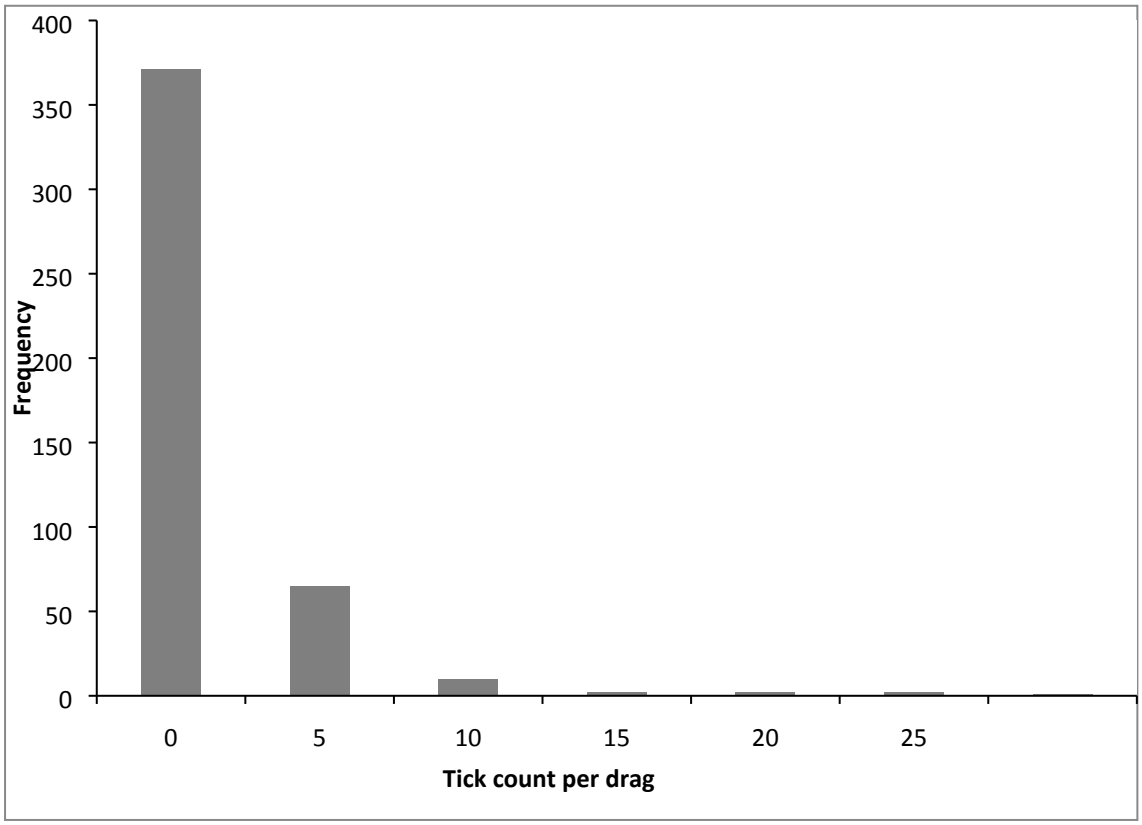

Figure 6 Histogram showing tick counts per drag across Bethecar Farm

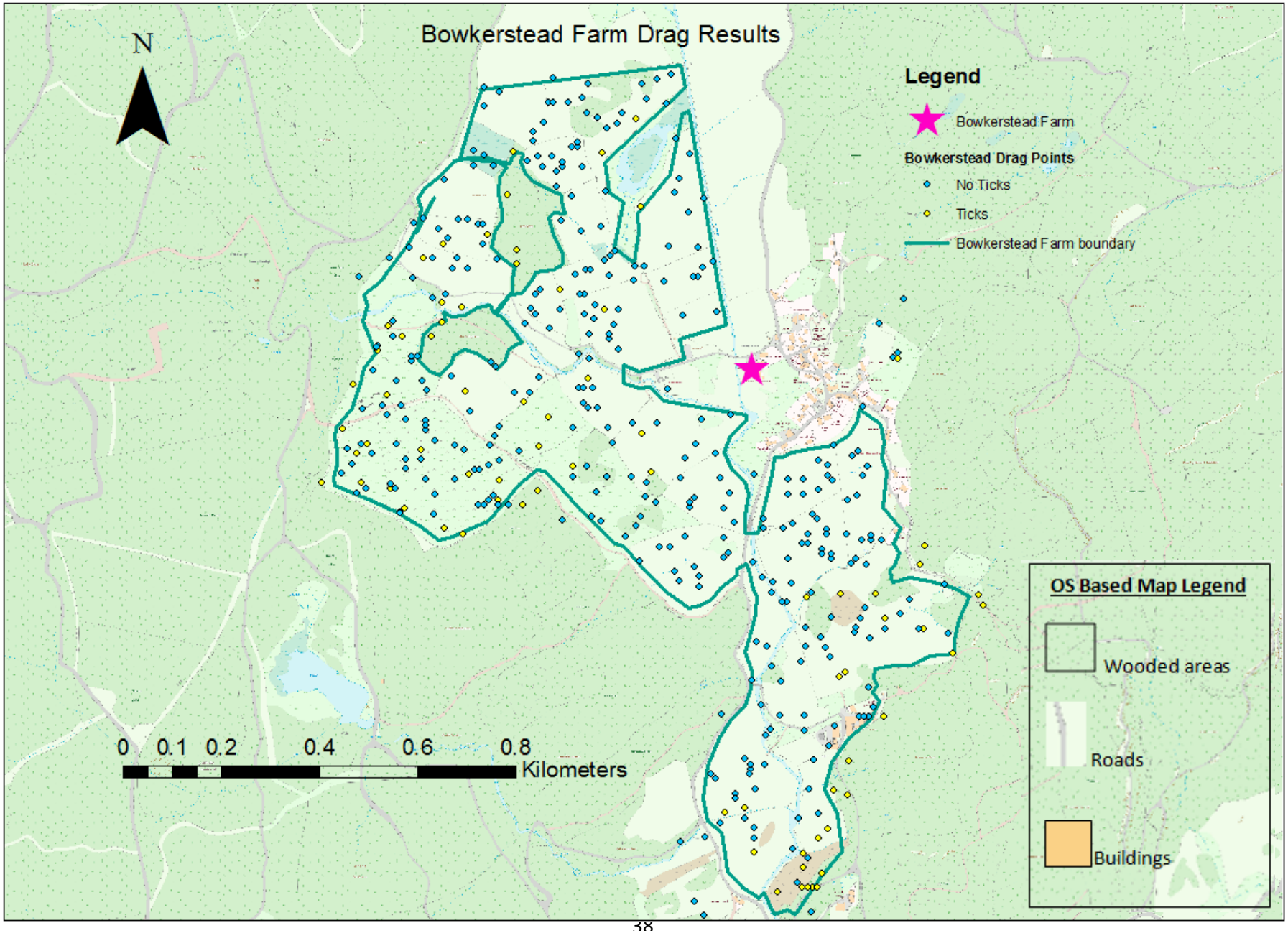

Figure 7 ArcGIS map showing drag points with and without ticks across Bowkerstead farm 1:9,000

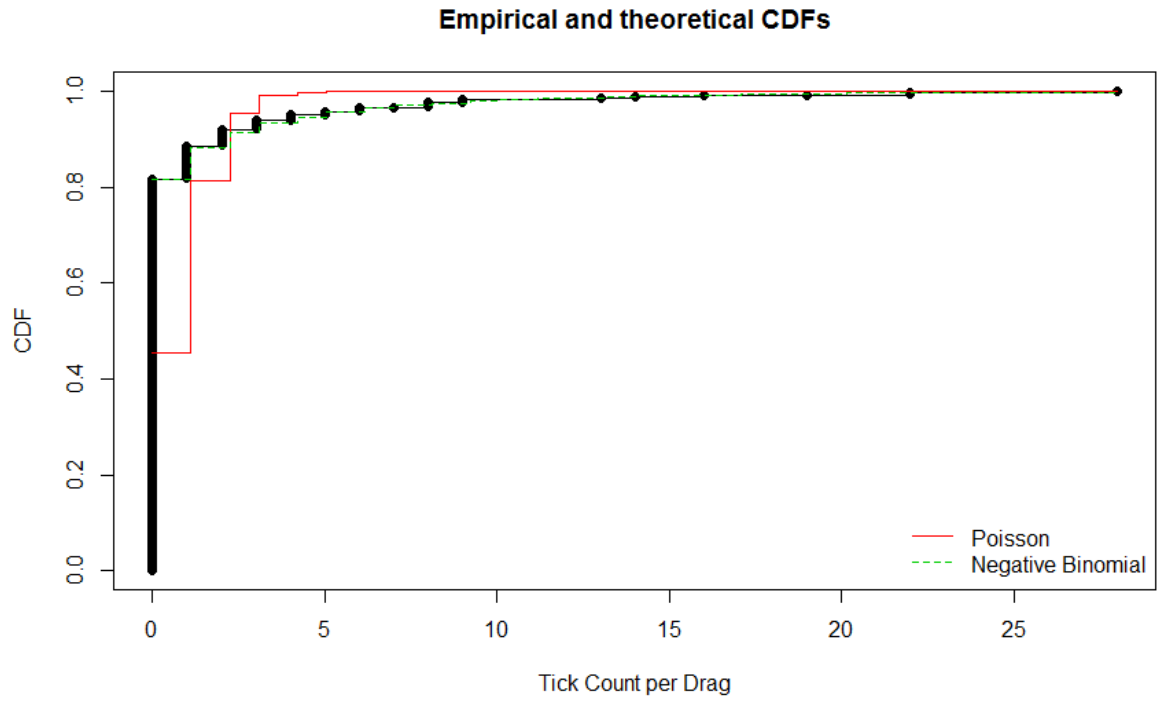

Figure 8 Graph comparing Poisson and negative binomial distribution fits of Bowkerstead Farm drag data, using empirical and theoretical Cumulative Distribution Functions (CDFs). The theoretical negative binomial values in green, align much more closely with the empirical values in black, than theoretical Poisson figures in red.

2.3.3 Mapping tick distribution

Tick dragging surveys provided the data to create a 'heat-map' of *I ricinus* distribution across Bowkerstead farm; the resulting chloropleth map (Figure 9), displays *I. ricinus* distribution across the farm. Areas of high tick density are displayed in red and low tick density in green.

2.3.3.1 NDVI

Sentinel 2 data produced an NDVI map of the farm (Figure 10). Where 0 represents no green vegetation and closer to +1 (0.8, 0.9) demonstrates the highest possible density of green leaves. Pixel values for each drag point were extracted and recorded.

2.3.1.2 Slope

LiDAR data was used to create a slope map of the farm, using degrees, in ArcMap. With greater inclines represented in darker shades, and more even ground in lighter shades. Drag points were overlaid on to the image (Figure 11), and pixel NDVI values were extracted for each drag point.

2.3.1.3 3D Mapping

Collated LiDAR data was also used to create a 3D map of the farm, over which each of the layers could be draped in ArcScene (Figure 12). The ticks were observed in flatter, lowland areas of the farm, alongside a smaller area of higher tick density in the higher, rougher pasture.

2.3.1.4 Distance to woodland

The distance from each drag point to each bit of woodland, both within and sharing a boundary with Bethecar moor was also calculated in ArcMap (Figure 13). This was then fed in to a larger dataset, collating all potential explanatory variables, to be analysed as potential predictors of tick density.

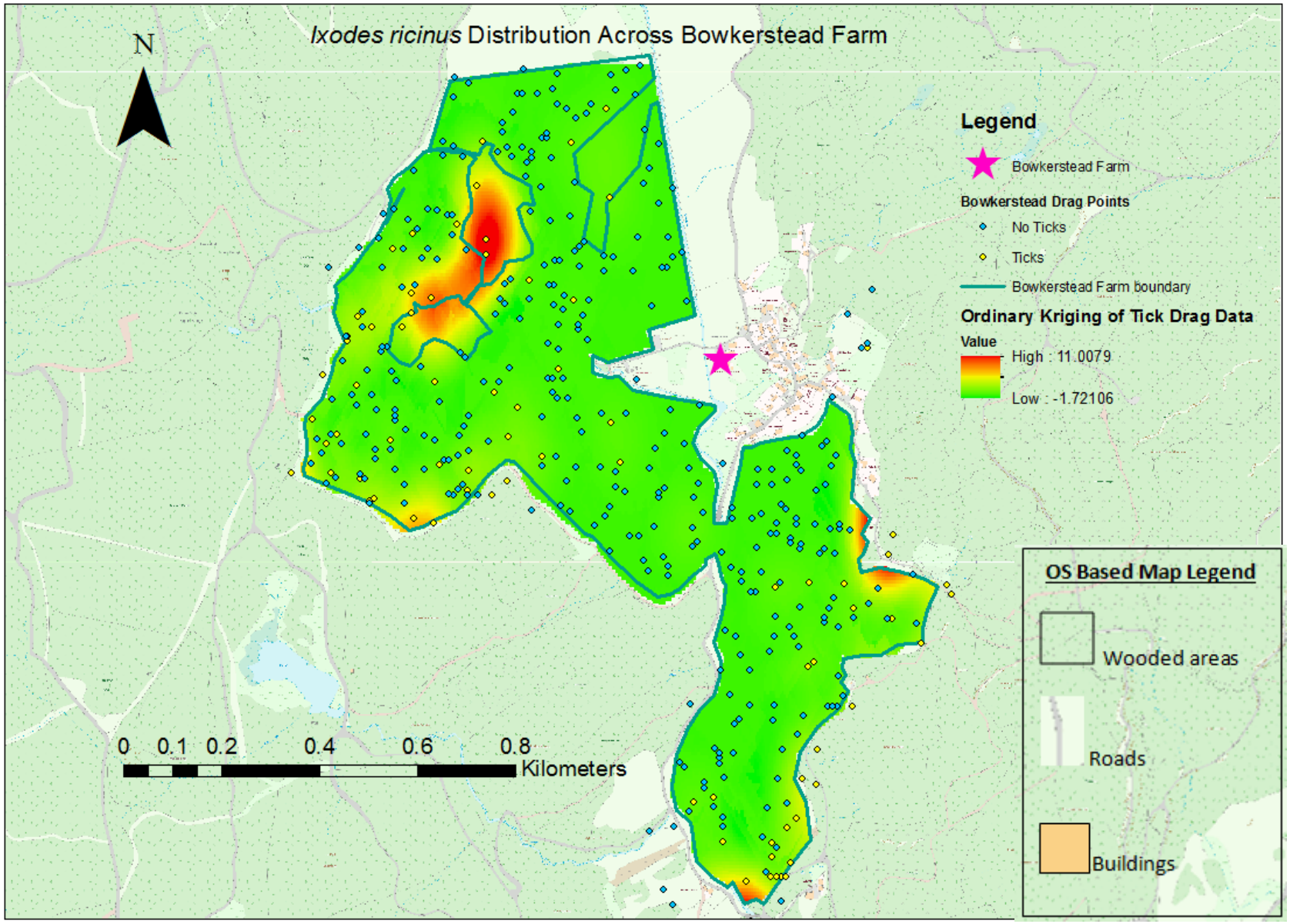

Figure 9 ArcGIS map showing Kriging layer for questing *I.rinicus* across Bowkerstead farm, generated from Sentinel-2 data. 1:9,000

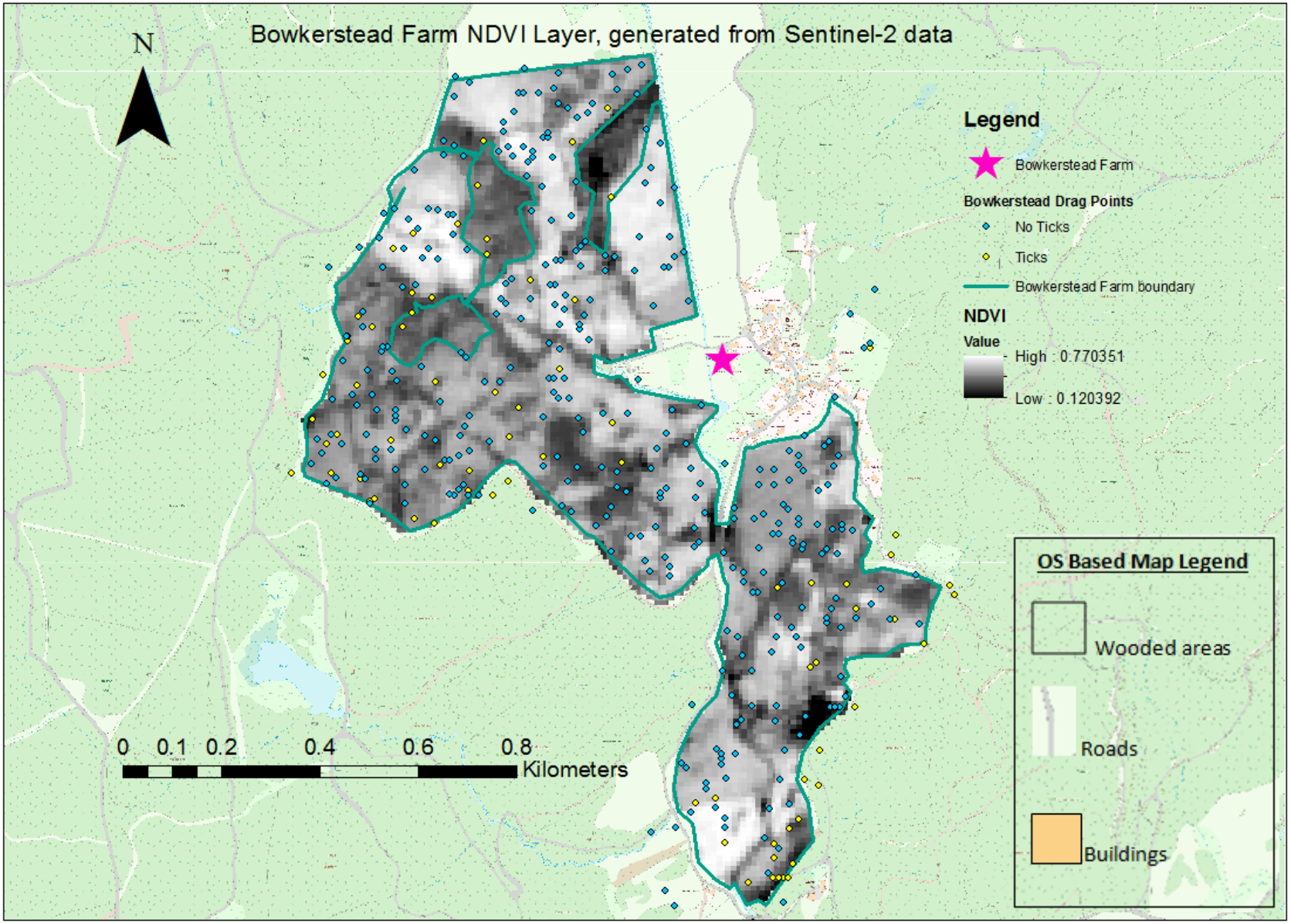

Figure 10 ArcGIS map showing NDVI image layer for Bowkerstead farm, generated from Sentinel-2 data. 1:9,000

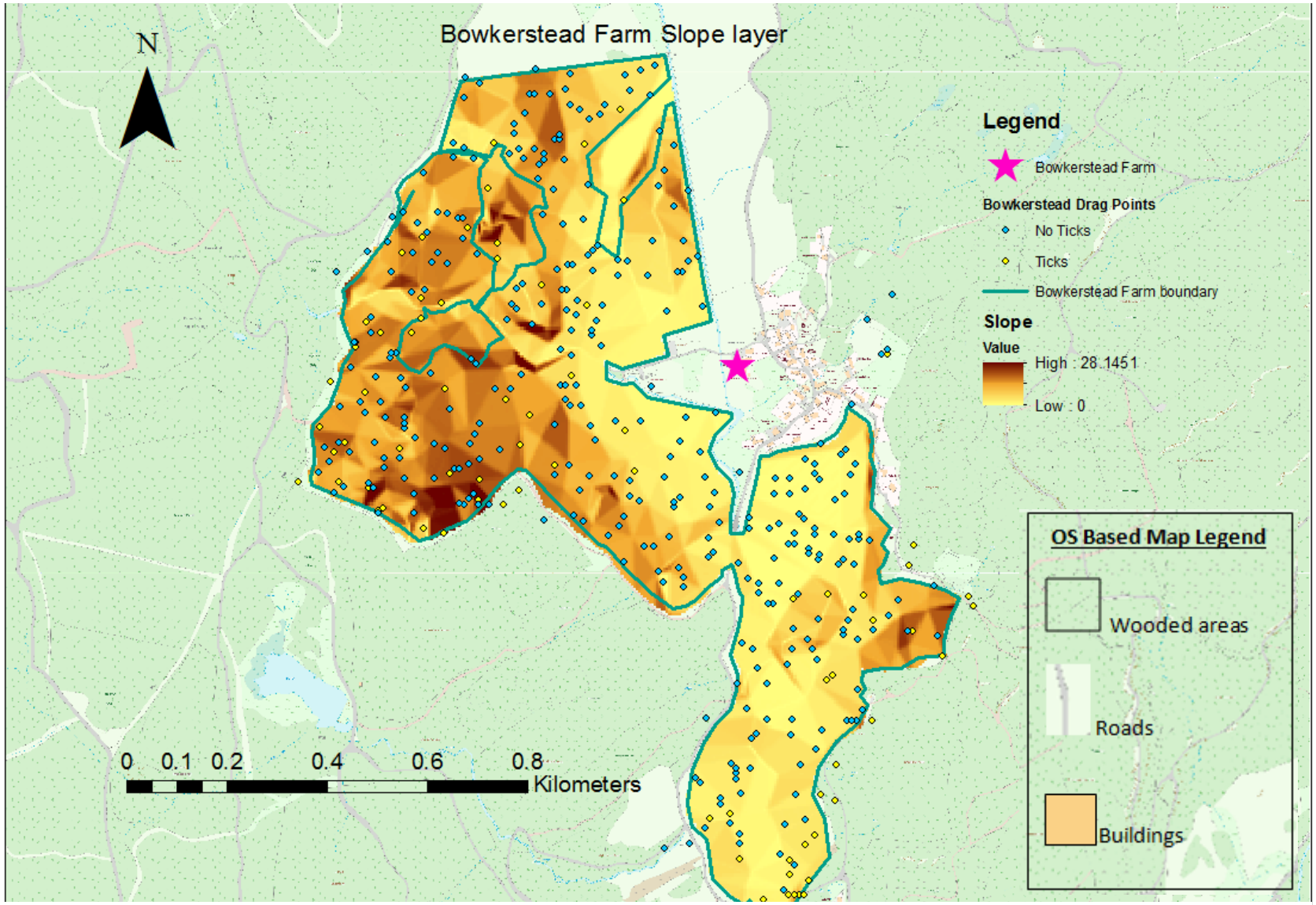

Figure 11 ArcGIS map showing slope layer of Bowkerstead farm, generated from LiDAR data. 1:9,000

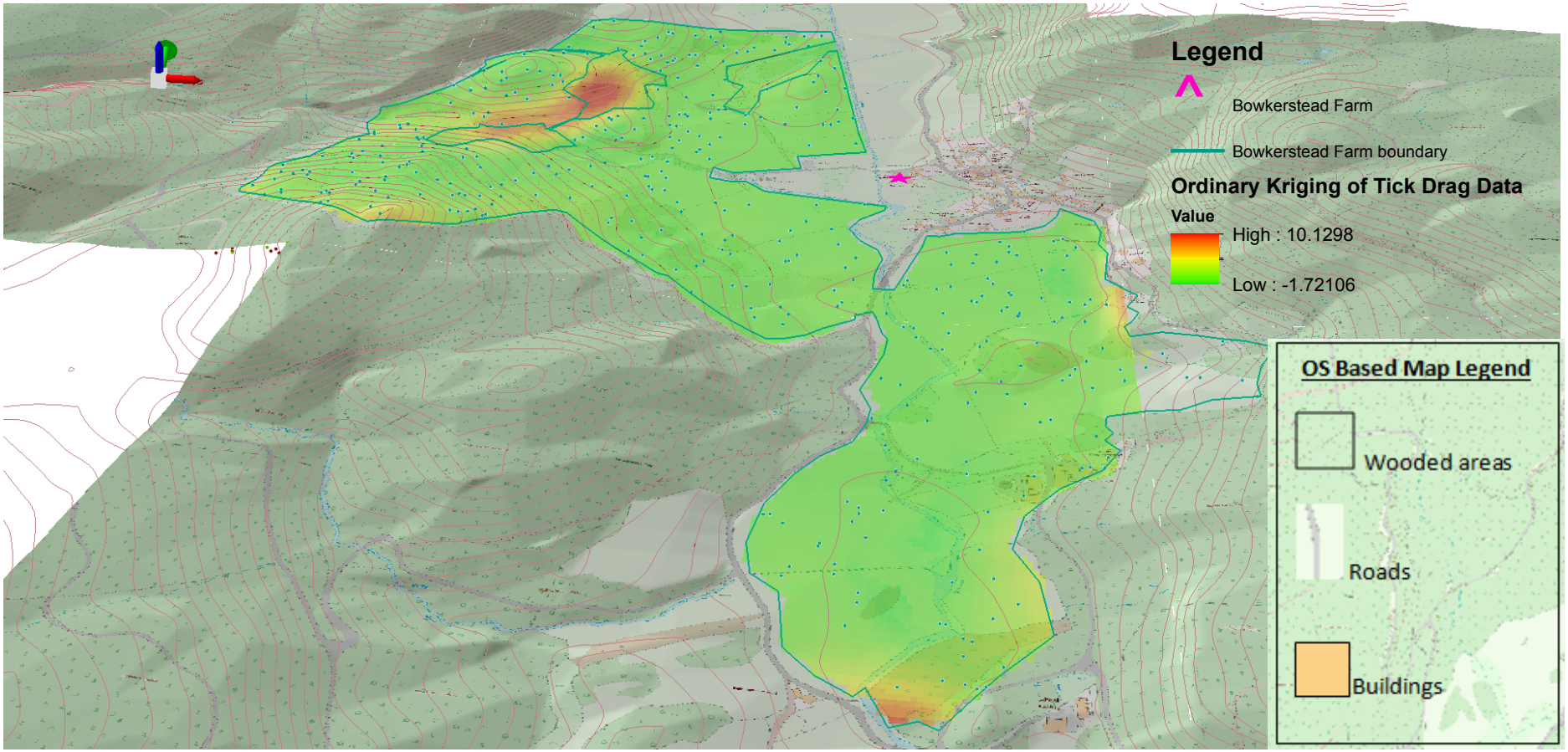

Figure 12 ArcScene 2D exported image showing 3D terrain map

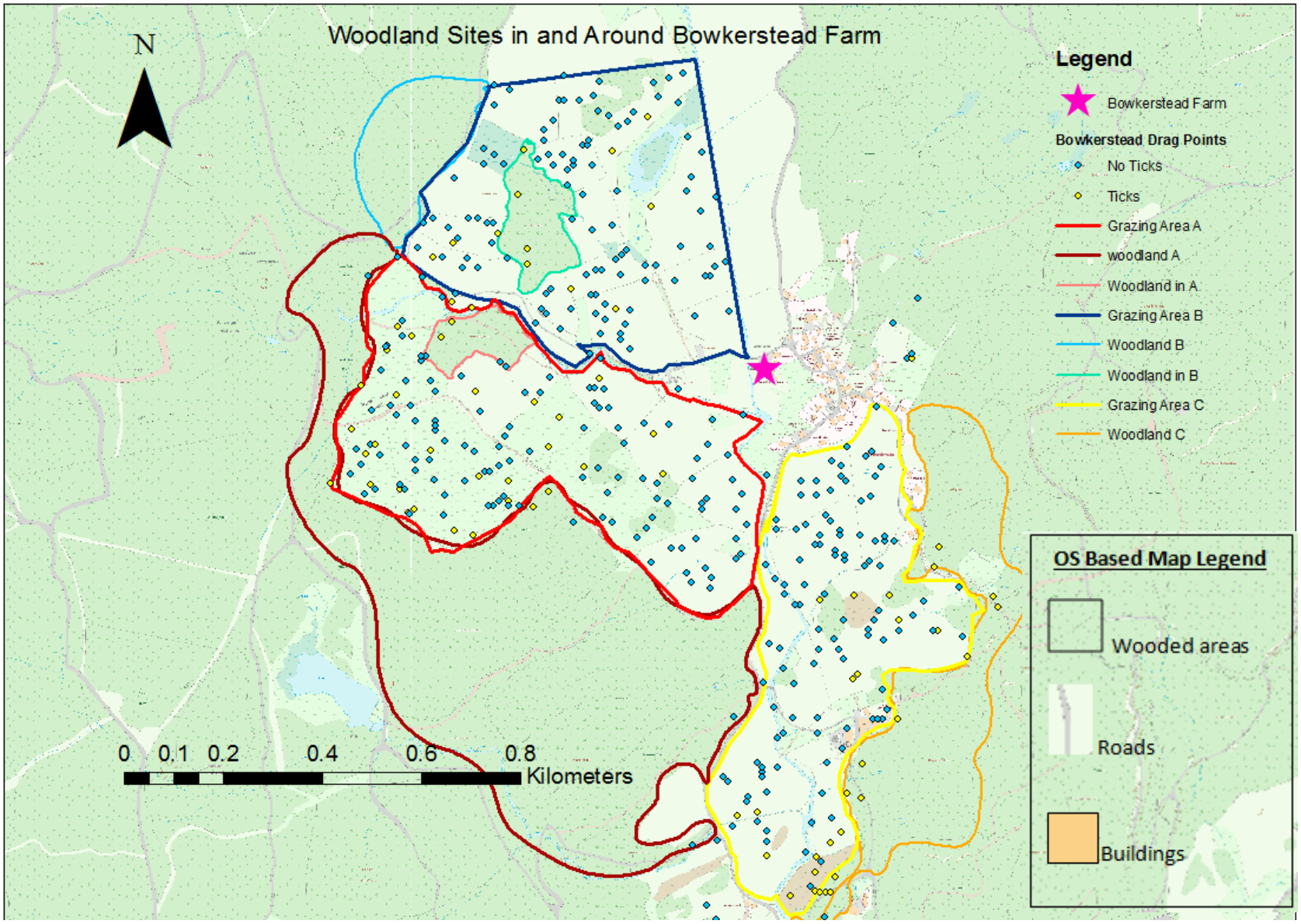

Figure 13 ArcMap map showing woodland surrounding and enclosed by Bowkerstead farm, 1:9,000

2.3.4 Fitting the Model

GLM analyses showed that distance to woodland and NDVI significantly influenced the probability of *I. ricinus* being observed on a drag (Table 3). Drags conducted in closer proximity to woodland were significantly more likely to yield ticks, along with drags conducted in areas with lower NDVI values.

Table 3 Bowkerstead Farm Negative Binomial GLM outputs, of factors associated with *I. ricinus* abundance (nymphs and adults). All variables included. Intercept is Grazing area A

	Estimate	Std. Error	z value	P
Questing *I. ricinus*				
Intercept	3.118135	0.885978	3.519	0.000432
Distance to woodland	-0.018210	0.002658	-6.850	**7.37e-12**
NDVI	-4.251547	1.551440	-2.740	**0.006137**

Plotting tick count per drag against distance to woodland (Figure 14) shows there are a lot of drags, carried out at varying distances from the woodland, which yielded zero ticks. However, the data still show a weak, but significant, negative correlation with a Spearman's Rank value of -0.26, (P< 0.001), confirming that the further away from the woodland the drag was, the less likely it was to yield ticks.

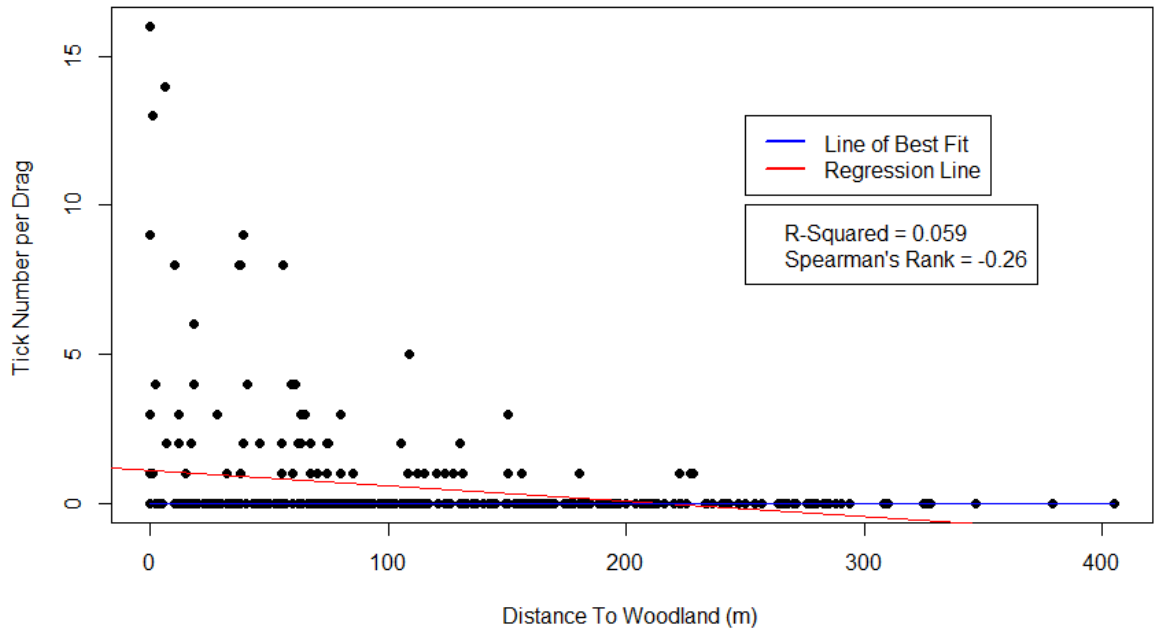

Figure 14 Scatter plot showing tick number against distance to woodland, including lines of best fit, R squared and Spearman's Rank values

2.4 Discussion

Blanket dragging across the farm demonstrated that *I.ricinus* ticks were indeed present in the area. The count numbers were highly variable and the daily variability of tick counts over a short period of time has been discussed in literature (Dobson; Randolph), a large number of drags (450) were conducted across the site in an effort to compensate for some of the noise, and provide a more accurate description of the tick distribution across Bowkerstead farm. Farmer 1 had stated that he believed area A to be frequently infested with ticks, where he grazed the hardier Swaledale breed; and area B to be free of ticks, where the mules were kept (Figure 13). By allowing his Swaledales to lamb on the 'dirty' side of his land, he believed they were able to develop some immunity to the tick borne pathogens. His method is in keeping with established husbandry methods, allowing naïve flocks to acquire immunity whilst suckling (Milne, 1950b). His flock rarely suffer from tick borne diseases other than occasional "stiffness", likely caused by tick pyaemia.

Kruskal-Wallis tests state that there was no significant difference in tick density between the three areas of the farm ($p=0.505$). This observation differed from the farmer's anecdotal evidence that the upland area of Bowkerstead Farm housed a larger tick population. This also contrasts previous studies, which found ticks to be more abundant in rougher pasture where conditions were more favourable (Milne, 1946) (Macleod, 1934).

Further analysis of the number of ticks collected on drags conducted in the different types of habitat observed across Bowkerstead Farm demonstrated that there was a significantly higher number of ticks observed in the woodland than there was in rough pasture or low pasture. This compliments the GLM outputs and is in keeping with previous studies (Milne, 1950a, Gilbert, 2016). Again, there was no significant difference between the number of ticks observed on drags in rough and low pasture. This is in contrast to what has been previously reported (Milne, 1946) (Macleod and Gordon, 1933) (Gilbert, 2016), where higher tick densities in rough, upland pasture have been reported than in managed pasture; due the more favourable conditions.

The 'rough' pasture observed at Bowkerstead Farm was comprised of large areas of upland calcerous grassland, bog, rocky outcrops, heather, small patches of *Pteridum aquilinum* (bracken) and tall rushes. There was a notable lack of bracken across most of the farm, with small outcrops near woodland boundaries and copses of trees; though there was an abundance observed in the woodland and its boundaries between the grazing pastures. Bracken has been implicated in the maintenance of tick populations previously, both within and bordering woodland (Dobson et al., 2011).

The presence of bracken bordering woodland combined with the GLM output demonstrating that proximity to woodland was a predictor for *I. ricinus* density across Bethecar Moor, suggests that border bracken control could reduce tick numbers across the farm.

Additionally, the number of sheep observed between the low and rough pasture at Bowkerstead is relatively even. With approximately equal numbers of Mules and Swaledales grazing on areas A and B respectively at the same times of year, with all animals grazing in area C during the mating season. If sheep are driving the tick population observed across Bowkerstead Farm then this could explain the similarity in densities between sites. This would need exploring via tick infestation counts on sheep, which was unfeasible to conduct at this site during this study.

This chapter concludes that the use of kriging in the production of a tick 'heat-map' is an effective and efficient way of communicating the *I. ricinus* distribution across the farm. The map reveals a patchy distribution of ticks, with several 'hot-spots' identified across the farmland. All of the hot-spots are in close proximity to a boundary of the farm, though they are only observed in areas where the boundary meets the woodland edge. There are ticks seen in all three areas of the farm, though the population in area A does appear to be more spread across the grazing land, as opposed to area B, where a couple of drags in the fenced off woodland appear to be skewing the results. The overlaying of this data on to DEM's provides another way of visualising and communicating this data. The resulting heat-maps could also be used to predict areas of high or low tick density across the site, though this would require validating via additional sampling in areas of high and low predictive prevalence, and considering a temporal component, which was outside of the scope of this project.

From the combined visual tick data in the form of chloropleth maps, and GLM output; proximity to woodland increases tick density at Bowkerstead farm. This is in keeping with previous studies (Milne, 1946; (James et al., 2013); Gilbert (Gilbert, 2016), 2015, 2017). There were some drags that were located in the woodland enclosed on, and surrounding, the farm. These drags had large numbers of ticks on them, due to the much more favourable habitat. Though sheep themselves would not routinely be found in these areas, the higher tick densities coupled with sightings of deer in these sites, demonstrate the importance of maintaining sufficient barriers between woodland, pasture, wildlife and livestock.

The visual representations of results obtained throughout this study were shared with the Farmer, so there was a mutually beneficial knowledge exchange, as part of a citizen science approach. He stated he was surprised by the tick 'hot-spots' in areas B and C of Bowkerstead Farm. Further discussions prompted the farmer to inspect his deer fencing in the woodland enclosed in the farm, alongside considering vegetation management where the boundary of his farm meets woodland or bracken. The production of the maps in this instance could help guide the farmer in targeting his efforts, and make for a more effective means of controlling the *I. ricinus* tick population across his land.

Proper management of boundaries to prevent the movement of deer, or sheep in to areas of higher tick density and bracken management, alongside a robust acaricide scheme should dramatically decrease the incidence of ticks observed on the sheep at Bowkerstead farm.

Chapter 3: Integrating GIS and sheep tracking, to explore the environmental hazard posed by *Ixodes ricinus* and the exposure rate to livestock in southern Cumbria.

3.1 Introduction

Results generated in Chapter 2, demonstrated that *I. ricinus* exhibit a patchy distribution on mixed pasture used for sheep grazing in Southern Cumbria. Furthermore it appeared that this distribution was driven primarily by the availability of suitable tick habitat. This Chapter aimed to extend the study to survey tick distribution across a much larger area of rough pasture, as is commonly used for sheep grazing across much of the United Kingdom, to further assess the drivers of this distribution. Additionally, the study aimed to combine this information with individual sheep movement data to explore the extent to which an individual sheep's tick burden is a product of its "behaviour" in a landscape presenting a heterologous tick hazard. Motivation for this work resulted primarily from anecdotal reports suggesting that different farmers observed markedly varied tick densities on their animals despite these animals sharing common upland grazing pasture; and that outbreaks of disease caused by tick-borne pathogens were restricted to individual farms.

Better understanding and mapping of the spread of *I. ricinus* (and changes in its abundance) is, however, essential to assess the risk of the spread of infections transmitted by this vector species.

The uplands cover 2.2 million hectares (17%) of England and are dominated by livestock farming. Hill farming is the primary driver behind the uplands' economy; housing 44% of breeding ewes and 40% of beef cows in England (Natural England, 2009). Hill farming also contributes via purchases of feed and machinery, as well as supply and marketing of food and processing of products through abattoirs (Institute for European Environmental Policy, Land Use Consultants and GHK Consulting, 2004)

The dense vegetation found in upland regions provides the ideal habitat for *I. ricinus,* numbers of which are reportedly increasing; following climatic changes, changes in land management and decreasing stock numbers (Estrada-Pena et al., 2004, Medlock et al., 2013, Cayol et al., 2017). Upland grazing plots are often bordered by woodland, which also provides suitable habitat for *I. ricinus*, this is set to increase as government policy has pledged to increase woodland cover in England from 10% to 12% by 2020 (https://publications.parliament.uk/pa/cm201617/cmselect/cmenvfru/619/61905.htm).

The uplands of the UK support a diverse range of open, semi-natural habitats. These include large expanses of blanket bog and heathland, moderate tracts of inland rock outcrop, mountain heaths and willow scrub, upland flushes, and upland calcareous grassland. Such habitats are not suitable or fertile enough for crops though are fit for purpose for the farming of hardy breeds of sheep for wool and meat production.

Milne and Macleod explored the association between ticks and hill farming, in a series of studies throughout the 1940's (Milne 1943;1945;1946), stating that the tick remains confined to hill pastures as this habitat meets the definite conditions of vegetation and humidity required for tick survival and development (Macleod, 1934). Macleod also stated that sheep were the main agents in spreading ticks in this scenario, likely leading to universal cover of ticks across the landscape, as they drop at random from the host, which is free to roam the moorland, which is considered suitable tick habitat (providing dense vegetation and high humidity). Macleod believed that habitat was the overriding factor, as ticks are not able to propagate in heavily stocked, lower pasture despite an abundance of hosts. Given a suitable habitat, the understanding which factors drive population densities of disease vectors is an important step in assessing disease risk.

As tick cover was anecdotally patchy across the Bethecar Moor landscape, with sheep and other hosts, such as deer, free to roam across it; we wanted to explore the idea that risk is a product of environmental hazard and exposure rate (Dobson et al, 2011).

This study aimed to take a holistic approach by: surveying the habitat; to establish the environmental hazard of ticks across the Moor. Sampling sheep; monitoring individual movements to provide data on exposure rate, in a population where intrinsic susceptibility factors such as breed and sex are not a feature. In addition to exploring the role of deer; which have been implicated as essential in the maintenance of tick populations (Jaenson et al., 1992; Wilson et al., 1998).

This study aimed to take a holistic approach to determine the ecological drivers behind tick infestation risk of sheep across Bethecar Moor, with implications for tick borne disease risk and agriculture economics. The desired outcomes of this study were to (i) determine whether questing *I. ricinus* ticks were evenly distributed across the fell via blanket-dragging and creating heat-maps of the questing tick population. (ii) Identify the ecological drivers behind the questing tick population via the use of general linear models (GLM's). (iii) Determine if tick burden was equal for all sheep by assessing individual sheep for ticks. (iv) Understanding if exposure rates were shaped by individual sheep movement across the more by exploring relationships between tick burden and GPS collar data for movement. (V) Establish if tick burden on sheep correlated with the environmental risk posed by the area of the Moor on which they grazed.

3.1.2 Bethecar Moor

Bethecar Moor, on which sheep from all farms included in this study graze, covers approximately 500 hectares and lies between the Coniston and Rusland valleys (Lat/Long: 54.31,-3.06) (Figure 15). The Moor rises to a peak at Arnside Barrow, which is 302.8m above sea level. The Moor consists of large expanses of upland heathland and bog, with tracts of inland rock outcrop and scree habitats. The moor is dominated by the presence of *Pteridium aquilinum* (bracken) and upland grasses. The Moor is predominantly one large grazing area, with several areas fenced off (shown by boundary lines in red, Figure 15) for crops and/or managed woodland. These were deemed to be inaccessible to livestock.

Anecdotally, Bethecar Moor is known to harbour a large population of ticks, with several farmers commenting on this and reporting sporadic outbreaks of louping ill and “stiffness” (tick pyaemia). In addition, when describing the Moor, farmers clearly identified what they considered 'ticky' or 'dirty' parts of the Moor; areas would change over time, but were mainly associated with the presence of bracken.

In addition to sheep, the Moor supports a range of wildlife species; of particular relevance to this study, red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*) cross the Moor daily. The Farmer’s involved in this study both stated that they felt the populations of both deer species had been increasing in recent years; with sightings of larger groups becoming more frequent when tending to their flocks across the Moor. The Farmer’s and Forestry Commission (FC) rangers also commented that Bethecar Moor had seen an increase in people visiting the fell in recent years, with the benefits of outdoor recreation being widely promoted.

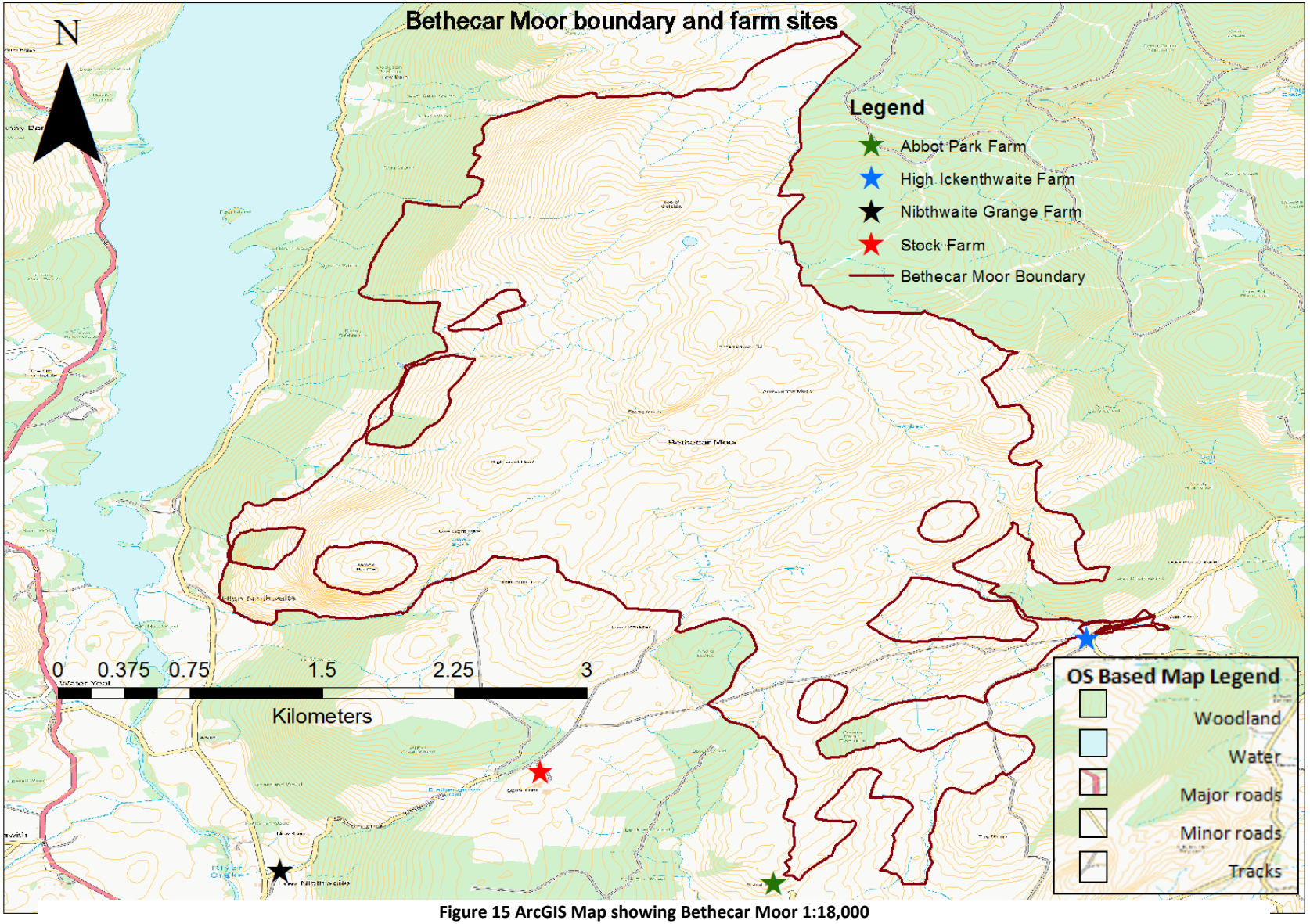

Figure 15 ArcGIS Map showing Bethecar Moor 1:18,000

3.1.3 The Farms

At the beginning of the study aims were discussed with colleagues in the Cumbrian Forestry Commission, and asked for their help with identifying relevant farms on which we could base our work. We also consulted with officers of the Bethecar Moor Commoners Association. Four farms have grazing right on Bethecar Moor, namely Stock Farm, Abbott Park Farm, Nibthwaite Grange Farm and High Ickenthwaite Farm (Figure 15)

3.1.4 Stock Farm

Stock Farm lies to the south of Bethecar Moor above the village of Nibthwaite (lat/long: 54.288517, -3.069384 (Figure 14) and is managed by managed by Farmer 2. Livestock on Stock Farm includes a small number (<40) of suckler cattle, 500 breeding Swaledale ewes and 12 Swaledale rams. Approximately 500 lambs are born into the flock each year, with females being retained to replace old ewes and males being sent to slaughter. The flock has access to the rough, common "fell" grazing area of Bethecar Moor (500ha), where they spend most of the year. The farmer reports that although his sheep are free to roam across the entire Moor, and a small portion of his flock will do so, they tend to avoid its north western and south eastern extremities as sheep from other farms are "hefted" (conditioned to occupy) these areas. The sheep are maintained almost solely on the natural vegetation of the Moor, although, they receive supplements prior to breeding, after lambing, and in severe weather (e.g. snow). The flock is rounded up just five times a year for various husbandry practices, as described below:

January: Sheep are collected for ultrasound scanning to determine how many of the ewes are pregnant. At this point all sheep are treated with closantel (Flukiver) for endoparasite control. Scanning is typically completed over the course of a day, with sheep being returned to the Moor immediately after scanning.

March: At the end of March, approximately three weeks before lambing, sheep are brought down into the pastures immediately surrounding the farm (by-land). All animals are vaccinated against *Clostridium* species, *Mannheimia haemolytica* and *Pasteurella trehalosi* (Heptivac P). Shearlings (1 year old ewes) are also vaccinated against louping ill virus (LIV). All pregnant ewes are then held in by-land for lambing apart from those having difficulty or those with twins, which are taken indoors.

Mothers and lambs are usually returned to Bethecar Moor within 48 hours of the birth. Lambs receive cypermethrin (Dysect) as an ectoparasite control before release.

July: Sheep are rounded up mid-July for shearing. This takes place in the farm yard over 3-4 dry days, weather dependant, after which sheep are immediately returned to Bethecar Moor.

September: Sheep are collected into the by-land so that young males can be separated from the flock to be sold or sent for slaughter. Some males remain on the farm, but indoors, where they are fattened up over winter for sale the following spring. Adult sheep and ewe lambs treated with Flukiver and Dysect, and ewe lambs are vaccinated with Heptivac P.

November: The flock is rounded up and lambs are given a second HeptivacP vaccination. All animals are also treated with Flukiver and receive albendazole (Albex) to further control endoparasites. Lambs are released back onto the Moor, but breeding ewes remain on the by-land for about 4 weeks during which time they will mate ("tup"). These ewes return onto the Moor in mid-December.

Stock Farm do not maintain records pertaining to individual sheep, thus information relating to the burden of infectious diseases, in particular tick-borne infections, to the farm is solely anecdotal. However, the farmer reported historical episodes of tick-borne diseases and perceives their ongoing threat to be sufficient to warrant the continued use of acaricides (Dysect) and LIV vaccination.

3.1.5 Abbott Park Farm

Abbot Park Farm lies to the south east of Bethecar Moor, above the village of Brandrake Head, (lat/long: 54.280471, -3.056304) (Figure 15). The farm maintains a variety of sheep breeds including about 150 breeding Texel-Swaledale crosses, 40 Swaledales, two Zwartbull crosses, one Jacob and three Texel rams. At the start of the study, all sheep were restricted to enclosed pasture surrounding the farm; however, during the second year of the study, the sheep were released onto Bethecar Moor to graze. Despite being free to roam across the entire moorland, sheep from Abbott Park Farm are strongly "hefted" to the Farm thus predominantly remained in the south-eastern portion of the Moor. Although the older sheep spend the majority of their time on the Moor, they were rounded up far more frequently than those from Stock Farm, as described below.

March: Shearlings returned to Moor after spending the winter months inside. Pregnant ewes are rounded up and held in by-land for lambing, bar those with complications, which are moved inside. All animals treated with Flukiver and a pour-on acaricide (various formulations/brands used). The ewes and lambs stay in the by-land until late summer.

April & May: All animals (on Moor and in by-land) treated with the anthelmintics closantel and mebendazole (Suparverm).

June: Sheep on the Moor moved onto by-land. All sheep sheered and treated with Supaverm and pour-on acaricide.

July & August: Sheep on Moor moved onto bi-land. All animals again treated with Suparverm. Male lambs removed from flock be sold or sent for slaughter. Some males remain on the farm, but indoors, where they are fattened up over winter for sale the following spring. Ewes and female lambs released onto Moor.

September: All animals rounded up and subjected to acaricide shower/dip. Animals also treated with Flukiver. The ewe lambs vaccinated with HeptivacP.

October: Once again, all sheep herded off Moor for treatment with Flukiver. Ewe lambs (now shearlings) separated from flock and kept on by-land then indoors over winter. Older ewes held in by-land for tupping.

November: All sheep treated with Flukiver

December: Inseminated ewes again treated with Flukiver and returned to the Moor.

January: Sheep on Moor rounded up and treated with Flukeiver.

February: In late February, ewes are brought down from the Moor into the by-land in preparation for lambing. All animals, including shearlings that are being prepared to return to the Moor, receive Heptivac P and flukeiver.

Akin to Stock Farm, Abbott Park Farm do not maintain records pertaining to individual sheep, thus information relating to the burden of infectious diseases, in particular tick-borne infections, to the farm is solely anecdotal. The farmer could not record any confirmed cases of tick-borne disease even though her sheep were not vaccinated against LIV. However, she suspected cases of tick-borne fever.

3.1.6 Nibthwaite Grange Farm

Nibthwaite Grange Farm is also partially served by the Moor, lat/long: 54.284321/-3.082317. This farmer had a flock of 180 Cheviot ewes, which were hefted to the north west of the fell. This farmer did not take part in the study.

3.1.7 High Ickenthwaite Farm

High Ickenthwaite Farm is the fourth farm partially served by the Moor, lat/long: 54.296394/-3.041193. This farmer did not keep sheep, but had a small herd of 12 Belted Galloway cattle. Farming was not the occupier's primary livelihood, so he was not included in the survey.

3.2 Methods

3.2.1 Survey of questing ticks on Bethecar Moor

Preliminary random dragging was conducted to confirm the presence of ticks. Once this was established, drag points were randomly generated across the Moor using ArcMap (Figure 16). The drag points were then uploaded to a Garmin GPS as waypoints, following manufacturer's guidelines and software (MapSource) to allow for location of points on site. The accessibility of each drag point was assessed by the use of Ordnance Survey (OS) maps and visiting points in the field. Points deemed unaccessible due to slope and other physical barriers were removed from the study.

Blanket dragging was conducted using the method described in Chapter 2. The number and stage of ticks per drag were recorded. Larvae were not collected. All field work took place on dry days during the tick season between April-September 2014-2016.

3.2.2 Survey of ticks on sheep grazing on Bethecar Moor

Sheep were rounded up in line with participating farmers' husbandry methods and a random selection was corralled for inspection. Each sheep was turned over individually under supervision of the farmer or an experienced farm hand. The sheep was then inspected for the presence of ticks for 2 minutes, with particular attention paid to the parts of the body lacking wool (armpits, groin, legs, ears, neck and face). All ticks encountered were removed into 70% ethanol using a silicone tick remover.

In the event of high tick burden, sampling continued until all visible ticks were removed, this took no longer than 4 minutes. Tick location, sheep identification number, breed and age were also recorded on sampling sheets in the field.

Identification of all ticks collected was verified by reference to taxonomic keys (Hillyard 2006).

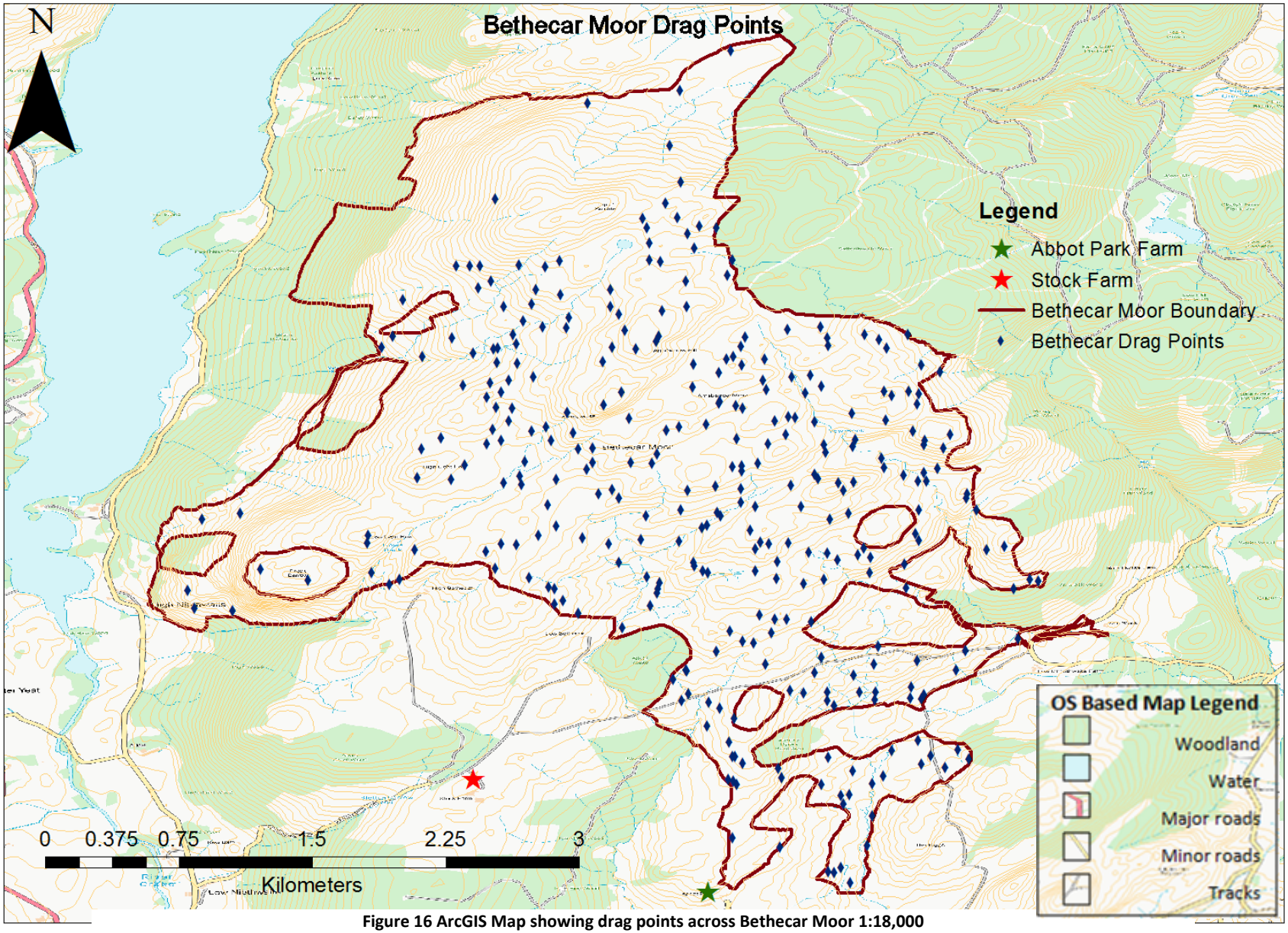

Figure 16 ArcGIS Map showing drag points across Bethecar Moor 1:18,000

3.2.3 Mapping

3.2.3.1 Mapping tick populations

Distribution 'heat-maps' of questing *I. ricinus* ticks were produced in ArcMap10. These were generated from empirical field data. Each drag point was assigned its tick count number in Excel then exported to ArcMap to undergo spatial analysis via kriging as described in Chapter 2. The resulting chloropleth maps were then clipped to Moor boundaries using shape files drawn in ArcMap10, using the draw tool and converted to shape files.

3.2.3.2 Mapping deer movement across the fell using expert knowledge

As discussed above, populations of red deer and roe deer are routinely monitored across our study region by Forestry Commission (FC) rangers. As a result of this, rangers have an intimate knowledge of deer movements in the area, which was used to inform a 'deer heat-map' of Bethecar Moor. A grid system was generated and overlaid on to the landscape with points every 250m in ArcMap (Figure 17). For each of these points, two local FC rangers and the Stock and Abbott Park farmers were asked to assess the distribution of deer using a semi-quantitative scale (Table 4,) averages of their given values for each point were taken and used to generate a heat-map via kriging.

Table 4 showing values and descriptors used by farmers and rangers to assess deer movement across Bethecar Moor

Value	Descriptor
0	Deer are never sighted here
1	Deer are rarely seen here. If so, they are usually solitary or have been disturbed
2	Deer are occasionally observed here, but do not graze or spend much of their time here
3	Deer are often sighted here, in small groups. Will occasionally graze.
4	Deer frequently observed in the area, will often graze.
5	Large numbers of deer frequently seen here, will spend reasonable time grazing or resting

3.2.3.3 Mapping sheep movement across the Moor

Each of the farmers had an intimate knowledge of their flocks grazing patterns across the Moor, which was used to inform a 'sheep heat-map' of Bethecar Moor. Using the same grid system and semi-quantitative scale as the deer movement data; both farmers were asked where they saw sheep across the Moor, inclusive of their own and other farmers' flocks.

To track individual sheep movement across the Bethecar Moor, Retrieva tracking collars (Retrieva Ltd, Hertfordshire, UK) were fitted to sheep. A total of 50 collars were used in the study, and were fitted to sheep at the time of survey for ticks. The collars were set up by Retrieva to record a GPS location every hour, with these data being stored on the collar. The estimated battery life of the collars under this set up was 30 days. Thus, the aim of this exercise was to obtain 30 day tracking data for at least 250 sheep during the course of the study.

3.2.3.4 Remote Sensing & NDVI

Remote sensing images of Bethecar Moor on the sampling date were obtained via freeware from the ESA and their Sentiel2 satellite. Images from the sampling date were downloaded from the Scientific Data Copernicus Open Access Hub (https://scihub.copernicus.eu/) and processed in line with the methods previously described in Chapter 2 to produce NDVI images.

Terrain and elevation data was compiled for the areas of interest from archived open access government data (http://environment.data.gov.uk/ds/survey/index.jsp#/survey?grid=SD39). The LIDAR Digital Terrain Models (DTMs) at 2m spatial resolution were collated and unified in ArcScene to create a 3D mosaic of the area, as described in Chapter 2. In addition to a 3D model, the elevation dataset was used to produce a Digital Elevation Model in ArcMap, which was manipulated with the spatial analyst slope tool to generate a 2D representation of slope across Bethecar Moor.

3.2.3.5 Distance to Woodland

The distance from each drag point to the surrounding woodland was also calculated in ArcMap. To do this, a near table was generated in ArcMap, which displayed all of the distances from each point to each bit of woodland, both within and sharing a Bethecar Moor boundary (Figure 18).

3.2.4 Statistical analyses

All statistical analyses were carried out using R Studio (version 1.0.136, RStudio, Inc., Boston, MA). Following initial analysis and distribution fits; all potential explanatory variables were tested in a Generalized Linear Model (GLM) as previously described in chapter 2. Variables considered in this study were: Distance to woodland, woodland site, deer density, slope, and NDVI.

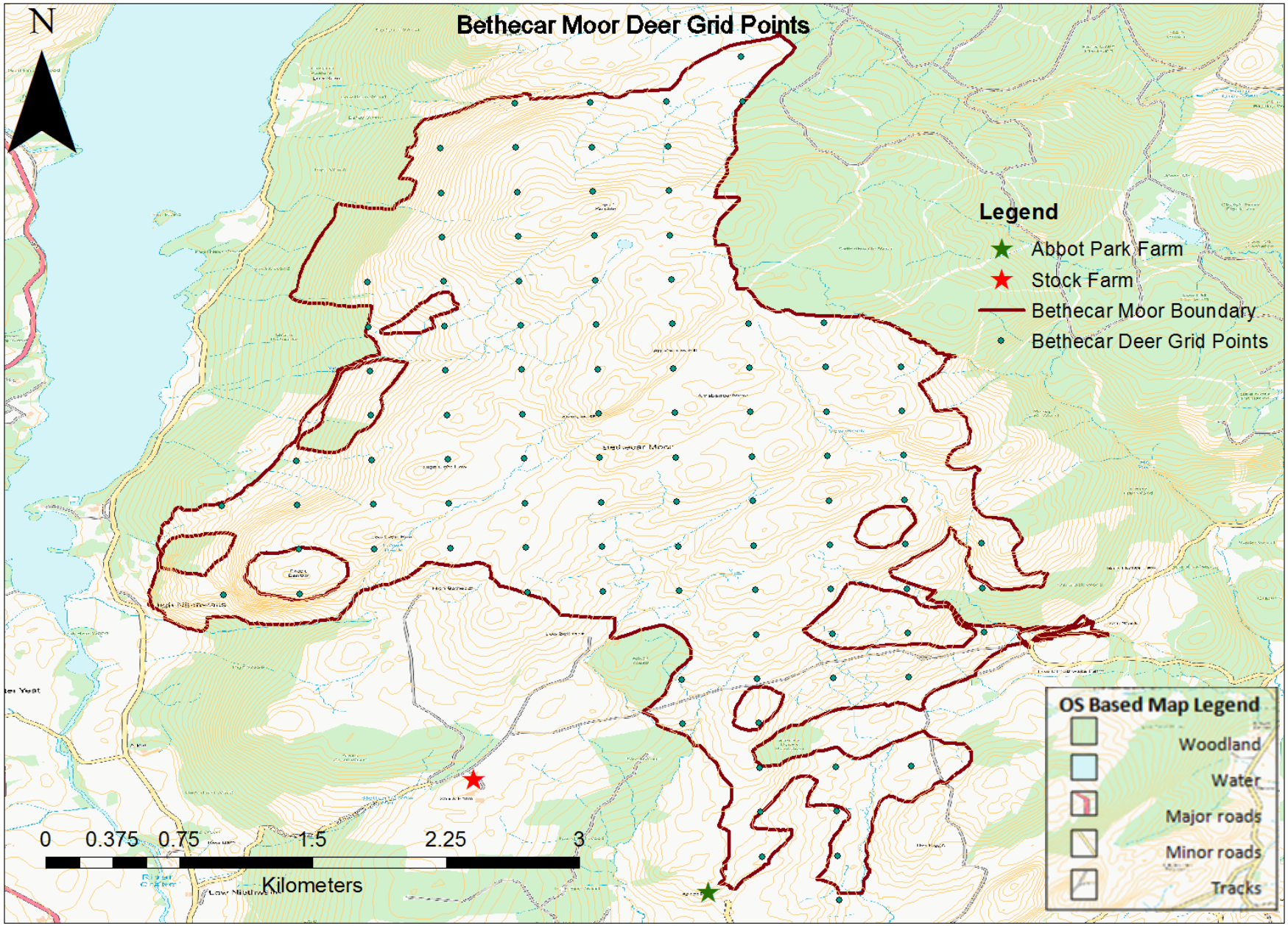

Figure 17 ArcGIS Map of Bethecar Moor showing Grid Points for Deer and sheep maps 1:18,000

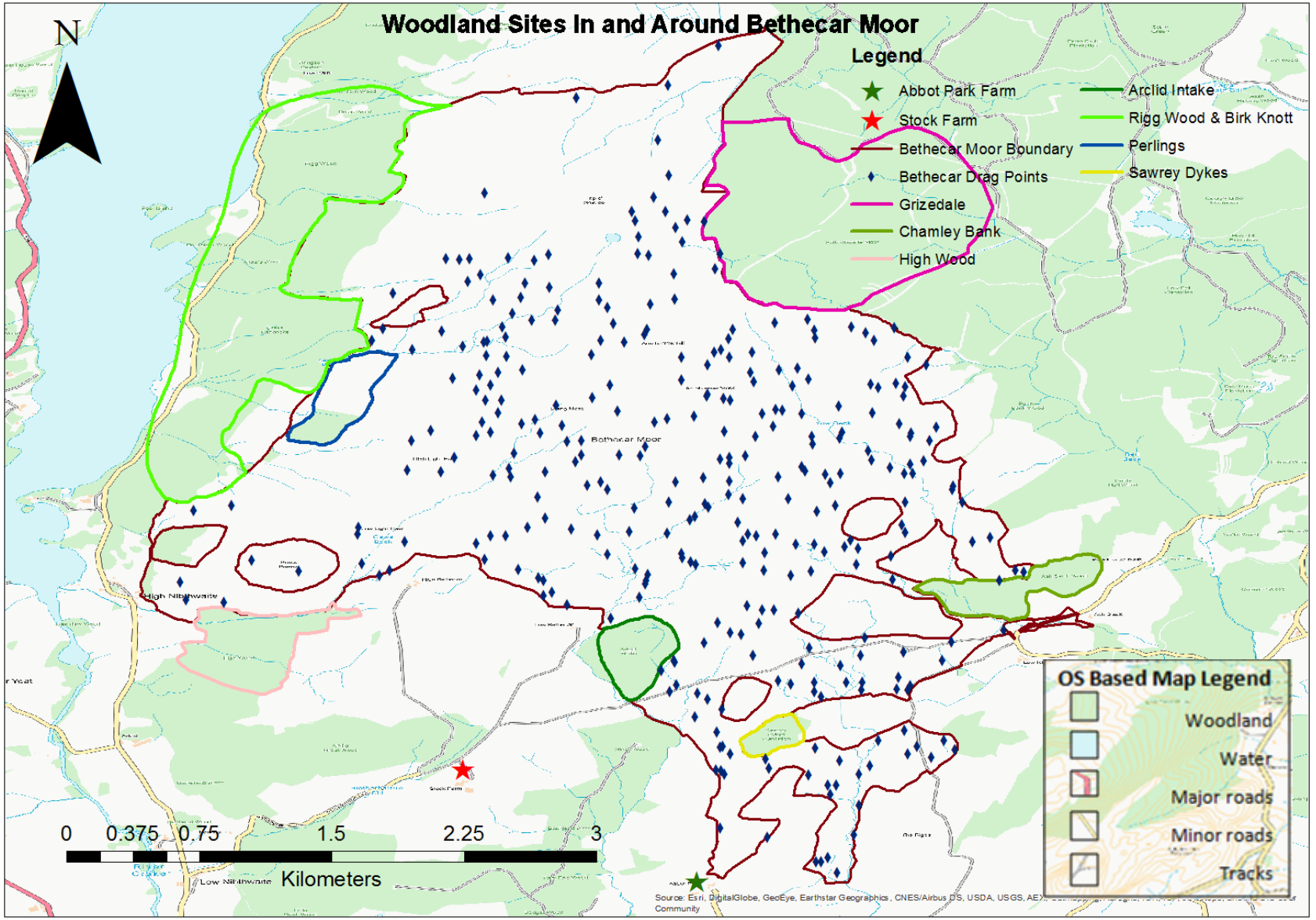

Figure 18 ArcGIS Map showing woodland surrounding and enclosed by Bethecar moor

3.3 Results

An initial spatial survey of Bethecar Moor was conducted in June 2015. However, by this point in the summer, *Pteridium aquilinum* (bracken) which grew on many parts of the Moor had grown to a height of about 1 meter, making survey of questing ticks by blanket dragging not feasible or reliable. Thus, a second attempt was made in April 2016, before the bracken had grown above other Moorland vegetation. This second survey was carried out on a warm (22^{o}C) dry. A total of 328 drags were completed, yielding 389 ticks, all of which were identified as *Ixodes ricinus* (Figure 19). Of these, 348 were nymphs, 20 were adult males and 21 were adult females.

3.3.1 Tick Distribution

A histogram of tick count per drag shows that there was a large positive skew in the data, due to many drags yielding no ticks (Figure 18). Comparative analysis between negative binomial and Poisson distribution confirmed that the tick count data were displaying a negative binomial distribution (Figure 20).

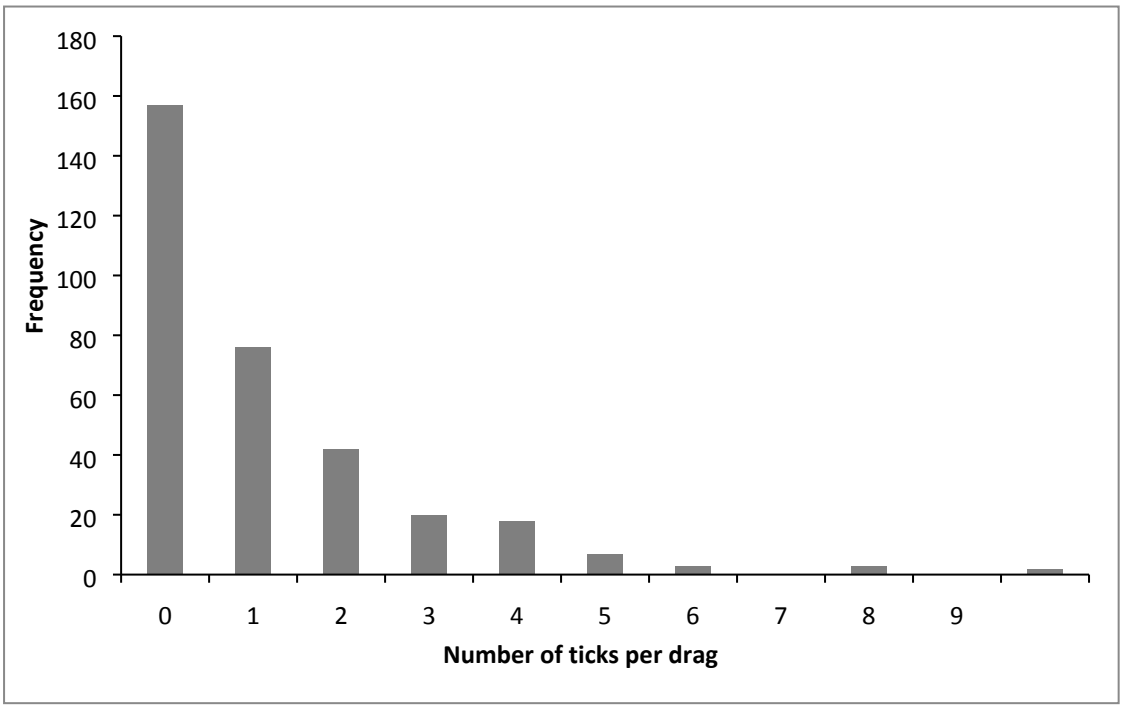

Figure 18 Histogram showing tick counts per drag across Bethecar Moor

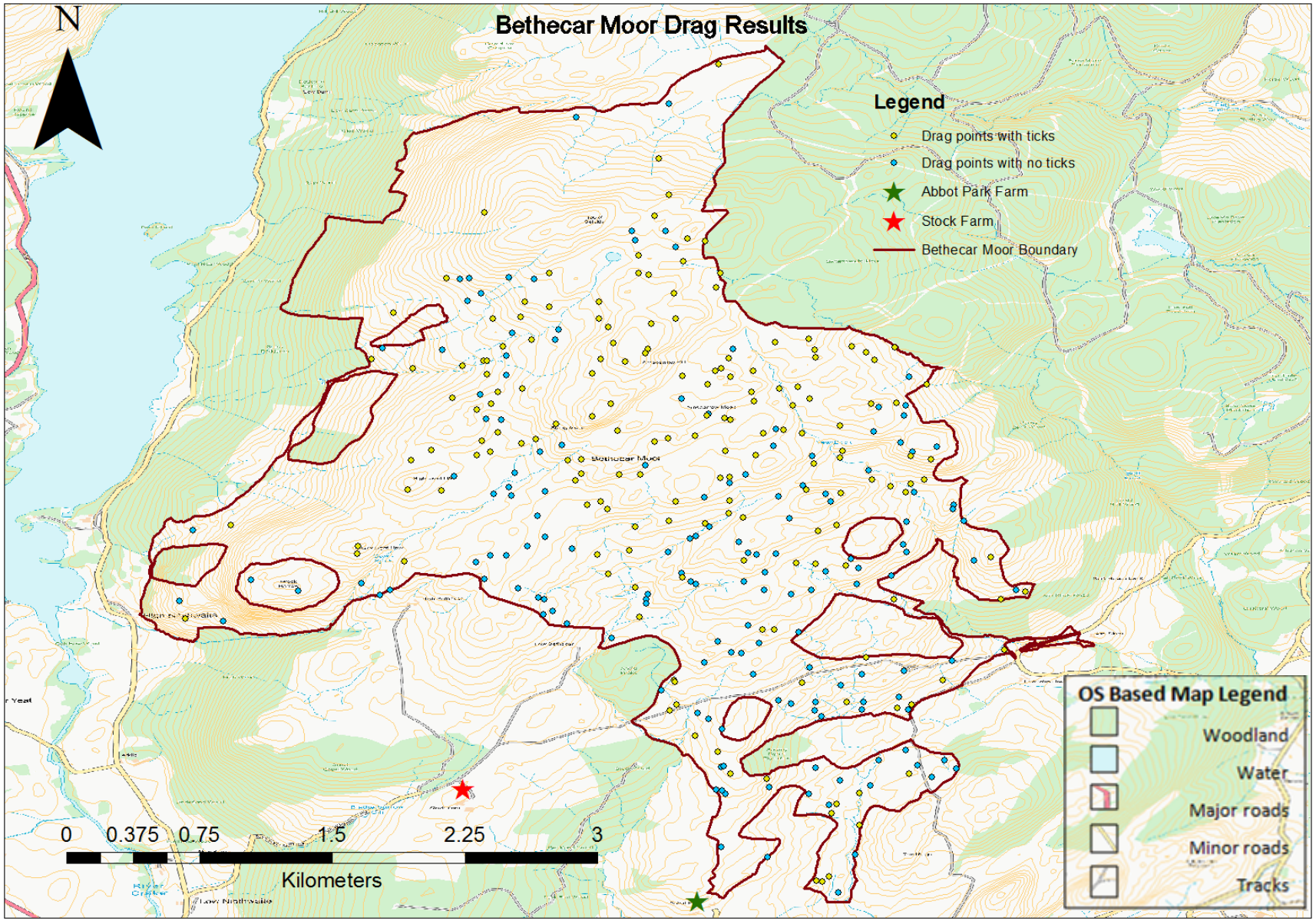

Figure 19 ArcGIS Map showing drag points with and without ticks across Bethecar Moor 1:18,000

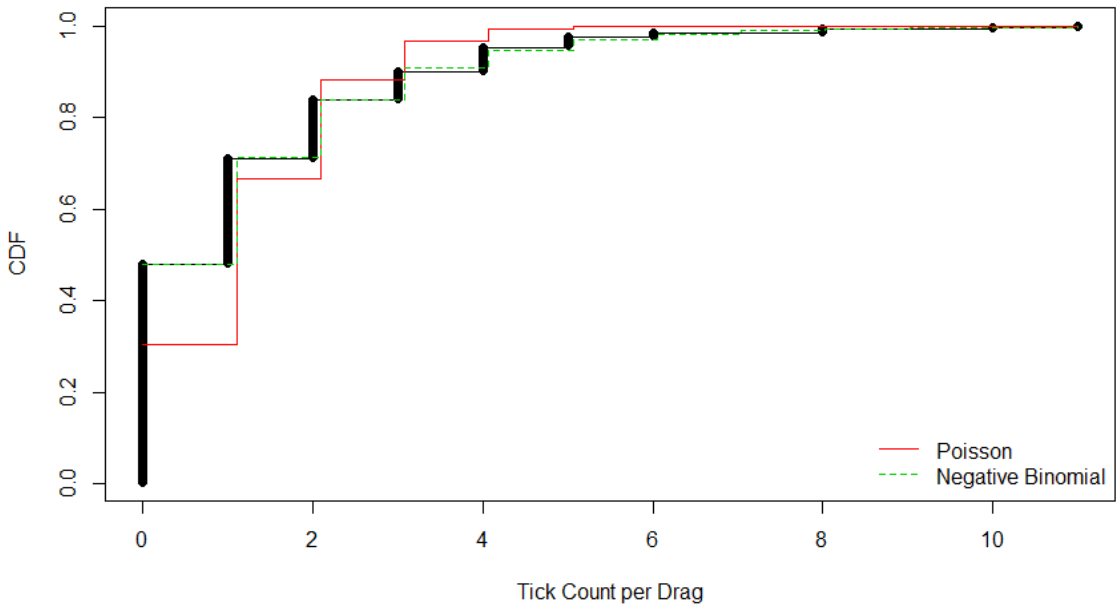

Figure20 graph comparing Poisson and negative binomial distribution fits of Bethecar Moor drag data, using empirical and theoretical Cumulative Distribution Functions (CDFs). The theoretical negative binomial values in green, align much more closely with the empirical values in black, than theoretical Poisson figures in red.

3.3.2 Mapping Tick Distribution

Tick dragging surveys provided the empirical data to create a 'heat-map' of *I. ricinus* distribution across Bethecar Moor; the resulting chloropleth map (Figure 21), displays *I. ricinus* distribution across the moor. Areas of high tick density are displayed in red and low tick density in green. A large red area, indicating a relatively high tick density can be observed on the north east section of the Moor; an area adjacent to a large expanse of woodland. This high density continues in a south westerly direction across the Moor, though the trail changes to orange as tick counts per drag decrease.

The rest of Bethecar Moor is predominantly green, indicating very few or no ticks; with the exception of moderately populated orange area on the north west of the Moor, to the east of Coniston Water.

3.3.2.1 Mapping deer movement across Bethecar Moor

The FC deer stalkers and local farmers provided the data to create a 'heat-map' of deer movement across Bethecar Moor. The resulting layer (Figure 22) displays perceived deer movement across the Moor, showing areas of high traffic in reds and oranges, and areas of the fell unlikely to have deer in shades of blue.

The map shows an area of high deer traffic in dark red, on the north east section of the Moor. Again, this area shares a boundary with the corner of Grizedale Forest. The higher density, red areas continue across the Moor in a south westerly direction, forming a 'corridor' between the bottom of Grizedale forest and a patch of woodland to the north east of Stock Farm called Arklid Intake. Though there is a high density throughout this corridor, it is highest in close to proximity to each of the woodlands.

The corridor is quite broad, with deer movement being sighted within a band of approximately 2km; deer density decreases and the band turns green then blue, as the distance from the centre of the band increases.

3.3.2.2 Mapping sheep occupancy of Bethecar Moor

Following in-depth discussions with the farmers regarding sheep occupancy on the Moor, no areas were identified where sheep did not graze, save areas of sheer rock, or those that were fenced off. Each farmer felt that though their flocks may cluster when being given supplemented feed, grazing from the four farms serviced by the Moor ensured sheep could be observed grazing right across it, and that time spent grazing in an area would change in response to environment, grazing quality and time spent out on the Moor. The farmer on Stock Farm felt his flock of 500 animals occupied much of the middle parts of the Moor, but that they would also disperse and smaller groups could be found anywhere on the Moor.

The farmer on Abbott Park Farm felt that her smaller flock of 200 animals generally kept to the south east of the Moor, but that again smaller groups would become more disperse and graze in a more central location.

Both farmers were in agreement that the other, smaller flock of 180 sheep belonging to Nibthwaite Grange Farm would generally graze in the North West section of the Moor, but that smaller groups would disperse and graze further south and east across Bethecar Moor. The Farmers were unable to identify any areas that were commonly grazed or ignored by the flocks, and so were free to move across the Moor (Figure 23).

3.3.2.3 NDVI

Sentinel 2 data produced an NDVI map of the Moor (fig xx). Where 0 represents no green vegetation and closer to +1 (0.8, 0.9) demonstrates the highest possible density of green leaves. Pixel values for each drag point were extracted and recorded.

There are areas of high and low NDVI values across Bethecar Moor. Two areas of fenced woodland are easily identifiable by their lighter colouration, representing NDVI values of approximately 0.7, on the south west and east of the Moor. Generally, the Moor has values in the region of 0.4, typically representing grassland; though it is not completely homogenous, with dark areas (NDVI of around 0.1) representing barren areas, or areas of bare rock.

3.3.2.4 Slope

LiDAR data was used to create a slope map of Bethecar Moor, using degrees, in ArcMap. With greater inclines represented in darker shades, and more even ground in lighter shades. Drag points were overlaid on to the image (Figure 24), and pixel slope values, in degrees, were extracted for each drag point.

The main body of the Moor is quite undulating, but not steep, represented by more subtle changes in shade. The exceptions to this are the west side of Bethecar Moor, which rises at a steep incline of up to 50 degrees in places from the east of Coniston Water. In addition, there is an identifiable steep incline in the centre of the Moor shown in brown; this is Arnsbarrow Hill.

3.3.2.5 Distance to Woodland

The distance from each drag point to each area of woodland, both within and sharing a boundary with Bethecar Moor was also calculated in ArcMap (Figure 25).

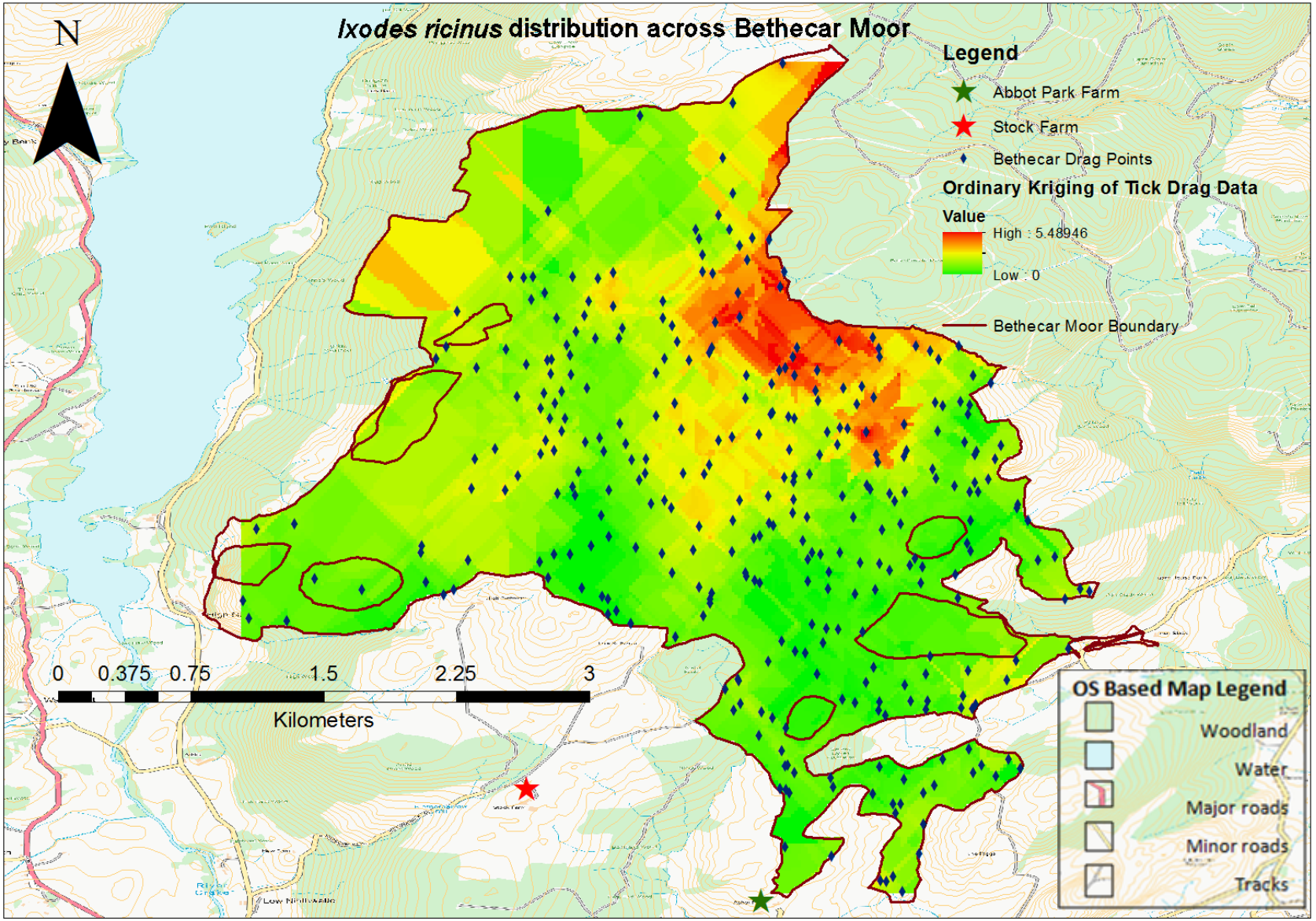

Figure 21 ArcGIS Map showing Kriging result of tick drag data and spatial distribution of *I. ricinus* across Bethecar Moor 1:18,000

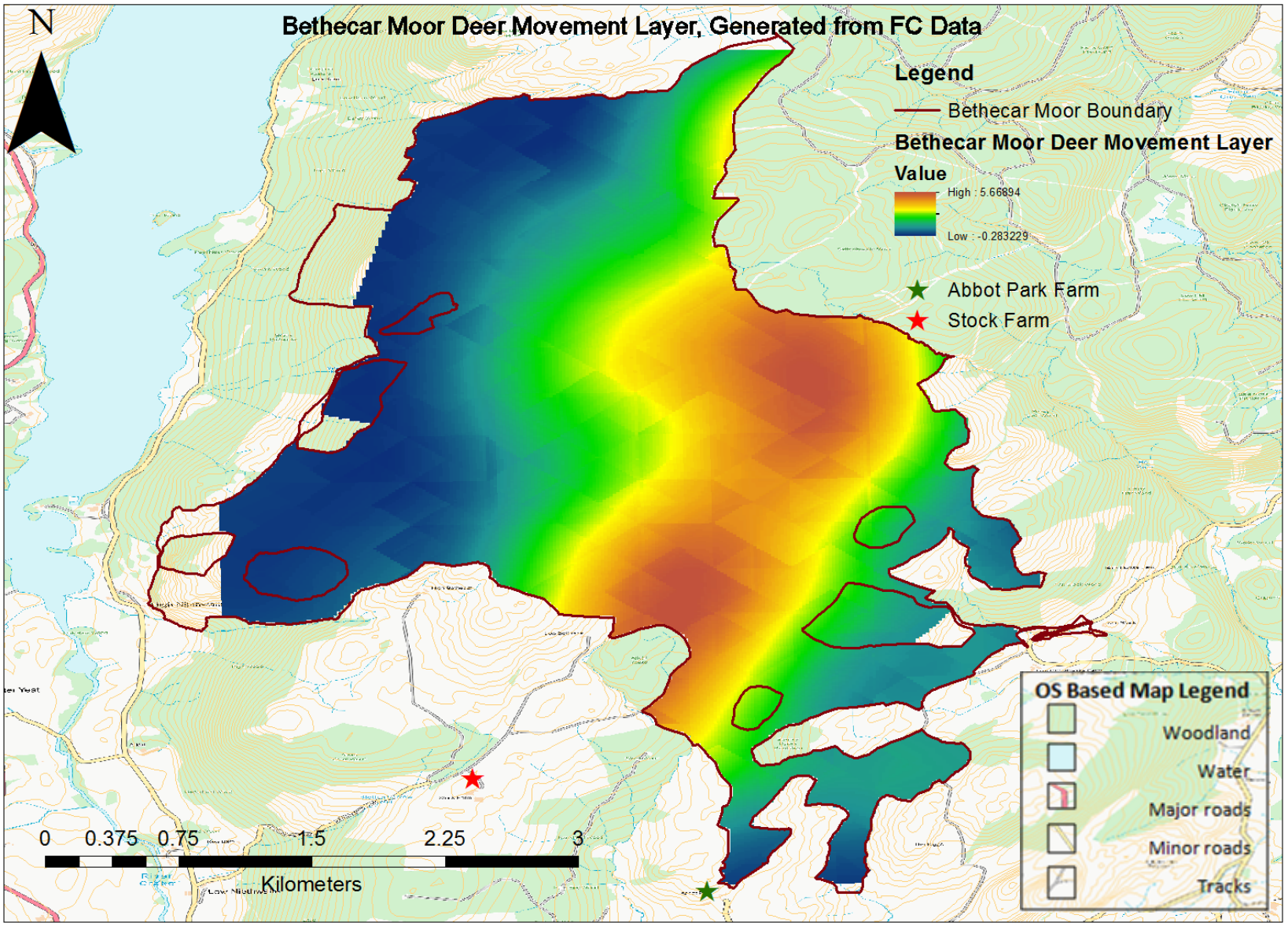

Figure 22 ArcGIS Map showing kriging results layer of deer movement across Bethecar Moor 1:18,000

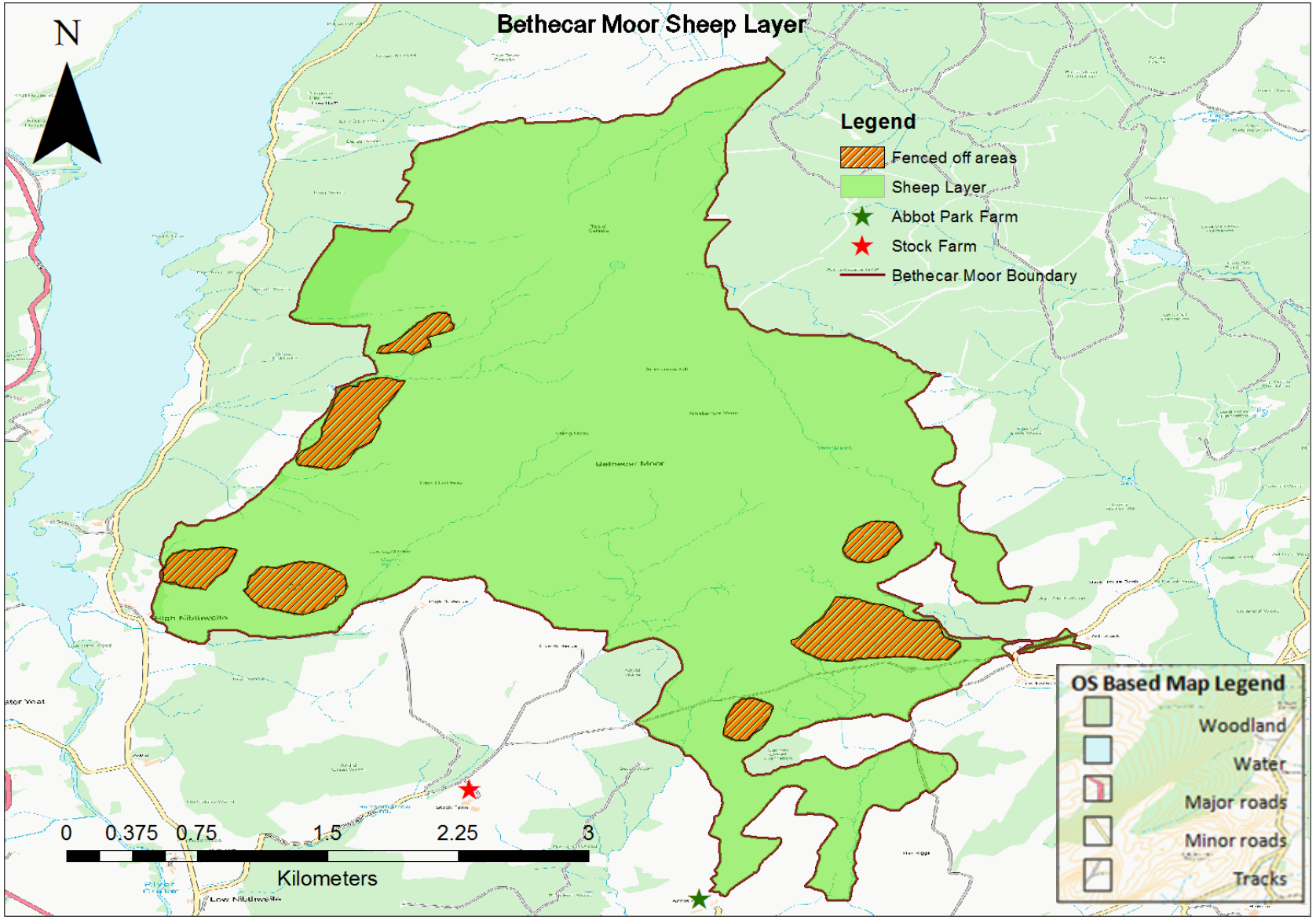

Figure 23 ArGIS Map showing where sheep graze on Bethecar Moor, 1:18,000

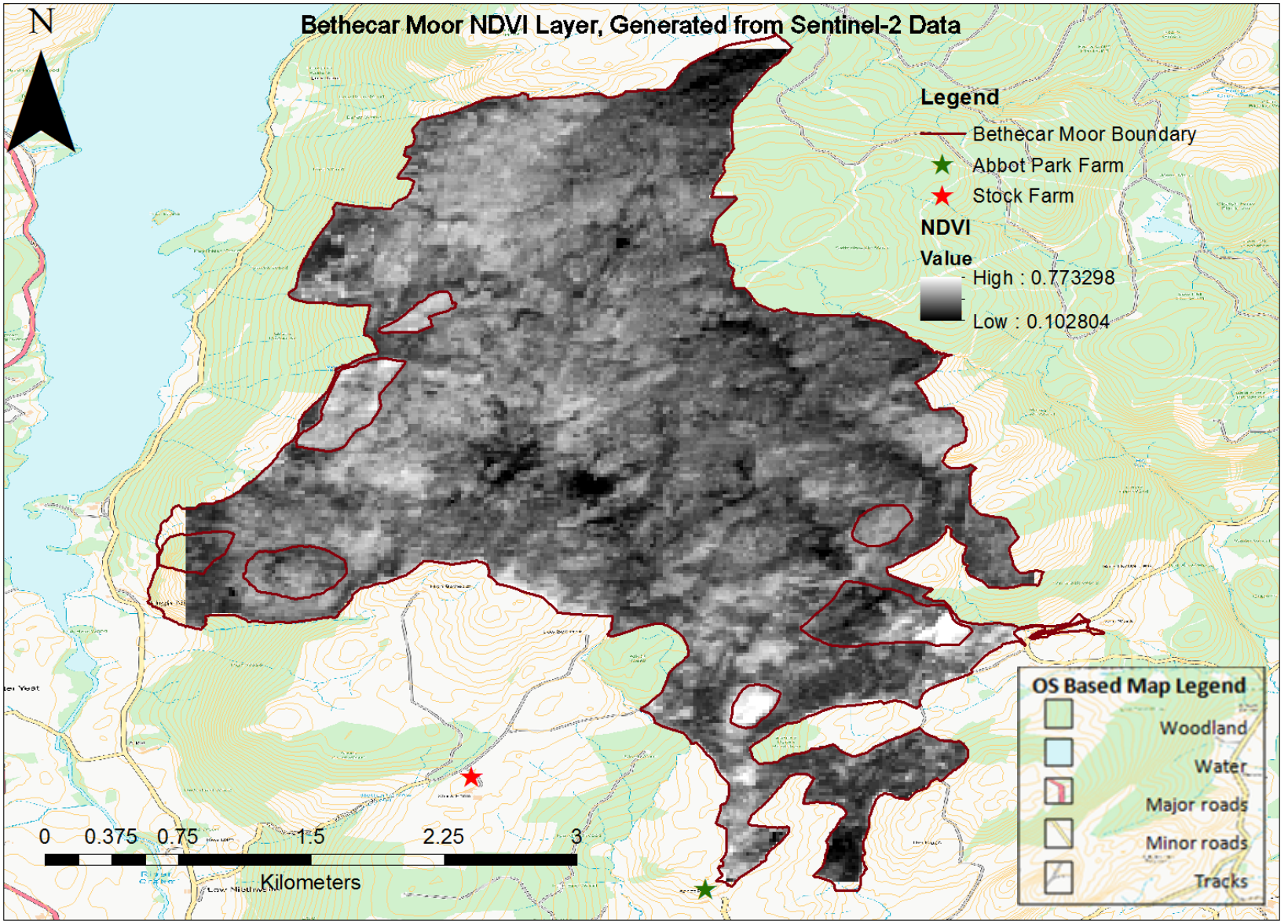

Figure 24 ArcGIS Map showing NDVI image layer for Bethecar Moor, generated from Sentinel-2 data 1:18,000

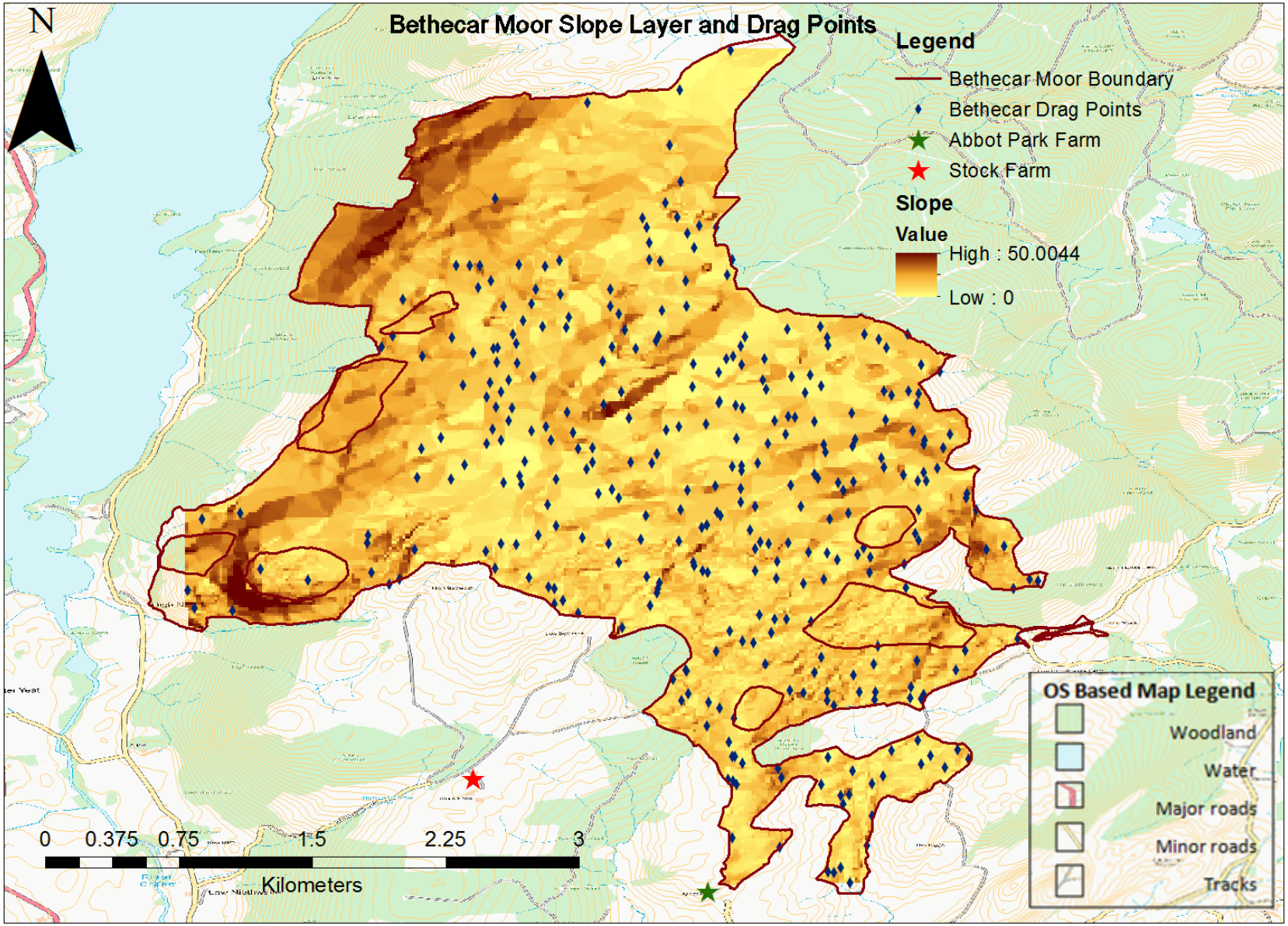

Figure 25 ArcGIS Map showing slope of Bethecar Moor, generated from LiDAR data, 1:18,000

3.3.3 Fitting the Model

Once a negative binomial distribution was confirmed, the potential explanatory variables were tested in a Generalized Linear Model (GLM). Initially, variables considered in the model were: NDVI, slope, which woodland was closest, distance to woodland and deer density (Table 5). Running the model confirmed that the closest woodland being Grizedale Forest was a significant predictor of tick density across Bethecar Moor ($p < 0.001$).

Table 5 Bethecar Negative Binomial GLM outputs, of factors associated with *I. ricinus* abundance (nymphs and adults). All variables included. Intercept is Chamley Bank

	Estimate	Std. Error	z value	p
Questing *I. ricinus*				
Intercept	0.053402	0.600251	0.089	0.9291
NDVI	-1.323664	1.166568	-1.135	0.2565
Deer Density	0.051386	0.056198	0.914	0.3605
Slope	0.018255	0.016099	1.134	0.2568
Grizedale	1.040128	0.259964	4.001	**6.31e-05**
Arklid	-0.112322	0.285287	-0.394	0.6938
Sawrey Dykes	0.006151	0.316322	0.019	0.9845
High Wood	-0.226969	0.554271	-0.409	0.6822
Perlings	0.487390	0.340791	1.430	0.1527
Rigg Wood & Birk Knott	0.631298	0.330488	1.910	0.0561
Classed distance to woodland	-0.013227	0.018180	-0.728	0.4669

The model was unable to fit to the fine scale data provided with individual distances to each woodland site, and was discarded.

To determine the model of best fit; slope, NDVI and deer density were eliminated from the model during the backwards and forwards step-wise procedures due to $p > 0.1$. As such, only closest woodland site remained in the model, with Grizedale Forest considered as significant in predicting tick abundance ($p < 0.001$) (Table 6).

Table 6 Bethecar Moor, Negative Binomial GLM outputs, of factors associated with *I. ricinus* abundance (nymphs and adults); testing site variables. Intercept is Chamley Bank

	Estimate	Std. Error	z value	P
Questing *I. ricinus*				
Intercept	-0.22826	0.20603	-1.108	0.268
Grizedale	1.01672	0.23976	4.241	**2.23e-05**
Arklid	-0.11658	0.27110	-0.430	0.667
Sawrey Dykes	-0.09265	0.29262	-0.317	0.752
Perlings	.033004	0.32268	1.023	0.306
Rigg Wood & Birk Knott	0.42608	0.30631	1.391	0.164

3.3.4 Plotting Sheep Movement

Due to complications and collar availability, we were only able to attach 128 collars in this time frame. Once attached, there were further problems with the collars including; early loss of battery life, physical collar damage due to extreme weather conditions, water-logging, missing data records, incorrect data records and huge losses of data due to server issues. Following analysis of empirical data, of the 128 attached, just 27 yielded a reasonable amount of reliable data; producing 1468 points of a possible 168,000 (0.87% success). The number of data points recorded for each collar ranged from 3 – 458, with some collars recording continuously rather than every hour. In addition, there was large variation in the number of days each collar lasted, from 1 – 24 days, with various levels of detail sent from the collars (Table 7).

These points were visualised in ArcMap (Figure 26), with the number of data points and length of time data was logged for each sheep recorded in Table xx. The data logged was sporadic and uneven, with the longest period of time monitored being 24 days, but yielding just 82 points. Conversely, a collar latched on to sheep number 716 logged 458 points over 8 days; these were not evenly distributed (Table 7). None of the collars worked to their full capacity, with 6 only beginning to log data days after latching.

However, the data that was captured, demonstrates that the sheep from Stock Farm move across the whole fell and do not appear to be entirely hefted to one area (Figure 26). There is a larger contingent that stayed around the Arklid Intake area, this could be a reflection on the poor battery life, and that the ewes had not had sufficient time to disperse before the collar was unable to record any more data.

Incorporating the data available from GPS collars and the *I. ricinus* distribution map shows that the sheep were spending time across the fell, both in areas where ticks were more abundant and where they were sparse or absent (Figure 27).

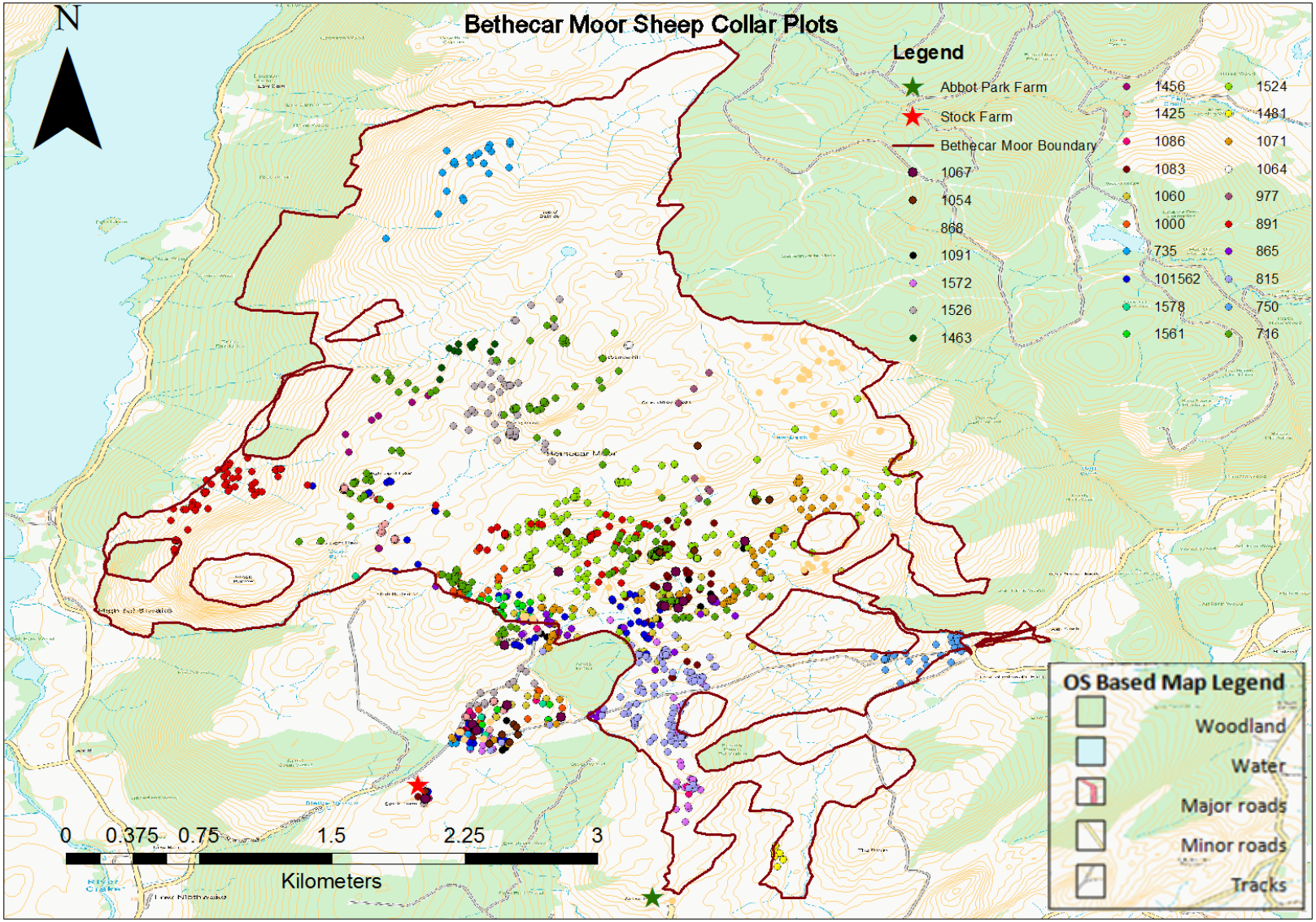

Figure 262 ArcGIS Map showing GPS collar data on sheep across Bethecar Moor 1:18,000

Table 7 cataloguing all sheep collar GPS data

Sheep no	Date on	Record start	Record end	Date off	No. of days	Data points	Comments
1578	28/07/2015	28/07/2015	11/09/2015	Sep-15	9	16	Sporadic, 2 points a day, all hours
1000	28/07/2015	28/07/2015	07/08/2015	Sep-15	10	80	Some days 1 or 2 points, most a few, sporadic. One day 52 points, every half hour
1083	28/07/2015	28/07/2015	11/08/2015	Sep-15	16	44	4-5 points a day 15 on one day
1425	28/07/2015	28/07/2015	11/09/2015	Sep-15	6	75	Most of these are over 2 days. The rest are sporadic
735	28/07/2015	29/07/2015	10/08/2015	Sep-15	14	35	4-5 a day, spread out
1060	28/07/2015	28/07/2015	13/08/2015	Sep-15	17	35	4-5 a day, spread out
1572	28/07/2015	28/07/2015	06/08/2015	Sep-15	10	39	16 on one day, the rest are sporadic and spread out
1456	28/07/2015	28/07/2015	05/08/2015	Sep-15	9	17	2-3 per day, spread out
1526	28/07/2015	28/07/2015	06/08/2015	Sep-15	10	67	Most days 10 + points. Every hour. Others sporadic, few points per day
1086	28/07/2015	28/07/2015	01/08/2015	Sep-15	5	10	Most 2-3 points, spread out a day
1064	28/07/2015	28/07/2015	04/08/2015	Sep-15	8	14	Few a day, sporadic
101562	28/07/2015	28/07/2015	08/08/2015	Sep-15	12	105	Mainly shared between 3 days, 46 on one day, sometimes taking a point each minute, then nothing for hours. Few sporadic points on other days
1071	28/07/2015	29/07/2015	10/08/2015	Sep-15	14	80	10-20 on 3 days, others sporadic few per day
1067	28/07/2015	28/07/2015	03/08/2015	Sep-15	7	14	3-5 a day, sporadic
815	28/07/2015	28/07/2015	15/08/2015	Sep-15	19	108	Approximately 20 on four days, almost every hour. Rest have a few a day, sporadic
1561	28/07/2015	28/07/2015	02/08/2015	Sep-15	6	19	10 on one day, almost hourly. Rest have a couple a day, sporadic
868	28/07/2015	28/07/2015	20/08/2015	Sep-15	24	62	4-5 points a day, sporadic all times
1091	28/07/2015	28/07/2015	04/08/2015	Sep-15	8	17	3-4 a day, sporadic, all times
1054	28/07/2015	28/07/2015	04/08/2015	Sep-15	8	15	3-4 a day, sporadic
977	18/04/2016	15/05/2016	16/05/2016	Jul-16	2	7	3 one day 4 other. Data points spread through the days
1463	18/04/2016	04/05/2016	04/05/2016	Jul-16	1	11	Collar stared recording in May. 11 points almost hourly.
865	18/04/2016	22/04/2016	23/04/1916	Jul-16	2	31	20 one day, 11 the other, range of time, spread out
750	18/04/2016	26/04/2016	27/04/1916	Jul-16	2	39	33 one day, 6 the next. Time clustered, then gap
716	18/04/2016	21/04/2016	28/04/2016	Jul-16	8	458	Hourly for first 6 days, then 242 points by the minute then 79 points next day

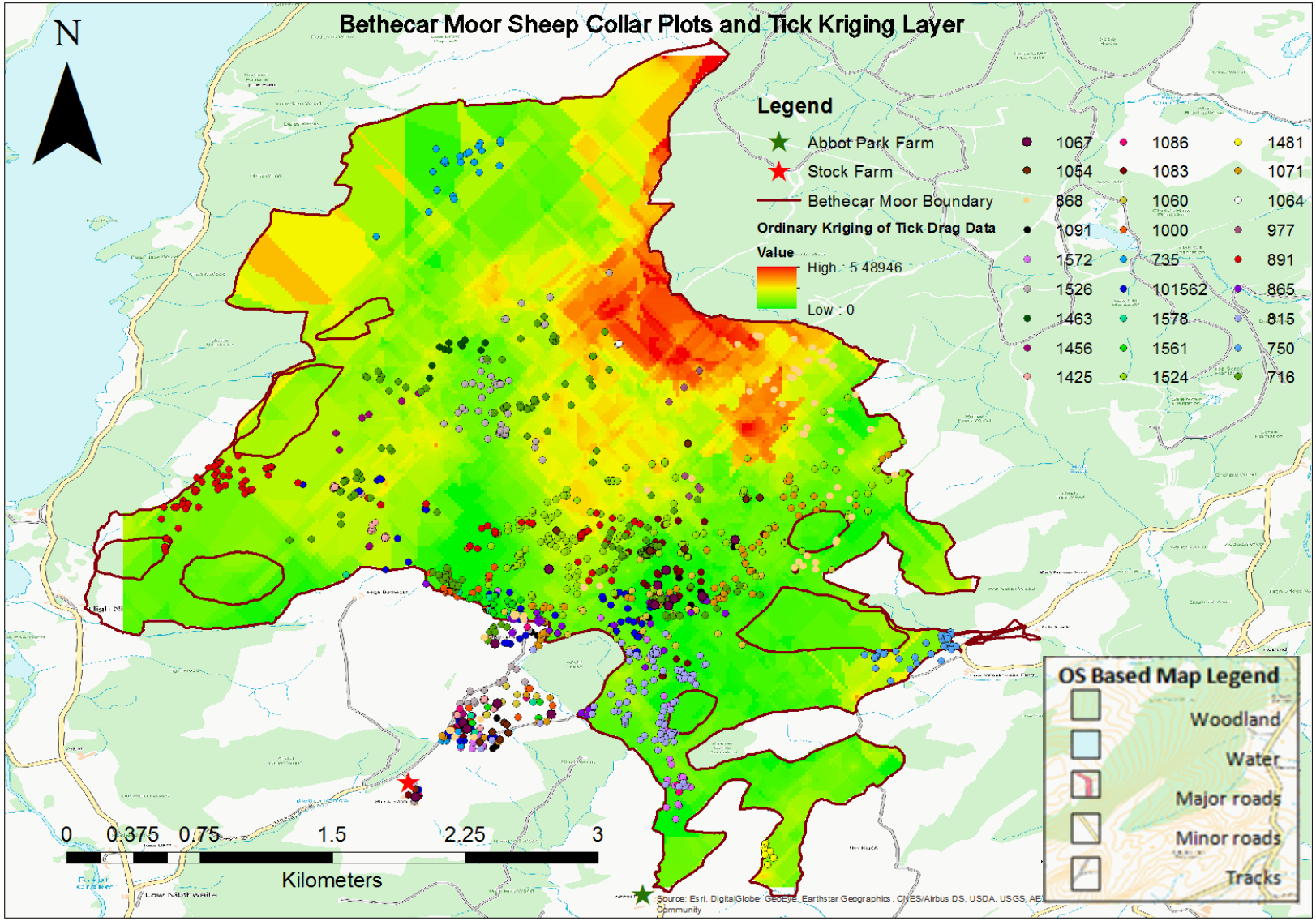

Figure 27 Map showing GPS sheep collar data and tick distribution across Bethecar Moor

3.3.5 Sheep Sampling

3.3.5.1 Stock Farm

Sheep were sampled for ticks a total of nine times between September 2014 and September 2016. During the two year sampling period, a total of 769 ticks were collected from 290 animals. Ticks were observed on 145 of these. 238 different sheep were sampled, of which 47 were resampled; 40 twice and 7 three times.

A histogram of tick count per sheep shows that there was a large positive skew in the data, due to lots of sheep with zero ticks (Figure 28). There were few individuals with many ticks, with the highest tick burden recorded as 36.

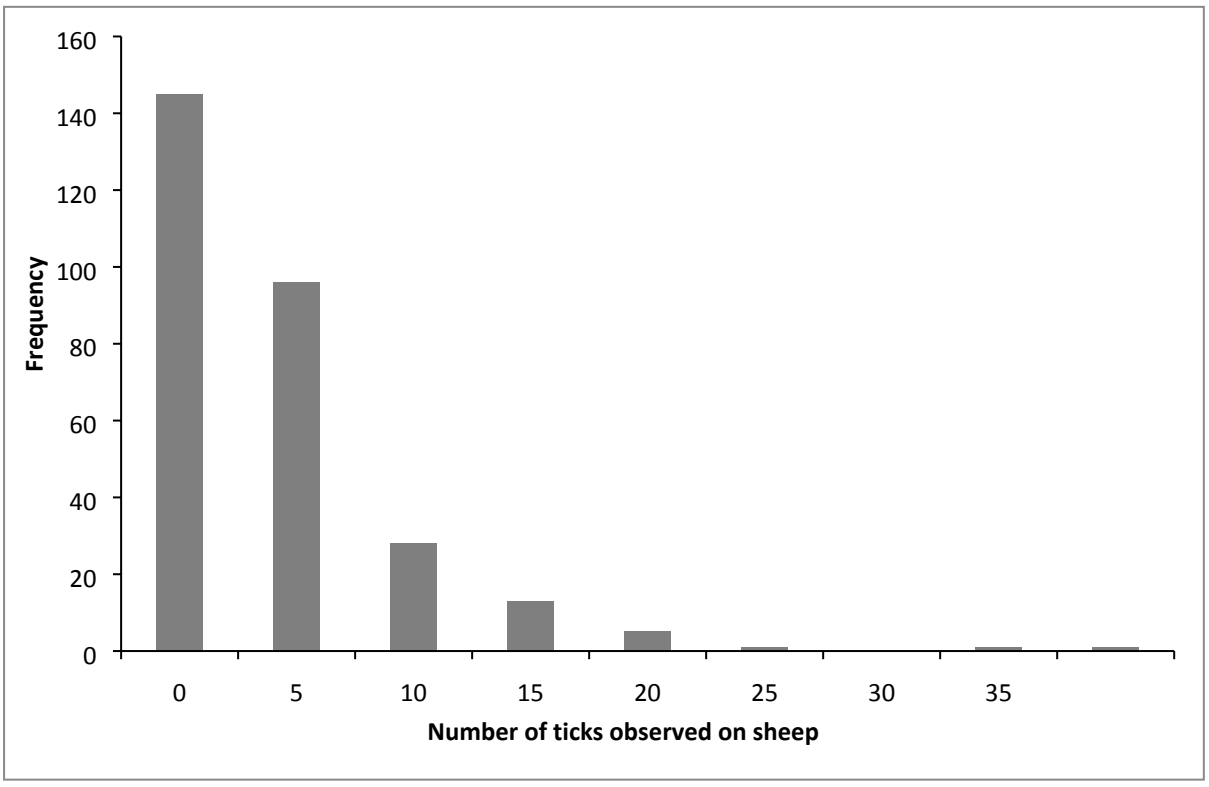

Figure 28 Histogram showing number of ticks taken off sheep at Stock Farm

There was large variation observed in the number of ticks collected at each visit (Table 8). Interestingly, no ticks were observed on sheep surveyed in May and July 2016.

Table 8 showing number of ticks observed on sheep during visits to Stock Farm. September 2014- September 2016.

	No. of sheep sampled				Ticks			
Sample date	With ticks	Without ticks	**Total**	**% of sheep infested**	Nymphs	Adult male	Adult female	**Total**
10/09/2014	47	1	**48**	97.92	1	54	357	**412**
10/11/2014	11	13	**24**	45.83	0	2	21	**23**
23/07/2015	8	63	**71**	11.27	0	0	11	**11**
11/09/2015	27	20	**47**	57.45	0	1	202	**203**
09/11/2015	25	13	**38**	65.79	0	1	87	**88**
09/05/2016	0	12	**12**	0.00	0	0	0	**0**
25/07/2016	0	21	**21**	0.00	0	0	0	**0**
12/09/2016	9	20	**29**	31.03	1	2	29	**32**
Total	**127**	**163**	**290**	**43.94**	**2**	**60**	**707**	**769**

In the sessions that ticks were observed on the sheep, there was marked variation between individuals, with many having 0 ticks (Table 8). The highest proportion of sheep infested with ticks was observed in September 2014, with 97.92% of animals infested.

3.3.5.2 Abbot Park Farm

Sheep were sampled for ticks a total of ten times between July 2015 and September 2016. During the sampling period, a total of 373 ticks were collected from 511 animals inspected. Ticks were observed on 208 of these (Table 9). 275 different sheep were sampled, of which 125 were sampled more than once; the highest number of times an individual sheep was sampled was 6.

A histogram of tick count per sheep shows that there was a large positive skew in the data, due to lots of sheep with zero ticks (Figure 29). The highest tick burden on one individual was nine.

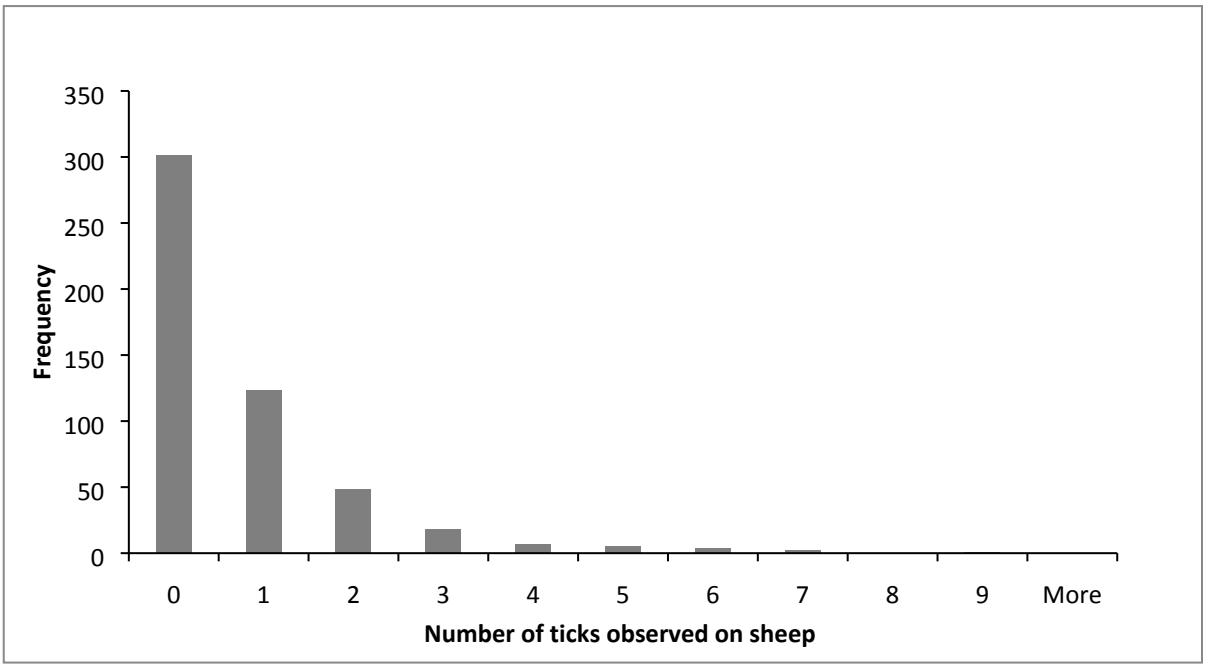

Figure 29 Histogram showing the number of ticks observed on sheep at Abbot Park Farm

There was large variation observed in the number of ticks collected at each visit (Table 9). Ewes could not be turned between the months of November and March due to lambing. The maximum number of ticks from a visit was 81, the minimum was four. There was no sampling period where zero ticks were observed.

Table 9 showing number of ticks observed on sheep during visits to Abbot Park Farm. July 2015- September 2016

	No. of sheep sampled				Ticks			
Sample date	With ticks	Without ticks	Total	% of sheep infested	Nymphs	Adult male	Adult female	Total
28/07/2015	17	37	54	31.48	1	1	25	27
20/08/2015	33	31	64	51.56	3	5	47	55
02/10/2015	43	24	67	64.18	0	0	76	76
30/10/2015	21	31	52	40.38	2	0	24	26
23/03/2016	27	26	53	50.94	6	5	54	65
27/04/2016	29	26	55	52.73	5	3	35	43
27/05/2016	6	33	39	15.38	0	0	6	6
08/07/2016	4	40	44	9.09	0	0	4	4
18/08/2016	24	26	50	48.00	4	9	47	60
22/09/2016	4	29	33	12.12	1	0	10	11
Total	208	303	511	40.7045	22	23	326	373

Looking at the sampling months individually, in the months that ticks were observed on the sheep, there is variation between individuals, with many having 0 ticks (Table 9). The highest proportion of sheep infested with ticks was observed in September 2015, with 64.20%. The lowest was 9.10%. July 2016, within two weeks of acaricide application at the end of June 2016.

3.3.6 Comparing data

To explore the drivers behind tick infestations on sheep, data from both farms were combined. As expected, a histogram of sheep tick counts showed that there was a large positive skew in the data, due to many sheep with zero ticks (Figure 30). The data were analysed to determine which distribution model fitted the data best. Comparative analysis between negative binomial and Poisson distribution confirmed that the tick count data was displaying a negative binomial distribution, with the theoretical negative binomial values in green, aligning much more closely with the empirical values in black (Figure 31).

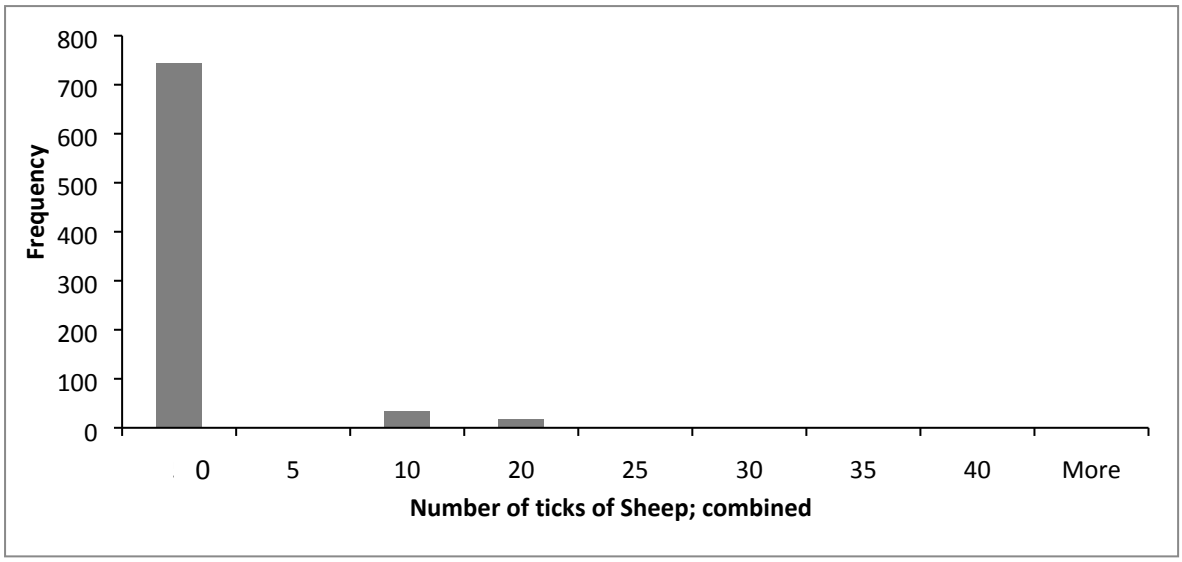

Figure 30 Histogram showing number of ticks taken off sheep and Stock and Abbot Park Farm

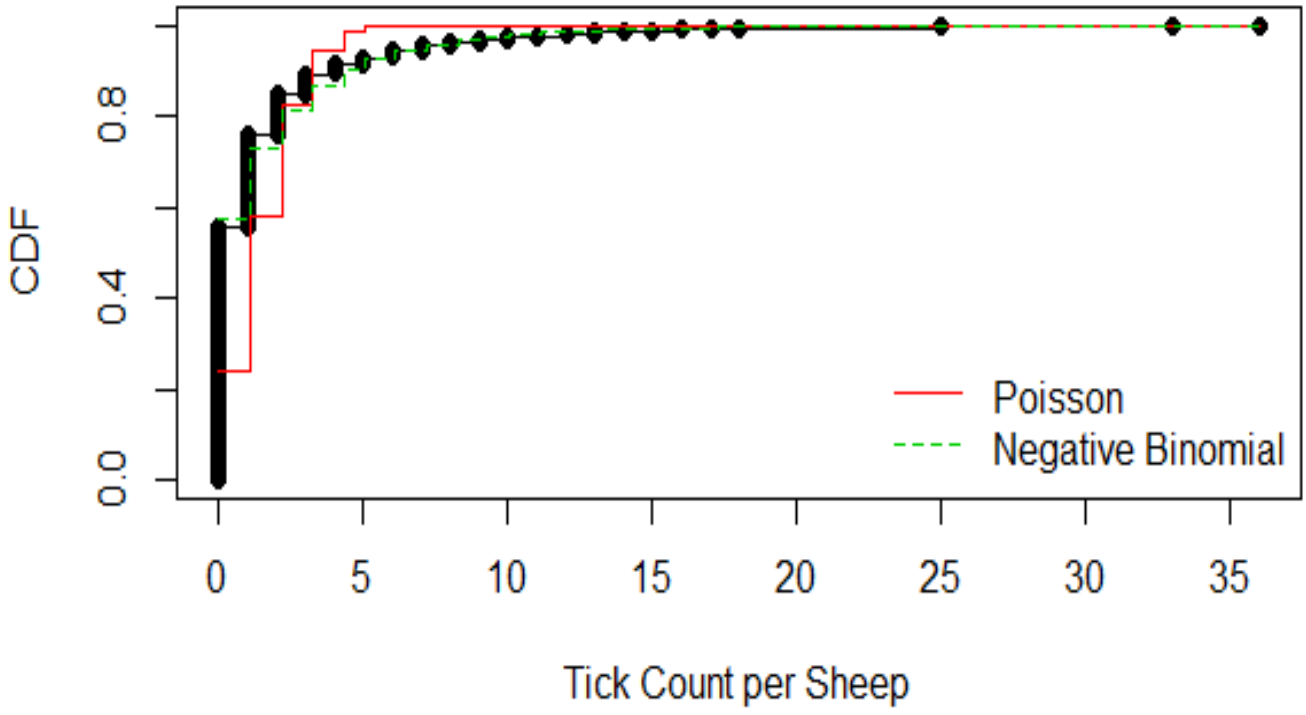

Figure 31 graph comparing Poisson and negative binomial distribution fits of tick infestation of sheep data, using empirical and theoretical Cumulative Distribution Functions (CDFs). The theoretical negative binomial values in green, align much more closely with the empirical values in black, than theoretical Poisson figures in red.

3.3.7 Fitting the model

Once a negative binomial distribution was confirmed, the potential explanatory variables were tested in a Generalized Linear Model (GLM). Initially, variables considered in the model were: site, number of months since last acaricide application, sampling season, the number of days spent in by-land prior to sampling and age group. Age groups were categorised as: 1= lamb, 2= shearling, 3= 2-4 years old, and 4= 5+. Running the model with all factors demonstrated that Stock Farm and the number of days spent on by-land prior to sampling were significant when predicting tick infestations on sheep. As the sheep that had been grazing on the by-land immediately before sampling had not been exposed to the same environmental hazard as other group, they were removed from the dataset, as they were effectively providing false negative results.

Table 10 tick counts on sheep. Negative binomial GLM outputs, of factors associated with *I. ricinus* abundance. All variables included. Intercept is Abbot Park

	Estimate	Std. Error	z value	P
Tick counts on sheep				
Intercept	-0.59041	0.28326	-2.084	0.0371
Age group	0.08013	0.07988	1.003	0.3158
Spring	-0.40250	0.17745	-2.268	0.0233
Summer	-0.32346	0.15640	-2.068	0.0386
By-land	-1.26268	0.10899	-11.585	**<2e-16**
Acaricide use	0.11703	0.01977	5.921	**3.21e-09**
Stock Farm	1.01847	0.18887	5.393	**6.95e-08**

Following this, the model was run again. To determine the model of best fit; age group and season were eliminated from the model during the backwards and forwards step-wise procedures due to P > 0.1. As such, only site and months since last acaricide application remained in the model. Stock Farm and acaricide use were considered as significant in predicting tick abundance (P < 0.001) (fig xx).

Table 11 tick counts on sheep. Negative binomial GLM outputs, factors are months since last acaricide application and site. Intercept is Abbot Park

	Estimate	Std. Error	z value	P
Tick counts on sheep				
Intercept	-0.39487	0.07166	-5.511	**3.58e-08**
Acaricide use	0.08719	0.02144	4.067	**4.75e-05**
Stock Farm	1.26148	0.19839	6.359	**2.04e-10**

3.4 Discussion

Both farmers stated that they believed there to be 'dirty' areas of the Moor that were infested with ticks, and 'clean', with no ticks; but that these areas would change over time. Generally, the farmers believed the presence of bracken was the driver behind tick distribution across the fell. Both farmers encouraged bracken control, and the spraying of the herbicide Asulox, across Bethecar Moor to mitigate the threat posed by *I. ricinus* and the pathogens they carry; in addition to acaricide use and in the case of farmer 2, Louping Ill vaccinations. Despite their efforts, both farmers frequently observe tick infestations on their flock along with occasional incidences of tick borne diseases, such as tick borne fever and Louping Ill during periods of vaccine shortage.

Blanket dragging across the fell demonstrated that *I. ricinus* ticks were indeed present on Bethecar Moor. The production of a tick 'heat-map' from this empirical drag data using ordinary kriging (Figure 21) revealed an uneven distribution of ticks; with a relatively densely populated red area following the boundary shared with the bottom of Grizedale Forest, and yellow coloured mid-density patches across the fell. There were also large areas of Bethecar Moor on which no ticks were found, represented in green. Although the choloropleth map confirms a patchy tick distribution of where ticks are most likely to be found, it is also important from an epidemiological and control aspect to know and understand why tick densities vary spatially.

In 1946, Milne stated that sheep were the main agent in spreading ticks. Preliminary GPS collar data and discussions with the farmers regarding the grazing patterns of their flocks confirmed sheep were grazing across Bethecar Moor; only areas with bare rock or unsuitable grazing were avoided. It is clear from the results that ticks are not uniformly distributed across the fell and sheep are therefore unlikely to be driving the patchy distribution. If sheep were the main agent, a universal cover of ticks would be observed as ticks drop randomly off their freely roaming host, and move no more than a few inches themselves (Milne, 1946). As the obtained GPS collar data was not as successful as hoped, it has been difficult to draw any clear conclusions regarding sheep movement across the Moor. As sheep are known to act as ‘tick-mops’ (Reid, 1975), any area where they spend a significant amount of time (E.g. sleeping, grazing) may be found to harbour more ticks. Farmer 2 had commented that he believed family groups within his flock to graze closer together; if this is happening, rather than solitary grazing, then foci of sheep could be identified across the fell (though they may move over time dependant of grazing availability) which may also create a foci of ticks which fall off once replete. This could be studying with a more comprehensive GPS collar data set, the use of drones, or sheep watching on the Moor.

A caveat to this would be the consideration of suitable habitat and vegetation for tick survival; they could be driven by the sheep, fall randomly while replete, but fail to establish a population due to unfavourable conditions. Initial observations of Bethecar Moor indicated that much of the landscape could provide a suitable tick habitat: with wet ground, long grasses, bracken, heather and thick vegetation matt observed. To begin to quantify this, NDVI images and pixel values were obtained and extracted, to be tested as a potential driver for tick density within a GLM.

The model shows single driver for tick density across Bethecar Moor was confirmed as Grizedale Forest being the nearest woodland to the drag point, with a strong positive correlation (0.869, $P<0.001$). None of the other bordering woodlands were demonstrated to be having an effect on tick abundance. Grizedale Forest contains both conifers and native deciduous trees, similar to many woodland sites surrounding Bethecar Moor, with the exception of Arklid and Perlings, which consist of managed coniferous trees. These results are not in keeping with previous studies which stated that solely proximity to woodland increases questing tick density (Gilbert, 2016). However, results from the GLM did not indicate NDVI as a driver for tick distribution. This was also the case for slope, relative deer density, or proximity to woodland. Previous studies (Gilbert, 2017) have demonstrated that proximity to woodland is important when considering *I. ricinus* distribution, with woodland encroachment on agricultural land suggested as increasing questing tick abundance and potentially exposure (Gilbert;2013;2016;2017). As there were many areas of woodland bordering the fell, it is likely that the model had trouble trying to fit to such fine scale data.

Though the GLM did not indicate relative deer density as a driver for tick abundance, the observed patchy distribution could still be impacted by resident deer populations in the surrounding woodland; as their presence, rather than abundance has recently been implicated in explaining tick densities (Hofmeester et al., 2017).

Deer have long since been implicated as essential for maintaining tick populations (Wilson et al., 1988; Jaenson et al, 1992), and can carry hundreds of ticks at any one time (Carpi et al., 2008);(Vor et al., 2010). There is a known resident deer population in Grizedale Forest, which is managed and culled by the FC. The data collated from the farmers and FC rangers, informed the deer movement chloropleth map (Figure 22) and indicated a deer population moving from the south of Grizedale forest towards Arklid Intake. Statements obtained from the FC rangers confirmed the presence of large resident roe and red deer populations in the forest. The deer which are stalked and culled use the fell as a corridor to access other surrounding woodland. Given that they are a prey animal, it is feasible that they are spending more time grazing closer to the woodland edge, specifically Grizedale. The spatial autocorrelation of clustered data accounted for by the kriging maps may have

been missed by the linear model. Further spatial analysis, comparing the map layers by geographically weighted regression could further elucidate this and potentially implicate deer as a driver for tick densities.

Due to time and financial resource restraints, it was not feasible to repeat this survey within the timescales of this project. If it were possible, it would be prudent to resample each of the drag points over time and several tick seasons. As shortcomings with blanket dragging, and without repeating on a three weekly basis, have been documented (Dobson, 2013)

This would enable the observation of spatial fluctuations in the tick population over time and, more importantly, if the areas of medium tick density (indicated in yellow) were maintained. This could also inform control strategies, such as livestock grazing areas and application of tick control agents in the environment.

As the vegetation and habitat appear suitable, host availability may be the primary driver for tick survival. The role of small animals such as birds and rodents in this system should also be explored as both food sources for the immature life stages of the tick and potential reservoirs for disease.

The tick surveys conducted on sheep demonstrated varying levels of tick infestation on sheep at the same farm, in keeping with previous studies (Ogden et al., 2002, Ladbury et al., 2008). In addition, there was a substantial number of sheep at any sampling point that were not infested with ticks. These stark variations in tick infestation were also demonstrated at site level, with sampling at Stock Farm in May and June 2016 yielding 0 infested sheep. The average tick burden combining the data from both farms was 1.89 ticks per sheep; which is much lower than would be expected to be observed on deer that were crossing Bethecar moor (Vor et al, 2010). This study recorded an average of 65 *Ixodes* ticks per deer, though there was still considerable variation in tick burden. Unpublished data following communications with colleagues, observed tick counts over 5,000 per deer in North East England. However, the number of sheep with access to the Moor as higher than the number of deer (900 from 3 farms, compared to an estimated 150 from FC rangers) and they spend more time on the Moor. Rough estimates taken by averages of husbandry for sheep and perceived time deer spend on the Moor from FC rangers, demonstrated values of 0.69 (250 full days out of 365) and 0.25 (91.25 full days of 365, from 8 hours a day for 273 days). Multiplying these numbers by populations (900 and 150 for sheep and deer respectively) and again by average tick burden (1.89 and 65) give index values of 1173.7 for sheep and 2437.5 for deer, providing a rough index for perceived importance of each host in carrying ticks. Indicating that deer are more important in terms of drivers of tick density across the fell than sheep within this system.

The observed difference in infestation between individual sheep from the same flock, breed and subsequently husbandry system, suggests tick burden could be a result of where individual sheep graze on the moor, their exposure rate, or due to some intrinsic susceptibility. This could be as a result of underlying illness, co-infection, or even breed resulting in an increase in susceptibility to tick bites and/or the pathogens carried.

To explore the variation in tick count per sheep, with a GLM was adjusted with potential explanatory variables; age class, last acaricide application, farm, season and time spent in by-land. It has been established in previous literature that ticks will only spend a few days at a time on a host (Milne 1943; Medlock et al, 2013). Sampling Farmer 2's sheep required us to conform research methods to occasional weather-dependent husbandry practices. As such, there were instances when we could only sample the sheep after they had spent between 1 and 3 days within the grazed pasture by-land, which is an unsuitable habitat to sustain a tick population. These sheep had not been exposed to the same environmental hazard (i.e. Bethecar Moor) immediately prior to sampling. Though they comprised 19% of the sample size, the cohort was not comparable to other individuals who had grazed the Moor immediately prior to sampling, and so were removed from the GLM.

Time spent in by-land was unsurprisingly shown to be the most significant predictor of tick burden on sheep (-1.26, $P<0.001$). Once these individuals were removed from the model, time since last acaricide application and Stock Farm were determined to be variables influencing tick burden on sheep.

Adjusting the model for site represented different breeds of sheep; Swaledales at Stock Farm compared to Texel's at Abbot Park, as well as the different husbandry employed by each farmer. Acaricide use was used differently at each farm, with Stock farm applying flock wide just once per annum in the September, compared to triannual flock wide application (March, June and September) at Abbot Park.

Previous studies have demonstrated breed-association resistance to tick infestation in cattle (Wambura et al., 1998) and sheep genetics have recently been implicated in tick burden in Norway (Sae-Lim et al., 2017). Whilst breed could be a factor in determining tick infestation in this study, it is unlikely to be the overriding cause, with differing husbandry and, therefore, acaricide use likely to be driving tick burden, along with exposure rate. No GPS collars were attached to any of the flock at Abbot Park. However, testimony of Farmer 3 is that the flock spend most of their time on the south east of Bethecar Moor. Their movements could also account for the lower tick infestation as they would be less likely to graze in the most densely tick populated areas of the fell.

Chapter 4: Molecular Detection & Characterisation of Tick Borne Pathogens in *Ixodes ricinus* and wildlife

4.1 Introduction

In addition to exploring tick distribution, the study aimed to determine the presence and diversity of tick-borne pathogens of (primarily) veterinary importance across southern Cumbria. This aim was achieved by survey of questing and feeding ticks and of blood collected from culled deer. Survey of sheep bloods was beyond the capacity of this study as it would have required Home Office licensing, for which the University of Salford is not currently able to apply.

Recent years have seen a growing scientific interest in bacteria of the genus *Anaplasma*, as a result of increased recognition of their pathogenic potential towards livestock, companion animals and humans (Ladbury et al., 2008, Stuen, 2007). Members of the genus are widely distributed across the northern hemisphere, but *A. phagocytophilum* is the only pathogenic member of the genus that is endemic in the UK (Heyman et al., 2010). With the pathogen causing severe pathology in an estimated 300,000 lambs in Norway annually (Stuen et al., 1998) the bacteria can have a severe economic impact on the farming industry across Europe. It has also been well documented in the UK, where an estimated 300,000 lams suffer tick pyaemia annually in the UK uplands (Brodie et al., 1986).

The detection of *A. phagocytophilum* DNA in a wide range of mammals and *Ixodes* species from across the globe suggest that it is a generalist parasite with the potential to exploit multiple hosts and vectors. However, there is increasing evidence that the species may, in fact, comprise of sub-populations adapted to specific hosts and/or vectors. In the USA, *A. phagocytophilum* genotypes solely associated with infections in deer have been reported (Massung et al., 2002, Massung et al., 2003).

Whereas in the UK, *A. phagocytophilum* genotypes solely associated with the rodent-specific tick *Ixodes trianguliceps* have been described (Bown et al., 2009). More recent studies in Europe have demonstrated four distinct ecotypes existing in a range of host species, without geographic clustering (Jahfari et al., 2014). These observations also carry some public health relevance as, for example, only sub-populations adapted to *I. ricinus* are likely to infect humans.

The role of sheep in the natural maintenance of *A. phagocytophilum* has been studied in extensively, and it is clear that in some circumstances they serve as its main reservoir. One such study in 2002 established that *A. phagocytophilum* was being maintained solely within flocks of fell-grazing sheep, in the absence of deer or rodents as other hosts (Ogden et al., 2002). The real epidemiological implications of *A. phagocytophilum* diversity have yet to be fully elucidated (Massung et al., 2002). This has been further compounded by the discovery of multiple strains circulating simultaneously within flocks (Ladbury et al., 2008) and wildlife (Bown et al., 2009). Strains associated and observed circulating in red deer populations have previously been implicated in causing pathology in livestock in a clinical setting, implying their status as reservoirs for livestock disease.

The Louping Ill virus (LIV) can also cause large losses to the farming industry, a debilitating disease with a vaccine which is not always readily available (Scotland-Vaccine shortage for hill famers (2009, April) www.farminguk.com/news/Scotland-Vaccine-shortage-for-hill-farmers._15308.html) (Balsom, A (2013 April 24) www.fwi.co.uk/livestock/hill-farmers-face-louping-ill-vaccine-shortage.htm). In addition to this, pathology caused by *A. phagocytophilum* in livestock can be compounded significantly with the co-infection of LIV which resulted in 100% mortality in a clinical setting (Reid et al, 1986)

Various *Babesia* species are also associated with livestock infections in the UK, and they too are transmitted by *I. ricinus*. While cattle are susceptible to *A. phagocytophilum,* the threat posed by *Babesia divergens* is much greater, as a much more severe pathology is displayed. *B. divergens* is the main agent of red water fever (RWF) in cattle in Europe with common symptoms including fever, fatigue, chills, anaemia, haemoglobinuria and possibly eventual death. It can cause severe and potentially fatal disease in immunocompromised humans (Hildebrandt et al., 2013). Deer are also known to act as reservoirs for various *Babesisa* species, including *B. venatorum* and *B. capreoli,* which have both been implicated as a potential emerging zoonosis (Herwaldt et al., 2003, Bos et al., 2017).

Recent research has demonstrated the presence of *B. divergens* in Red Deer (*Cervus elaphus)* (Michel et al., 2014). This could become of increasing public health and veterinary relevance with speculated increases in interactions between livestock and wildlife (deer) following proposed changes in land management, increases in deer populations, and decreasing stocks in uphill farming.

This part of the study aimed to explore the epidemiologies of infections caused by *A. phagocytophilum,* LIV and *Babesia spp.* across the study area that embraced the three farms described in earlier Chapters. Infections were sought in questing *I. ricinus* ticks, in ticks feeding in livestock (as proxies for livestock themselves) and in deer. There has been a nationwide increase in the deer population, potentially leading to increased interactions between wildlife and livestock, resulting in shift in infectious disease landscape.

4.2 Sample collection and processing

4.2.1 Ticks from the environment

Areas for tick sampling were Bowkerstead Farm and Bethecar Moor. Ticks were collected via blanket dragging as previously described in Chapters 2.

4.2.2 Ticks from sheep

Sheep were rounded up in line with participating farmers' husbandry methods and a random selection was corralled for inspection. Each sheep was turned over individually under supervision of the farmer or an experienced farm hand. The sheep was then inspected for the presence of ticks for 2 minutes, with particular attention paid to the parts of the body lacking wool (armpits, groin, legs, ears, neck and face). All ticks encountered were removed into 70% ethanol using a silicone tick remover.

In the event of high tick burden, sampling continued until all visible ticks were removed, this took no longer than 4 minutes. Tick location, sheep identification number, breed and age were also recorded on sampling sheets in the field.

Identification of all ticks collected was verified by reference to taxonomic keys (Hillyard 2006).

4.2.3 Blood samples from deer

Much of the land bordering the study farms is managed by the Forestry Commission for wood production or recreation. Sizable populations of red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*), roam freely across the region such that they frequent, probably on a daily basis, the grazing land used by the study farms.

The populations of each deer species are regulated by the Forestry Commission to limit deer damage to young trees, with individual animals being culled by shooting throughout most of the year. Between November 2014 and August 2016, Forestry Commission rangers agreed to collect blood samples from just-shot deer for the study.

Approximately 5ml of blood were collected soon after death from the body cavity during "gralloching" (removal of the alimentary tract in the field to avoid faecal contamination of the carcass). These samples were dated, packed and, accompanied by a brief questionnaire, sent to the laboratory by first class post. Samples were only collected on a Monday, Tuesday or Wednesday to ensure they were received by the laboratory within 48 hours of collection.

4.2.4 Extraction Techniques

4.2.4.1 DNA Extraction from ticks

Crude nucleic acid extracts were prepared from individual *I. ricinus* as previously described (Kurtenbach et al, 1998). Simply, ticks were removed from ethanol and placed into a 1.7ml sure-lock Eppendorf tube. Each tick was ground using a sterile pipette tip.

Once ground, ticks were immersed in 1.25% ammonium hydroxide (500µl per adult, 100µl per nymph) and placed on a heating block at 100°C for 15 minutes, lids closed. After which time, tubes were briefly centrifuged then placed back on the heating block, lids open, until 50% of the contents had evaporated. These crude DNA extracts were stored frozen at -20°C until required. In order to control for cross-contamination between samples, a "blank" (tube containing ammonium hydroxide but no tick) was co-processed with every five tick samples.

Though DNA extraction via other methods such as phenol chloroform and spin column kits have been perceived as more efficient and ammonium hydroxide, these methods were outside of the budget for this project given the total number of samples that were tested. Further to this, unpublished data (personal communications from Bown) demonstrated similar efficiencies when comparing extraction methods, which has been documented in published literature (Ammazzalorso et al, 2015). In addition, the use of this extraction method with ticks is well documented (Øines et al, 2012; Alekseev et al, 2001)

4.2.4.2 DNA Extraction from Blood

Crude DNA extracts were prepared from deer bloods using the protocol described above for ticks. A 50µl aliquot of blood was mixed with 500µl ammonium hydroxide. These crude extracts were diluted 1:4 before PCR. Blood is a know inhibitor for PCR, though unpublished data

(personal communications with Bown) demonstrated the efficacy of this method on blood samples, as long as extracts were diluted with PCR water, reducing the likelihood of false negatives.

4.2.4.3 RNA Extraction from Ticks

Simply, the crude tick homogenate was centrifuged at 13,000 x g for 5 minutes and the clear supernatant was transferred to a clean 1.5ml microcentrifuge tube with care taken not to transfer any of the fatty, upper layer. One volume of 70% ethanol was added to the supernatant, vortexed thoroughly, and then transferred to the HiBind® RNA Mini Column, care taken to not touch the column with the pipette tip. The column was centrifuged at 10,000 x *g* for 1 minute with filtrate discarded and collection tube reused to repeat until all supernatant was used. The column was then washed with 500µl Wash Buffer I, centrifuged for 30 seconds, flow through discarded and process repeated twice with 500µl Wash Buffer II, centrifuged for 1 minute.

Once column was completely dried, 70µl was added to the column for elution and centrifuged at 13,000 x g for 2 minutes. Presence of RNA was confirmed via Nano Drop and all samples were stored at -80°C until further processing.

4.2.5 Sampling for Pathogens

4.2.5.1 *Anaplasma phagocytophilum*

A previously described real-time PCR (q-PCR) (Courtney et al., 2004) was used, with modification as per (Bown et al 2007) to screen ticks for *A. phagocytophilum*, using primers targeting an *msp2* fragment (Table 1) of a conserved, multi-copy gene. This assay was used as it has been documented as comparable in terms of sensitivity to previously published nested PCRs (Courtney et al., 2004; Massung et al., Massung et al 1998). The assay has the ability to detect one-eighth of an infected cell, with efficiences similiar across *A. phagocytophilum* strains, with background DNA's having no significant effect on the sensitivity or specitivity of the assay, further reducing the chance of false negatives occuring.

Each reaction mix contained 10µl of 2x MyTaq Readymix, (supplied by Applied Bioline reagents Ltd), 1µl 10 ρmol/µl of the forward primer, 1µl 10 ρmol/µl of the reverse primer, 1µl 3.3 ρmol/µl fluorescent probe,-labelled at the 5' and 3' ends with 6-carboxy-fluorescein (6-FAM) and 6-carboxyl-tetramethyl-rhomadine (TAMRA), respectively, 5µl of water and 2µl of template DNA. Reaction

mixes were subjected to a thermal programme consisting of an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing-extension step at 60°C for 60 seconds. The fluorescence intensity of the mix was captured at the end of the extension step. All results were visualised via Opticon Monitor software.

All positive samples were further analysed using a previously published semi-nested *msp4* PCR (table 12), (Bown et al., 2009) to allow delineation of genotypes within the species.

For the first round, each reaction contained 10µl of 2x Red taq Ready mix; 1µl desired forward primer, 1µl respective reverse primer (both at 10 ρmol/µl), 6µl of water plus 2µl of template DNA. The assay involved an initial 3 minutes step at 95°C for, then 35 cycles of 95°C for 10 seconds, 58°C for 10 seconds, and 72°C for 50 seconds, followed by a final extension step of 72°C for 5 minutes. Cycling conditions for the second round were the same, though just 1µl of first round template was used, increasing the amount of water to 7µl.

Table 12 Primers used for amplification of *msp2* and *msp4* fragments and generic apicomplexan primers. Y = T or C, N = A, C, G or T.

Primer	Sequence
***msp2* F**	5'- ATG GAA GGT AGT GTT GGT TAT GGT ATT -3'
***msp2* R**	5'- TTG GTC TTG AAG CGC TCG TA -3'
***msp2* FAM**	(FAM)5'- TGG TGC CAG GGT TGA GCT TGA GAT TG -3'(TAMRA)
***msp4* F**	5'- ATG AAT TAC AGA GAA TTG CTT GTA GG -3'
***msp4* R**	5'- TTA ATT GAA AGC AAA TCT TGC TCC TAT G -3'
***msp4nov* F**	5'- CTA TTG GYG GNG CYA GAG T-3'
***msp4nov* R**	5'- GTT CAT CGA AAA TTC CGT GGT A -3'
BmF1	5'- GCG ATG TAT CAT TCA AGT TTC TG -3'
BmR1	5'- TGT TAT TGC CTT ACA CTT CCT TGC -3'
BmF2	5'- ACG GCT ACC ACA TCT AAG GAA GGC -3'
BmR2	5'- TCT CTC AAG GTG CTG AAG GA-3'

4.2.5.2 *Babesia spp.*

A nested PCR assay, generic for Apicomplexa, was used to screen nucleic acid extracts for the presence of Babesia DNA (Simpson et al., 2005) (Table 12).

For the first round, each reaction mix contained 10µl of 2x Red taq Ready mix, 1µl BmF1 forward primer at 10ρmol/µl, 1µl BmR1 reverse primer at 10ρmol/µl, 6µl of water plus 2µl template DNA.

Reaction mixes were subjected to a thermal programme that consisted of an initial denaturation step at 96°C for 5 minutes, then 35 cycles of denaturation at 96°C for 10 seconds, annealing 55°C for 10 seconds, and extension at 72°C for 50 seconds, followed by a final extension step of 72°C for 5 minutes.

Cycling conditions for the second round were the same, with 1µl of both BmF2 and BmR2 primers at 10ρmol/µl each and just 1µl of first round template was used, increasing the amount of water to 7µl. PCR products of approximately 600 base pairs (bp) were visualised using 1% agarose gel, as described below.

4.2.5.3 Louping Ill Virus

Testing for LIV was carried out by collaborators at The Animal Plant and Health Agency (APHA), Weybridge. The assay used was a one-step reverse transcriptase PCR, as previously described in (Marriott et al., 2006).

4.2.6 Agarose Gel Electrophoresis

PCR products were visualised following their electrophoretic resolution on 1% (w/v) agarose gel. 2.5g of agarose powder was added to 250ml of 10% Tris-Borate-EDTA (TBE) buffer (Severn Biotech Ltd) and heated until the agarose was completely dissolved. Once the molten agar had cooled to approximately 50°C, 250µl of GelRed (Cambridge Bioscience) was added. The agarose was poured into a gel tray with combs inserted producing wells, and then allowed to set for 60 minutes at room temperature. The solid gel was placed in an electrophoresis chamber and flooded with TBE buffer (approximately 2l). Each well in the gel was loaded with 5µl PCR product then the gel was subjected to electrophoresis at 220V for 60 minutes. The presence of PCR products was determined by visualisation under UV light. The size of PCR products was estimated by reference to a 1kb hyperladder (Bioline), run on each gel.

4.2.7 DNA Purification & Sequencing

PCR products were purified for sequencing using an Invitrogen PureLink PCR Purification Kit (supplied by ThermoFisher Scientific Ltd) and following manufacturer's instructions. Simply, 25µl of PCR product was added to 100µl of PureLink Binding Buffer (B2) and vortexed.

The sample was then added to the PureLink Spin Column, with care taken not to touch the filter and centrifuged at 10,000 x *g* for 1 minute. In an amendment to the protocol, the flow through was added to the column and centrifuged for a second time, after which it was discarded. Following this,

650µl of Wash Buffer with ethanol was added to the column and centrifuged at 10,000 x *g* with flow through discarded. Residual Wash Buffer was removed by centrifuging for a further 3 minutes.

Once the column was dry, 50µl of Elution buffer was added to the column, which was placed in a clean PureLink Elution Tube and incubated at room temperature for one minute. The column was centrifuged at maximum speed for 2 minutes and the column discarded, with purified PCR products stored at -20°C until further use. To confirm purification, an aliquot of each sample was run on an agarose gel as described above. Subsequent to purification, both strands of each product were sequenced commercially using Sanger sequences (Source BioScience or Macrogen) using the relevant second round primers that were used for amplification.

4.2.8 Analysis of DNA sequence data

Sequence data were returned from the commercial sequencing services in the form of .ab1 files. These files, which contained chromatograms, were visualised using ChromasPro software (version 1.7.7 Technelysium Pty Ltd). Chromas Pro was used to align chromatograms obtained for each strand of a PCR product, and this alignment was used to assess the accuracy of base calling on each strand and amend if necessary and to identify then remove primer sequences from extremities. If an unambiguous consensus sequence for the PCR product could be obtained in this manner, the sequence data were stored as ChromasPro file prior to export for use in other software.

Initial analysis of sequences was carried out using the NCBI BLAST sequence searching tool within Chromas Pro to identify the sequences within GenBank that shared highest similarity with those generated in this study. Comparisons of generated sequences were carried out using NCBI BLAST and phylogenetic analysis was conducted using MEGA (version 6) (Tamura et al., 2013)

4.2.9 Alignments & Phylogeny Constructs

The MEGA (version 6) software suite (Tamura et al., 2013) was used to establish databases of relevant DNA sequence data, to create and edit alignments of sequences and to infer the phylogenetic positions of the organisms from which sequences were derived.

All sequence alignments were generated using ClustalW Multiple Sequence Alignment Software in MEGA 6. This was then used to visualise the alignments and ensure the alignment remained in frame. Following this, a Neighbour-Joining phylogenetic tree was constructed; this was run with bootstrap test using 5000 repetitions in the first instance. The nucleotide substitution model was Kimura 2- parameter model, Gamma distributed with Invariant sites (G+I). All other settings remained as default.

4.3 Results

4.3.1 Bowkerstead Farm

From the spatial surveys of Bowkerstead Farm described in chapter 2, a total of 329 ticks were collected from 436 X 30m blanket drags. Of these, 307 were nymphs, 16 were adult males and 6 were adult females. Of the 329 *I. ricinus* processed, *A. phagocytophilum* DNA was detected in 13 using the partial *msp2* qPCR (12 nymphs, 1 adult). Dragging results were split into three different habitat categories: woodland, rough pasture (area A), and low pasture (areas B and C). This demonstrated *A. phagoctyophilum* prevalvances of 1.7, 2.4 and 7.3% respectively (Table 13).

Kruskal Wallis indicated that there was a significant difference in the prevalence of *msp2* observed on drags in the three habitats (χ^2= 9.6424, df =2, P<0.01). Pairwise Wilcox test stated that there was no difference in prevalence of *msp2* observed on drags in rough and low pasture, but that there was a significant difference in the prevalence of *msp2* observed between drags in the woodland and drags in rough and low pasture (p<0.05).

Chi-squared also indicated that there was a significant difference in the prevalence of *msp2* observed in questing ticks collected from each habitat. With woodland prevalence being significantly lower (χ^2= 6.1238, df =2, p<0.05).

A visible *msp4* amplicon was obtained from 10 of these 13 ticks, and unambiguous *msp4* sequence data were obtained from 8. Comparative sequence analysis indicated 8 alleles among these 8 sequences. All alleles had been previously encountered in ruminants across Europe (Table 14).

A total of 2 of 329 ticks yielded an amplicon when tested with the *Apicomplexan*-specific nested PCR. Unambiguous partial 18S rDNA sequences were obtained for both of these samples. Comparative sequence analysis indicated 2 alleles among the 2 sequences, which displayed high sequence similarity (98%) with previously recorded strains of *Babesia odocoilei,* and *B. venatorum,* both of which are deer associated species (Figure 38).

Both of these pathogens have been plotted across the farm, at the site of the drags from which they were collected (Figure 32). The pathogens have been observed in the fields of the farm and in the surrounding woodland, both in what was considered by the farmer to be the 'ticky' and 'non-ticky' areas. The majority of the pathogens are generally closer to woodland, as opposed to the large area of grazed pasture in the north east of the farm.

Table 13 dragging results and *A. phagocytophilum* prevalence, by habitat at Bowkerstead Farm

	No. Drags				Ticks observed and *A. phagocytophilum* positives (%)			
Habitat	With ticks	Without ticks	Total	% of drags infested	Nymphs	Adult male	Adult female	Total (%)
Woodland	22	15	37	59.46	169(1.7)	5(0)	2(0)	1.70
Rough pasture	22	115	137	16.06	75(2.4)	6(0)	3(0)	2.4
Low pasture	37	236	273	13.55	63(5.8)	5(20)	1(0)	7.3

Table 14 *msp4* alleles observed in different habitats at Bowkerstead Farm

Habitat	No. of ticks *A.phagocytophilum* positive	No. of samples sequenced	No. of alleles observed	Comments
Woodland	3	2	2	All different. All previously encountered: KU712183 sika deer. EU180060 roe deer and *I.ricinus* in Europe
Rough pasture	5	5	5	All different. 4 previously encountered: KF420116 ricinus, EF442008 sheep, KU712150 red deer, sheep, mouflon, goat, *I. ricinus.* Across Europe. One sample was 99% similar to other alleles recorded in ruminants across Europe
Low pasture	6	1	1	Previously encountered: EU240485 in sheep UK

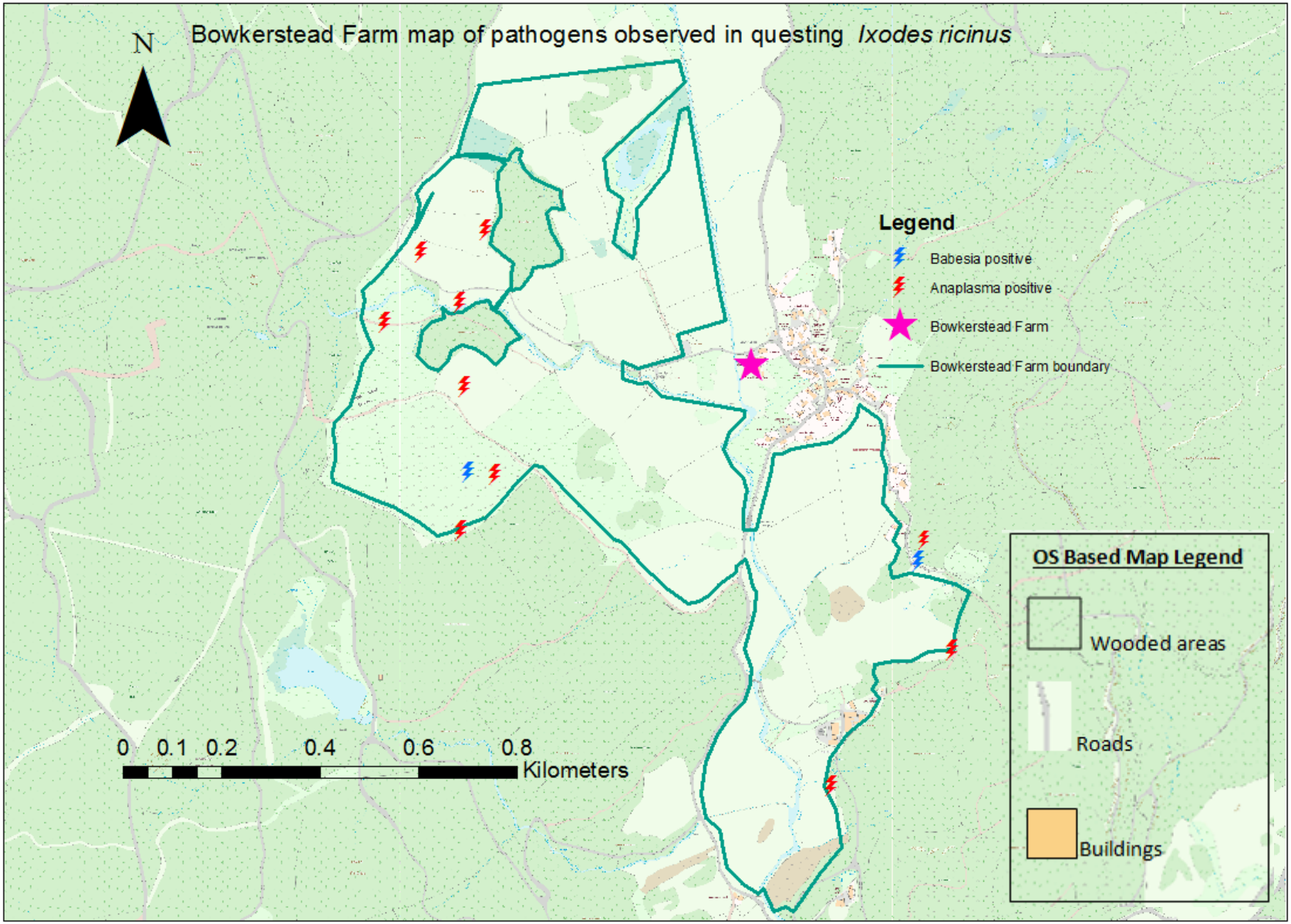

Figure 32 ArcMap showing distribution of pathogens in questing *I. ricinus* across Bowkerstead Farm, 1:9,000

4.3.2 Bethecar Moor

From the spatial surveys of Bethecar Moor described in chapter 3, a total of 389 ticks were collected. Of these, 348 were nymphs, 20 were adult males and 21 were adult females. Of the 389 *I. ricinus* ticks processed, 13 gave a positive result in the partial msp2 q-PCR, all of which were confirmed with nested *msp4* PCR. Following purification, 11 samples yielded sufficient amplicon to be sent off for sequencing; data was successfully generated for each tick sample, and confirmed by comparisons in BLAST. All sequence types observed had homology with sequences previously described in ruminants across Europe (Table 15). Construction of phylogenetic trees (Figure 35) demonstrated that all of the alleles observed across Bethecar Moor clustered together.

A. phagocytophilum has been plotted across the farm, at the site of the drags from which they were collected (Figure 33). The pathogens have been observed across the Moor and do not cluster together.

Table 15 *msp4* alleles observed across Bethecar Moor

Sample	Accession number	Previously described in
BET/012/NY/001	KU712150	red deer, sheep, mouflon, goat, *I. ricinus*
BET/042/NY/001	AY530198	cattle, sheep, *I. ricinus*
BET/067/AM/001	EU240467	sheep, mouflon, horse, red deer
BET/110/NY/001	n/a	99% homology with KU712150
BET/239/NY/001	KM205422	red deer, roe deer, sheep
BET/346/NY/003	KU712150	red deer, sheep, mouflon, goat, *I. ricinus*
BET/353/NY/001	KU712166	roe deer
BET/356/AM/001	n/a	99% homology with KU712150
BET/448/NY/001	n/a	99% homology with KU712150
BET/481/NY/001	EU240467	sheep, red deer, mouflon
BET/540/NY/001	EU240485	sheep

All questing *I. ricinus* were tested for *Babesia spp.* using the A*picomplexa*-specific PCR assay; no positive results were obtained.

The subset of 200 questing *I. ricinus* tested for LIV at the APHA, did not yield any positive results.

4.3.3 Culled Deer

A total of 82 deer (27 red deer and 55 roe deer) were culled by the FC and blood samples collected. *A. phagocytophilum* DNA was detected in 4 of these samples using the partial *msp2* q-PCR. Partial msp4 sequence data were obtained for 2 of these samples. Both sequence types observed had homology with sequences previously described in ruminants across Europe (Table 16). The construction of phylogenetic trees revealed one allele clustering with a divergent roe deer strain (Figure 36).

Table 16 *msp4* alleles observed in culled deer

ID number	Sex	Species	Previously described	Comments
G2001798	Female	Red deer	None 100%	99% homology to KU712173; divergent roe strain
G2001718	Female	Roe deer	KM205422	Previously observed in deer and sheep
G2001821	Female	Red deer	n/a	No sequence data was generated
G2001697	Female	Red deer	n/a	No sequence data was generated

Two samples yielded an amplification product in the Apicomplexan-specific PCR assay, and unambiguous partial 18S rDNA data was obtained from one sample. Comparative sequence analysis indicated the sequence displayed 98% similarity with previously observed sequences of *B. odocoilei;* a deer associated Babesia species (Figure 38).

The shoot locations of the culled deer and their pathogens were plotted using ArcMap (Figure 34); pathogens do not demonstrate any clustering. Deer were shot in the winter, spring and summer months of 2014-2016, all pathogens were detected in the winter 20014-2015 period (Table 17).

Table 17 *Anaplasma* and *Babesia* in culled deer, by season shot

Season	No. of culled deer bloods	*Anaplasma* positive (%)	CI	*Babesia* positive (%)	CI
Winter 2014-2015	41	4 (9.8%)		2 (4.9%)	
Spring/Summer 2015	15	0	n/a	0	n/a
Winter 2015-2016	21	0	n/a	0	n/a
Spring/Summer 2016	9	0	n/a	0	n/a

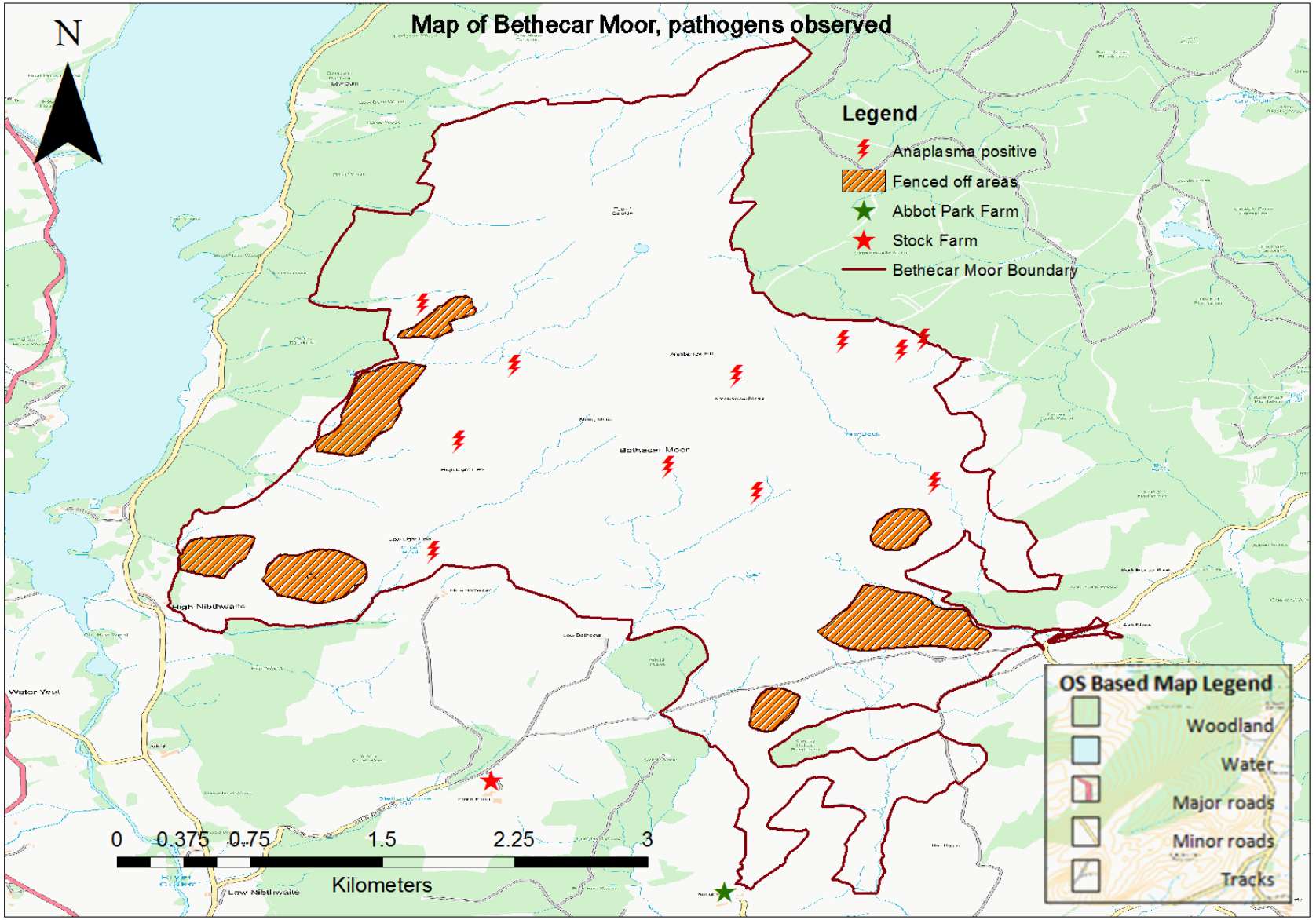

Figure 33 ArcMap showing distribution of pathogens in questing *I. ricinus* across Bethecar Moor, 1:18,000

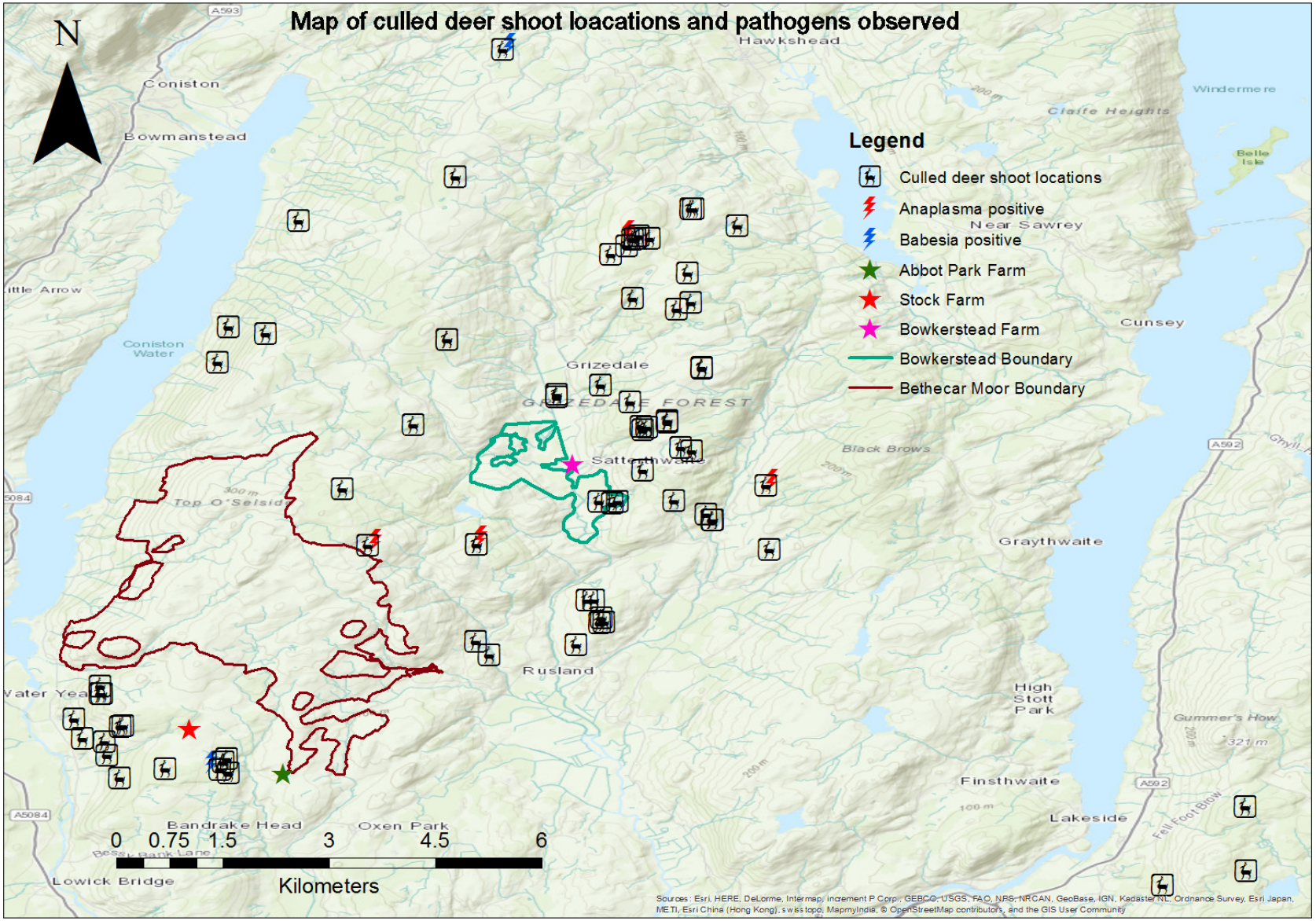

Figure 34 ArcMap showing pathogen distribution across study sites in questing *I. ricinus* and culled deer 1:45,000

4.3.4 Ticks from sheep

4.3.4.1 Stock Farm

Sheep were sampled for ticks a total of nine times between September 2014 and September 2016. During the two year sampling period, a total of 769 ticks were collected. *A. phagocytophilum* DNA was detected in 161 of these ticks using the partial *msp2* q-PCR. Partial *msp4* sequence data were obtained from 66 of these samples. Comparison of sequence similarity among alleles revealed all new alleles shared high levels of similarity (>80%) with previously reported alleles in ruminants across Europe (Table 18 & 19). Multiple ticks were observed on sheep and many were carrying *A. phagocytpohilum,* not all of the ticks off the same sheep were positive.

8 individuals with more than 1 tick observed had all their ticks yield a positive *msp2* result. 19 sheep infested with multiple ticks demonstrated *msp2* positive results in just one tick taken off them during a given sampling period. Ticks taken off 62 infested sheep contained no detectable *msp2* fragment in any tick. Multiple alleles were observed circulating in questing ticks and ticks taken off sheep at any one time. In instances where more than one tick was taken off the same sheep at the same time, the *msp4* sequences observed were not always identical.

None of the 769 ticks contained detectable apicomplexan DNA.

Table 18, part 1 of 2; *Anaplasma* positive and *msp4* alleles observed in ticks taken off sheep at Stock Farm

Sheep ID	Date Sampled	No. of ticks	*Anaplasma* +ve	No. of alleles sequenced	Alleles observed: previously described in
836	10/09/2014	36	6	2	KU712150 in both: red deer, sheep, mouflon, goat, *I. ricinus*
274	10/09/2014	33	3	0	
1031	10/09/2014	25	3	0	
979	10/09/2014	17	2	0	
856	10/09/2014	16	1	0	
1023	10/09/2014	16	4	0	
1065	10/09/2014	15	10	3	KP861636 in 2 ticks: sheep, *I. ricinus* One novel.
Lamb 3	10/09/2014	15	5	2	EF442010 in both: sheep
1044	10/09/2014	13	2	0	
1069	10/09/2014	13	3	2	EU240473 in both: sheep
1050	10/09/2014	12	2	0	
1043	10/09/2014	11	3	1	Novel
1067	10/09/2014	11	3	1	KF420110: fallow deer, *I. ricinus*
619	10/09/2014	10	1	0	
1036	10/09/2014	10	1	0	
998	10/09/2014	9	1	0	
1041	10/09/2014	7	1	0	
Lamb 7	10/09/2014	7	2	0	
776	10/09/2014	6	1	1	EU857671: red deer, sheep
859	10/09/2014	6	5	0	
249	10/09/2014	5	1	0	
870	10/09/2014	4	1	1	EU240479: sheep, cattle tick
Lamb 1	10/09/2014	4	3	1	Novel
1042	10/09/2014	3	2	1	EF442006: sheep
Lamb 2	10/09/2014	2	2	1	KU712150: red deer, sheep, mouflon, goat, *I. ricinus*
Lamb 3 Male	10/09/2014	1	1	0	
3033	10/11/2014	3	1	0	
893	23/07/2015	3	2	1	KU712150: red deer, sheep, mouflon, goat, *I. ricinus*
Lamb 27	23/07/2015	2	1	0	
634	23/07/2015	1	1	1	Novel
766	23/07/2015	1	1	1	Novel
739	11/09/2015	16	1	0	
1039	11/09/2015	14	2	0	
996	11/09/2015	12	9	2	KU712150 in one: red deer, sheep, mouflon, goat, *I. ricinus.* One novel

Table 19, part 2 of 2; Anaplasma positive and msp4 alleles observed in ticks taken off sheep at Stock Farm

Sheep ID	Date Sampled	No. of ticks	*Anaplasma* +ve	No of alleles sequenced	Alleles observed: previously described in
783	11/09/2015	8	2	1	Novel
976	11/09/2015	8	5	3	all 3 none 100 are they the same
1003	11/09/2015	7	3	1	KU712150: red deer, sheep, mouflon, goat, *I. ricinus*
1579	11/09/2015	6	2	2	2 none 100% are they the same
1029	11/09/2015	5	2	1	EU240488: sheep
1053	11/09/2015	5	2	0	
592	11/09/2015	4	1	0	
1023	11/09/2015	4	1	0	
Lamb 2815	11/09/2015	4	2	0	
Lamb 19715	11/09/2015	3	3	0	
Lamb 7915	11/09/2015	3	3	0	
Lamb 8915	11/09/2015	3	3	0	
271	11/09/2015	2	2	0	
990	11/09/2015	2	1	0	
1521	11/09/2015	1	1	0	
Lamb 6815	11/09/2015	1	1	0	
262	09/11/2015	9	3	3	KU712150 for one: red deer, sheep, mouflon, goat, *I. ricinus.* 2 novel
1013	09/11/2015	9	4	0	
2976	09/11/2015	7	4	3	EU240467: sheep, red deer, mouflon. EU712183: Sika. one novel
886	09/11/2015	5	5	3	EU2404 for 2: sheep, red deer, mouflon. one novel
256	09/11/2015	3	1	0	
312	09/11/2015	3	1	1	Novel
260	09/11/2015	2	1	1	KU712150: red deer, sheep, mouflon, goat, *I. ricinus*
296	09/11/2015	2	1	0	
301	09/11/2015	2	1	1	EU240467.1 sheep red mouflon
637	09/11/2015	1	1	1	Novel
1456	12/09/2016	9	2	2	HM028680.1 cattle. One none 100%
880	12/09/2016	8	5	5	EU240488 SHEEP in 3. 2 none 100%
1495	12/09/2016	5	2	2	Novel
1464	12/09/2016	2	2	1	KU712150: red deer, sheep, mouflon, goat, *I. ricinus*
1513	12/09/2016	2	2	2	Novel
1565	12/09/2016	2	1	1	Novel
2228	12/09/2016	2	2	0	
975	12/09/2016	1	1	1	Novel

4.3.4.2 Abbot Park Farm

Sheep were sampled for ticks a total of ten times between July 2015 and September 2016. During the sampling period, a total of 373 ticks were collected. *A. phagocytophilum* was detected in 110 of these ticks using the partial *msp2* qPCR, and unambiguous partial *msp4* sequence data were obtained from 41 of these. Comparison of sequence similarity among alleles revealed all new alleles shared high levels of similarity (>80%) with previously reported alleles in ruminants across Europe (Table 20 & 21). Though multiple ticks were observed on sheep and many were carrying *A. phagocytpohilum,* most of the time not all of the ticks off the same sheep were positive.

11 individuals with more than 1 tick observed had all their ticks yield a positive *msp2* result. 18 sheep infested with multiple ticks demonstrated *msp2* positive results in just one tick taken off them during a given sampling period. Ticks taken off 130 infested sheep contained no detectable *msp2* fragment in any tick. Multiple alleles were observed circulating in questing ticks and ticks taken off sheep at any one time. In instances where more than one tick was taken off the same sheep at the same time, the *msp4* sequences observed were not always identical.

Results from the nested apicomplexan PCR showed five positive bands of around 600bp; due to financial restrictions of the project, these samples were not sequenced.

Table 20, part 1 of 2; *Anaplasma* positive and msp4 alleles observed in ticks taken off sheep at Abbot Park Farm

Sheep ID	Date Sampled	No. of ticks	*Anaplasma* +ve	No of alleles sequenced	Alleles observed: previously described in
Lamb 14	28/07/2015	1	1	0	
Lamb 17	28/07/2015	1	1	0	
765	28/07/2015	1	1	0	
970	28/07/2015	2	1	0	
975	28/07/2015	2	2	2	EU240467: sheep, red deer, mouflon. One novel
978	28/07/2015	1	1	1	KU712150: red deer, sheep, mouflon, goat, *I. ricinus*
981	28/07/2015	2	1	0	
982	28/07/2015	3	2	1	KU712166: roe deer
Lamb 1	28/07/2015	1	1	1	EF442008: sheep
359	20/08/2015	6	2	0	
552	20/08/2015	2	2	1	KU712173: roe deer, divergent strain
769	20/08/2015	2	1	0	
785	20/08/2015	1	1	1	EU857666: cattle, sheep
786	20/08/2015	2	1	0	
975	20/08/2015	2	1	1	KU712164: roe deer, cat, goat, *I. ricinus*
359	02/10/2015	5	5	1	KU712184: sika deer
559	02/10/2015	2	2	0	
576	02/10/2015	1	1	0	
691	02/10/2015	3	1	0	
705	02/10/2015	3	1	0	
712	02/10/2015	2	1	0	
718	02/10/2015	2	1	0	
720	02/10/2015	4	2	0	
767	02/10/2015	3	1	0	
786	02/10/2015	1	1	0	
977	02/10/2015	1	1	0	
978	02/10/2015	4	2	0	
981	02/10/2015	1	1	0	
352	30/10/2015	1	1	0	
693	30/10/2015	1	1	0	
397	23/03/2016	2	1	0	
797	23/03/2016	1	1	0	
4932	23/03/2016	4	3	0	
4957	23/03/2016	2	2	1	EU240479: sheep cattle *I. ricinus*
4967	23/03/2016	2	1	0	
5087	23/03/2016	2	2	0	
5092	23/03/2016	3	3	1	EU857671: red deer, sheep
5097	23/03/2016	5	1	0	

Table 21, part 2 of 2; Anaplasma positive and msp4 alleles observed in ticks taken off sheep at Abbot Park Farm

Sheep ID	Date Sampled	No. of ticks	*Anaplasma* +ve	No of alleles sequenced	Alleles observed: previously described in
5108	23/03/2016	6	5	1	Novel
108525	23/03/2016	1	1	1	EU857671: red deer, sheep
Notag8	23/03/2016	1	1	0	
393	27/04/2016	5	1	0	
394	27/04/2016	1	1	0	
395	27/04/2016	1	1	1	KU712150: red deer, sheep, mouflon, goat, *I. ricinus*
400	27/04/2016	1	1	0	
797	27/04/2016	2	1	1	Novel
798	27/04/2016	1	1	0	
1164	27/04/2016	1	1	0	
1483	27/04/2016	1	1	1	EF442008: sheep
1957	27/04/2016	1	1	0	
4909	27/04/2016	1	1	1	KU712150: red deer, sheep, mouflon, goat, *I. ricinus*
4950	27/04/2016	2	2	0	
4967	27/04/2016	1	1	1	EU857671: red deer, sheep
5087	27/04/2016	1	1	1	Novel
5092	27/04/2016	1	1	1	Novel
Notag1	27/04/2016	1	1	1	KU712166: roe deer
Notag2	27/04/2016	2	2	2	both novel
Notag5	27/04/2016	1	1	0	
Notag6	27/04/2016	5	3	3	KU712150: red deer, sheep, mouflon, goat, *I. ricinus*
Notag9	27/04/2016	2	2	1	Novel
4950	08/07/2016	1	1	1	KU712150: red deer, sheep, mouflon, goat, *I. ricinus*
559	18/08/2016	1	1	0	
966	18/08/2016	1	1	1	EU240479: sheep, cattle, *I. ricinus*
975	18/08/2016	3	2	1	KU712150: red deer, sheep, mouflon, goat, *I. ricinus*
976	18/08/2016	9	1	0	
982	18/08/2016	3	3	0	
1288	18/08/2016	4	1	0	
2533	18/08/2016	3	2	2	KU712184: sika deer
3065	18/08/2016	1	1	0	
3716	18/08/2016	2	1	0	
4950	18/08/2016	1	1	1	EF442010: sheep
5108	18/08/2016	1	1	1	Novel
lamb 1	18/08/2016	2	2	2	KU712150: red deer, sheep, mouflon, goat, *I. ricinus* in both
Notag 1A	18/08/2016	1	1	0	
967	22/09/2016	6	4	0	

4.3.5 Epidemiology of *A. phagocytophilum* infections across the study region

Both Bowkerstead Farm and Bethecar Moor were found to have the same prevalence of *msp2* positives in questing ticks collected from these sites (3.3%). Questing tick data from both sites was combined, resulting in a prevalence of 3.4% in 655 questing nymphs (Tables 22).

There is a much higher prevalence of *msp2* in ticks taken off sheep at Stock and Abbot Park Farm's, 20.9% and 29.5% respectively, than in questing ticks and the culled deer bloods, which yielded a prevalence of 4.88% (Table 23).

Combining the data from ticks taken off sheep demonstrates feeding nymphs as having the highest prevalence (66.7%), with adult females second (28.9%) and finally adult males (14.6%) (Table 23).

Given the differences in *A. phagocytophilum* prevalence in ticks taken off sheep between the two farms, a chi-squared test was conducted and revealed that there was a significant difference in the prevalence observed between each site (χ^2= 28.17, d.f = 1, P <0.001) and that Abbot Park Farm had a higher prevalence of Anaplasma in ticks taken off sheep. When the data was manipulated to consider one positive tick on a sheep as a positive individual, the results were not significant (χ^2= 2.37, d.f = 1, P >0.1)

Table 22 comparing pathogen prevalences in questing ticks and ticks removed from sheep

Survey	No. of samples	*Anaplasma* positive (%)	Exact binomial 95% Confidence Interval (%)	*Babesia spp.* Positive (%)	Exact binomial 95% Confidence Interval (%)
Bowkerstead questing *I. ricinus*	329	11 (**3.3%**)	1.7-5.9	2 (**0.6%**)	0.07-2.2
Bethecar questing *I. ricinus*	389	13 (**3.3%**)	1.8-5.6	0	0-0.9
Culled deer bloods	82	4 (**4.9%**)	1.3-12.02	3 (**3.7%**)	0.77-10.32
Stock Farm sheep *I. ricinus*	769	161 (**20.9%**)	18.11-23.99	0	0-0.4
Abbot Park Farm sheep *I. ricinus*	373	110 (**29.5%**)	24.9-34.4	5 (**1.3%**)	0.44-3.1

Table 23 comparing pathogen prevalence in questing ticks, between tick life stages, and culled deer bloods

Sample	No. of samples	*Anaplasma* positive (%)	Exact Binomial 95% Confidence Interval (95%)
Questing *I. ricinus* nymphs	655	22 (3.4%)	2.1-5.04
***I. ricinus* adult male off sheep**	82	12 (14.6%)	7.8-24.17
Feeding *I. ricinus* adult female	1010	292 (28.9)	26.13-31.81
Feeding *I. ricinus* nymphs	6	4 (66.7)	22.28-95.67
Culled deer bloods	82	4 (4.9%)	1.3-12.02

4.3.6 Sequence data analysis

4.3.6.1 *A. phagocytophilum*

A total of 127 unambiguous *A. phagocytophilum* partial *msp4* sequences (301bp) were obtained in this study. The diversity of these sequences was put into context by comparison with all *A. phagocytophilum msp4* alleles present in GenBank. As of 1st June 2017, a total of 49 alleles were delineated among the 127 msp4 sequences present. Sequences for inclusion in this analysis were selected from GenBank using the search terms "*Anaplasma phagocytophilum*" and "*msp4*".

A total of 49 alleles were observed among the 127 sequences obtained in this study, 24 of which had been previously characterised, 25 of which were new. Comparison of sequence similarity among alleles revealed all new alleles shared high levels of similarity (>80%) with previously reported alleles. Some of the alleles (8) were only encountered once, but 16 alleles were encountered on multiple occasions, with the most commonly-encountered allele occurring in 21 samples (Table 24).

Phylogenetic inference of relationships among the *A. phagocytophilum* strains possessing the new *msp4* alleles and those with previously reported *msp4* alleles suggested that all strains encountered in this study fell within the previously-recognised spectrum of *A. phagocytophilum* diversity (Figure 35). Almost all strains from this study lay with a populous cluster of *A.phagocytophilum* strains associated with a wide variety of wildlife and domesticated species, and humans, in Europe and beyond. However, two strains, infecting a roe deer and a questing *I. ricinus* tick, lay within one of the outlying clusters within the *A. phagocytophilum* spectrum, shared with strains infecting roe deer across Europe (Figure 35).

A total of 127 samples were successfully sequenced from culled deer, questing ticks and ticks taken from sheep from around the study sites; all of which were compared to previously reported sequences in BLASTn. From the 127 samples, 49 different alleles were observed; 25 of these were novel sequences, obtained from 47 different samples. These novel sequences displayed high levels of homology (99%) to sequence types previously observed in deer, cattle, sheep and *I. ricinus* ticks. Phylogenetic inference of evolutionary relationships between these sequence types and those previously observed, indicated the clustering of all the newly encountered strains with those already available on GenBank (Figure 37).

The remaining 80 samples demonstrated 24 different sequence types between them, all of which had been previously recorded in GenBank. All of these sequence types had previously been observed in ruminants and questing *I. ricinus* across Europe (Table 24).

Further analysis via the construction of phylogenetic trees (Figure 36) revealed that two of the samples, one from a culled deer and another from a tick taken off a sheep at Abbot Park Farm, were part of a divergent strain, previously observed in roe deer in Europe.

Multiple alleles were observed circulating in questing ticks and ticks taken off sheep at any one time. In instances where more than one tick was taken off the same sheep at the same time, the *msp4* sequences observed were not always identical.

Table 24 showing all *msp4* alleles previously described (100% sequence similarity), observed throughout this study

GenBank reference	Previously observed	Frequency observed in this study	Observed in this study
AY530198.1	*I. ricinus,* sheep, cattle	1	Questing, Bethecar
EF442006.1	Sheep	1	Stock sheep
EF442008.1	Sheep	4	Questing Bowkerstead, Stock sheep, Abbot Park Sheep (x2)
EF442010.1	Sheep	4	Abbot Park sheep, Stock sheep (x3)
EU180060.1	*I. ricinus,* roe deer	1	Questing Bowkerstead
EU240467.1	Sheep, red deer, horse, mouflon	8	Questing Bethecar (x2), Abbot Park sheep, Stock sheep (x5)
EU240473.1	Sheep	2	Stock sheep (x2)
EU240479.1	*I. ricinus,* sheep, cattle	6	Stock sheep (x3), Abbot Park sheep (x3)
EU240485.1	Sheep	2	Questing Bethecar, questing Bowkerstead
EU240488.1	Sheep	5	Stock sheep (same sheep)
EU857666.1	Sheep, cattle	2	Questing Bowkerstead, Abbot Park sheep
EU857671.1	Sheep, red deer	4	Stock sheep, Abbot Park sheep (x3)
HM028680.1	Cattle	2	Stock sheep, Abbot Park sheep
KF420110.1	*I. ricinus,* sheep	1	Stock sheep
KF420116.1	*I. ricinus*	1	Questing Bowkerstead
KJ832664.1	*I. ricinus*	1	Abbot Park sheep, Stock sheep (x3)
KM205422.1	Sheep, red deer, roe deer	2	Questing Bethecar, Culled Red deer
KP861636.1	*I. ricinus,* sheep	2	Stock sheep (same sheep)
KU712150.1	Red deer, sheep, mouflon, goat, tick	21	Questing Bowkerstead, Questing Bethecar (x2), Abbot Park sheep (x8), Stock sheep (x10)
KU712164.1	*I. ricinus,* roe deer, goat, cat	1	Abbot Park sheep
KU712166.1	Roe deer	3	Questing Bethecar, Abbot Park sheep (x2)
KU712173.1	Roe deer (divergent)	2	Abbot Park sheep, culled Roe deer blood
KU712183.1	Sika deer	2	Questing Bowkerstead, Stock sheep
KU712184.1	Sika deer	3	Abbot Park sheep (2 from same sheep)
Novel sequences	>80% sequence similarity with these alleles	47	All sites

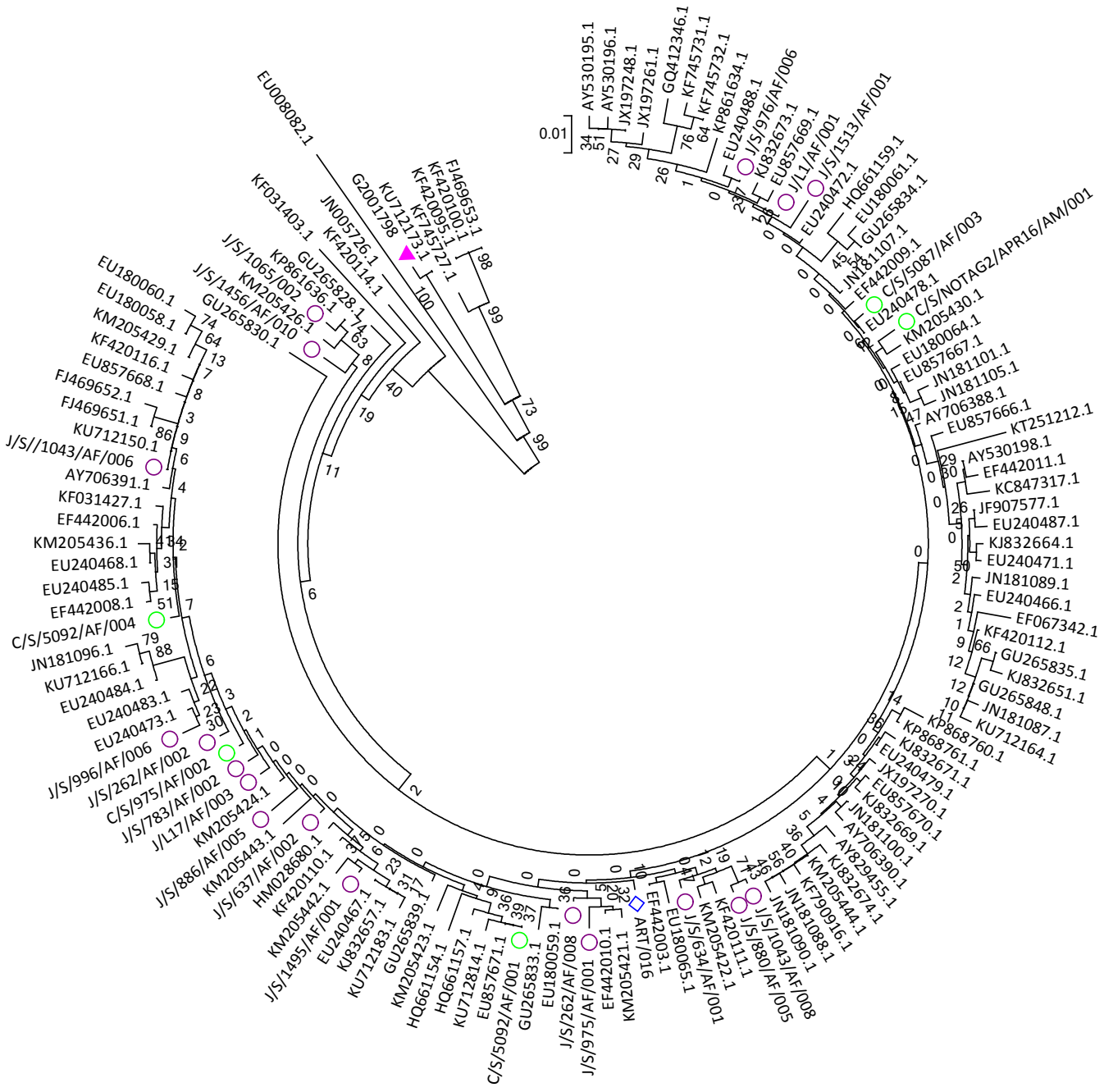

Figure 35 Evolutionary relationships of taxa. With reference sequence types obtained from GenBank and sequence types observed in this study indicated by: O for ticks taken off sheep at Stock Farm, O for ticks taken off sheep at Abbot Park Farm, ◊ for ticks collected via blanket dragging at Bowkerstead Farm, and ▲ for culled Roe deer bloods. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (5000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method. Evolutionary analyses were conducted in MEGA6.

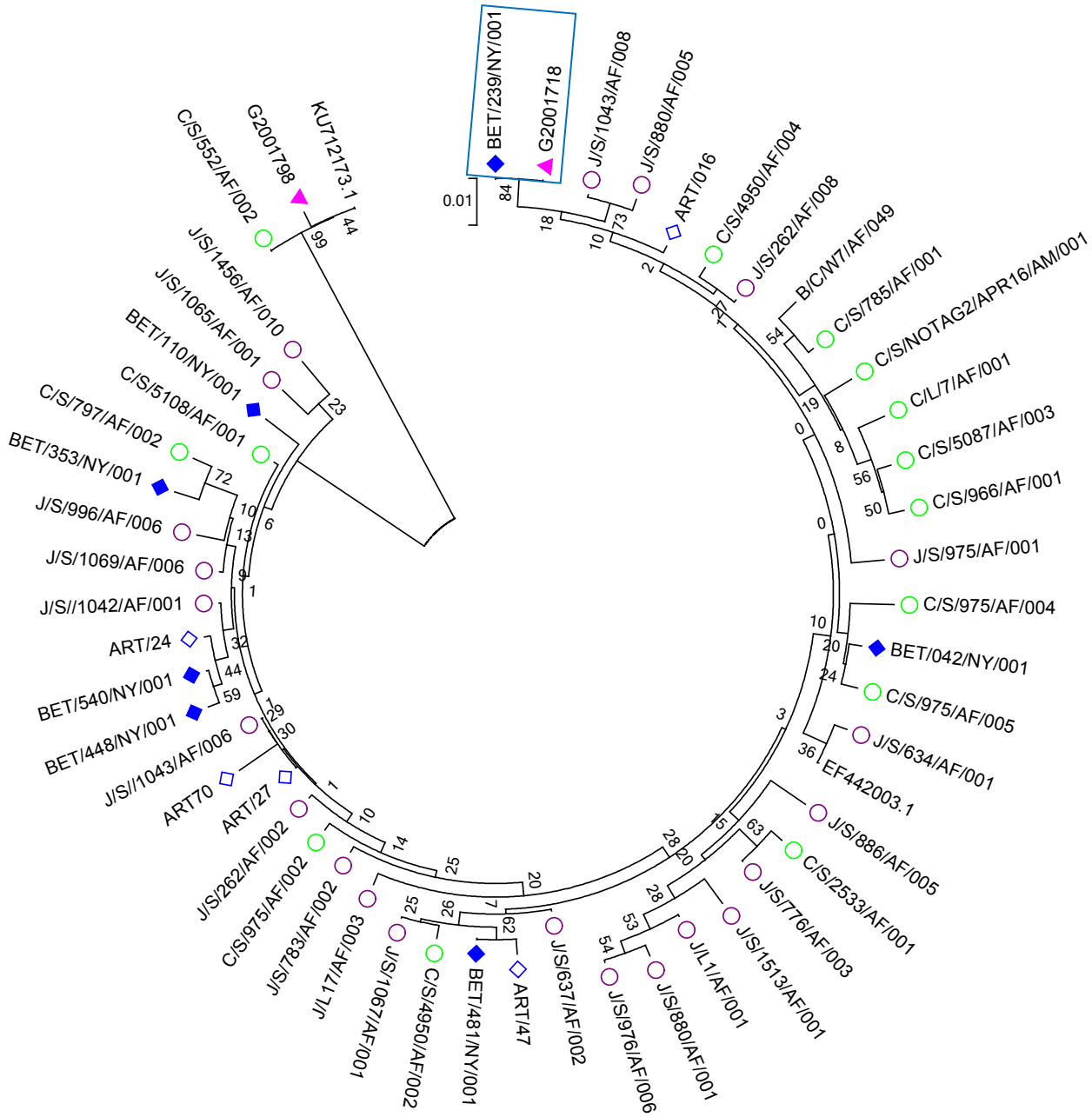

Figure 36 Evolutionary relationships of taxa. With Sequence types observed in this study indicated by: O for ticks taken off sheep at Stock Farm, O for ticks taken off sheep at Abbot Park Farm, ◊ for ticks collected via blanket dragging at Bowkerstead Farm, ◆ for ticks collected via dragging across Bethecar Moor and ▲ for culled deer bloods. The deer blood samples boxed in red come from a Roe deer sample, where the deer sample boxed in blue is from a Red deer. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (5000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method. Evolutionary analyses were conducted in MEGA6.

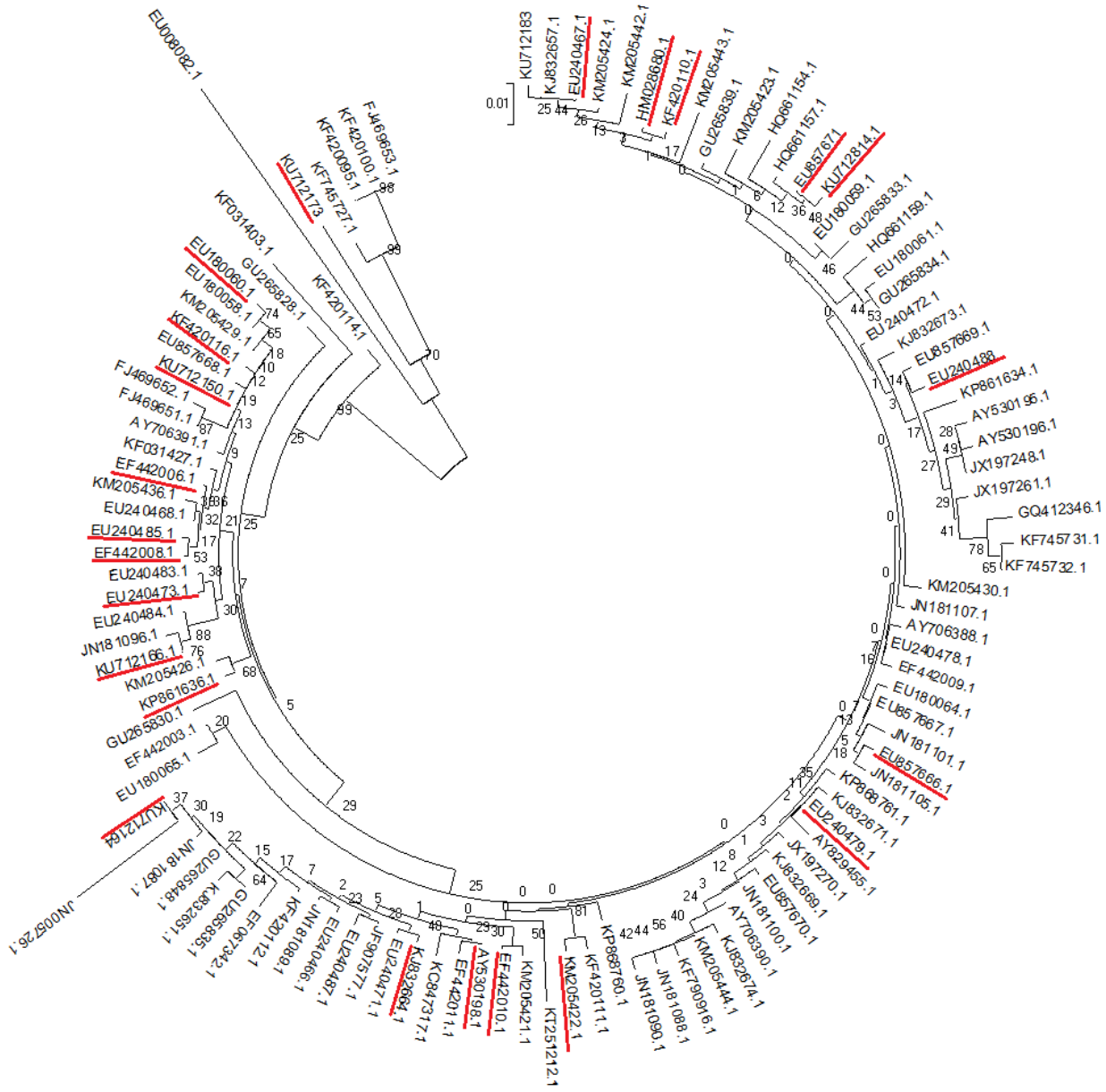

Figure 37. Evolutionary relationships of taxa; Showing all alleles obtained from GenBank, with sequence types observed in this study underlined in red. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (5000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method. Evolutionary analyses were conducted in MEGA6.

4.3.6.2 *Babesia sp.*

A total of three samples were successfully sequenced from culled deer and questing ticks from Bowkerstead Farm; all of which were compared to previously reported sequences in BLASTn. Each of the three alleles observed were novel sequences, though they displayed high sequence similarity (98%) with previously recorded strains of *B. odocoilei* and *B. venatorum,* two deer associated species (Figure 38).

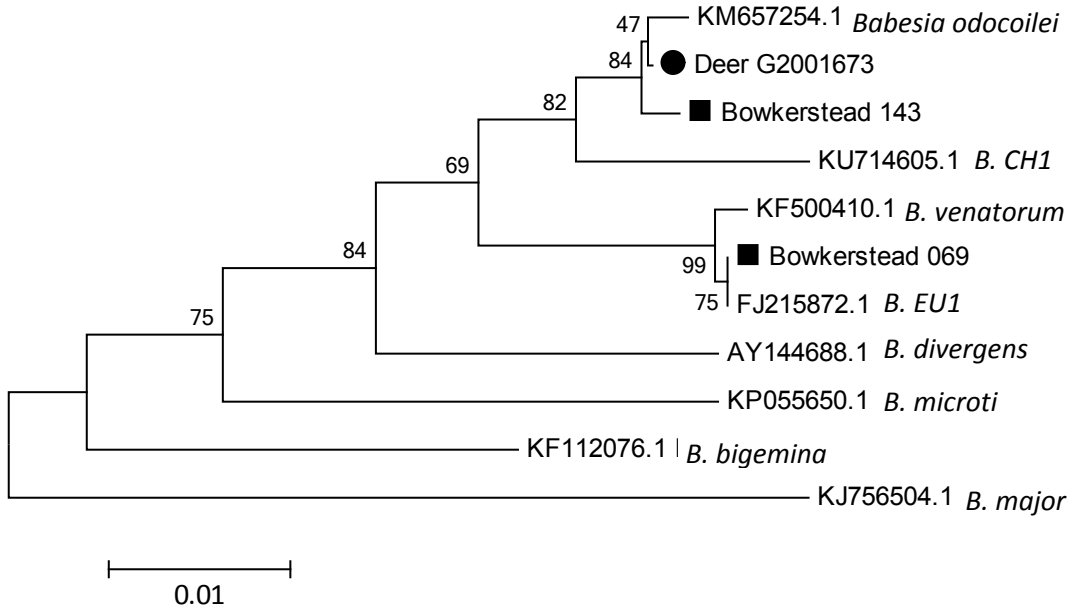

Figure 38 Evolutionary relationships of taxa. Showing all alleles obtained from GenBank, with sequence types observed in this study represented by ● for Roe deer blood and ■ for questing ticks from Bowkerstead farm. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (5000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method. Evolutionary analyses were conducted in MEGA6.

4.4 Discussion

PCR assays demonstrated that *A. phagocytophilum* was endemic in the area in questing *I. ricinus* ticks, ticks taken from sheep, and culled deer bloods. The pathogen was observed in ticks taken from the environment across both sites, and did not exhibit any clustering. Each of the sites had a prevalence of 3.34%, with a combined prevalence of 3.36% in questing nymphs. These numbers are similar to what has previously been reported in questing *I. ricinus* across Europe (Overzier et al., 2013) (Matei et al., 2015) (Sormunen et al., 2016) including Scotland (Walker et al., 2001). This is significantly higher than the 0.7% previously reported in north east England (Bown et al, 2009), which was conducted in the absence of sheep.

Sheep have previously been implicated as being able to maintain *A. phagocytophilum* cycles (Ogden, 2002) in the absence of deer; though there was a large deer population in the study area and its surroundings, which were frequently observed crossing each of the sites in this study. A prevalence of just 4.88% infection with *A. phagocytophilum* was observed in culled deer blood samples. This is much lower than other areas of Europe, which have recorded high prevalences upwards of 70% in both red and roe deer (Stuen et al, 2013; Jahfari et al, 2014; (Jouglin et al., 2017). This, in part, could be due to blood samples being handled poorly, leading to degradation. As not all bloods were received within 24-48 hours of being shot, some were as old as a week before they were processed.

However, it could also suggest that sheep are responsible for maintaining *A. phagocytophilum* at the study sites. The prevalence in ticks taken off sheep at Stock Farm and Abbot Park Farm was 20.94% and 29.49% respectively, this is considerably lower than the 66% previously reported in ticks taken off sheep in The Netherlands (Jahfari et al, 2014), which considered a much more dynamic system with an abundance of various hosts. However, this is much closer to the 20% prevalence observed in ticks taken off sheep in an upland system in North Wales, where they were considered the only suitable hosts available in sufficient numbers to circulate the pathogen (Ogden et al, 2002). In addition, statistical analysis at Bowkerstead Farm demonstrated that there was a significant difference in the prevalence of *msp2* fragment observed on drags conducted in the woodland than those on the Farmland. With a Alongside the low prevalence in deer, the hypothesis that sheep are the main hosts in maintaining *A. phagocytophilum* at the study sites is further supported by the prevalence of infected male and feeding female ticks observed. A total of 14.63% of the male ticks searching for a mate on the sheep host were confirmed as PCR positive, with that number almost doubling, to 28.91% in feeding females. If the sheep were not responsible for circulating the pathogen, similar frequencies would be observed.

There were insufficient numbers of feeding nymphs obtained from sheep (6) to compare *A. phagocytophilum* prevalence with that recorded in questing ticks collected from the study site, and so this could not be used to estimate sheep infection status as per Jahfari and colleagues (2014).

Previous studies (Ogden et al, 2002) stated that neither prevalence or intensity of infection in engorged ticks feeding on sheep were related to a blood PCR result, therefore, we cannot assume the infection status of the sheep solely from the tick data collected. Though the study highlighted that sheep carrying higher numbers of adult female ticks were less likely to be blood PCR positive, this is unlikely to be occurring in this study. In most instances, low numbers of feeding ticks were observed infesting the sheep, with the majority of infested sheep housing fewer than ten ticks. The implications of this suggest that there is potential for a high prevalence of *A. phagocytophilum* infection in sheep in this study area. Though further studies and analysis of blood samples taken from sheep would be needed to confirm this

The purpose of this study was to characterize the diversity of *A. phagocytophilum* strains circulating in a natural multi-host, multi-vector system and to determine whether the observed diversity had any ecologic basis. A total of 49 msp4 alleles of *A. phagoytophilum* were observed circulating the study sites, all of the samples were shown to demonstrate high sequence similarity (>97%) with previously documented strains alleles analysed with BLAST. This dynamic system, of multiple strains circulating in the same time and space, has been previously documented in flocks (Ogden et al., 2002; Ladbury et al 2008).

Not all of the alleles observed in ticks taken off the same sheep at the same time were identical, perhaps being more representative of an infected tick rather than an infected sheep. However, two ticks taken from one sheep at Abbot Park (identification number 2533) were found to be infected with identical sequence types, which had previously been recorded in Sika deer, accession number KU712184 (unpublished data). This could be as a result of the sheep being infected with that particular sequence type, or as a result of co-feeding transmission; as these ticks were observed and removed in close proximity to each other on the sheep (Figure 39). This could be confirmed with parallel sheep blood sampling and PCR.

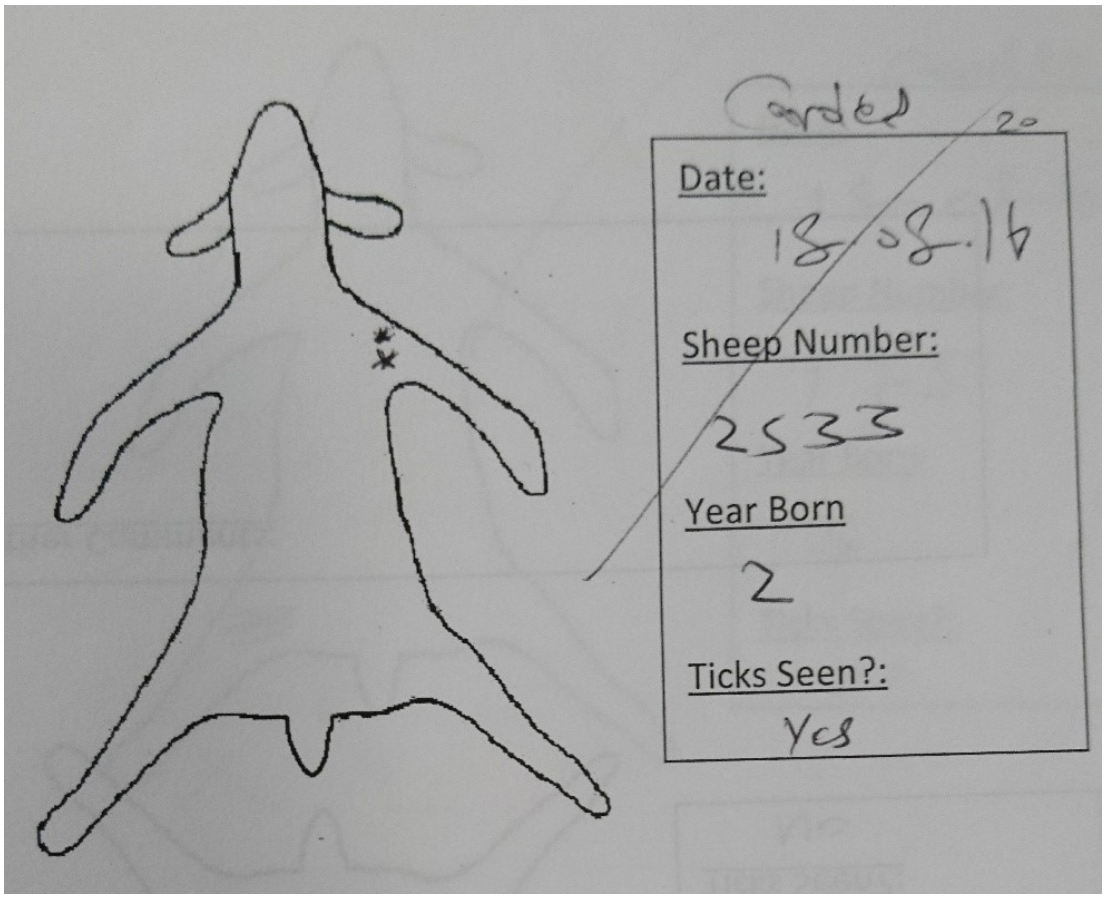

Figure 39 Field work sample sheet, showing location of feeding ticks on sheep number 2533

Phylogenetic analysis demonstrated that all *msp4* positive tick and deer blood samples were shown to be carrying *A. phagocytophilum* strains associated with ruminants, and belonged to the same incredibly large and diverse clade, which contains strains observed in deer, cattle, goats and humans among others (de la Fuente et al., 2008, Bown et al., 2009).l

Two of the *msp4* positive samples clustered outside this clade, along with a divergent Roe deer associated strain detected in Germany (De la Fuente et al 2005). One of these samples was from deer blood, the other was observed in a tick taken from one of the sheep at Abbot Park Farm.

It has been suggested that the divergent roe deer strain does not cause pathology in sheep (de la Fuente et al., 2008), though this project has demonstrated that this strain is present in the area and coming in to contact with the flock, its low incidence suggests that it is not circulated by the sheep in this system and is unlikely to be of veterinary importance for livestock.

Its presence is notable, suggesting that there is no geographical clustering of ecotypes at the finer scale, and that they circulate within the same space in this study; as has been previously demonstrated across the UK and Europe (Bown et al., 2009; Jahfari et al, 2014).

The results presented in this chapter align with previously published studies (De la Fuente et al., 2005; Bown et al., 2009), supporting the observation that most of the strains circulating in Europe are associated with ruminants. Other than the divergent allele, *msp4* sequences observed across all four sites display great homology and appear to exist within the same cluster. Provisional sequence analysis states that the same strains are circulating at the different sites and there does not appear to be differences between breeds or age groups. This is in line with previous studies (Ogden et al 2002, Bown et al 2009).

B. divergens was not observed at any of the study sites, despite the presence of cattle and anecdotal evidence from farmers suggesting that red water fever was present in the area. Each of the three *Babesia* positives observed demonstrated high homology (98-99%) with previously described *Babesia* sequences that were available on GenBank, and were all different. They were all wild ruminant associated species, which have previously been observed across Europe (Duh et al., 2001);(Michel et al., 2014); Hamšíková et al, 2014).

The two questing ticks that were confirmed positive for *Babesia* by sequencing were both from Bowkerstead Farm. One of the samples was confirmed as *B. venatorum*, also known as *B. EU1,* which is of note, as it has, to the best of my knowledge, only been documented once before in the UK (Smith et al., 2013, Kauffmann et al., 2017). As a severe human pathogen, and potential emerging disease, its discovery in a new area of the UK is of potential medical importance.

The second questing tick was confirmed to be carrying strain *Babesia* sp. OO-2012,which has been put forward as being part of a sister clade to *B. odocoilei,* a species which primarily infects white tailed deer in North America (Waldrup et al., 1990);(Hamsikova et al., 2016) and may represent a geographical variant of this species. The third sample was obtained directly from a deer culled in the area, due to imprecise record keeping from the FC rangers; the species of the deer is unknown. The sample was confirmed to be positive for *Babesia* and *B. odocoilei*- like*,* the strain has previously been observed in Germany (Wiegmann et al., 2015);(Kauffmann et al., 2017). Though the genetic analysis carried out here is not detailed enough to make a species specific identification, it is unlikely to be a species observed in North America, and will most likely be closer to *B. capreoli and B. CH1,* further genetic analysis would be needed to confirm this. To the best of my knowledge, this is first time that *Babesia* sp. OO-2012 and this *B. odocoilei*- like strain have been observed in the UK, this has veterinary implications as they are known to cause disease in wild ruminants and could be a threat to livestock. Their zoonotic potential is currently unknown, though they are closely related to *Babesia CH1,* a species known to infect red deer and presumed to be non-pathogenic to humans, was first described in Switzerland 2006 (Hilperthauser et al 2006).

None of the 200 questing ticks from Bethecar Moor were positive for Louping Ill. Previously published data on the prevalence of LI in questing ticks (Watts et al, 2008), demonstrated values across six site between 1.8% (S.E 1.2) and 15.3% (S.E 3.9). Using these values, I would expect to observe between 3 and 30 infected ticks in a sample size of 200 ticks. In addition, using the median prevalence of 0.7% in a proportion power calculation in R stated a sample size of 53 would presumably be sufficient in detecting LI in ticks at this site. Therefore, a sample size of 200 is deemed adequate in this study.

Most of the ticks from blanket dragging for this assay were obtained from the areas of the Moor which has previously been identified as harbouring the most ticks (Chapter 3), due to time constraints and requiring a large number of ticks quickly. It could be that these ticks were on the fell as a result of deer movement, and therefor unlikely to have been exposed to the pathogen as they are unsuitable hosts. Additionally, the pathogen may be truly not present in the area, though as Abbot Park Farm do not vaccinate against it, then it may well return. Further work should consider and test ticks taken from sheep for LI, as well as questing ticks collected from across the Moor.

A total of 82 deer were culled by the FC and blood samples collected; 27 red and 55 roe. Of the 82 bloods processed, four gave a positive result in the partial msp2 q-PCR, two of which were confirmed with nested msp4 PCR. Following purification, two of these samples yielded sufficient amplicon for sequencing; data was successfully generated for each sample and confirmed by comparisons in BLAST. Results from the nested apicomplexan PCR showed two positive bands of around 600bp; one of which was confirmed to belong to the *Babesia* genus via sequencing. The shoot locations of the culled deer and their pathogens were plotted using ArcMap (Figure 34). The deer have been culled within their ranging distance of Bethecar Moor and Bowkerstead Farm, so it is feasible that they would have crossed the study sites during their lives. The deer that tested positive for either pathogen were typically culled within 1km of one of the study sites, making it likely that they would have crossed one or both sites in their lifetime.

Chapter 5: Exploring the role of citizen science in the surveillance of tick borne disease in Cattle

5.1 Introduction

The impact of ticks and tick borne disease to cattle and livestock has been discussed throughout this thesis, in Chapters 1-4. This part of the study aims to explore the potential of how citizen science could work to improve our knowledge and surveillance of diseases of veterinary importance. Specifically, this chapter will look at the two previously discussed pathogens, *Anaplasma phagocytophilum* and *Babesia* species. In addition, this chapter will embrace the 'one health initiative' and begin to incorporate a human health aspect in sampling for *Borrelia burgdorferi*: the causal agent of Lyme disease.

Volunteers have contributed to the wider scientific understanding of the natural world for centuries (Pocock et al. 2015). Recent advances in technology and the advent of the internet as a tool for recruitment, have seen drastic increases in both the number of studies conducted and the scales at which they are being carried out (Franzoni, C. & Sauermann, H. 2014; Palmer et al. 2017).

Citizen science has considerable potential for helping to detect and track infectious disease (Crowl et al. 2008) and has been recognised as a useful tool in obtaining samples for scientific processing as well as engaging, and educating the general public on current scientific research within an ecological and public health setting (Silvertown J, 2009; Dickinson et al, 2010; Toerpe K, 2013).

More recent studies have utilized this increase in interest to begin address its usefulness as a tool for public health and vector borne disease; collecting large numbers of samples without the need for complex field work (Curtis-Robels et al, 2015; Hall et al, 2017). Veterinary practices have also been utilized, as a source for companion animals, to map and monitor ticks and tick borne disease (Smith et al. 2011).

This section of the study looked in to the feasibility of expanding this method and idea to the farming industry; another aspect of the one health sphere. Zoonotic disease can be endemic, in upland farming; this study aimed to test the feasibility of citizen science within a farming context, to investigate the potential of using citizen science in facilitating disease surveillance. Ultimately, this was to explore if citizen science could be beneficial to researchers by: obtaining and processing a large number of samples from an area where conducting labour intensive field work is not always plausible due to accessibility right of way, permissions, physicality, legislations, ethics and animal handling. But also to farmers; by making them aware of the pathogens that are circulating in the environment with their livestock, both of veterinary and medical importance.

5.1.2 Strathairlie Farm

Further to the details of the farmers provided in Chapters 2-4, the Forestry Commission (FC) identified Farmer 4 as a candidate to participate in our study. Strathairlie Farm is located in north Arnside, within the Arnside and Silverdale Area of Natural Beauty (AONB) Lat/Long, 54.199912, -2.810649.

Farmer 4 is a beef cattle farmer with a unique husbandry, and the founder of 'The Morecambe Bay Local Grazing Scheme'. Farmer 4 has developed the scheme over the last 15 years, evolving out of a small, organically certified, livestock business that has been able to integrate commercial livestock farming into a successful conservation grazing scheme in the Morcambe Bay area.

Farmer 4 has a closed herd of 160 cattle from calf to 5-6 year old steers ready for slaughter, breeding heifers and bulls. His herd is a mix of breeds including: Red Poll cattle, Blue Grey Cattle, Beef Shorthorn cattle.

At present, the cattle are routinely moved around a network of sites, covering over 1000ha of land, representing more than 30 separate conservation sites in 3 counties in North West England; most of them designated for their biological importance under UK and EU law. The vast majority of this land is limestone grassland, though there are also 80ha of more fertile restorative reedbed and 8ha of meadows, which are cut for hay and silage.

Within this complicated system, the cattle are moved in response to each of the sites individual grazing and conservation needs; nearly all of them rely on grazing to control the advance of scrub and contain the spread of bracken. In addition, many of them provide habitats for key butterfly populations, such as the High Brown Fritillary.

Farmer 4 was an ideal candidate for this study, which could facilitate disease surveillance in new, previously investigated areas; at minimal effort to the farmer, and eliminating the need for complex, in labour intensive field studies in the first instance.

5.2 Methods

5.2.1 Sample Collection

Discussions were had with Farmer 4 regarding what was feasible in terms of data collection. It was determined that Farmer 4 would remove ticks from his cattle opportunistically from his cattle while he visited grazing sites, or moved his herd. Data regarding site, date and cattle sample size were also to be recorded, with as much detail as feasible, during collection.

Following this, Farmer 4 collected ticks off the cattle opportunistically, while visiting each of his grazing sites, as and when he observed ticks. Ticks were removed from cattle and stored in 70% ethanol at room temperature, until they were sent to The University of Salford for processing.

5.2.3 Sample processing

All of the ticks removed from cattle were identified to species level, using the methods described in Chapter 2. Following identification, they were processed individually for DNA extraction and tested for *Anaplasma phagocytophilum* and *Babesia* species using the methods and assays described in Chapter 4.

5.2.4 Sampling for *Borrelia burgdorferi spp.*

The same previously described real-time PCR (q-PCR) (Courtney et al 2004) was used (as described in Chapter 4) to screen ticks for *B. burgdorferi*, using primers targeting the 23S rRNA gene (Table 25). Each reaction mix contained 10µl of 2x MyTaq Readymix, (supplied by Applied Bioline reagents Ltd), 1µl 10 ρmol/µl of the forward primer, 1µl 10 ρmol/µl of the reverse primer, 1µl 3.3 ρmol/µl fluorescent probe,-labelled at the 5' and 3' ends with 6-carboxy-fluorescein (6-FAM) and 6-carboxyl-tetramethyl-rhomadine (TAMRA), respectively, 5µl of water and 2µl of template DNA. Reaction mixes were subjected to a thermal programme consisting of an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing-extension step at 60°C for 60 seconds. The fluorescence intensity of the mix was captured at the end of the extension step. All results were visualised via Opticon Monitor software.

Table 25 primers used for amplification of 23S rRNA

Primer	Sequence
Bb23Sf	5'- CGA GTC TTA AAA GGG CGA TTT AGT -3'
Bb23Sr	5'- GCT TCA GCC TGG CCA TAA ATA G -3'
Bb23Sp-FAM	(FAM)5'- AGA TGT GGT AGA CCC GAA GCC GA -3'(TAMRA)

5.2.5 Reverse Line Blot (RLB)

RLB was used to delineate *B. burgdorferi* strains detected by qPCR into distinct *Borrelia* genospecies. The genospecies tested for were those previously recorded in the UK, namely *B. garinii, B.valaisiana, B.afzelli* and *B. burgdorferi sensu stricto.* The accuracy the qPCR was also assessed by incorporating a probe for *B. burgdorferi sensu lato* into the RLB (i.e. a probe with the same specificity as the qPCR). RLB PCRs were performed following a published protocol (Alekseev et al., 2001). Each 25µl reaction mix contained 12.5µl of 2XMasterMix, 9µl dH_2O, 0.5µl of each primer (both at 50 ρmol/µl), and 2.5µl of DNA. Mixes were exposed to a thermal cycle that consisted of an initial denaturation step of 10 minutes at 94°C, followed by two cycles of 20 seconds at 94°C, 30 seconds at 65°C, and 30 seconds at 72°C, then two cycles with conditions identical to previously, but with an annealing temperature of 63°C. During the subsequent two cycle sets, the annealing temperature was lowered by 2°C until it reached 55°C. This was followed by a further 20 cycles of 20 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C. These cycles were then repeated 20 more times, though with an alteration from 55°C up to 63°C. The thermal cycle was completed with a final elongation for 7 minutes at 72°C. Products were stored in the freezer until use, where they would be denatured at 100°C for ten minutes then used immediately. To help prevent contamination and confirm true results, a positive control containing all genomospecies observed was run, along with a negative control of just reagents.

5.2.5.1 Primers & Probes

The primers and probes used in this study were taken from previously established studies (Alekseev et al., 2001)

Table 26 primers and probes; showing probes used in the RLB. With primers used in the amplification of target

Probe	Sequence
Sensu lato	5'-CTTTGACCATATTTTTATCTTCCA-3'
Sensu stricto	5'-AACACCAATATTTAAAAAACATAA-3'
Garinii	5-'AACATGAACATCTAAAAACATAAA-3'
Afzelii	5'-TATATCTTTTGTTCAATCCATGT-3'
Valaisiana	5'-CAAAAACATAAATATCTAAAAACATAA-3'
Gane	5'-TCAAGATTTGAAGTATAAAATAAAA-3'
Primer	**Sequence**
23sBOR	(Biotin)5'-GAGTTCGCGGGAGAGTAGGTTATT-3'
B-5sBOR	5'-TCAGGGTACTTAGATGGTTCACTT-3'

5.2.5.2 Stocks & Solutions

The following stock solution of buffer was prepared:

20X SSPE stock:	0.2M	NaH_2PO_4	24g/l (anhydrous)
	3.6M	NaCl	175.3g/l
	20mM	EDTA	7.4g/l

The pH was adjusted to 7.4 by adding 10M NaOH or HCl, then autoclaved. Stock 20XSSPE was then diluted with dH_2O and SDS at varying concentrations was added to give working solutions, which were required at varying temperatures:

500 ml 2 x SSPE/0.1% sodium dodecyl sulphate (SDS) (5 ml of 10% (10g/100ml) SDS in 250 ml, 50 ml 20x SSPE and 445 ml dH_2O) at 50°C

1000 ml 2 x SSPE/0.5% SDS (25ml of 10% SDS in 500ml, 50ml of 20x SSPE, and 425ml dH_2O) in two 500ml volumes at 50°C and 55°C

500ml 2 x SSPE (50ml of 20x SSPE and 450ml dH_2O) kept at room temperature (RT)

A working concentration of 100ml 20mM EDTA at pH8 from stock (100mM) was also made. This could be stored for a week before use (RT). 0.5M of $NaHCO_3$ (pH 8.4) was made up and used to dilute the oligo probes; 1490µl $NaHCO_3$ + 10µl of probe (to 10 ρmol/µl)

100ml 0.1M NaOH and 10ml 16%, N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC) solution (1.6g in 10ml) were made freshly before use.

5.2.5.3 Membrane activation and hybridization

Following the preparation of buffers and probes, Biodyne C membrane (PALL) was cut to size then carefully placed (wearing gloves and using forceps) into a Tupperware container and covered with the fresh 16% (w/v) EDAC, a condensing reagent used for amine bonding with primary amines. The container was placed on a rocker for 15 minutes at room temperature, after which the membrane was rinsed in dH_2O for 2 minutes. Once rinsed, the membrane was placed into the mini-blotter, kept straight and slightly overlapping the first lane to ensure all probes and samples were run. A mini-blotter sponge was placed over the top of the membrane to support and the rest of the mini-blotter was put together, ensuring screws were done tightly enough. Any bubbles or left over liquid were aspirated off, then undiluted drawing pen ink was added to the first two and last lanes to aid with orientation. The diluted probes were then added to the slots of the blotter (≈ 130µl), taking care to run each sample down to the end of the membrane, and to not get any bubbles in the grooves. These were then left to incubate, covalently bonding the probes to the membrane for one minute at room temperature. The probe solutions were then removed from the blotter in the order in which they were added and the membrane was removed using forceps and washed in fresh 0.1M NaOH at RT whilst shaking to inactivate the membrane. Next both the blotter and membrane were rinsed with dH_2O. To remove any excess probe, the membrane was then washed in 250ml 2x SSPE/0.1% SDS for 10 minutes in a hybridization oven at 50°C whilst rocking.

A further wash followed, with gentle shaking for 15 minutes in 100ml 20mM EDTA (pH8) at RT. At this point the membrane could be stored in the fridge, wrapped in Clingfilm for future use. To continue, a second wash in 250ml 2x SSPE/0.1% SDS was carried out, this time at 55°C to increase stringency, with gentle rocking. The membrane was then placed back in the mini-blotter, though this time turned 90° from its previous position, with ink markers running horizontally. Care was taken to ensure that the first probe was fully exposed to all lanes, with ink lines running above the wells. After tightening blotter, any residual fluid was once more aspirated away.

The slots were then filled with 130µl of diluted PCR product (140µl 2x SSPE/ 10µl DNA) that had been kept on ice having been denatured. Again, care was taken to run each sample to the end of the membrane and to not introduce any bubbles. The entire blotter was then placed lying flat, into the hybridization oven for 45 minutes at 50°C, after which all PCR products were aspirated off and membrane was washed twice in 250ml 2x SSPE/0.5% SDS at 60°C with gentle rocking.

The oven was then cooled to 42°C and the membrane with the hybridized PCR products, placed into new Tupperware and allowed to cool to prevent inactivation of peroxidase. Once cooled, 5µl of streptavidin – peroxidase conjugate and 25ml 2x SSPE/0.5% SDS was poured over membrane and left to incubate in solution for 30 minutes at 42°C with shaking. The membrane was then washed twice in 2x SSPE/05%SDS for 10 minutes at 42°C to ensure removal of any excess streptavidin, and rinsed twice at RT with rocking in 2x SSPE to remove any SDS. Lastly, the membrane was incubated at room temperature in a small quantity of ready-to-use CPD-star detection liquid for 1 minute with hand shaking.

5.2.5.4 Developing the Membrane

Under infrared light, x-ray film (Amersham) was placed in the film cassette over the membrane and the top corner was folded, to once again aid with orientation. The cassette was firmly closed and left to expose for 15 minutes. After exposure, the film was developed under infrared light (developer and fixer made up to manufacturer guidelines) for between 1-2 minutes, until some results could be seen. This was then washed in a stop bath of water for around 30 seconds before fixing for 1 minute or until film became clear.

5.3 Results

A total of 670 ticks were collected from cattle at 17 separate grazing sites during the collection period of May 2014-March 2015. All ticks were confirmed as *I. ricinus* and comprised of; 529 feeding adult females, 134 adult males and 7 feeding nymphs.

The detail recorded during collections was primarily site; month of collection was also recorded for the majority of samples (558/670). Multiple sample tubes were collected and returned from each site and date recorded. There was no indication of cattle sample size, or if all ticks observed in one tube were from one, or multiple individuals.

Each of the 670 ticks were processed individually; results from the nested apicomplexan PCR showed 2 positive bands of around 600 base pairs (bp); the bands produced were faint and there was not sufficient amplicon for sequence data to be generated.

A. phagocytophilum was detected in 120 of these ticks using the partial *msp2* qPCR; due to financial restrictions of the project, these samples were not sequenced.

The 120 partial *msp2* fragments were observed in ticks removed from cattle at 15 of the 17 sites. 9 of these sites were sampled from twice and one site, 3 times. Samples collected from 2 of these sites were recorded as *msp2* positive in the first sampling period, with *msp2* not detected during the second sampling period.

Pooling the tick data together demonstrated a prevalence of 14.3%, 11.9% and 21.4%, in nymphs, adult males and adult females feeding on cattle, respectively (Table 27). There was a significant difference between the prevalence of *Anaplasma* observed in adult males and feeding adult females removed from cattle ($\chi^2 = 6.055$, df= 1, $P<0.05$).

B. burgdorferi was detected in 6 of these ticks using the 23S rRNA qPCR. 3 were adult males and 3 were feeding adult females; further delineation using the RLB confirmed 4 of the 6 ticks were positive for *B. afzelli.*

Pooling the tick data together demonstrated a prevalence of 0%, 2.2% and 0.6%, in nymphs, adult males and adult females feeding on cattle, respectively (Table x). There was no significant difference between the prevalence of Bb23S observed in adult males and feeding adult females removed from cattle (χ^2= 3.332, df= 1, P>0.05).

Table 27 showing *msp2* and Bb23S prevalence in feeding ticks removed from cattle

Sample	No. of samples	*Anaplasma* positive (%)	Exact Binomial 95% Confidence Interval (95%)	*Borrelia* positive (%)	Exact Binomial 95% Confidence Interval (95%)
***I. ricinus* adult male off cattle**	134	16 (11.9)	7.0-18.7	3(2.2)	0.4-6.4
Feeding *I. ricinus* adult female	529	113(21.4)	18.0-25.1	3(0.57)	0.1-1.6
Feeding *I. ricinus* nymphs	7	1(14.3)	0.4-57.9	n/a	n/a
Total	670	120(17.9)	15.1-21.0	6(0.9)	0.3-1.9

5.4 Discussion

The presence of *A. phagocytophilum* in the ticks removed from cattle was entirely expected. There appears to be a high prevalence in adult females, of 21.4%. Though this is not indicative of prevalence or infection status in the herd, as it is unknown how many cattle were sampled at each site and how many were found to be infested with ticks. Ticks were not collected from the environment in this study, though the prevalence observed in adult males was not subject to the same bias as feeding females, and are more representative of their environment.

The prevalence of *msp2* in adult males was 11.9%; given the prevalence of 3.4% observed in questing ticks in Chapter 4, and the lower prevalence generally observed in questing nymphs throughout Europe (Bown et al, 2009;(Overzier et al., 2013, Matei et al., 2015, Sormunen et al., 2016, Walker et al., 2001)) it would be reasonable to assume that nymphs are acquiring high levels of *A. phagocytophilum* infection when they take a blood meal, as adult males represent a fed nymph. Further to this, the prevalence observed in feeding adult females was significantly higher than in adult males, suggesting that cattle are amplifying *A. phagocytophilum.* This needs to be quantified, though cannot be done with current data.

For cattle to be playing a role in amplifying Anaplasma, the nymphal stage of the tick could be of ecological relevance, due to their much higher numbers. Also, adult females die once replete, whereas nymphs moult and take another blood feed. However, feeding preferences of the life stages may not implicate cattle as a main food source for the nymphal tick. Adult female ticks feed on cattle and a tick that was infected at nymphal stage may pass on infection during a blood meal, though an uninfected adult tick which becomes infected during its blood meal may result in a dead end for the pathogen, due to trasnsovarial trasnmission is thought not to occur (Stuen et al 2013).

Low numbers (7) of nymphs were removed from the cattle, providing an *msp2* prevalence of 17.9%. The low numbers could be indicative of life-stage feeding preferences, as the immature stages typically feed on smaller hosts; or it could be representative of collection bias by the Farmer, who may not have noticed nymphs during a quick inspection unless they were fully replete, due to their small, unengorged size (1-1.5 mm). Further work is needed to quantify the role nymphs may be playing in this system, including more thorough training of farmers or people involved in citizen science, to mitigate any bias towards the larger life stages.

Two of the ticks yielded amplicon product in the Apicomplexa- specific PCR assay, there was not sufficient amplicon for sequencing. Again, as cattle sample size was not recorded and it is unknown which individual which tick came from, prevalence cannot be accurately inferred. The results suggest that *Babesia* species were absent from 15 of the 17 sites which were sampled. However, all ticks collected during each period could have been removed from one disease free individual.

Moreover, as sequence data was not obtained, the samples could be containing non-pathogenic species to cattle, such as *B. venatorum* and *B. odocoilei*- like, as observed in Chapter 4.

6 of the ticks taken from cattle tested positive for *B. burgdorferi* using the Bb23S rRNA qPCR. These ticks were taken from five different sites, and so at least represented a sample size of five cattle. *B. afzelli* was confirmed via RLB, in 2 adult males and 2 feeding adult female *I. ricinus* ticks. There was no significant difference in the prevalence of Borrelia between males and females, though this is likely due to the small sample size.

The detection of *B. afzelli* in feeding adult females is of potential note, as cattle are not considered to be reservoirs for disease (Gray et al., 1992, 1995; Jaenson and Talleklint, 1992). Further to this, cattle complement was observed to kill all genospecies except *B. burgdorferi sensu stricto* (including *B. afzelli)* in laboratory studies (Kurtenbach et al, 1998). This suggests that the results from in vitro assays do not seamlessly translate into the field, and that further work is needed to quantify the role cattle may play in the circulation of Borrelia. This has also been documented following studies on red deer; in vitro studies demonstrated red deer complement killed all genospecies of *B. burgdorferi* (Kurtenbach et al, 1998), while field studies in the Netherlands demonstrated a *B. burgdorferi* prevalence of 0.7% in feeding female *I. ricinus.* However, as PCR only detects DNA; the feeding ticks could have been infected with Borrelia during a previous blood meal and cattle complement is indeed killing the parasite. This could be confirmed with serology on the cattle, though as cattle have not been implicated as a host for Borrelia previously, it is unlikely that they are hosts in this system.

B. afzelli is a small mammal associated genospecies of Borrelia, and is considered to be pathogenic to humans (Canica et al, 1993), demonstrating the potential veterinary, human and one health impact of future participation studies. The presence of a human pathogen in this study was of importance to the farmer, as he is at risk of contracting Lyme disease in the areas that he works. This was communicated to the farmer so that he was able to take necessary precautions, and benefit from the findings.

Following sample processing, the results were discussed with Farmer 4. He felt that the collection of ticks from cattle had been feasible for him to undertake whilst managing his herd and was happy to continue to do so. The level of detail recorded regarding number of cattle sampled and individual results was also discussed in depth. Farmer 4 agreed that collecting more detailed records was important for the development of the study, and was open to contributing to a more quantitative study.

The collaboration and partaking in participation science by Farmer 4 allowed for the sampling of 670 ticks removed from cattle, from 17 separate sits, located through the North West. Without the invaluable input of Farmer 4, this level of field work would have otherwise been outside the scope of this project. As field work is costly, time consuming, and requires meticulous planning. In addition, Farmer 4 agreed that his contribution- though necessary- was low input, and so did not affect his husbandry or working day; an attractive prospect when considering reaching out to engage potential future participants.

Though this study provided some thought –provoking insights, it is not without its shortcomings. The opportunistic sampling allowed for a large bias, with number of cattle surveyed unknown, limiting this study's capacity to quantify the observed results. However, discussions with Farmer 4 following the results of this study were able to highlight where the greatest changes should and could be made. Farmer 4 stated that he would be willing to continue collecting ticks feeding on his cattle at each of his sites. He also confirmed that as the input was low intensity, it was feasible for him to begin to keep more detailed records of his sampling; including keeping ticks removed from one animal in separate tubes with ethanol, and status of the animal regarding sex, breed and age. All of which have been demonstrated as factors associated with intrinsic disease susceptibility (Jaenson et al., 1992; Wilson et al., 1998).

Therefore, this study demonstrates the potential, and willingness, within participation science regarding the surveillance of tick borne diseases of veterinary and human health importance.

Chapter 6: General Discussion

6.1 Chapter summary

- **Chapter 2**
  - Questing *I. ricinus* ticks were found across Bowkerstead Farm, differing to anecdotal evidence. Though they did exhibit a patchy spatial distribution.
  - Habitat, rather than sheep grazing site, was found to be driving the tick population
  - Demonstrated the use of kriging to produce tick 'heat-maps' and their potential in complimenting tick control programmes.

- **Chapter 3**
  - Questing *I. ricinus* ticks were found to exhibit a patchy spatial distribution across Bethecar Moor
  - Proximity to a particular woodland site was found to be a significant predictor in tick density
  - Deer abundance was not found to be a significant predictor in tick density
  - Tick burden was not equal for all sheep of the same age, sex and breed
  - Farm was found to be an indicator for tick burden; suggesting differences in husbandry or breeds
  - Improvement of record keeping and co-ordinating acaricides reccommended

- **Chapter 4**
  - High prevalence (3.3%) of *A. phagocytophilum* in questing ticks
  - Relatively low prevalence (4.9%) *A. phagocytophilum* in deer
  - Higher prevalence of *A. phagocytophilum*, observed in questing ticks in farmland than in woodland
  - No *B. divergens* observed
  - No geographic clustering of pathogens or ecotypes observed
  - Multiple *msp4* alleles observed circulating
  - *B. venatorum* observed in the UK for the second time
  - *B. odocoilei*-like and sister clade *Babesia* sp. OO-2012 observed in the UK for the first time

- **Chapter 5**
  - Use of citizen science to sample a large study area
  - No *B. divergens* observed
  - High prevalence of *msp2* in feeding males removed from cattle, indicating *Anaplasma* prevalence of the environment
  - *B. afzelli* observed in feeding ticks removed from cattle

6.2 Major findings

The major objective of this thesis was to further understand the spatial distribution of questing *Ixodes ricinus* ticks across farms in Southern Cumbria; as well as to attempt to understand the epidemiological and ecological factors that have a significant influence on the patterns of infections in livestock. Key to understanding and resolving these complex ecological issues involved a multidisciplinary approach, embracing ecology-based field work, molecular methods; such as genotyping, and exploiting GIS.

Though the project was able to provide an insight in to the complex ecology and epidemiology of tick borne disease in livestock, it was not without its shortcomings. To allow the surveying of multiple sites within the financial and time constraints of the project, repeat sampling was not feasible; particularly across Bethecar Moor. Though the disadvantages of blanket dragging as a proxy to infer tick densities without repeat sampling have been documented (Dobson, A. 2013), it was outside the scope of this project.

Ticks were removed from sheep, as well as the areas they graze; GLM output identified Stock Farm as a variable influencing tick burden on sheep. The differences between the sites represented different breeds of sheep; Swaledales at Stock Farm compared to Texel's at Abbot Park, as well as the different husbandry employed by each farmer. Previous studies have demonstrated breed-association resistance to tick infestation in cattle (Wambura et al, 1988) and sheep genetics have recently been implicated in tick burden in Norway (Sae-Lim et al, 2017).

In addition to varying infestation levels between sites, there was also variation in tick burden in sheep from the same site. Site was also an indicator of breed and subsequently husbandry system; this suggests that tick burden could be a result of where individual sheep graze on the moor (their exposure rate), or due to some intrinsic susceptibility. This could be as a result of underlying illness, co-infection, or even breed resulting in an increase in susceptibility to tick bites and/or the pathogens carried.

The study demonstrated a high prevalence of *msp2* observed in ticks removed from sheep and ticks collected via Blanket dragging from grazing. Whereas a lower prevalence was observed in deer bloods and ticks taken from woodland, these data suggest that the sheep are the driving force behind circulating the pathogen. The higher tick densities in closer proximity to woodland could mean that sheep are involved in maintaining the tick population, but that only certain areas of their grazing fields have habitat suitable for tick survival.

While the GLM did not indicate deer as a driver for tick density, previous studies have demonstrated that they are (Gray et al., 1992);(Ruiz-Fons and Gilbert, 2010).

Further to this, there appears to be some correlation between the map layers. While the methods used here extracted pixel values for multivariate analysis in GLM's, it would be worth while exploring spatial statistics to see if any correlations can be inferred between the generated layers.

While they were tested for, the absence of *B. divergens* and Louping Ill virus is notable, as anecdotal evidence suggested they were present in the area.

The two questing ticks that were confirmed positive for Babesia by sequencing were both from Bowkerstead Farm. As a severe human pathogen, and potential emerging disease, the discovery of *B. venatorum* in a new area of the UK is of potential medical importance. The second questing tick was confirmed to be carrying strain *Babesia* sp. OO-2012, and the third was *B. odocoilei*, to the best of my knowledge, this is the first recorded incidence of these pathogens in the UK.

The detection of *B. afzelli* in feeding adult females feeding on cattle is important, and a good example of the mutual beneifts that can be acheived via citizen science; the detection of the human pathogen was of importance to the farmer, as he is at risk of contracting Lyme disease in the areas that he works, from a research perspective, hundreds of samples were collected by the farmer which I subsequently processed, something that otherwise would have been outside the scope of this project.

6.3 Further work

This study has provided a basis for understanding the threat posed to livestock by tick borne diseases in Southern Cumbria. The methods used here, could be used to map tick densities across the UK.

Due to time and financial resource restraints, it was not feasible to repeat this survey within the timescales of this project. If it were possible, it would be prudent to resample each of the drag points over time and several tick seasons. As shortcomings with blanket dragging, and without repeating on a three weekly basis, have been documented (Dobson, A. 2013)

One obvious extension of this study would be to obtain a license from the Home Office to collect blood samples from the animals at the study sites. This could then be utilized to infer the efficacy of using feeding ticks taken of sheep as a natural syringe and a means of xenodiagnoses. Building on previous studies (Ogden et al., 2002, Ladbury et al., 2008, Stuen, 2013) and comparing *msp4* alleles

observed in ticks taken from the environment, ticks taken from sheep, sheep bloods and deer bloods.

The results obtained in this study allowed for multivariate analysis and the fitting of GLM's to identify the drivers behind tick densities. Though this has been a useful tool, this could be further built upon using a mechanistic approach based upon mathematical modelling. This is likely to offer much greater insight into potential transmission processes. The motivation behind this work was to identify critical control points at which transmission to livestock could be perturbed; mathematical models could also be used to simulate a variety of control strategies and could be linked to economic models.

One objective that was not met in this study was the tracking of individual sheep movement to determine exposure rate to ticks and the diseases they carry. Based on the data that was collected in this study, the methods applied here could be easily replicated and used to provide a more insightful dataset, given more robust and superior equipment.

Collection of more detailed host movement data could then be used within more advanced spatial statistics. Further work could use this data as part of a coupled map lattice, dividing grazed moorland in to squares and running a series of ordinary differential equations in each testing a different hypothesis, with movement between the squares generated from the collar data.

In addition to advancing the tick density aspects of the project, further work could be done involving *A. phagocytophilum.* The results generated in this study demonstrated no spatial clustering of observed *msp4* alleles, including those which belonged to a divergent strain. This is synonymous with other studies (Jahfari et al., 2014) (Bown et al., 2009) (Ogden et al., 2002) (Ladbury et al., 2008), though not all studies utilize the *msp4* fragment. One area which would be interesting to develop would be the usage of the haplotype viewer used by Jahfari (2014) to analyse the sequences generated in this study and to see if the *msp4* alleles observed here, clustered in the same way as the *groEL* alleles observed in Europe (Jahfari et al. 2014).

Chapter 5 demonstrated the potential of using citizen science as a way to collect large field samples without the need for labour intensive field studies. While there were a large number of ticks collected by the farmer in this study (670), the level of detail recorded could definitely be improved upon. Discussions with the farmer, confirmed that he would be willing to partake in this, which could help in the surveillance of tick borne diseases across a large area in the north west of England. The methodology employed here, could be used anywhere that there are willing participants.

Incorporating the testing for pathogens of importance to human health, was attractive to the farmer in this study, ensuring his participation

The flexibility of this study and the potential to incorporate the testing of many pathogens of interest, with limited field work, is an exciting prospect; with the potential to contribute invaluable information to the science and improve our knowledge and understanding within the 'one health' sphere.

6.4 Concluding remarks

During this thesis I have successfully planned and carried out multiple field work visits to various sites in southern Cumbria to quantify the presence of ticks. I have then used this empirical data in conjunction with GIS to produce 'heat-maps' at a fine scale, to individual grazing plots.

This has provided farmers with an easily interpreted tool for visualising tick densities, and therefore environmental hazard, across their grazing land. Through the use of multivariate analysis, I have been able to identify drivers for these tick populations at each site and discuss them with the farmers and how this information could be translated in to efficient control strategies, such as targeted vegetation management, co-ordinated acaricide programmes and boundary management.

There was a large knowledge transfer between the farmers, FC Rangers and myself; which was paramount to the success of this project. Illustrating the potential power of utilizing Citizen Science within a farming setting. Discussions with the farmer's with grazing rights to Bethecar Moor provided and invaluable insight to the husbandry and efforts undertaken to minimise the threat of ticks. It was surprising to me that there was no co-ordinated approach between the farmers, or that losses were not recorded and shared. Though the politics of subsidies via stewardship schemes was a delicate subject, which did not necessarily result in harmony between grazers. It is my belief that a co-ordinated acaricide programme would help with the issue of ticks. In addition, the accurate recording and sharing in losses would be valuable; as Farmer 3 does not vaccinate against Louping Ill virus, nor was it observed in any of the ticks collected and tested, it may be prudent for Farmer 2 to stop vaccinating his flock and save funds. However, the local view is that the pathogens circulate every 7 years and it may be difficult to get farmer 2 to change his husbandry methods so drastically, based on a 3 year study.

I have also quantified the tick borne diseases circulating in ticks at these sites using molecular methods and demonstrated that *A. phagocytophilum* is endemic. I have argued that sheep are the main drivers for the pathogen within this system and that deer abundance does not appear to be driving the pathogens circulating, or the general tick population. Though tested for, I did not find *B. divergens* or Louping Ill virus, despite anecdotal reports suggesting their high incidence in the area.

I have also found *B. afzelli* in ticks feeding on cattle, and *B. venatorum* in deer bloods highlighting the importance of a one health approach when considering disease ecology and epidemiology.

The integration of field work, GIS, molecular methods and citizen science, with the truly multidisciplinary nature of this project, has demonstrated the need for a holistic approach when considering the ecology and epidemiology of tick borne disease; whilst contributing novel research and findings to the scientific community.

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