Investigating the presence of tick-borne encephalitis virus in the United Kingdom

by

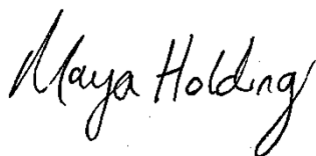Maya Holding

March 2021

Authors Declaration

I declare that the work in this thesis was carried out in accordance with the regulations of the University of Liverpool. The work is original except where indicated by special reference in the text and no part of the dissertation has been submitted for any other degree.

Louping ill virus hemagglutination inhibition (LIV HAI) assays (included in Chapter 2) were performed by Moredun Research Institute. Genomic sequencing (included in Chapter 2 and Chapter 3) was performed by colleagues in the Public Health England (PHE) Genomics group and phylogenetic tree constructed by Roger Hewson, PHE Virology & Pathogenesis group. Technical assistance (Chapter 2 and Chapter 3) was provided by colleagues in Virology and Pathogenesis group. Colleagues PHE Medical Entomology group assisted with some tick collections (Chapter 3). Individuals are acknowledged in the acknowledgement section.

Any views expressed in the thesis are those of the author and in no way represent the University of Liverpool.

The thesis has not been presented to any other University for examination either in the United Kingdom or overseas.

Signed: *Maya Holding*   Date: 31 March 2021

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Abstract

Louping ill virus (LIV), a member of the tick-borne encephalitis virus (TBEV) serocomplex, had been the only known zoonotic tick-borne flavivirus endemic to the UK. LIV is closely related to and serologically reacts with TBEV, which is much more pathogenic to humans; causing around 3,000 cases in the European region annually. TBEV is endemic across much of Europe, where it is increasing in range, but was thought to be absent from the UK. It is important to understand the potential for TBEV to be emerging in the UK undetected.

A large-scale sentinel deer serosurveillance study was conducted to identify regions of exposure to TBEV-serocomplex pathogens. Serum samples were tested for specific antibodies to TBEV as a measure to indicate exposure. In addition, submitted tick samples removed from deer, close to an ELISA positive sample, were tested for TBEV and LIV RNA by RT-PCR. Overall, 4% of samples were ELISA-positive for the TBEV serocomplex. The Thetford Forest area in England had both the highest proportion (47.7%) of seropositive samples, and importantly no previous reports of LIV infection in livestock which could cross-react with the TBEV serology assays. Of 2,041 tested ticks from areas near seropositive deer, five were positive by TBEV/LIV RT-PCR, all within the Thetford Forest area. From 1 tick, a full-length genomic sequence of TBEV-Eu was identified.

A two-year extensive ecological study collecting questing ticks was conducted in sites associated with high rates of exposure to TBEV-serocomplex virus. A total of 7,085 questing ticks were collected in Thetford Forest, with TBEV being detected in 6 sites out of 24 sites surveyed over 2018 and 2019. In addition, 3,205 questing ticks were collected and tested from 7 sites in the New Forest and bordering areas, resulting in the detection of TBEV in one site on the Hampshire/Dorset border.

These results demonstrate that TBEV has been detected in the UK for the first time - in two geographically distinct locations 200 miles apart. High seropositivity and presence in questing ticks suggests it is being maintained in enzootic cycles in these areas. This finding is of public health significance and requires rapid dissemination of findings to health professionals in the UK.

Contents

Abbreviations

Abbreviation	Description
A&O	Ancient and ornamental (woodland)
ABLC	Slowly permeable seasonally wet slightly acid but base-rich loamy and clayey soils
APHA	Animal and Plant Health Agency
AWF	Ancient woodland stands
B	Broadleaved
BABS	Bovine serum albumin in borate saline
BDS	The British Deer Society
BPT	Breakpoint temperature
C	Conifer
CCHFV	Crimean Congo haemorrhagic fever
CI	Confidence intervals
CNF	Central nervous system
Ct	Cycle threshold
CWD	Chinese water deer
DIN	Density of infected nymphs
DON	Density of nymphs
ELISA	Enzyme-linked immunosorbent assay,
FC	Forestry Commission
FDSAL	Freely draining slightly acid loamy soils
FDSAS	Freely draining slightly acid sandy soils
FDSB	Freely draining sandy Breckland soils
FDVASL	Freely draining very acid sandy and loamy soils
GGEV	Greek goat encephalitis virus
GPS	Global Positioning System
HAI	Hemagglutination inhibition assay
HRS	Home range size
IgG	Immunoglobulin G
IgM	Immunoglobulin M
kb	kilobases
LI	Louping ill
LIV	Louping ill virus
MIR	Minimum infection rate

MMB	Mixed mainly broadleaved
MMC	Mixed mainly conifer
NCA	New Forest National Character Area
NCVC	National Vegetation Classification
NHS	National Health Service
OS	Ordnance Survey
PCR	Polymerase chain reaction test
PHE	Public Health England
PRNT	Plaque reduction neutralisation test
RBCs	Goose red blood cells
RH	Relative humidity
SAC	Special Areas of Conservation
SALCID	Slightly acid loamy and clayey soils with impeded drainage
SAT	Saliva activated transmission
SD	Saturation deficit
SGEV	Spanish goat encephalitis virus
SLOCL	Shallow lime-rich soils over chalk or limestone
SSEV	Spanish sheep encephalitis virus
SSSI	Site of Special Scientific Interest
STANTA	Stanford Training Area
TBE	Tick-borne encephalitis
TBE-Him	Himalayan TBEV subtype
TBEV	Tick-borne encephalitis virus
TBEV-Bkl	Baikalian TBEV subtype
TBEV-Eu	European TBEV subtype
TBEV-FE	Far Eastern TBEV subtype
TBEV-Sib	Siberian TBEV subtype
TSEV	Turkish sheep encephalitis virus
UTR	Untranslated region
VIEU/ml	Vienna units/ml
WVASL	Naturally wet very acid sandy and loamy soils

Chapter 1: Introduction- tick-borne viruses of the UK and Europe that pose greatest risk to the UK human population.

1.1: Ticks

Ticks (Ixodida) are blood sucking arthropods that are found across much of the world and are important vectors of pathogens to both humans and animals (Pfäffle et al., 2013). There are three families within the Ixodida suborder, namely Ixodidae (hard ticks), Argasidae (soft ticks) and Nuttalliellidae, with over 700 species, approximately 200 species and one species, respectively (Baneth, 2014; Dantas-Torres et al., 2012).

In the UK, the most common tick species is *Ixodes ricinus* although at least another 19 tick species are also endemic (Medlock and Leach, 2015). *I. ricinus* is of great medical and veterinary importance, transmitting a large number of pathogens; it has a very broad host range, feeding on mammals, birds and reptiles. As a three host tick, *I. ricinus* feeds on a different host in each parasitic stage, maximising opportunities to transfer pathogens between hosts and ticks (Pietzsch et al., 2008; Labuda and Nuttall, 2004). Humans are incidental hosts of ticks, with most cases of human tick bite relating to the Ixodidae family. The focus will be on this family, as they pose the greatest risk to public health in the UK (Dantas-Torres et al., 2012).

1.1.1: Tick lifecycle

The typical lifecycle of an ixodid tick follows three distinct life stages with a blood meal between each active life stage, as shown in *Figure 1:1*. Eggs hatch into larvae, which moult to nymphs and then to adult males or females. At the time of their third and final feed as part of the life cycle, the adult female ticks will mate with an adult male; mating occurs while attached to the host, after which females a take blood meal then drop off and find a suitable place to lay thousands of eggs in vegetation (Estrada-Peña and de la Fuente, 2014).
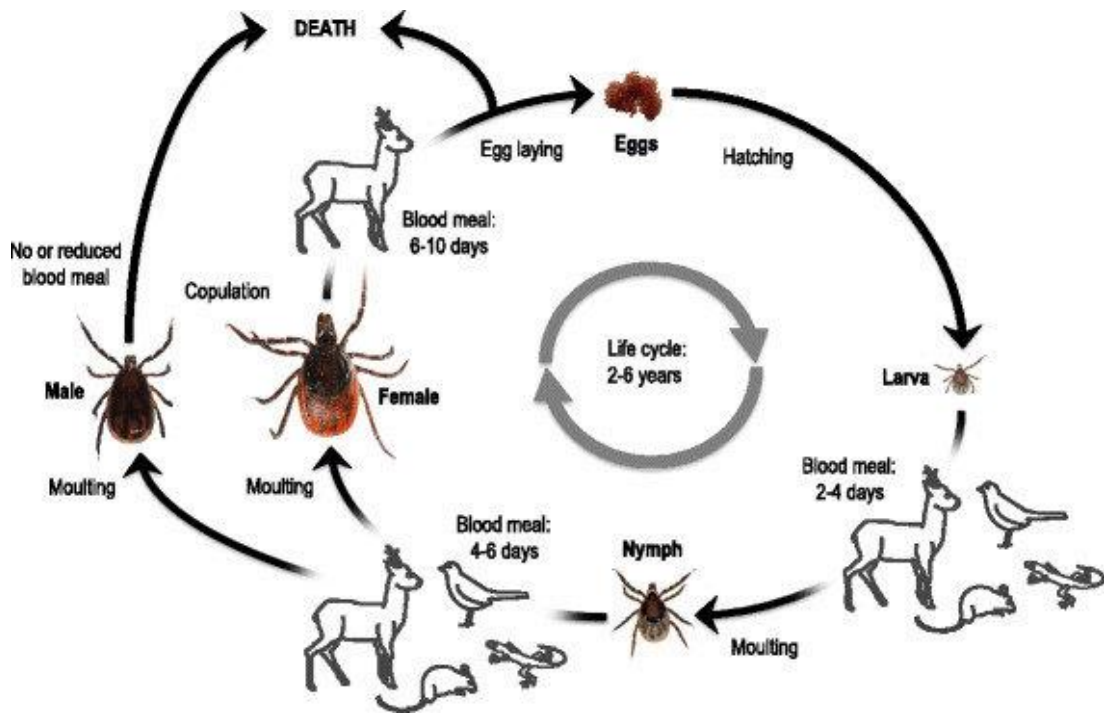
Figure 1:1: *Ixodes ricinus life cycle. Extracted from Herrmann and Gern, 2015* (Herrmann and Gern, 2015)

Feeding behaviours vary between different species of ixodid ticks, some being one host ticks, remaining on the same host through all three life stages such as *Rhipicephalus decoloratus* (Estrada-Peña and de la Fuente, 2014; Estrada-Peña et al., 2004). Other species are two host ticks; such as the medically important *Hyalomma marginatum and Hyalomma rufipes* (Chitimia-Dobler, Schaper, et al., 2019); they remain on one host as larvae and nymphs then following engorgement, the nymph drops off and locates a second host once moulted to an adult(Flick, 2007). Three-host ticks which are most common in Europe, such as *I. ricinus,* will feed on a new host during each life stage; these ticks are particularly effective as vectors of pathogen transmission due to this feeding behaviour (Jongejan and Uilenberg, 2004; Labuda and Nuttall, 2004).

It can take as little as one year but up to six years for *I. ricinus* to complete their lifecycle, the timing being dependent on environmental conditions (Labuda and Nuttall, 2004). The behaviour of *I. ricinus* is strongly influenced by seasonality; those in more extreme climates having more restricted periods of activity. The biological process of diapause results in minimal development during winter months, due to low temperatures and reduced photoperiod (Medlock et al., 2013).

The combination of lengthening daylight hours and rising spring temperatures then trigger synchronised development of moulting ticks, resulting in the emergence of large numbers of active ticks. This is the seasonal cycle of the *I. ricinus* in the UK, enabling them to maximise their success in seeking a host, feeding and moulting when conditions are optimal (Estrada-Peña and de la Fuente, 2014). In hotter climates there is not the same seasonal requirement to pause life-stage development, leading to faster maturation, providing there is sufficient access to a humid environment which is critically important to survival (S.E. Randolph, 2004).

1.1.2: The impact of climate and climate change

Following a period of questing, *I. ricinus* need to rehydrate by returning to a humid base layer of vegetation. A factor in determining the habitats in which *I. ricinus* can survive is its need for relative humidity levels of at last 80% (Gray et al., 2009). In consequence tick lifecycles are inherently sensitive to climate changes, impacting survival of individual ticks, duration of development and host-seeking activity (Medlock and Leach, 2015). Tick sensitivity to climate change, in terms of mild winters and warmer springs can reduce the period of diapause and extend the period of questing, leading to an expansion of the *I. ricinus* population (Medlock et al., 2013), and thus increased risk of exposure to tick bite to the general public. In the UK, endemic populations of *I. ricinus* already occur from the SW tip of Cornwall (no records on Isles of Scilly) up to Orkney (not thought to be established on Shetland) (Medlock and Leach, 2015). However further afield, climatic changes are impacting expansion in geographic range at high latitudes with cold winter temperatures (Hvidsten et al., 2020; Gray et al., 2009).

1.2: Ticks as vectors of disease

Ticks transmit a great number of pathogens, being important vectors of viruses, bacteria including rickettsia, helminths, fungi and protozoa, to both humans and animals (Baneth, 2014; Dantas-Torres et al., 2012; Heyman et al., 2010; Jongejan and Uilenberg, 2004). *Borrelia burgdorferi sensu lato* genospecies complex*,* the causative agent of Lyme disease, is endemic in the UK, much of Europe and other parts of the Northern Hemisphere such as the USA and northern Asia (S. E. Randolph, 2004). It is the most common tick-borne pathogen to cause disease in

humans in Europe, including the UK, with recent UK estimates suggesting it causes 1.95 cases per 100,000 (Tulloch et al., 2019). *I. ricinus* transmits *B. burgdorferi* s.l. and a wide range of other pathogens that can cause disease in humans. These include *Anaplasma phagocytophilum,* which can cause human granulocytic anaplasmosis and *Babesia* spp. (such as *Babesia divergens* and *Babesisa microti*), which can cause human babesiosis (Heyman et al., 2010). In addition, *Coxiella burnetii* which is the causative agent of Q fever, *Francisella tularensis* causing tularemia and *Rickettsia* spp., from the spotted fever group infections such as *Rickettsia helvetica* (WHO, 2004) can be transmitted by *I.ricinus*. In recent decades some microorganisms that had been detected in *I.ricinus* have subsequently been found to also cause human disease, such as *Neoehrlichia mikurensis* and *Borrelia miyamotoi* (Springer et al., 2020).

The medical importance of tick-borne diseases has continued to increase in recent decades, due to the increasing geographical range of tick-borne pathogens and growing incidence of disease in humans (Estrada-Peña and de la Fuente, 2014). These increases have been facilitated by increased globalisation, with goods, people, animals and plants being moved around the world, with arguably insufficient control, creating new opportunities for the spread of vectors and pathogens. Factors affecting the success of invasive pathogens are complex but include climate change and environmental issues that together are contributing to changing patterns of vector borne disease distribution (Medlock and Leach, 2015; Gould et al., 2006).

The maintenance and transmission of tick-borne pathogens is complex, involving the vector, pathogen and host. Some pathogens are maintained throughout their lifespan, being transstadially (horizontally) transmitted through the moult between each life stage of the tick. Some pathogens can be transovarially (vertically) transmitted from adult female ticks to offspring, albeit at a low transmission efficiency; this can still be an important transmission route between ticks (Brackney and Armstrong, 2016; Labuda and Nuttall, 2008). The ability of a virus to be transmitted either horizontally or vertically is key to a tick species being a competent vector (Gaff and Gross, 2007).

1.3: Louping ill virus

1.3.1: Virology

LIV is in the Family *Flaviviridae* genus *Flavivirus,* closely related to tick-borne encephalitis virus (TBEV), both being part of the TBEV serocomplex. Louping ill virus (LIV) is the only zoonotic tick-borne virus isolated from ticks in the UK (Marriott et al., 2006). The LIV genome consists of a single-stranded positive-sense RNA molecule, approximately 11 kilobases (kb) in length. It was the first arbovirus isolated in Europe, found in Scotland in 1929 (Jeffries et al., 2014; Greig et al., 1931). LIV has a typical flaviviridae conserved genome structure, made up of a structural capsid, pre-membrane, envelope and 7 non-structural genes. The envelope protein is a primary target of neutralising antibodies, which also exhibits antigenic cross-reactivity with the envelope proteins of other tick-borne flaviviruses such as TBEV (Jeffries et al., 2014).

Four LIV subtypes are present in the British Isles, with <5% nucleotide divergence within the envelope coding sequence; these are characterised by the molecular phylogenetic patterns of their envelope gene: genotype 1 (Scotland and England), genotype 2 (Scotland), genotype 3 (Wales) and genotype 4 (Wales) (Jeffries et al., 2014). In addition, further variants of LIV-type virus that cause disease in sheep and goats are present, these are Spanish sheep encephalitis virus (SSEV), Spanish goat encephalitis virus (SGEV), Turkish sheep encephalitis virus (TSEV) and Greek goat encephalitis virus (GGEV) (Clark et al., 2020).

LIV in the British Isles represents the far western range of the TBEV serocomplex of related virus distribution. Molecular clock analysis suggests that LIV was first introduced to Ireland over 800 years ago and divergence occurred over the last 300 years (Jeffries et al., 2014). Genetic diversity within LIV appears to be low sharing around 98% mean amino acid identity and around 96% nucleotide identity. LIV has less genetic variability than the European TBEV subtype (TBEV-Eu) which has higher variability within the 3' Untranslated region (Clark et al., 2020).

1.3.2: Geographic distribution

Louping Ill virus (LIV), which mostly causes disease in sheep and red grouse (*Lagopus lagopus scotica*), is predominantly found in the British Isles, having a very

limited distribution; however, LIV or LIV-like viruses have been reported in Turkey, Norway, Greece, Spain, Denmark, Bulgaria, the Russian Far East and Japan (Clark et al., 2020; Balseiro et al., 2013; Ytrehus et al., 2013; Dobler, 2010; Randolph and Rogers, 2006; Skarphédinsson et al., 2005; Anon, n.d.). LIV was first isolated in Selkirkshire (Scotland) in 1929 (Jeffries et al., 2014; Greig et al., 1931); however much of the distribution of LIV within the British Isles, and further afield, links with the history of sheep farming and livestock movements over the last 300 years (Jeffries et al., 2014; Randolph and Rogers, 2006). For example, the Negishi (Japanese) and Primorye (Russian Far East) strains both are linked back to a single British LIV subtype, suggesting that there was a single introduction to the Far East likely after 1860, possibly through livestock movements during World War I or II to Primorsky Krai (Russia) (Clark et al., 2020; Anon, n.d.). In Norway, LIV was first isolated on the southern coast in 1978 in goats, and 1982 in sheep. Interestingly sheep were exported from Britain to Norway during the 19th century which may explain the possible route of introduction (Ytrehus et al., 2013; Randolph and Rogers, 2006).

LIV tends to be found in upland grazed areas of the British Isles in which *I. ricinus,* the natural vector, and suitable hosts are both abundant. Within the British Isles LIV has been more commonly reported in Ireland, Scotland and Wales, with more localised distributions in England, mainly in areas of upland sheep grazing in northern England and the south west (*Figure 1:2*) (Jeffries et al., 2014). The surveillance of the distribution of LIV in the UK is limited to voluntary submissions of symptomatic livestock, with no nationwide surveillance studies conducted in recent years.

1.3.3: Clinical disease in humans

Humans are incidental hosts for LIV and infection can occur without illness. Serological surveys carried out in the mid to late 20th century found that between 8 and 18% of tested abattoir workers were positive for LIV (Davidson et al., 1991). Although rare, approximately 45 clinical cases causing potentially fatal encephalitis of the central nervous system have been reported in the UK since 1934; this is reflected in its Advisory Committee on Dangerous Pathogens (ACDP) classification

of Hazard Group (HG) 3 (Jeffries et al., 2014; Health and Safety Executive., 2013; Walkington et al., 2013). No confirmed cases have been reported in the last 20 years; however, a suspected case of fatal Louping Ill (LI) disease was recorded in 2013 (Jeffries et al., 2014; Walkington et al., 2013). Unlike the majority of zoonotic tick-borne diseases, LI cases have tended not to be as a result of tick bite, but predominantly as a result of occupational exposure to the virus in a laboratory setting or to infected livestock; with veterinarians, stockmen, abattoir workers and butchers being at risk (Davidson et al., 1991).

Just over half of patients with LIV infection experience a single febrile phase of illness which tends to resolve within a week, characterised by influenza-type symptoms with fever, muscle stiffness, headache, dizziness, and anorexia. Approximately 48% of patients experience a more serious biphasic illness, in which following the initial febrile stage, a brief period of resolution occurs followed by an encephalitic phase. This encephalitic phase is characterised by severe headache, stiffness of the neck, tremor of the head and limbs, drowsiness, vomiting and fever (Davidson et al., 1991; Davison and Neubauer, 1948).

Up to 60% of encephalitis cases in the UK are of unknown cause (Kennedy et al., 2017). It has been hypothesised that some LI cases may have gone undiagnosed, particularly considering the lack of awareness of the disease by medical professionals and the resultant lack of testing (Jeffries et al., 2014).
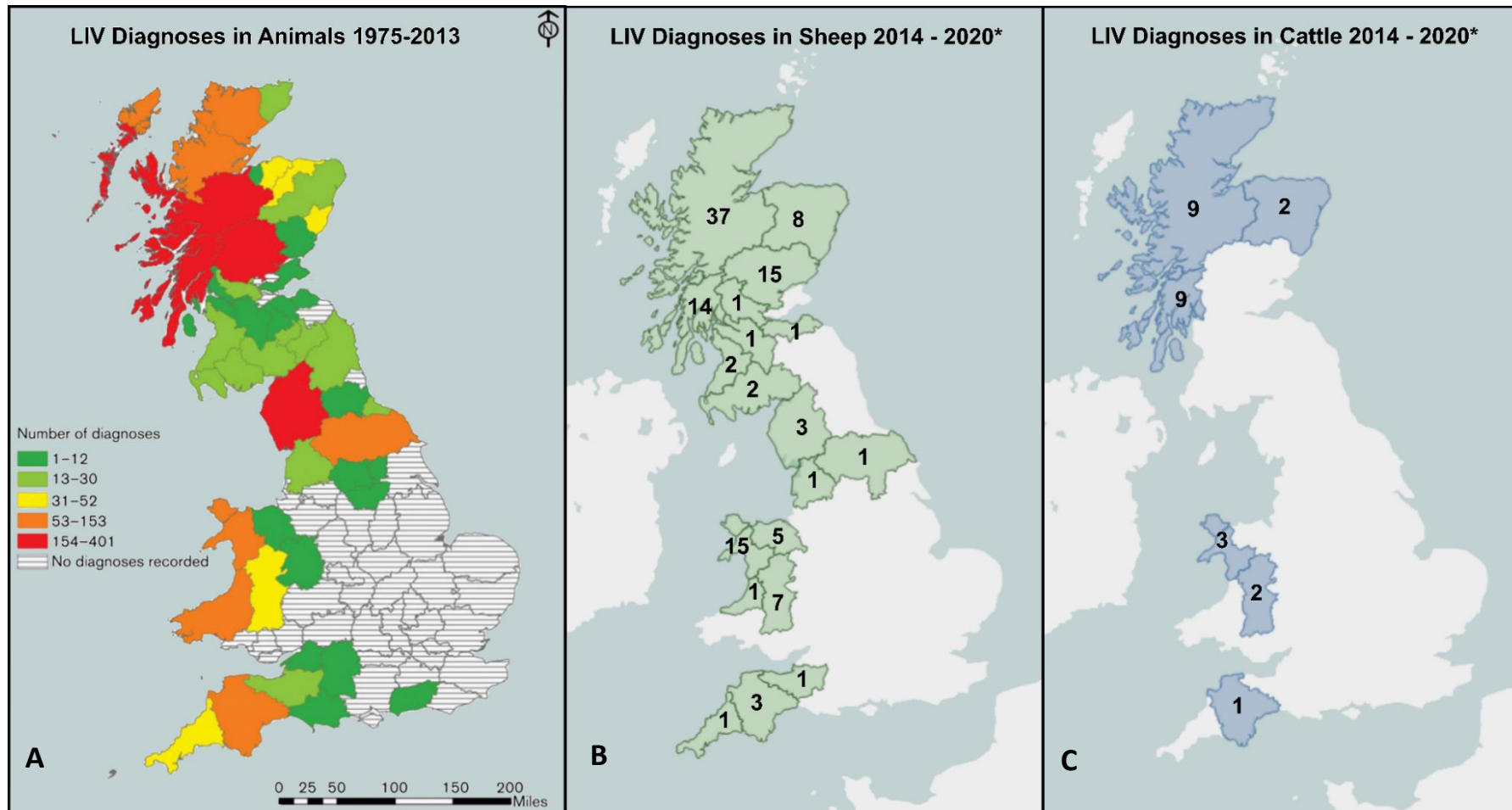
Figure 1:2: *A) Animal LIV diagnoses in Great Britain by county. 1975-2013 map adapted from Jeffries et al. 2014* (Jeffries et al., 2014).*B) 2014-2020 display number of diagnoses adapted from GB Cattle Disease Surveillance Dashboard and GB C) Sheep Disease Surveillance Dashboard*(Animal & Plant Health Agency; Scotland's Rural College, 2020b, 2020a). **Data extracted 25 Nov 2020*

1.3.4: Clinical disease in animals

LIV is named in reference to the uncoordinated gait produced in diseased sheep which is derived from an old Scottish phrase 'to loup' which means 'to leap'(Buxton and Reid, 2017). LIV predominantly causes disease in sheep, red grouse (*Lagopus lagopus scotia)* and cattle; however, clinical disease has also very occasionally been reported in goats, horses, alpacas, llamas, pigs, roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), hare (*Lepus europaeus*) and a pine marten (*Martes martes*) (Summers, 2018; Jeffries et al., 2014; Macaldowie et al., 2005). Sheep, red grouse and mountain hare (*Lepus timidus*) are considered the natural transmission hosts of the virus (Laurenson et al., 2003).

1.3.4.1: Red grouse (Lagopus lagopus scotica*)*

In areas where LIV is endemic, mortality of up to 80% can occur in red grouse (Moseley et al., 2007). They can be infected through the bite of an infected tick, or through ingestion of infected ticks whilst grooming. It is thought that the latter may be an important route of infection (Zintl et al., 2017), with clinical disease and rapid death occurring 5-7 days after infection (Moseley et al., 2007). In addition to red grouse, high mortality as a result of LI infection also occurs in other upland moorland/tundra species such as the rock ptarmigan (*Lagopus mutus*). Conversely, species that tend to inhabit woodland, such as capercaillie (*Tetrao urogallus*), black grouse (*Tetrao tetrix*) and ring-necked pheasant (*Phasianus colchicus*) are known to experience sub-clinical infection. Therefore, moorland/tundra species appear far less than species that inhabit woodlands to adapt and generate an effective immune response (Buxton and Reid, 2017; Gilbert, 2016). Evidence suggests that LIV is likely to have only moved from an ancestral woodland TBE-like virus around 400 years ago, around the time that extensive sheep farming started in the Scottish Borders and Northumberland (Buxton and Reid, 2017). Therefore, it could be hypothesised that moorland/tundra species are still adapting to being relatively new hosts of LIV.

1.3.4.2: Sheep

A disease of sheep causing LI has been known for over 200 years, the specific cause was unidentified for over a century; both the etiological agent and the transmission of this from *I. ricinus* to sheep were identified in 1931 (Dobler, 2010). Even before

the identification of the etiological agent, shepherds had identified that the disease in sheep was limited to specific pastures, with disease more often occurring in March and April (Buxton and Reid, 2017). In historic records from the 1930s, infected and 'clean' pastures are often just a short distance apart even when within similar habitats. Farmers found despite their farms lying in a similar habitat, within a 12km radius, some were 'clean' with disease occurring if sheep were moved from these clean farms to land where ticks were prevalent. It was also noted that farms at the top of watersheds seem to be 'clean' (Leiper et al., 1933).

Lambs acquire humoral immunity from their mother's colostrum (first milk) and subsequent maternal milk feeds that produces comparable antibody titres in the young, which is maintained for the first year (Davidson et al., 1991; Saunders, 1948). Following this, they are then susceptible to infection, with disease tending to occur in weaned lambs and yearlings; however, the highest mortality is in mature sheep naive to the virus. Sheep that have been reared on 'clean' pastures, who are naive to LIV and subsequently moved to 'affected' pastures during periods of tick activity are prone to developing disease, suffering high levels of mortality (Buxton and Reid, 2017; Davidson et al., 1991; Leiper et al., 1933; Pool et al., 1930). This tends to occur in rams brought in to improve stock or when naive flocks are moved, for example when farms change hands (Pool et al., 1930). This is illustrated by the stark statistic of a mortality rate of 60% in introduced stock, which is reduced to 5-10% in those reared on infected pastures (Zintl et al., 2017). The period in which heaviest losses occur fluctuates to some extent from year to year, but generally falls between the middle of March to the middle of May. A second period of disease occurs in autumn in some areas such as the Western Highlands; however, this is less severe than in spring/early summer (Leiper et al., 1933; Pool et al., 1930).

Whilst many infections in sheep are sub-clinical, most clinical infections are fatal (Moseley et al., 2007; Davidson et al., 1991). Disease follows an 8-13 day incubation period (Jeffries et al., 2014). The disease tends to be bi-phasic, with a primary viraemic febrile stage, in which titres are sufficient to infect ticks for 2-3 days. Symptoms in this phase are 'dullness' and fever, with this phase frequently going unnoticed by farmers. The second encephalitic phase is when the virus enters the

central nervous system (CNS) when symptoms are overt; however, once this stage is reached the disease is usually fatal (Zintl et al., 2017; Davidson et al., 1991). In this second stage, depression, nibbling and panting initially occurs followed by uncoordinated movement, muscle tremors, particularly of the head, salivation and circling which lasts from a few hours to two days (Jeffries et al., 2014; Davidson et al., 1991; Pool et al., 1930). This is followed by further deterioration in which the animals are unable to stand, being recumbent on one side and unable to change position but able to vigorously kick, followed by coma and death (Pool et al., 1930).

There remains no treatment for LI and a previously available effective vaccine for sheep is now no longer available (Zintl et al., 2017). Therefore despite great advances in knowledge about the disease, ecology and vector control, including a long period of vaccination of sheep over the last century, LI is still an important disease resulting in losses of sheep, cattle and red grouse in many upland areas (Jeffries et al., 2014). Although work has been carried out studying LIV prevalence in animal hosts using serology, as discussed by Jeffries *et al* (2014), there is a need for improved surveillance in wildlife and in symptomatic livestock in addition to a new vaccine for livestock (Jeffries et al., 2014). In addition, co-infection with *Anaplasma phagocytophilum*, the causative agent of tick-borne fever, can increase the severity of disease; experimental studies have also shown T*oxoplasma gondii* infection may also increase mortality (Barrett et al., 2012; Reid et al., 1982; Leiper et al., 1933).

1.3.5: Ecology

The distribution of LIV exhibits an extremely focal nature, with areas of high LIV prevalence found bordering areas which are LIV free (Gilbert, 2016). Knowledge of the ecology of LIV is limited to that of Great Britain, and even then the detailed nature of the transmission cycle is not fully understood, still requiring further extensive investigation (Dobler, 2010). Sheep, red grouse and mountain hare are considered the reservoir/transmission hosts of the LIV (Gilbert et al., 2020). Absence of the virus in areas where the reservoir hosts and vector are present, particularly when adjacent to a LIV focus, remains unexplained. This may be due to lack of importation of the virus to the area or specific ecological conditions that are required for LIV foci maintenance, that are not met (Gilbert, 2016).

Higher seroprevalence rates have been reported in sheep farms with more growing degree days (day-by-day sum, over a year, of the mean number of degrees by which the air temperature is more than 5.5∘C) resulting from a warmer climate. The plant growing season is a good measure of warmth which aligns to the period of tick activity, thus warmer weather can increase tick abundance, activity, development and oviposition rates (Gilbert et al., 2020). LIV is typically found in upland areas, such as heather moorlands, particularly grouse moors and crofting areas of rough grassland which are utilised for low-productivity sheep grazing, for example on the west coast of Scotland (Gilbert, 2016). Unimproved rough grassland produces a higher seroprevalence in sheep when compared to lowland improved grassland, which is likely to be as a result of the suitability of the upland habitat for LIV transmission hosts (red grouse and mountain hare), along with higher tick densities (Gilbert et al., 2020).
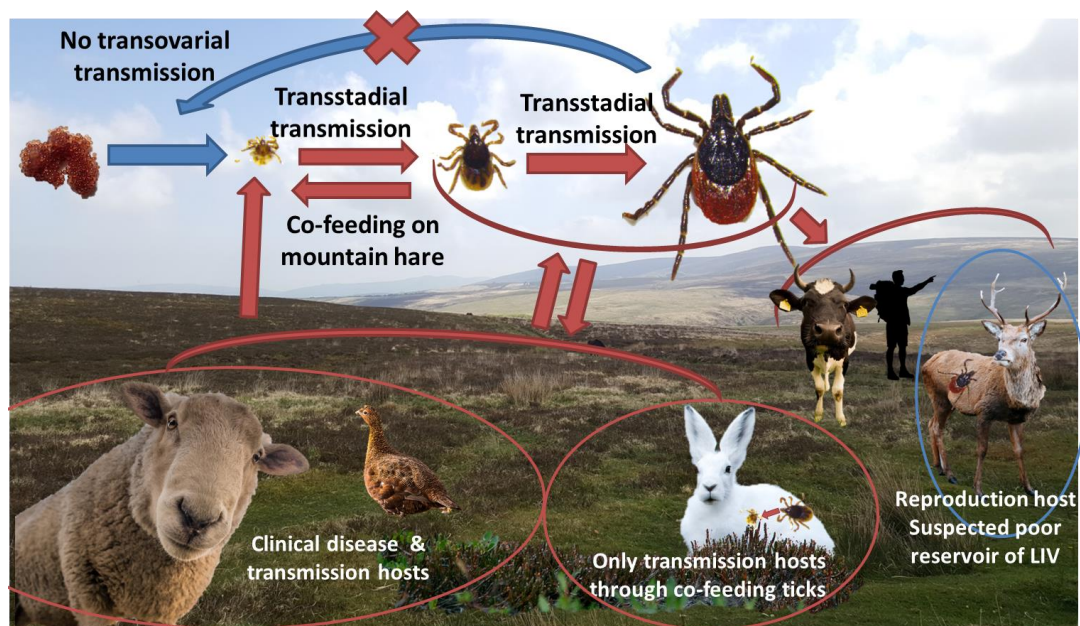
Figure 1:3: LIV enzootic cycle, including transmission to humans as incidental hosts.

1.3.5.1: Viraemia of LIV

1.3.5.2: Sheep

Only two species, sheep and red grouse, are able to maintain a level of viraemia sufficient to act as amplification hosts (*Figure 1*:3). Sheep are key transmission hosts of LIV, both in that they have a more efficient post-infection viraemia due to lower mortality than grouse, which can die very quickly, and also that sheep support all life stages of ticks (Gilbert, 2016; Gilbert et al., 2001). Unvaccinated

sheep, which are not treated with acaricides, have been demonstrated to be able to maintain a natural enzootic cycle of LIV infection in the absence of any other LIV transmission hosts(Gilbert et al., 2000). The LIV sheep vaccine, had been demonstrated as an effective measure to dramatically reduce LIV incidence, was withdrawn in 2018; however, it has been recently announced that research is underway to develop a new vaccine (Moredun Research Institute, 2020; Laurenson et al., 2007).

1.3.5.3: Red grouse (L. lagopus scotia)

Despite grouse developing a post-infection viraemia, the rapid mortality that results can limit the potential for transmission to ticks (Gilbert, 2016; Gilbert et al., 2001). Considerably more infections in young grouse are as a result of tick ingestion (73-98%) rather than tick bite (2-27%) (Gilbert et al., 2004). Adult ticks do not feed on red grouse; however, in the absence of other transmission hosts, the presence of a non-LIV competent adult tick host, in addition to red grouse, can be sufficient to maintain LIV. This was demonstrated by Laurenson *et al*. (2007), where despite 5 years of vaccination and acaricidal treatment of sheep, it was not possible to fully eradicate the virus. The area studied was a grouse moor; therefore grouse are likely to have supported a low level of infection (Gilbert, 2016; Laurenson et al., 2007). At slight odds to this weak maintenance, a separate ecological study found a high seroprevalence rate of 46% in red deer (*Cervus elaphus*) in an area in which red grouse were the only known transmission hosts (Gilbert, 2016).

1.3.5.4: Lepus timidus (Mountain hare)

Mountain hare are not LIV hosts which develop viraemia, but are important as LIV can be transmitted between ticks whilst co-feeding on the hares (Gilbert et al., 2001; Jones et al., 1997). Mountain hare only develop either a low or undetectable level of viraemia when exposed to LIV infected ticks; however, laboratory experiments have demonstrated transmission via co-feeding. These studies found up to 56% of recipient nymphs acquired LIV infection when co-feeding with eight LIV infected adult female ticks (Jones et al., 1997). Mountain hare carry all three feeding life stages of tick and they have been shown to be important transmission hosts in field experiments. In a field experiment in a LIV focus, where red grouse

and mountain hare were the only LIV competent transmission hosts, mountain hare were culled to very low numbers, resulting in LIV seroprevalence being reduced to very low levels in red grouse (Laurenson et al., 2003). As a result, culling mountain hare has been adopted on some grouse moors as a LIV control measure (Gilbert, 2016).

1.3.5.5: Non-competent tick hosts

Laboratory experiments have demonstrated no evidence of either viraemic or non-viraemic transmission of LIV via red deer or rabbits *(Oryctolagus cuniculus)* (Gilbert et al., 2001; Jones et al., 1997). Although clinical disease has been previously reported in roe and red deer, this is extremely rare and laboratory experiments have shown cervids to be incompetent reservoirs of infection (Gilbert, 2016). Despite not being implicated as transmission hosts for LIV, deer have been shown to still have an important impact on its ecological maintenance. Even in very low densities, deer from an area of high hare density that move into an area free of hares can still be sufficient to allow LIV to persist in the hare-free area, possibly by transporting ticks between these areas (Watts et al., 2009). Higher red deer densities may result in increased LIV prevalence, this is likely to be due to the higher tick densities that deer support (Gilbert et al., 2020).

UK small mammals are not thought to be implicated in LIV transmission cycles. Despite previous studies indicating that this should be explored, contemporaneous studies have indicated small mammals are unlikely to be involved in the transmission of LIV. A field study in Ayrshire, Scotland, conducted in 1962 and 1963 isolated LIV from the brain and/or spleen of 7.7% of wood mice (*Apodemus sylvaticus*) and 1.9% in common shrew (*Sorex araneus*) (Smith et al., 1964). The authors also reported when a wood mouse *w*as inoculated with LIV, viraemia occurred on all days but the fourth day, out of five days post inoculation; the mouse survived and produced an antibody response. However the highest titre in the blood was 1.1 log mouse LD_{50} 0.03ml, which may not be sufficient to infect feeding ticks (Smith et al., 1964). A subsequent laboratory study found that when inoculated with LIV, 70% of small mammals seroconverted. However, a field study conducted in known LIV areas in 1998 found no evidence of antibodies in 81

trapped small mammals (field vole, common shrew, wood mouse and bank vole) (Gilbert et al., 2000). In addition, a low abundance of small mammals, with a low density of ticks on those caught, was found. No evidence of transmission by ticks co-feeding on small mammals was found in ticks removed during these field trials or through additional laboratory tests (Gilbert et al., 2000).

1.3.5.6: Ticks
There is only one published study investigating LIV prevalence in questing ticks. The study was conducted by Watts *et al.* 2009, who tested a limited sample size of 1,063 ticks from across 6 Scottish upland moor sites (average 177 ticks per site) (Watts et al., 2009). A prevalence of between 1.8% and 15.3% was detected. Further work is needed to establish whether this can be consistently found. Anecdotal evidence (unpublished work by Animal Plant Health Agency and Moredun Research Institute in LIV endemic areas), in addition to preliminary studies carried out as part of this project which involved testing >2840 ticks, indicate LIV can be difficult to detect and the former reported prevalence by Watts *et al.* 2009, may not be representative of most UK LIV foci. In addition, estimates of LIV prevalence in ticks in a variety of associated habitats and ecologies are required.

1.4:   Tick-borne encephalitis virus
1.4.1: Virology
TBEV is a flavivirus that is present in much of Europe and parts of Asia. It is one of the most important tick-borne viral diseases in Europe, with several thousand European cases annually (Jeffries et al., 2014; Süss, 2011). It has been suggested that TBEV first emerged in the Far Eastern area or in Siberia and moved west and southwards with new subtypes and viruses evolving during this process (Labuda and Nuttall, 2004).

Like LIV and the rest of the flavivirus genus, TBEV is an icosahedral enveloped 50 nm virus; the genome is a single stranded positive sense RNA molecule, approximately 11 kb in length (Ruzek et al., 2017; Lindquist and Vapalahti, 2008). It is a membrane enveloped virus composed of surface envelope (E) proteins in a dimer formation and the smaller membrane (M) protein. The envelope glycoprotein is the major antigenic determinant of the virus. It initiates receptor binding and

membrane fusion (Kellman et al., 2018). A protective immune response is mounted against the envelope protein following infection (Labuda and Nuttall, 2004). Contained within the envelope is the viral nucleocapsid which contains multiple copies of the capsid (C) protein packing a single RNA genome.

1.4.2: Geographic distribution and epidemiology in Europe

TBEV is endemic in areas of the Far East, including Mongolia, Japan and northern parts of China, also across much of Russia and in eastern, central and northern Europe (Valarcher et al., 2015). TBEV is increasing in prevalence and range in Europe, and with increasing cases of TBE over the last 20 years, it is now found in over 27 European countries; having most recently been detected in the Netherlands for the first time (Jahfari et al., 2017; Bogovic and Strle, 2015; Lindquist and Vapalahti, 2008). The interplay between a combination of sociological and ecological factors, in addition to improved diagnostics and medical awareness, are thought to be responsible for the increase in case numbers (Bogovic and Strle, 2015). There were 3,092 confirmed TBE cases in EU/EEA countries in 2018 with a notification rate of 0.6 cases per 100,000 population, which has remained stable over the preceding three years. The notification rate varies vastly between countries; in 2018 Lithuania, Slovenia and Czech Republic reported the highest rates in EU/EEA at 13.6, 7.4 and 6.7 per 100 000 population, respectively (ECDC, 2019).

Three classic subtypes of TBEV have been recognised for many years, the European (TBEV-Eu), Siberian (TBEV-Sib), and Far Eastern (TBEV-FE). Recently, two additional subtypes have been proposed, namely the Baikalian subtype (TBEV-Bkl) and the Himalayan subtype (TBEV-Him) (Dai et al., 2018). The subtypes tend to be divided in distribution corresponding with the location of their most common vector (*I. ricinus* for TBEV-Eu and *I. persulcatus* for the other subtypes) as illustrated in *Figure 1:4*. There is an overlap between these distributions. TBEV-Eu is the dominant subtype in Western Europe where it is primarily transmitted by *I. ricinus* ticks. Interestingly, the European subtype of TBEV has a higher degree of genetic homology to LIV than to the Far Eastern or Siberian subtypes (Lindquist and Vapalahti, 2008). TBEV-Eu has also been identified in a wider area such as Siberia and western Urals. In addition to

TBEV-Sib being found in Siberia, it has also been identified in the Baltics and as far west as Northern Finland (Bogovic and Strle, 2015; Jääskeläinen et al., 2006).
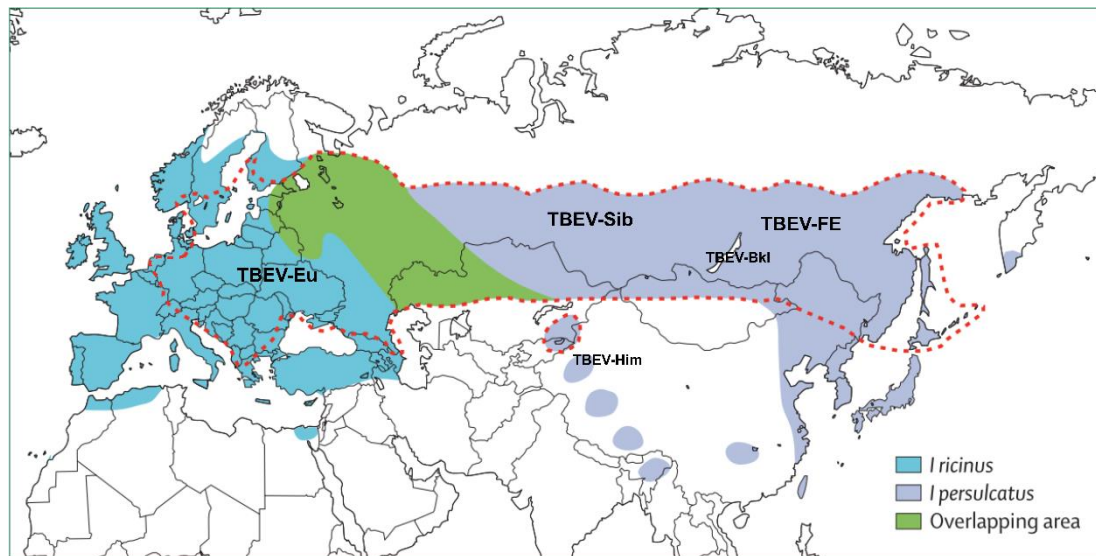
Figure 1:4: *The distribution of TBEV endemic areas, signified by the red dashed line. The general indication of areas where each subtype is most prevalent is marked, though multiple subtypes are found crossing over in numerous areas. The distribution of the key TBEV vector species Ixodes ricinus and Ixodes persulcatus are shown. Figure updated and adapted from Lindquist and Vapalahti (2008)* (Lindquist and Vapalahti, 2008)*.*

In people, overall TBE is more common in males (ratio 1.5 M: 1 F); however, the sex ratio of disease varies greatly with countries studied and by year. This variation is also true for the age profile of disease, with the largest proportion of TBE cases being in individuals aged 45-64 years at 0.8 cases per 100 000 population. The lowest rates tend to be among children aged 0-4 years at 0.2 per 100 000 population (ECDC, 2019; Süss, 2003).

Seasonal peaks are experienced in line with peaks in activity of the main vectors; in Europe TBE cases are usually reported from May to November, peaking in June to August with 59% of cases occurring in this period (ECDC, 2019).

1.4.3: Risk of exposure/high risk activities
In addition to specific ecological conditions enabling the formation and maintenance of TBEV foci, variations in human activities in these areas have a crucial impact on exposure to the virus and the resultant case numbers. Social and economic factors are important drivers affecting type and level of human activity in high risk areas and habitats (Randolph, 2010). Affluent countries can experience higher numbers of cases resulting from exposure in endemic woodlands during

leisure time, where there is a higher level of recreation activity including various sports, hunting and fishing (Süss, 2003). Activities can differ in lower income countries where woodlands are often accessed for berry and mushroom picking and wood collecting (Randolph, 2010) though these are also leisure activities in some wealthy countries. Occupational groups such as forestry workers, hunters and farmers are at high risk. In addition new housing developments in TBE risk areas are linked to an increase in infections (Süss, 2003). Changes in forest structure, for example a decreased ratio of coppice to high stand forest can also impact case numbers due to resultant impact on rodent and deer numbers. Patterns and changes in TBE case numbers tend to result from a complex interaction of socio-economic, biotic and abiotic factors (Randolph, 2010).

Transmission of TBEV via tick-bite is most common; however, alimentary infection can occur from consumption of unpasteurised milk or milk products from a viraemic goat or cow which can lead to small local outbreaks (Cisak et al., 2010). Extremely infrequently, TBEV can be transmitted through blood transfusion or to an infant via breastfeeding (Lindquist and Vapalahti, 2008); there has also been reported infection through slaughtering a viraemic goat (Süss, 2003).

1.4.4: Clinical disease

TBEV results in clinical disease in an estimated 2-30% of those infected; the course of the disease is biphasic in 72-87% of patients (Bogovic and Strle, 2015; Lindquist and Vapalahti, 2008; Kaiser, 1999). The median time between the tick bite and onset of first clinical symptoms is 8 days (range 4-28 days) (Kaiser, 1999). During this first phase, the most common symptoms are fever (99%), fatigue (63%), general malaise (62%), headache, aching back and limbs (54%), catarrhal symptoms (28%) and gastrointestinal symptoms (21%) (Mickiené et al., 2002). This first phase lasts for a median of 5 days (range 2-10 days). A symptom free interval occurs after this, lasting a median of 7 days (range 1-21 days) (Lindquist and Vapalahti, 2008). Two thirds of patients do not experience a second biphasic course of disease (Valarcher et al., 2015), when the virus spreads to the central nervous system. This second acute phase varies in clinical course ranging from mild meningitis to severe encephalitis; spinal paralysis and myelitis can also concurrently occur (Valarcher et

al., 2015; Lindquist and Vapalahti, 2008). Symptoms that may be experienced during this phase include headache, tremor, paresis, photophobia, anorexia, fever, visual disturbances, altered consciousness, ataxia, spinal nerve paralysis, sensory impairment, cranial nerve paralysis, seizures, dysphasia and hemiparesis. Mortality can occur as quickly as within a week following onset of clinical disease (Valarcher et al., 2015; Lindquist and Vapalahti, 2008). Just under 2% of TBEV-Eu clinical cases, not including asymptomatic cases, result in fatality and up to 46% of patients experience long term morbidity including both cognitive and physical symptoms (Bogovic and Strle, 2015; Suss, 2008). Approximately 10% of patients experience a severe neurological deficit (Bogovic and Strle, 2015), with more serious disease often associated with increasing age (Kaiser, 1999).

The different subtypes are associated with the severity of disease, with the TBEV-Sib subtype tending to result in a more serious aetiology than TBEV-Eu; with case fatality rates as high as 6-8% occurring in the former (Suss, 2008). The TBEV-FE subtype results in the most severe form of central nervous system (CNS) disease with case fatality rates up to 20-40% being recorded (Suss, 2008).

1.4.5: Prevention and treatment

There is no specific treatment for TBE, which puts particular importance on the prevention. Treatment provided is supportive, this can include intensive care and ventilation (Lindquist and Vapalahti, 2008). TBE is a vaccine preventable disease with six vaccines currently available; two using TBEV-Eu strains and four TBEV-Fe. The vaccination policy and rates by country can have an important effect on case numbers. Austria is highly endemic for TBE and has a strong vaccination uptake. A study found 88% of the population there had history of TBE vaccination and this high uptake has prevented an estimated 4,000 TBE cases and 20 deaths between 2000 and 2011 (Pöllabauer and Kollaritsch, 2019).

1.4.6: Virus-tick interface: Pathway of TBEV infection in ticks and transmission to host

In order for tick-borne viruses to persist and replicate in their vectors, they must evade the tick's immune response and cross a number of barriers (Kazimírová et al.,

2017), for example RNA interference through RNAse activity within tick cells which may inhibit virus infection (Garcia et al., 2005).

Upon ingestion of an infected blood meal, the virus must pass through the midgut infection barrier and midgut escape barrier, the dissemination barrier through which the virus disseminates through the body presumably in the haemocoel. The virus may evade the tick's immune system through infecting the tick's haemocytes, and via this route, travel to the salivary glands. Once the salivary glands have been reached, the salivary gland infection barrier is traversed; crossing the salivary gland escape barrier enables the virus to be released into the salivary ducts and be secreted within its saliva. There is evidence that some tick-borne viruses may not need to infect the salivary glands to transmit to a host; results from an experimental study suggests it may pass rapidly from haemocoel to saliva (Nutall, 2014). This process through which the virus enters the tick to travel to the saliva is called the extrinsic incubation period where during this time the tick is not able to transmit the virus to a new host. With TBEV, the salivary glands are infected prior to feeding commencing, indicating that transmission can occur as soon as fluid secretion into the host begins. The nature of the virus when infecting ticks is an overlooked area, i.e. whether infection occurs within the blood meal as extracellular virions or as infected cells. TBEV does infect Langerhans cells; however, the timing of the uptake may affect the state of the virus in the blood meal. It remains to be determined whether either state specifically aids infection of the tick (Nutall, 2014).

Once a tick is infected, generally it is thought that TBEV is passed on very effectively through the tick's life stages transstadially (Nuttall and Labuda, 2003). The virus must survive the moulting process of the tick, which is a hostile environment as a result of the histolytic enzymes and tissue replacement. Evidence has suggested that virus titres fall within the tick within the ecdysis process, which then rise in the new tissues following completion of moulting (Labuda and Nuttall, 2004). Some evidence has indicated that a proportion of *I. ricinus* ticks may lose TBEV infection during moulting, although more research is required to determine this (Slovák et al., 2014).

1.4.6.1: Cyclical TBEV titres in questing and blood-fed ticks

It has been long observed that TBEV prevalence in questing ticks is usually low, and often not detected even in known foci (Stefanoff et al., 2013). The prevalence in questing ticks in Europe tends to range between 0-5% (Süss, 2003). The difference in prevalence in fed ticks compared to questing ticks can be considerable; for example, a Bavarian study found the prevalence in *I. ricinus* removed from humans was 21 times higher than in questing ticks (Diller et al., 2006). There has been some debate regarding the causation of this disparity, including the finding that TBEV infection in ticks results in a more aggressive questing behaviour (Belova et al., 2012). This does not seem a plausible explanation for the difference as the flagging method simulates a host moving through vegetation, therefore would surely equally attract more 'aggressive' TBEV questing ticks.

The process of a tick taking a blood meal has been shown to result in a faster and more intensive virus replication in experimentally infected *I.* ricinus compared to those infected and left unfed. The virus titre increased 500 times in feeding ticks compared to no change in those unfed (Belova et al., 2012).

Perhaps the biggest breakthrough in this long-discussed quandary is the finding that TBEV has been shown to have a temperature-sensitive riboswitch altering genomic folding and preventing translation at lower temperatures. The specific TBEV strains have different threshold temperature of genomic unfolding. This indicates that seasonality of questing tick surveys may be important. It has been suggested that the climate in which the virus was isolated may be responsible. TBEV-Torö, which had a lower breakpoint temperature (BPT) of 23.68°C, originates from the Swedish island of Torö which experiences cooler spring and summer months, when compared to Neudorfl, isolated from Austria (BPT 30.08°C) and 263, isolated from Czech Republic (BPT 26.88°C) (Elväng et al., 2011). This effect of temperature is supported by an experimental study from 1979, which found lower virus titres in ticks that went through diapause (induced by both or either light or temperature) than those that did not. Interestingly, when temperature was maintained and light alone induced diapause, this also had a detrimental impact on the replication of TBEV (Mishaeva and Erofeeva, 1979).

The combined effect of thermoregulation along with the normal drop in viral titres to very low levels as a result of moulting followed by behavioural diapause, would result in a much-reduced detectable prevalence over winter. Titres in many ticks might be at low enough levels to be below the limit of detection for the regular assays used. The reported BPT thresholds will only be passed in each locality, during late spring at the earliest, or when the questing tick begins a blood meal on a host, when the RNA may then unfold and virus replication commences (Elväng et al., 2011).

Supporting this, temporal field studies have reported TBEV prevalence in questing ticks dropping over winter, with no TBEV-infected questing ticks detected before the month of May (Zöldi et al., 2015; Perez-Eid et al., 1992). A field study of the Alsatian TBEV focus in France, reported only detecting TBEV in questing ticks between May and October, and two-thirds of virus isolations occurred between August and October (Perez-Eid et al., 1992). The field study in a TBEV focus in Hungary detected positive questing ticks between May and August (Zöldi et al., 2015). One study suggests that the virus may overwinter in a low proportion of ticks; however, the temperature riboswitch theory would appear to also be a plausible explanation (Zöldi et al., 2015).

1.4.6.2: Virus transmission between ticks

For the successful maintenance of TBEV, the virus must be passed by an infected tick to at least one uninfected tick, therefore the R_0 must be at least 1 or higher (Labuda and Randolph, 1999). TBEV can be transmitted to other ticks through four different mechanisms (*Figure 1:5*); i) viraemic, ii) non-viraemic, iii) transovarial and iv) sexual transmission. Viraemic transmission occurs when a tick becomes infected through feeding upon a host with sufficient levels of virus present in its blood. In key reservoir hosts, viraemia tends to last for a very short period of less than 9 days (Michelitsch et al., 2019). The transmission mechanism in which a tick is infected by feeding on a viraemic host is only marginally effective for the maintenance of the

virus in nature, with estimates suggesting that this produces a R_0 of 0.98 (Labuda and Randolph, 1999).
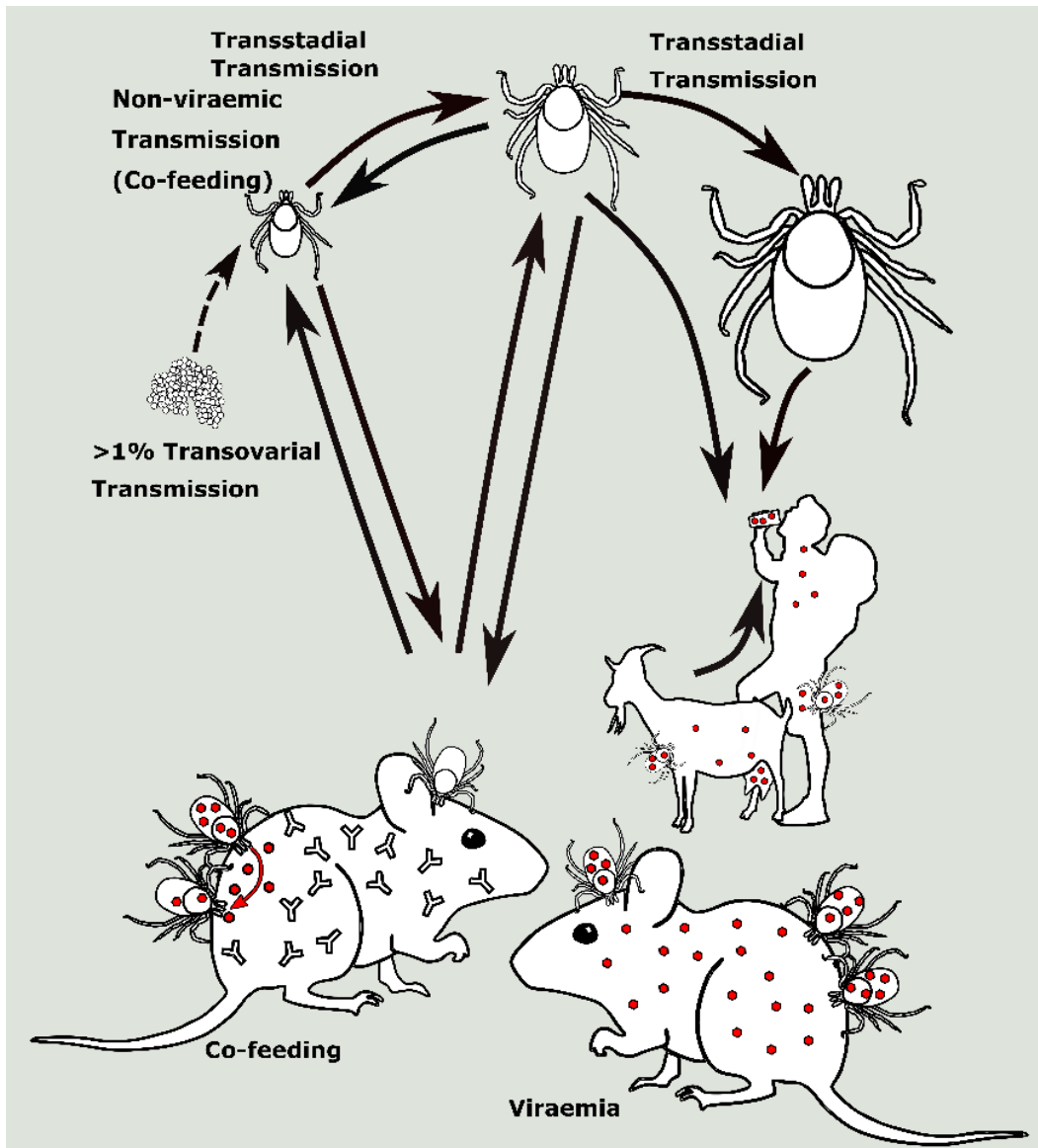
Figure 1:5: *TBEV enzootic cycle, including transmission to humans as incidental hosts. Co-feeding image adapted from Michelitsch et al. 2019*

A tick may also be infected via non-viraemic transmission between co-feeding ticks. Co-feeding is a mechanism by which the virus can be transmitted in the absence of systemic viraemia between infected and uninfected ticks whilst feeding on certain species of animal host. It is possible for the virus to be transmitted, even in the presence of an antibody response to TBEV in the host. This mechanism in ticks was first described in 1987 for Thogoto virus (Jones et al., 1987). Co-operative activity in ticks enhances their abilities to overcome the host's protective systems. During feeding, ticks secrete saliva containing a great diversity of pharmacologically active

substances that have properties which include: immunosuppression, anti-haemostasis , vasodilation and anti-inflammation (Labuda and Nuttall, 2004). The substances secreted in saliva can also indirectly promote arthropod-borne pathogen transmission, known as saliva activated transmission (SAT). Studies have found inoculation with tick saliva or salivary gland extract, in addition to virus, increases transmission compared to ticks inoculated with virus alone (Nutall, 2014). Co-feeding experimental studies have demonstrated the importance of SAT promoting transmission of TBEV between co-feeding ticks with effective demonstration of TBEV transmission between infected and uninfected ticks on an immune rodent, whereas immunisation with antigen derived from ticks reduced transmission (Nutall and Labuda, 2008). Due to the key reservoir hosts for TBEV mainly supporting just larval and nymphal tick life stages, and most larvae being uninfected, co-feeding between infected nymphs and a large number of potentially infectible larvae is required for the maintenance of TBEV (Randolph, 2001). Suitable conditions must exist in order for synchronous feeding of large numbers of larvae and nymphs, with both questing periods occurring simultaneously (Randolph and Rogers, 2000). The addition of non-viraemic transmission between co-feeding ticks has a great impact on transmission efficiency, producing a 50% higher amplification (R_0=1.65) than viraemic transmission alone (Labuda and Randolph, 1999). Consequently, a high prevalence of co-feeding between larvae and infected nymphs is thought to be the most critical factor that underpins the establishment of a TBEV foci and for it to be maintained (Esser et al., 2019).

In addition, the virus may be passed on via transovarial transmission, where the virus is passed onto eggs by an infected female. This occurs very infrequently with an estimated <1% of eggs infected, therefore the majority of larvae are uninfected prior to taking their first blood meal (Slovák et al., 2014). There is debate regarding the importance of this mechanism due to the low transmission rate; however, it has been argued that this may still be of potential significance, particularly given the number of larvae that co-feed together (Süss, 2003; Danielová et al., 2002).

The fourth and least studied mechanism is via sexual transmission, in which infected male ticks transmit the virus to females via infected saliva and/or seminal

fluid. Starved male ticks have been shown to contain high titres of virus in their saliva, this mechanism may increase subsequent transovarial transmission through infecting more females (Chitimia-Dobler, Mackenstedt, et al., 2019; Pettersson et al., 2014).

1.4.7: Natural transmission cycles

TBEV serocomplex viruses require a combination of factors to coincide to enable its maintenance. Comparatively, there has been very limited research conducted on the ecological requirements of LIV as compared to TBEV (Gilbert, 2016; Labuda and Randolph, 1999). Due to the need for a precise combination of conditions, the presence of either TBEV or LIV, tends to be localised to specific areas, which are known as 'foci' (Randolph et al., 1999), defined as the presence of a pathogen within specific geographical boundaries (Süss, 2003). Both biotic and abiotic factors contribute to maintenance of a foci which can broadly be classified into the following requirements: i) reservoir host, ii) vector, iii) climate/seasonality, iv) microclimate and v) habitat. In addition, other non-reservoir animal tick hosts can also impact the ecology of TBEV. The following section will describe how these important factors interlink, as illustrated in *Figure 1:6*.
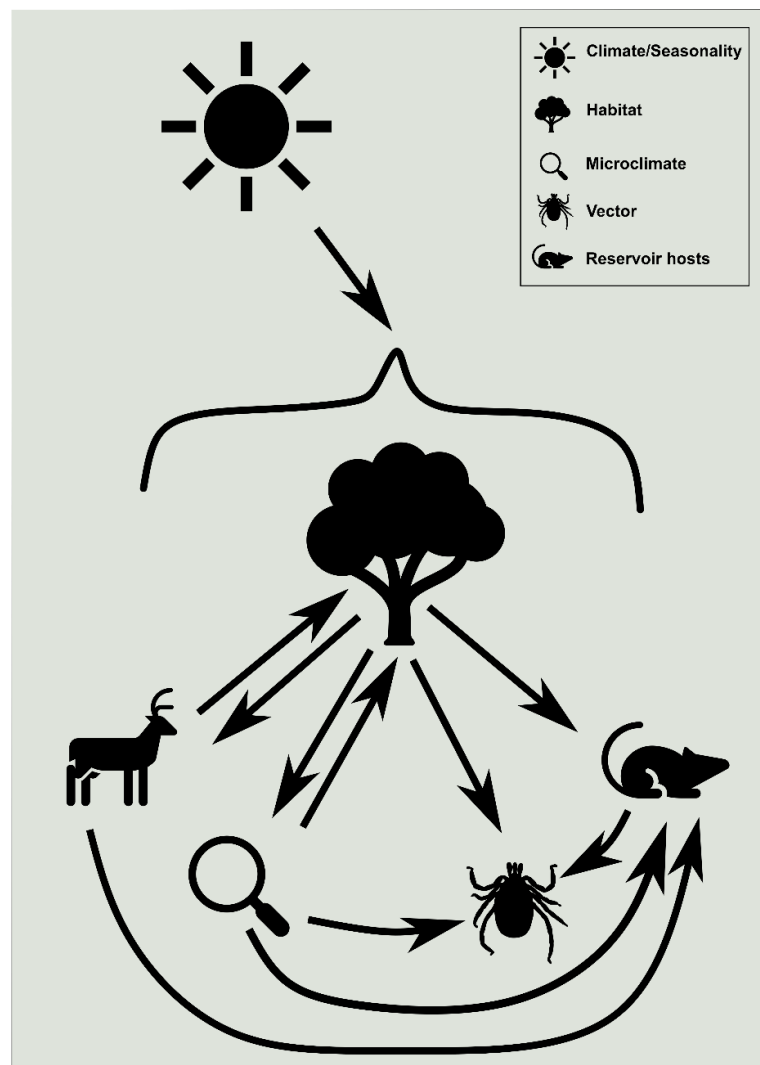
Figure 1:6: *Key interlinking factors important for the maintenance of TBEV foci and their relationship and impact on one another.*

1.4.7.1: Reservoir hosts

Further research is required to investigate natural cycles for maintenance of TBEV through reservoir hosts, as there is limited contemporaneous research on this area. There is a need to investigate the potential roles of the full range of small mammal species and other wildlife (Michelitsch et al., 2019), particularly due to the limited research conducted since the identification of non-viraemic transmission.

Not all tick hosts produce viraemia, with only a limited number producing sufficient viral titres required to infect ticks. Additionally, few hosts are thought to be competent for the transmission of TBEV through co-feeding ticks. There is variable efficiency in viraemic and non-viraemic transmission between species in which it does occur. The number of ticks able to complete a blood meal on a host, and the proportion that become infected differs between species.

The long lifespan of the tick vectors, and ability to maintain the virus overwinter, and transstadially in addition to non- viraemic transmission to other ticks, suggests ticks themselves are also fitting reservoirs; however amplifying host reservoirs are also required for successful maintenance (Michelitsch et al., 2019).

Current knowledge identifies rodents as the key reservoir hosts for TBEV; however, only a limited number of species have been shown to produce a sufficient viraemia to infect ticks; crucially these species are also competent for non-viraemic transmission (Valarcher et al., 2015). Within Europe, the rodent genera *Apodemus* and *Myodes* are important in maintaining TBEV within natural cycles (Bournez et al., 2020). The yellow-necked mouse *(Apodemus flavicollis)* is thought to be the most competent species for TBEV transmission; in addition the bank vole (*Myodes* glareolus) is also important in the maintenance of the virus, among other rodents (Michelitsch et al., 2019). There has been suggestion that the European hedgehog (*Erinaceus europaeus*) may be a reservoir(Schönbächler et al., 2018); however, evidence suggests that their potential transmission efficiency to ticks is low, therefore it is unlikely they are an important reservoir host (Michelitsch et al., 2019; Schönbächler et al., 2019; Labuda and Randolph, 1999).

Although yellow-necked mice develop a low level viraemia, an *in vivo* study found that 68% of engorged ticks and 46% of all exposed ticks became infected (Labuda and Randolph, 1999; Labuda et al., 1997; M. Labuda et al., 1993). On the other hand, the bank vole develops a higher level of viraemia but just 28% of engorged ticks became infected and fewer were able to complete their blood meal due to the bank vole's strong immune response to ticks resulting in just 13% of all exposed ticks being infected (Labuda et al., 1997). In comparison, *in vivo* experiments found that hedgehogs, blackbirds, pheasants, and goats did not efficiently support transmission via co-feeding (Labuda and Randolph, 1999; Milan Labuda et al., 1993).

 Yellow-necked mice have a short lifespan rarely exceeding a year and averaging just 3-4 months. They breed from April-October, though can sometimes breed year-round if conditions/climate/environment are favourable. Yellow-necked mice produce up to three litters a year, of between two to eleven young. They are active year-round and have just one single period of daily activity, which is at night (The Mammal Society, 2020). Bank voles have a relatively short life span of 6-18 months. As mentioned, they have a slightly longer breeding period than yellow-necked mice, breeding between March and October. Their breeding period may also be extended with sufficient food availability. They produce three to six litters during this time, of three to five young in each litter. Females reach sexual maturity after six weeks, therefore those born earlier in the season can breed the same year. Like yellow-necked mice they are active year-round; however, they are active during two periods, which are dawn and dusk (The British Trust for Ornithology, n.d.; The Mammal Society, n.d.; The Wildlife Trusts, n.d.). The short life span of these species, together with the high reproductive rate, enables breeding throughout the peak questing tick period, ensuring that there is a continuous supply of naive hosts able to produce viraemia (Michelitsch et al., 2019).

As a result of the short life span and breeding period, primarily older individuals are present in February to April, with the population then increasing in May, when large numbers of young are born and become active. This is a key time for viraemic infection with TBEV, of large numbers of naive young which become viraemic, at a

time when questing tick populations are also starting to peak (Perez-Eid et al., 1992). Despite the important phenomena of transmission of TBEV by co-feeding ticks on immune hosts occurring, there is much higher transmission efficiency to ticks, for both of the key host species, in non-immune animals. The efficiency of transmission via co-feeding for yellow-necked mice is 72% in naive animals versus 24% in immune hosts, and 42% and 24% respectively for bank voles (Randolph et al., 1999).

In order for TBEV transmission via co-feeding to occur on small mammal hosts, specific conditions need to be met for larvae and nymphs to feed in sufficient numbers together. Although larvae largely feed on small mammals, nymphs tend to feed on medium sized hosts, although some conditions result in more feeding on small mammals instead (Burri et al., 2011).

The level of infestation of small mammals by nymphal ticks is a key factor in transmission via co-feeding and varies geographically (Randolph and Storey, 1999). An ecological study in Southern Hesse, Germany found 98% of yellow-necked mice were parasitised by at least one *Ixodes spp*. larvae whereas this was just 68% of bank voles (Kiffner et al., 2011). Similarly, despite a smaller proportion of small mammals being infested, a study in Hampshire, England, found more yellow-necked mice infested with ticks than bank voles (86.0% and 76.9%, respectively), where 98.4% of ticks collected were larvae (Cull et al., 2017). A study in Trento, Italy found a similar proportion of ticks collected were larvae (98.8%) (Rosà et al., 2019). There is also considerable inter-year (Rosà et al., 2019) variation in co-feeding between larvae and nymphs, highlighted by a study in the Alsace region of eastern France finding 57.8% of larvae fed with nymphs in 2013 and just 2.7% in 2012. In 2012 a mean of just 2.2 larvae per rodent were found and just 4.5% of rodents were infested with nymphs (Bournez et al., 2020).

Tick behaviour is described as following a 20/80 rule, by which 80% of ticks feed on 20% of reservoir hosts (Bournez et al., 2020; Michelitsch et al., 2019; Rosà et al., 2019). This high degree of aggregation of ticks feeding on a small number of reservoir hosts can increase the R0 of a pathogen. The host contact rate is one factor that affects the aggregation of ticks on small mammals, if their population is

low this results in higher densities on individual hosts. The density of nymphs questing and the proportion of these that are infected, known as the density of infected nymphs (DIN), is an important parameter to monitor (Bournez et al., 2020).

Understanding the characteristics of the 20% of the reservoir host population that support the majority of the feeding juvenile ticks is central to understanding and monitoring the disease ecology (Kiffner et al., 2011). Both adult yellow-necked mice and bank voles have been shown to carry higher larval tick burdens than sub-adults. This is likely linked to the 'body size' hypothesis; heavier rodents have been shown to carry more *Ixodes* spp*.* larvae than lighter individuals (Kiffner et al., 2011).

A number of studies have found that there is a higher tick infestation prevalence in male vs female rodents, with males carrying more co-feeding groups. This may be due to high testosterone levels in males impairing their ability to mount an immune response to the ticks, the increased home range in males, and behaviour. There is also evidence that their larger body size is also a factor, with heavier males carrying more ticks and also being more likely to survive winter; therefore being present to support nymphs and larvae in the spring peak questing period (Rosà et al., 2019; Boyard et al., 2008). However, this phenomena has not always been identified, with Kiffner *et al*. (2011) finding no support for this sex-bias hypothesis (Kiffner et al., 2011).

In addition to ticks aggregating on a small proportion on hosts, ticks aggregate on the host itself in certain locations, tending to feed together behind the ear. Up to 100 larvae may feed alongside a small number of nymphs. This aggregation of ticks both on a small number of hosts and also in a specific locality on the host is beneficial to promote transmission through co-feeding (Michelitsch et al., 2019).

Despite a simultaneous increase in the total number of feeding larvae with increasing rodent density, a threshold density of approximately 10 yellow-necked mice/ha has been identified for increasing co-feeding groups. Greater densities of yellow-necked mice then results in a decline in co-feeding groups due to the finite number of ticks in the environment and their long lifecycle limiting their ability to rapidly increase population in response (Rosà et al., 2019).

There is a close relationship between fluctuations in small mammal numbers and tick populations. Increases in rodent populations can decrease tick density per host (Rosà et al., 2019). This dilution effect applies to both yellow-necked mice and bank voles (Kiffner et al., 2011). A longitudinal study in the Alsace region of France found that there was a reduced level of co-feeding and aggregation of ticks in a year with increased abundance of small mammals. This in turn reduced the TBEV prevalence in questing nymphs the following year (Bournez et al., 2020). In years in which small mammal populations are low, they produce an immune response much earlier in the year, as there is a greater aggregation of ticks on a smaller number of hosts (Bournez et al., 2020).

The population of *Apodemus* and *Myodes* genus species are not stable over time, varying both seasonally but also annually. In temperate forests multiannual fluctuations occur, often largely due to changes in oak (*Quercus* spp.) and beech (*Fagus sylvatica)* seed crop production.

1.4.7.2: Ecology of tree fruitification

Trees are considered to be masting when abundant volumes of seeds are produced in a flowering year. This phenomenon can occur in a wide range of tree species and generally in trees at least 30-50 years old (Nussbaumer et al., 2016). The rodent population peaks the year following a beech mast year, therefore also resulting in higher larval densities due to the increased host availability. Consequently, it is in the second year after the mast year, when higher nymph densities occur, which could increase frequency of TBE cases in that year. Beech fruitification has been shown to drive regular 2-3 year oscillations in the TBEV transmission cycle between small mammals and ticks. Models that study TBE case oscillations and beech fruitification have found a link between increases in TBE cases in the second year after a mast year on 64% of occasions (Rubel and Brugger, 2020; Rubel et al., 2020).

A beech forest can be considered to be "in mast" when a third of the trees have abundant fruitification. The age of the trees in the forest has an impact on the level of fruitification produced, with older trees producing a greater abundance of seeds. Older trees optimal for abundant fruitification tend to be over 60 years and at a minimum 49 years old (Reil et al., 2015).

Factors that affect seed production include climatic and light conditions, also tree age and size. Trees that produce the most seeds are those positioned to receive ample quantities of light, are dominant and have large crowns (Harmer, 1995).

Flower buds are initiated in the year preceding flowering and fruiting with the whole process taking 12-18 months. Initiation of flower buds begins in May, and pollen formation is then arrested over winter and completed for both beech and oak before flowering, as new leaves are produced in spring (Harmer, 1995). Fruit development takes place firstly in beech in September, closely followed by oak in September to October (Nussbaumer et al., 2016). Both oak and beech display bimodal normal masting where a large amount or no/low levels of seed produced occurs in non-mast years, with this phenomena being more pronounced in beech (Nussbaumer et al., 2016).

Evidence of the weather required in the year preceding fruiting and mast years is well established for beech. Trees produce little wood when the weather is sunny, warm and dry, which is then conducive to flowering the following year. For the year preceding fruiting, in the northern hemisphere there is a positive relationship between the mean temperatures in June and July, and a negative relationship with precipitation in these months on the subsequent yield of beech. Periods of drought in the preceding summer have been shown to produce the heaviest flowering (Harmer, 1995).

Spring frosts, hail or damp weather may damage flowers or inhibit pollination. Beech fruit development is adversely affected by drought, and ripening affected in cold wet summer conditions. In the case of the oak, acorns are often unable to mature in cold summers, which benefit from warm temperatures in summer with sufficient rain. Fruiting in oak and beech is irregular and difficult to predict (Harmer, 1995).

Oak masting is sporadic, occurring every 2-6 years, with the largest crops just every 6-9 years in Britain. Frequency of masting in beech also varies, in Germany this occurs in a two year pattern (Reil et al., 2015); however, this can be much longer with 15 year intervals between full masts and partial masting in the years in

between (Harmer, 1995). Increases in frequency of mast years have been identified which may be affected by increasing temperatures during the vegetation period, changes in nitrogen deposition and water/precipitation availability (Nussbaumer et al., 2016). The warmer the climate, the more frequent the oak crop production tends to be. This varies from 8-10 years in colder climates to 5 years or sometimes far less, at 3-4 year frequencies such as was found in one study in South Moravia in the Czech Republic (Čepelka et al., 2020). The seed years appear to remain the same over time, although the size of the acorn crop is increasing in western Europe (Čepelka et al., 2020).

Common beech tends to display a two to three-year mast occurrence with production depending on cross-pollination via wind. There is a high degree of mast synchrony within species; however, even within geographically close areas, years of mast can vary. Beech also show a high within-plot synchrony. The frequency of mast years have increased greatly between 1991 and 2010 in Great Britain (Nussbaumer et al., 2016).

In contrast, in Great Britain pedunculate oak (*Quercus robur*) and sessile oak (*Quercus petraea)* have both been found not to show a synchronised mast pattern unlike beech. Oak displays a low within plot synchrony, where neighbouring trees do not align in masting. In Great Britain, oak also does not show strong masting synchrony with Central European countries which tend to be quite synchronised (Denmark, Flanders, Switzerland and Germany). There has been no trend displayed for changes in recent years in oak mast frequency in Great Britain(Nussbaumer et al., 2016).

1.4.7.3: Reservoir host population dynamics

The volume of seeds produced by trees has a strong effect on rodents in temperate forests, impacting the degree of competition between species feeding on this food source (Amori et al., 2015). Fruit-fall can impact population densities and winter survival of both yellow-necked mice and bank voles, with good mast years optimising conditions for rodents to overwinter and start the breeding season early the following spring (Reil et al., 2015; Flowerdew and Ellwood, 2001). If mast crops and other food sources are in short supply, the reproduction potential in both

yellow-necked mice and bank voles can be reduced, both affecting overall population size and reducing breeding season length (Flowerdew and Ellwood, 2001). If there is a high food resource availability, the home range and dispersal of yellow-necked mice and bank voles is reduced (Casula et al., 2019). Their populations swell the year after a heavy mast year and then crash the following year. Due to slightly different diet and ecologies between these two species this food–induced population cycle can affect the species non-uniformly (Bournez et al., 2020). Within oak forests, acorn crop production has been shown to have a positive effect on yellow-necked mice, wood mice and bank voles, but only yellow-necked mice demonstrated a significant relationship between crop size and population (Čepelka et al., 2020). There is a stronger relationship between a good mast year and the increased reproduction of yellow-necked mice than with bank voles. The spring following a good acorn crop, yellow-necked mice reproduce earlier than bank voles, with the former also reproducing longer into the autumn (Čepelka et al., 2020). Cyclical fluctuations in rodent population patterns can be observed not only on an inter-annual but also intra-annual basis, similar to that observed with masting trees. Such a pattern was demonstrated in Hungary, with 3-4 year peaks being demonstrated in bank vole densities (Horváth and Wagner, 2003).

1.4.7.4: Impact of reservoir host population dynamics on TBEV prevalence
The varying fluctuations between yellow-necked mice and bank voles, resulting in change in host availability and relative species ratios can impact TBEV transmission. This is due to the hosts varied transmission efficiency. The population fluctuations also has a knock-on effect on TBEV transmission from nymphs to larvae, impacted as a result of variations in tick aggregation on reservoir hosts (Bournez et al., 2020).

The seasonal dynamics of these small mammals are an important factor influencing the feeding dynamics of larval and nymphal ticks on small mammals, in turn affecting the possibility of a TBEV focus developing with a resultant raised level of TBEV prevalence in ticks. A focus may be maintained but at a weaker prevalence in suboptimal conditions for TBEV (Randolph and Storey, 1999). For example, the Alsace region of France has a TBEV focus which is persistent but of low TBEV prevalence, where there is an even smaller proportion of nymphs feeding with one

hundred times more larvae (Bournez et al., 2020; Randolph and Storey, 1999). Interestingly, it has been shown that seasonal questing of larvae and nymphs does not match the infestation of rodents, with a much longer period of activity being shown on the rodents than is detected by questing tick surveying (Burri et al., 2011).

1.4.7.5: Reservoir host habitat

The habitat has an important effect on TBE incidence affecting both the small mammal and tick populations. As might be expected, a higher TBE incidence is found in areas with known habitat for ticks, providing sufficient humidity, cover and food for their hosts (Vanwambeke et al., 2010). Habitats that incur high tick abundance include woodland with thick undergrowth, particularly canopies consisting of oak/common hornbeam (*Carpinus betulus*), oak/beech or beech/spruce (*Picea*) and understory of hazel (*Corylus avellana*), elder (*Sambucus nigra*) and bramble (*Rubus fruticosus*). Also ground layer plants such as ferns (*Polypodiopsida spp.*), dog's mercury (*Mercurialis perennis*), nettles (*Urtica diocia*) and bluebells (*Hyacinthoides non-scripta*) are important.

Oak groves and forests and common hornbeam/oak forests, and also forests with tree height variation of at least five meters are particularly suitable (Zeimes et al., 2014; Minár, 1992). Some alluvial plain landscapes also provide the habitat required (Vanwambeke et al., 2010; Süss, 2003; Nosek and Blaškovič, 1973).

The landscape configuration is significant, with large forest areas and a high mean shape index producing a larger ecotonal perimeter area which is optimal for tick abundance (Vanwambeke et al., 2010). These are transitional areas between two biological habitats where species may integrate. These edge zones have an important effect on wildlife including tick hosts, providing a greater complexity of vegetation and availability of multiple landscape elements and microclimates (Pfäffle et al., 2013), although this is not the case where the area abuts agricultural land.

Areas with wide landscape diversity, and those with high proportions of natural forest regeneration, broad-leaf or mixed forest, particularly well connected oak,

birch and pine forests with ecotonal clear-cut or open areas and marshlands or river meadows, have all been demonstrated to be favourable landscapes for TBEV presence (Zeimes et al., 2014; Vanwambeke et al., 2010).

In Austria, there have been changes in forestry from spruce monocultures to more species-rich deciduous and mixed forests due to warming climates. The latter create a more suitable habitat for *I. ricinus*, with the increase in areas of beech forests, combined with the rise in temperature, leading to more frequent mast seeding and several TBE peaks in the last decade (Rubel et al., 2020).

In contrast, arable agricultural land is particularly unfavourable for TBEV presence, as is land left fallow. Even forestry surrounded by agricultural land is less favourable; larger areas of surrounding arable land, further lower the likelihood of the occurrence of TBE. Greater tree height also reduces the probability of TBEV, which is likely to be related to these areas having increased proportions of coniferous trees which tend to be taller than deciduous trees. In addition, clear cutting, where all the trees in an area are felled, may have an adverse effect on ticks and rodent hosts, therefore affecting TBEV presence (Zeimes et al., 2014).

Despite some comprehensive studies investigating TBEV presence on a landscape scale (Zeimes et al., 2014; Vanwambeke et al., 2010), a very limited number of studies have specifically investigated the detailed habitat composition of TBEV foci or microfoci. Therefore, examination of the habitats of the key TBEV reservoir hosts, yellow-necked mice and bank voles could provide further insight into the ecological niches required for TBEV presence; in particular, to identify microfoci and the habitat impact on rodent population fluctuations.

It must be born in mind that a combination of these environmental conditions may not be sufficient to create the required complex habitat needs for establishment of foci, as the necessary climatic and microclimatic conditions are also needed to be suitable for co-feeding on these key small mammal hosts; these are discussed in 1.4.7.7: 1.4.7.8: (Zeimes et al., 2014; Vanwambeke et al., 2010).

Both yellow-necked mice and bank voles are forest species, which avoid open agricultural areas, particularly favouring deciduous woodlands, though to different extents (Schlinkert et al., 2016).

Mature deciduous woodland, particularly ancient woodland is the favoured habitat of the yellow-necked mouse; it is less likely to be found within conifer forests (Flowerdew and Ellwood, 2001). Mature diverse woodland planted in the last 50-100 years are also populated; however, as larger seasonal population fluctuations may occur in these less mature woodlands, they are a less suitable habitat (Marsh et al., 2001). Yellow-necked mice are the only rodent species to prefer older coppiced woodland, benefiting from fallen trees for shelter and nesting sites (Marsh and Harris, n.d.). Hedgerows can also provide an important habitat for this species.

Yellow-necked mice prefer woodlands with a good canopy cover and shrub understory which includes a high diversity of trees that produce hard seed/fruit, particularly hazel in the understory (Flowerdew and Ellwood, 2001). It is mainly granivorous, with a broad diet, feeding on fruits from trees and seeds with insects making up about 20% of their diet (Vukićević-Radić et al., 2006). Therefore, they are highly dependent on mast from trees, particularly beech, oaks and hazel with a slightly higher preference for the latter (Jensen, 1985). A study on Mount Avala, Serbia found an increased abundance of yellow-necked mice in habitats with sessile oak, flowering ash (*Fraxinus ornus*) and common hornbeam (*Carpinus betulus*) present (Vukićević-Radić et al., 2006).

Within its limited range in the UK, the yellow-necked mouse is widespread within suitable habitats (Marsh et al., 2001), although restricted to southern England. There is a correlation of the current yellow-necked mice distribution with the southern Domesday woodland and historical 19th century coppice (Marsh, 1999). Analysis of the distribution of yellow-necked mice in the UK found that a minimum summer temperature had the greatest impact, with areas experiencing maximum summer temperatures of above 20°C being favourable. Woodlands which don't meet this threshold may have diminished tree seed diversity and production (Marsh et al., 2001). Despite Marsh *et al* (2001) not finding an association with soil

moisture or pH, the authors suggested maximum summer temperature may correlate with soil moisture deficit; therefore this may support a previous study's findings of yellow-necked mice being limited to areas with drier soils (Marsh et al., 2001; Montgomery, 1978). It was also found that wet fen-type soils were a particularly unfavourable habitat for yellow-necked mice, very rarely supporting this species (Marsh et al., 2001).

Bank voles are found in a wider variety of habitats than yellow-necked mice, preferring mature broadleaved and mixed woodland, but also inhabiting conifer plantations, field margins, hedgerows and road verges, with a preference for low bush cover (Amori et al., 2015). Contrasting to the yellow-necked mice, a more open canopy/understory promoting good ground cover is beneficial to the bank vole, providing both cover and food source (Flowerdew and Ellwood, 2001). Bank voles are omnivorous or facultative granivorous species, feeding on a very wide range of food sources, including insects, fruits, herbaceous material, with a preference for dicotyledonous plant species, including broadleaved trees and their seeds (Čepelka et al., 2020; The Mammal Society, 2018). Beech seeds are preferred, however acorns and hazel nuts are also eaten (Jensen, 1985). They also feed on the fleshy parts of shrub layer fruiting species, for example hawthorn (*Crataegus monogyna*), elder (*Sambucus nigra*), spindle (*Euonymus europaeus*), and blackberry (*Rubus fruticosus* agg.) and vegetation from ground layer plants such as dog's mercury nettles and bluebells (Bush et al., 2012). Flowers, grasses and mosses are also eaten (The Mammal Society, 2018).

1.4.7.6: *Dilution hosts*
There is a complex relationship between deer density, ticks feeding on rodent host reservoirs and also TBEV foci. Deer feed all life stages of *I. ricinus* ticks and are one of the most important hosts for the adult life stage; however, they are not competent for TBEV transmission (Randolph et al., 1999). Deer enable adult *I. ricinus* to complete their lifecycle, thus increasing tick numbers, which initially can increase numbers of ticks feeding on rodents and therefore the potential for TBEV prevalence. However once a threshold is reached, higher densities of (reservoir incompetent) deer can divert ticks, including immature life stages, from feeding on

reservoir competent rodents. As a result, high densities of deer can in fact act as dilution hosts, decreasing TBEV prevalence in ticks (Jaenson et al., 2012). Therefore, both tick burden on rodents and TBEV prevalence in ticks exhibit a bell-shaped curve, with an initial increase of both factors with increasing deer numbers, followed by a steep decline (Cagnacci et al., 2012; Pugliese and Rosà, 2008). However, a different mathematical modelling study has shown there is a positive relationship between roe deer abundance and co-feeding on rodent hosts (Rosà et al., 2019). This finding may be specific to the locality studied and host populations present and the densities of these; this would need further investigation.

1.4.7.7: Seasonality and climate

Climate and seasonality are further critical factors that determine the nature of a particular microclimate which affect the distribution of ticks and TBE foci. In addition to habitat, the rodent species present and the abundance of both reservoir hosts and non-reservoir tick hosts, will together influence the characteristics of a potential TBEV focus (Hofmeester et al., 2017). Specific climatic conditions that result in synchronised seasonal nymphal and larval activity, is a key factor in the development and maintenance of TBEV foci. This synchronised activity does not always occur throughout the geographical range of *I. ricinus*. TBEV foci occur within areas which exhibit these specific climatic conditions that lead to the phenomenon of co-feeding (Randolph, 2001; Labuda and Randolph, 1999).

Climatic conditions that pertain from spring to early summer, and in late summer to early autumn, have a particular impact on the potential for TBEV foci establishment (Bournez et al., 2020). The autumnal cooling rate has been found to be a critical ecological driver for co-feeding transmission of TBEV and the maintenance of a TBEV focus. Rapidly cooling temperatures in autumn prompts cessation of questing, with larvae and nymphs entering behavioural diapause, overwintering whilst unfed (Rosà et al., 2019). This autumnal cooling has a crucial impact on the seasonal synchrony of ticks the following spring (Rosà et al., 2019). An above average rate of autumnal cooling, in relation to the midsummer peak, is consistently found in areas of synchrony of larvae and nymphs in TBEV foci (Randolph, 2001). Lindgreen and Gustafson found that in Sweden, a long 'mild autumn', between 5-8°C, promoted

tick survival and resulted in increased TBE cases (Lindgren and Gustafson, 2001). Despite being classified as a 'mild autumn', it is of note that Sweden experiences far harsher autumns and winters than some other TBE endemic countries and these temperatures would not be considered mild for autumn in many other areas.

A very low winter temperature can be detrimental to tick survival. In countries that experience particularly cold winters, such as Sweden and Switzerland, 'mild winters', with temperatures between 0°C and -7°C, lead to increases in TBEV by allowing nymphs to quest earlier and also increased tick survival rates (Jaenson et al., 2012; Burri et al., 2011; Lindgren and Gustafson, 2001). However, over winter temperatures in countries that have more temperate climates are less likely to have this impact. Cooler winter temperatures, that allow sufficient temperature gradient to generate a rapid rise in spring, promotes synchronous emergence of larvae and nymphs from behavioural diapause (Jaenson et al., 2012).

Evidence suggests that the titre of virus in infected fed nymphs drops during the winter diapause. In unfed ticks, the titre of virus is at its highest level for six weeks, following moulting from the previous life stage, which then gradually drops (Perez-Eid et al., 1992). It appears that TBEV is maintained in just a small proportion of ticks over winter. Spring breeding of rodents results in an expansion of the population of young naïve rodents that have not yet been exposed. Concurrently when ticks become active in spring, the small number of infected ticks begin to transmit the virus to the young naïve rodents (Bournez et al., 2020; Zöldi et al., 2015). This is supported by field studies detecting no infected ticks before May (Zöldi et al., 2015; Perez-Eid et al., 1992) and a higher prevalence of TBEV in autumn than in spring (Bournez et al., 2020).

Nymphs become active in spring when temperatures rise to between 5-7°C, whereas larvae have a higher threshold of 10°C. Therefore, for sufficient numbers of the two immature life-stages to co-feed on the same hosts, to enable non-viraemic transmission of TBEV, a rapid rise in spring temperatures is needed to stimulate larvae and nymphs to simultaneously start questing (Andreassen et al., 2012; Jaenson et al., 2012; Burri et al., 2011; Lindgren and Gustafson, 2001). The rate of spring warming, and the mean January minimum temperature, can be used

to explain the distribution of TBEV foci (Andreassen et al., 2012; Randolph and Sumilo, 2007). At a microclimatic level, humidity must be sufficient during this period to enable tick survival; however, drier conditions promote questing lower in the vegetation which is more humid, increasing contact with rodent hosts (Randolph and Rogers, 2000; Randolph and Storey, 1999). More gradual increases in spring temperatures result in an initial rise in questing nymphs with a larval peak following a few weeks later (Jaenson et al., 2012). This condition decreases the chances of sufficient levels of co-feeding between these two immature life stages.

Larval questing peaks from May to early July, and questing nymphs peak from April/May to early July in the Alsace region of France, until recently the western-most TBEV foci for many years (Bournez et al., 2020). Favourable conditions occur for TBEV transmission during early May; the rodent population is still low but increases in larvae and nymph numbers result in better opportunities for higher density co-feeding to occur (Bournez et al., 2020; Perez-Eid et al., 1992). In addition, later in May numbers of older overwintering rodents reduce; young naïve rodents then make up a large proportion of the population, and are most efficient for both non-viraemic and viraemic transmission (Perez-Eid et al., 1992). Co-incident peaks then occur during June and early July, when the small mammal population peaks concurrently with those of questing larvae and nymph densities (Bournez et al., 2020; European Centre for Disease Prevention and Control, 2012). Examples of similar periods of peak activity (April to July) were found in the Danube, Slovakia when 86% of *I. ricinus* detected on small mammals were found feeding during this peak (Randolph et al., 1999).

Temperatures need to be high enough over the summer to allow for rapid tick development. In some conditions, such as have been found in Switzerland, larvae that feed between April and May are able to go on to quest as nymphs between July and October. This may explain the higher prevalence of questing nymphs often found in autumn, compared to spring (Bournez et al., 2020). Larvae that are unable to complete moulting, prior to the drop in autumn temperature must overwinter in morphogenic diapause whilst engorged, and then moult in the following spring, before beginning to quest as nymphs (Rosà et al., 2019).

1.4.7.8: Microclimate

Within an ecological context, the microclimate is defined as the climatic conditions in the first metre or so of the earth's surface, within a relatively small area (Kearney, 2018). These conditions include soil temperature, air temperature, relative humidity (RH) and saturation deficit (SD). Microclimatic conditions vary on a smaller scale than weather patterns; these localised conditions are affected by both biotic and abiotic factors such as the vegetation, soil type, latitude, elevation and aspect. For example, sandy soils and other coarse, loose and dry soils experience larger temperature extremes than heavy, wet clay soil types. Temperature variability can be reduced by vegetation coverage which can also insulate soil (Rafferty, n.d.).

Microclimate impacts tick survival, development rate and behaviour such as seasonal activity. Shielded habitats with vegetation cover and leaf litter produce a more stable microclimate and benefit the development and maintenance of tick populations in these areas (Pfäffle et al., 2013).

Tick development relies on the combination of suitable temperatures, particularly seasonal temperature cycles, and relative humidity (RH), which combine to affect development rates of moulting between life stages, which in turn also affects the seasonal dynamics (Andreassen et al., 2012; Randolph, 2002; Labuda and Randolph, 1999). Ticks can become desiccated which affects their survival rate; for example, *I. ricinus* requires a humidity of at least 80%. Therefore moisture availability is essential for tick survival, with regular dew being found to be the most important climatic factor during dry periods in the summer (Zöldi et al., 2015; Andreassen et al., 2012; Randolph, 2002; Labuda and Randolph, 1999). There is a fine balance between all of the factors outlined above to enable optimal conditions that support co-feeding larvae and nymphs on small mammals. Ticks cease questing activity at RH <70%; however, conflicting results have been demonstrated on the impact of RH on *Ixodes* spp. larval tick burden and this needs further investigation (Kiffner et al., 2011).

An experimental study found that prolonged dry and hot conditions can cause mortality in ticks, and in the short term this can cause nymphs to quest lower in the

vegetation increasing the likelihood of contacting rodents. However, this did not have the same effect for larvae, with fewer questing than in more humid conditions. Larvae have a greater sensitivity to small changes, so they cease questing at increased saturation deficit (SD) earlier than nymphs (Andreassen et al., 2012). In Norway, sites with the highest RH and lowest mean SD produced the highest TBEV prevalence in ticks (Esser et al., 2019; Andreassen et al., 2012). If a high SD does result in the reduction of larvae on small mammals, this will result in increases in nymphs becoming infected. This is due to nymphs questing lower in vegetation, so resulting in more feeding on small mammals rather than on larger hosts. However overall, there will be reduced amplification in the tick population due to the limited number of larvae feeding and becoming infected. Therefore, enzootic TBEV transmission efficiency may be reduced in warm and dry microclimatic conditions due to reduced incidence of co-feeding (Andreassen et al., 2012; Randolph and Storey, 1999). Where there are sustained conditions of high SD and low RH, as well as high temperatures for consecutive springs, the TBEV prevalence may decrease or the foci may disappear (Burri et al., 2011).

Areas with a very high number of feeding nymphs can increase the number of infected adults. Despite being less important for the maintenance of sylvatic cycles due to adults mainly feeding on non-competent hosts; high number of infected adults does increase the risk of TBEV exposure to humans (Labuda and Randolph, 1999).

Conversely, field experiments in Germany and France have demonstrated larval tick burdens were higher under drier conditions (82-89% vs 91-98%) (Kiffner et al., 2011; Boyard et al., 2008). This therefore suggests a higher SD is optimal for TBEV transmission as it results in nymphs questing lower in vegetation, so more often contacting rodents, alongside large numbers of larvae. Nymphs quest higher in the vegetation during more optimal RH, so do not encounter small mammals as often (Jaenson et al., 2012; Burri et al., 2011).

Therefore, field and experimental studies are in agreement that drier habitats increase the number of nymphs feeding on small mammal hosts (Bournez et al., 2020; Kiffner et al., 2011; Randolph and Storey, 1999). Further studies are required

to clarify the effect of humidity levels on larval tick burdens on small mammals. It is possible that ticks from different geographical areas may be more acclimatised and adapted to different climatic conditions, therefore responding differently to specific microclimatic conditions compared to ticks from another geographical location (Randolph and Storey, 1999).

In addition to the impact on tick behaviour, humidity impacts on the maintenance of TBEV within ticks, influencing its replication. Higher RH promotes replication of virus and lower RH can result in disappearance of virus from a tick. This phenomenon can in turn influence TBEV prevalence on a tick population scale (Andreassen et al., 2012).

The above illustrates just some of the microclimatic factors that impact tick behaviour and therefore interaction of ticks with their rodent hosts, and co-incidence between the activity of immature life stages. RH, SD, temperature, altitude and vegetation presence are all important variables that can affect tick survival, longevity, development, questing behaviour, seasonality, hosts and virus maintenance. These factors at a local scale can enable the development and maintenance of areas of presence of TBEV infected ticks, and in turn, reservoir hosts can be found in highly localised areas, called 'microfoci'. The quality of this interaction can vary over a relatively small geography creating microfoci within foci due to a varying microclimate (Labuda and Randolph, 1999). It is of note that despite specific habitat and climatic conditions being crucial, they are not always sufficient. A study in Slovakia found all foci matched specific conditions of mean annual rainfall of 800mm, in 8°C annual isotherm and within mixed oak and black locust forests, yet there were other localities which also fulfilled these conditions yet TBEV was not present (Labuda et al., 2002).

1.4.7.9: Vectors

In order for TBEV to establish within an area appropriate tick vector(s) must be present. At least 18 species have been reported to be competent for TBEV transmission (Slovák et al., 2014). Secondary vectors include *Ixodes arboricola*, *Ixodes trianguliceps*, *Ixodes hexagonus*, *Haemaphysalis concinna*, *Dermacentor marginatus* and *Dermacentor reticulatus* (Chitimia-Dobler, Mackenstedt, et al.,

2019). Just *I. ricinus* and *Ixodes persulcatus* have been reported as primary vectors. This is likely to be due to a number of specific ecological criteria being required, which *I. ricinus* meets in Europe as follows: i) the relatively long-life cycle means that a tick infected as a larva, will carry the infection into the subsequent year for feeding and onward transmission; ii) the life cycle must allow for synchrony in the larval and nymphal questing periods with sufficient numbers of each life stage active at the same time (Randolph, 2002) and iii) host relationships that result in feeding of larvae and nymphs on small mammal reservoir hosts efficient for TBEV transmission (Labuda and Randolph, 1999).

Both *I. ricinus* and *D. reticulatus* are competent TBEV vectors that feed on small mammals; however, notably they have different preferences with *D. reticulatus* preferentially feeding on bank voles and *I. ricinus* feeding on yellow-necked mice respectively; the latter being a more efficient TBEV reservoir (Randolph et al., 1999). *D. reticulatus* also differs in having a slightly shorter life cycle, often completed in a year - or two years if the adults overwinter. As a result of the short life cycle, there is just a brief period in which there is the opportunity for larvae and nymphs to co-feed (Földvári et al., 2016). Despite this, a study in Poland of ticks collected from six districts found that TBEV infection prevalence in *D. reticulatus* ranged from 0 to 14.3% with an overall prevalence of 10.8%, whereas TBEV prevalence in *I. ricinus* ranged from 0 to 4.3% with overall minimum infection rate (MIR) of 1.6% (Wójcik-Fatla et al., 2011). Földvári *et al.* (2016) highlighted that *D. reticulatus* can have dominance over *I. ricinus* on cattle hosts in regions in which the former is endemic, with the authors suggesting cattle may be implicated as a TBEV reservoir in some situations (Földvári et al., 2016). *D. reticulatus* has a very limited UK distribution mostly in coastal dune environments in parts of Wales and the South West of England and some urban parks in Essex (Medlock et al., 2018).

1.4.7.10: TBEV foci and prevalence in questing ticks
In addition to a usually low prevalence in ticks within a focus (usually 0.1-5% in Europe) (Burri et al., 2011), active foci have been shown to be infrequent and difficult to identify. Foci tend to be small and localised due to the limited range of both the vectors and their main rodent reservoir hosts, typically around 100m^2. The

food and shelter availability for rodents can affect the distribution of the virus. For example, if rodents need to travel further for these resources, the virus can be transported tens of metres through carriage of infected ticks (Zöldi et al., 2015).

Foci can be sometimes unstable in location and impact square metres (Rosà et al., 2019), and microfoci which can just be a few m^2 have also been shown to move within foci. This is due to movements of infected rodents, infected ticks detaching at different localities within their range, and also infected larvae hatching from a cluster of eggs laid by an infected female. Infected adult ticks may aid the spread of the virus to further neighbouring non-focal areas, through attaching to larger wider ranging hosts such as deer (Zöldi et al., 2015). Seasonal and annual variation of prevalence of TBEV in ticks has been demonstrated (Bournez et al., 2020; Burri et al., 2011; Perez-Eid et al., 1992). A temporal study in the Alsatian TBEV focus, one of the closest in proximity to the UK, found that two thirds of infections in ticks are found between August and October, with the virus not being detected before May (Perez-Eid et al., 1992).

1.5: Importation risks of ticks and tick-borne viruses

LIV is the only zoonotic tick-borne virus currently known to be present in the UK. However in addition to TBEV being endemic in many areas of Europe, Crimean-Congo haemorrhagic fever virus (CCHFV) is also an important emerging tick-borne virus, being highly pathogenic to humans, and increasing in range in Europe (Sorvillo et al., 2020).

In order for an emerging tick-borne virus to successfully establish within the UK, two key factors need to be met. Firstly, the tick vector must either already be endemic or be able to both endure the UK climate, and find suitable hosts to establish and maintain a colony. Secondly the emerging tick-borne virus would need to be introduced to the UK and to infect the tick population and reservoir hosts, and find suitable climatic and ecological conditions for it to establish.

An example of exotic tick importation to the UK was reported by Hansford *et al*., (2014) following accidental importation events of *Rhipicephalus sanguineus*, the dog kennel tick (Hansford et al., 2014). Although the climate is not currently

suitable in the UK for *R. sanguineus* to live outside, there have been numerous reports of house and kennel infestations (Medlock and Leach, 2015). In addition, *Hyalomma spp.* are not resident species in the UK, despite evidence suggesting that they are being transported to the UK every spring (Jameson et al., 2012). However, there have been two separate occurrences identified in England in which ticks appeared to have moulted and overwintered to commence questing the following season in 2018 (McGinley et al., 2021; Hansford et al., 2019). There were also similar reports in the same year, in Germany (Chitimia-Dobler, Schaper, et al., 2019), Austria (Duscher et al., 2018) and the Netherlands (RIVM, 2019). Although *Hyalomma* spp. ticks were identified to have successfully wintered in various localities in western and north western Europe in 2018; the cooler climate affecting metaporphosis of nymphs to adults is thought to be a major factor limiting establishment of *Hyalomma* spp. in the UK (Hansford et al., 2019).

Although the UK is an archipelago of islands which gives a greater degree of protection than mainland countries, there are various routes that a vector and pathogen may take to reach the UK. These may be via migratory birds, migratory bats, imported livestock, companion animals returning from foreign holidays with owners and also the movement of humans (Hasle et al., 2009; Gould et al., 2006).

Birds frequently travel great distances, crossing geographical barriers, such as oceans, deserts and mountains in a very short time. Passerines are very effective ixodid tick hosts, particularly carrying the smaller life stage larvae and nymphs (Hasle, 2013). Some of these birds are migratory, and are therefore able to transport virus-infected ticks to uninfected areas (Kazarina et al., 2015).

Autumn is one of the UK's two key bird migratory periods. During this time there is a complex movement of winter visitors travelling to the UK from Northern European countries such as Sweden, Norway and Iceland, also with passage visitors stopping en-route to southern Europe and Africa. Visitors include *Turdus* spp. such as song thrush (*Turdus philomelos*), fieldfare (*Turdus pilaris*), redwing (*Turdus iliacus*), and also goldcrest (*Regulus regulus*) and European robin (*Erithacus rubecula*) arriving to overwinter in the UK. At the same time, UK breeding birds are migrating south to warmer climates for the winter. These movements are all

reversed during spring, the UK's other key migratory period in which birds overwintering in southern Europe and Africa return to the UK for the summer breeding season, or use it as a stop-over point (Flegg, 2004).

There is well founded theory that TBEV could be dispersed across uninfected areas to establish new TBEV foci via transportation of infected *I. ricinus* on passerines during migration (Rizzoli et al., 2014). Waldenström et al. (2007) found evidence that this was a likely possibility, finding TBEV-infected ticks on birds migrating to Sweden during both the spring and autumn migration (Waldenström et al., 2007). A total of 13,260 birds were screened during spring and autumn with 3.4% of birds sampled infested by ticks. Four out of 326 tick infested birds screened during autumn were found to have TBEV positive ticks. Although this appears a small number, several hundred million birds visit Sweden on migration each year, thus the number of TBEV positive ticks being imported each year is likely to be substantial.

1.6: Objectives of this PhD research

In the UK, TBE has for many years been considered an imported disease with limited opportunities for the virus to become established. This is principally because UK climate and forests were not thought to support the necessary overlap of small rodent hosts and tick life stages for TBEV to become endemic (Gould et al., 2006) along with the need for importation, given the UK is an archipelago. Whilst there is ongoing surveillance work on imported ticks (Hansford et al., 2018) this has not focused on TBEV. There has been very little investigation to confirm that TBEV has not already been introduced into the UK. This gap in research is particularly pertinent, based on TBEV's increasing range in Europe, notably its identification in the Netherlands for the first time in recent years. The main European vector *I. ricinus* is widespread in the UK (Jahfari et al., 2017) and reservoir hosts are present over large areas (NBN Atlas, 2021), therefore this is an area that is a priority for investigation.

In order to understand the potential of TBEV emerging in the UK and understand the distribution of LIV, the aim of this PhD is to test the hypothesis that:

"There is ecological and epidemiological evidence of TBEV in the UK causing foci that present a risk to public health"

The hypothesis will be answered through the following objectives:

Objective 1: Serological screening of sentinel animals (deer) to identify regions of exposure to TBEV-serocomplex pathogens (Chapter 2).

Objective 2: Testing of ticks collected from sentinel animals from seroprevalent sites for presence of TBEV-serocomplex virus (Chapter 2).

Objective 3: Ecological survey and collection of questing ticks from sites associated with high rates of exposure to TBEV-serocomplex virus (Chapter 3).

Chapter 2: Serological screening of UK deer for TBE serocomplex viruses and testing of ticks removed from deer in risk areas

2.1: Introduction

Whilst LIV is known to be present in the UK, its prevalence across the country has had limited research attention, with UK estimates of prevalence and distribution based on voluntary submission from symptomatic livestock. Most surveys of LIV have focused on areas in Scotland and parts of the North of England where the virus is known to be present in the animal population (Jeffries et al., 2014; Laurenson et al., 2007; Adam et al., 1977) and only very limited information is available on the prevalence of LIV in ticks or data on the ecology of the virus (Harrison et al., 2010; Watts et al., 2009; Laurenson et al., 2003; Hudson et al., 1997). The most recent study from 2009 reported between 1.8% and 15.3% prevalence of LIV in 1,063 ticks collected across two years from Scottish upland grouse moors (Watts et al., 2009).

In Europe more extensive research has been conducted investigating TBEV prevalence in ticks and seeking to understand its ecology. The detectable observed prevalence of TBEV in questing ticks in Europe tends to be very low, even in areas of high incidence in humans, rarely exceeding 1% (Imhoff et al., 2015; Stefanoff et al., 2013; Gaumann et al., 2010). Stefanoff *et al.* (2013) (Stefanoff et al., 2013) demonstrated that detection of TBEV in questing ticks is not always reliable, even in regions of known TBEV presence and when relatively large numbers of ticks are tested. This research supports the theory that TBEV tends to be highly localised in small foci with defined borders where the factors that govern the maintenance, locality and defined borders of these foci are largely unknown (Michelitsch et al., 2019). Therefore, this can lead to difficulties in defining survey sites if not guided by specific information such as local human cases or animal seroprevalence studies.

Sentinel animals are important tools for TBEV surveillance and can act as an 'early warning system' where they are of great value for use in areas where TBE has not yet been reported in humans or TBEV detected in ticks. This was demonstrated in the Netherlands where TBEV-neutralising antibodies were detected in deer serum

samples collected 6 years prior to the first human cases (Jahfari et al., 2017). Tick collections and screening can then be targeted on any areas where samples from seropositive sentinels were collected.

Deer are often used as sentinels for TBEV surveillance and have been utilised for this purpose for many years (Gerth et al., 1995). They are proven, reliable sentinels due to 1) presence in large numbers 2) generally having a wide distribution and3) frequenting a variety of habitats; thus lending themselves to serosurveillance studies that aim to cover a wide geographical area, including national studies. The home range of deer mean that they are less suitable to study very small areas and microfoci; conversely, it does mean they are ideal when there is a requirement to cover a large area.

Deer are not thought to be important in the maintenance of TBEV, this is due to the low level of viraemia present for a very short time (Gerth et al., 1995). However, crucially deer have a high susceptibility to tick bite, including by *I. ricinus,* and produce an antibody response to TBEV and LIV (Imhoff et al., 2015; Adam et al., 1977).

Due to the high population of deer in the UK, it is necessary to conduct deer management by culling a proportion of the population each year. This lends itself to sampling for large scale studies as this enables collection of samples from a wide geographic distribution from many different sites. Such a comprehensive level of sampling would not be feasible for a research team to collect working alone. In the UK, deer management tends to be performed by a mixture of recreational and professional deer stalkers, who most often take responsibility for the management of deer on an area of land as agreed by the landowner.

The UK is home to six species of free ranging deer, all of which exhibit specific combinations of distribution, biology, ecology and behavioural characteristics (Carne, 2000). The between-species variation of these factors is beneficial in increasing the geographical regions and habitats covered in sentinel sampling. These factors must also be taken into account in subsequent analysis of data to seek to narrow down the possible area in which a seropositive deer may have been

exposed to an infected tick. As the home range size (HRS) varies considerably between the different deer species (*Table 2:1*), understanding the HRS gives an indication of the possible radius of the seropositive deer cull site and where the deer may have been exposed, as illustrated in *Figure 2:1.*

 Due to the large HRS of fallow and red deer, they are useful in locating general areas where foci may be present due to their wide-ranging behaviour giving an increased chance of encountering a focus. However, pinpointing the location of the exact focus from the deer cull location may be more challenging than for the more hefted deer species.
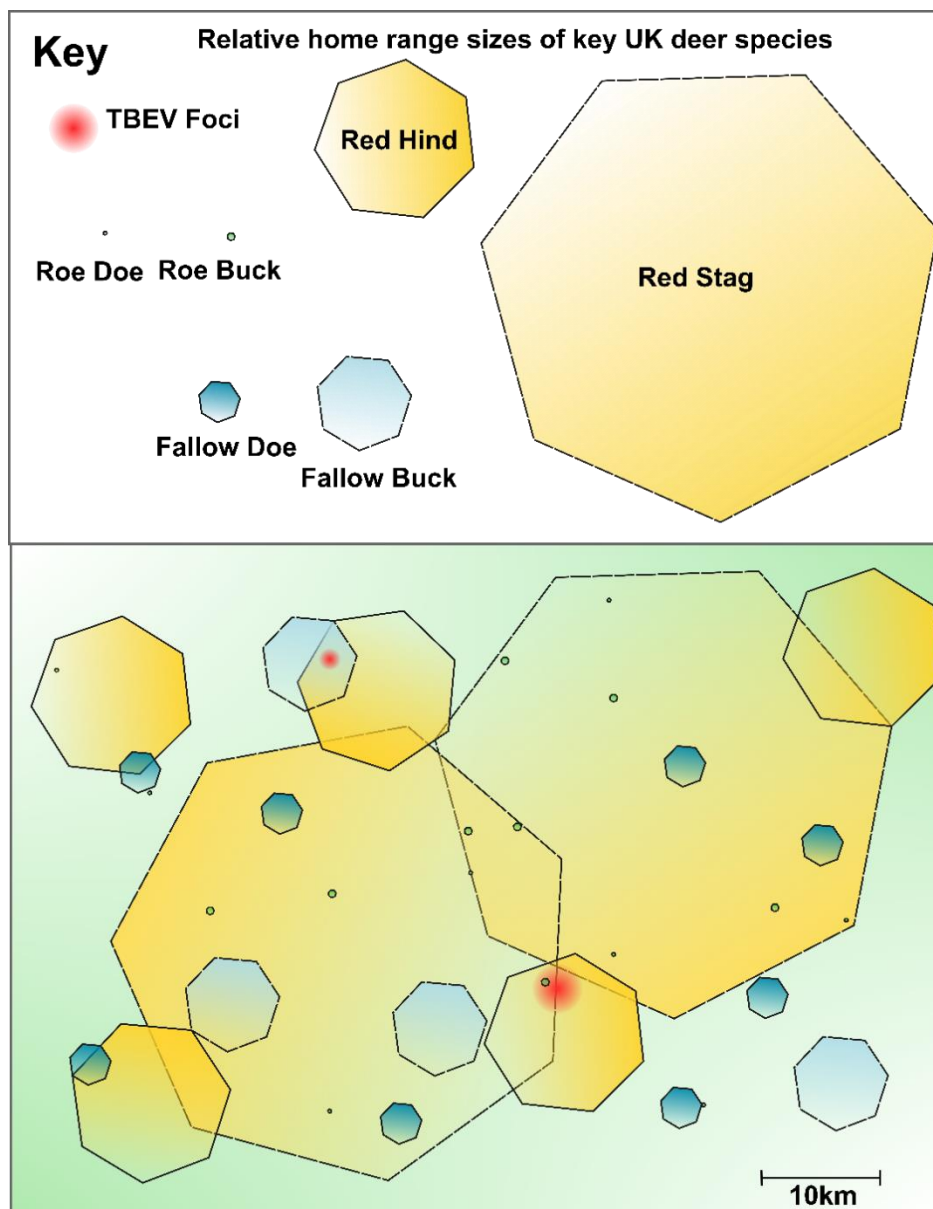
Figure 2:1: *Illustration of variation in relative home range size between deer species, and how these may affect their utility as sentinels*
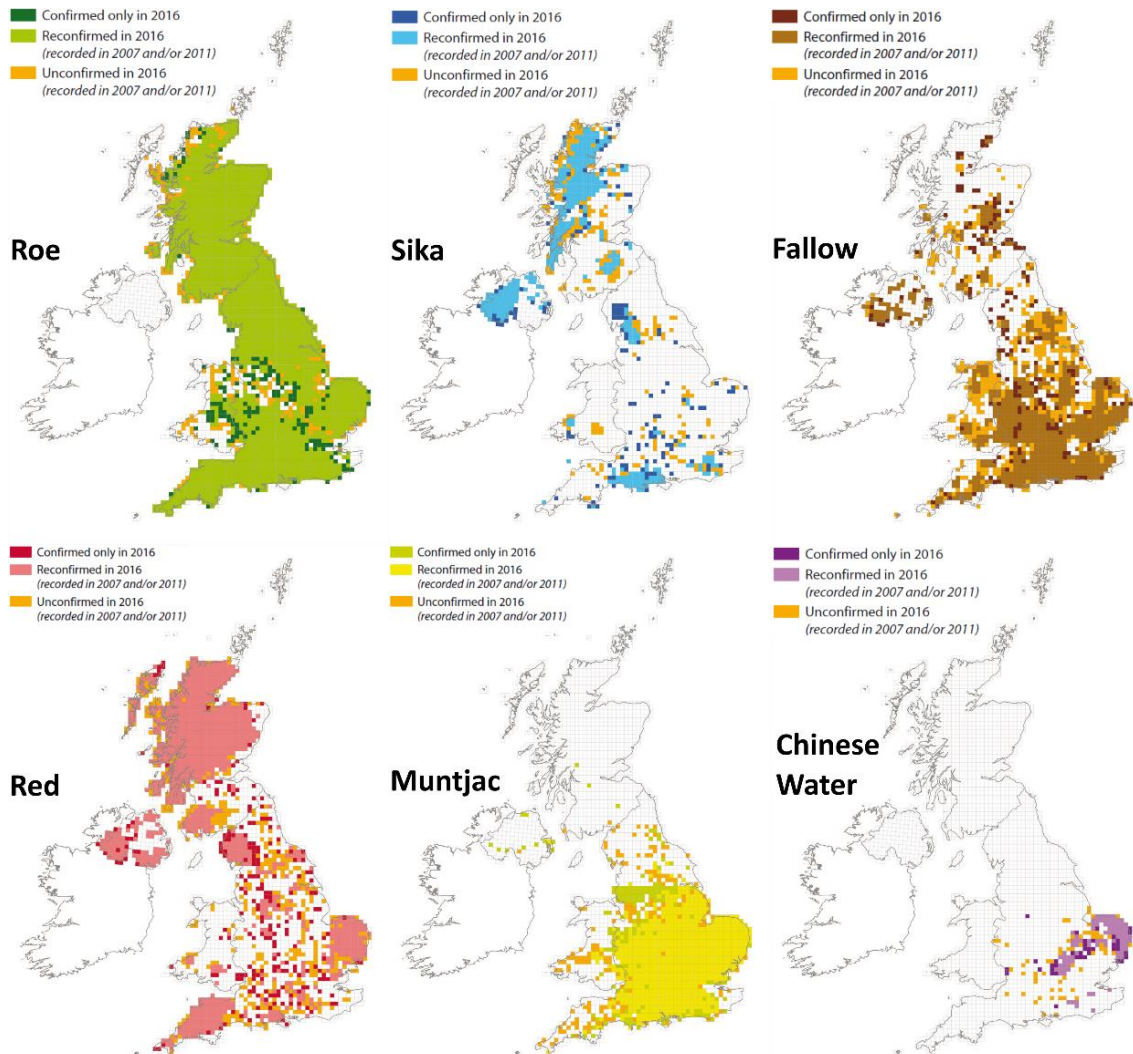
Figure 2:2: *Deer species distribution in the United Kingdom (Image adapted from British Deer Society 2016 Deer Distribution Survey)* (British Deer Society, 2020)

2.1.1: Deer species found in the UK

2.1.1.1: Red deer (Cervus elaphus)

Red deer (*Cervus elaphus*) are native to the UK, with a population of approximately 346,000, around 98% of which are located in Scotland where they are widely distributed (The Mammal Society, 2018; Battersby, 2005). The distribution in England is sparse, with populations predominantly residing in Cumbria, East Anglia, and parts of the South West. There is a very limited population in Wales (British Deer Society, 2020). Geographic location has an impact on habitats occupied; they thrive in areas with diverse woodland near to open heath, moorland or farmland. However, red deer have also adapted well to live in the open upland hill habitats of Scotland, though woodlands are still utilised where available for shelter and food

(The Deer Initiative, 2008a; Carne, 2000). They are a herding species, feeding opportunistically, principally grazing but also browsing. They have seasonal home ranges which vary in size through the year; some are migratory, travelling long distances (18km sometimes) between these ranges, with others just travelling small distances to their seasonal grounds (Luccarini et al., 2006). Stags range quite widely with varying annual HRS, often 10-67km^2; hinds are usually more sedentary with HRS from 1-26km^2; subadults of both sexes tend to use relatively small home ranges (Reinecke et al., 2014; Luccarini et al., 2006; Szemethy et al., 1998).

2.1.1.2: Roe deer (Capreolus capreolus)

Roe (*Capreolus capreolus*) are the deer species in Europe that are most frequently used as TBEV or LIV sentinels. Roe deer, one of two native deer species in the UK, are one of the most abundant species with an estimated population of 296,000 (The Mammal Society, 2018). They are also the most widely distributed across the country, present in most areas with the exception of Northern Ireland, Kent and parts of Wales (*Figure 2:2*) (British Deer Society, 2020). Described as browsers, they generally reside in woodlands of all sizes from large forestry to small thickets, with a penchant for those with abundant shrub understory. Roe also utilise hedgerows and can be found resting in cultivated fields. In some areas, such as the hills in Scotland, they have adapted to living on more open ground (Carne, 2000). They are territorial as a species, usually reliably hefted to their home range and tending to be found alone or in small groups. Roe young are born during May and June (*Figure 2:3*). The home range of the female, known as the doe, are often shared or overlap with other does, or in the summer, with the males, the bucks. Bucks maintain their territory, more frequently holding exclusive home ranges (The Deer Initiative, 2008b). Sex, season, roe density and habitat structure, specifically the edge density all have notable effects on roe deer HRS which are reported to vary with the above influences averaging approximately 0.45-1.10km^2 for bucks and 0.20-0.70km^2 for does (Saïd et al., 2009; Saïd and Servanty, 2005; Kjellander et al., 2004; Chapman et al., 1993) Of note, the HRS for both sexes is at its largest during winter, when tick activity is at its lowest. Displacement by other deer species and yearlings pushed away by territorial bucks can also affect the range that roe cover (Hemami et al.,

2004). The mean HRS of does tends to be below 0.25km^2 during the periods of peak tick activity (spring/summer) (Saïd et al., 2009; Saïd and Servanty, 2005).

The edge density is an index measuring the habitat network, which is the sum of the length of all contacts between different patches of landscape, divided by total area. This is a key factor in influencing roe deer's home range size, due to this area being a rich food resource supporting the roe deer's browsing feeding behaviour (Saïd and Servanty, 2005). Roe deer are the species most commonly used as sentinels for TBEV in Europe, having been successfully utilised in multiple studies across Poland, France, Czech Republic, the Netherlands, and Serbia (Jahfari et al., 2017; Frimmel et al., 2016; Balling et al., 2014; Cisak et al., 2012; Zeman and Januška, 1999). Their relatively small HRS gives a good indication of locality of TBEV foci to the cull location from which seropositive samples are obtained.

2.1.1.3: Fallow deer (Dama dama)

The fallow deer (*Dama dama)* population is large, with approximately 264,000 in the UK (The Mammal Society, 2018). They are a naturalised herding deer species in the UK, widespread in England and Wales, but patchy in Scotland (*Figure 2:2*) They frequent woodlands, favouring established deciduous woodland with thick understory; however they do also colonise coniferous plantations. Woodlands with adjoining arable, meadow or pasture land in which they graze are preferred (Carne, 2000). The movement pattern of fallow deer is complex; males and females tending to remain in separate herds for the majority of the year, with males entering female ranges during rutting, the young being born in June and July (Putman, 1986). Fallow buck home ranges average around one fifth larger than that of females, with males' annual home range averaging 6-10km^2 and females 2-5km^2 (Borkowski and Pudełko, 2007; Davini et al., 2004; Ciuti et al., 2003). Both sexes often occupy two to four smaller different ranges over the four seasons, so the distance travelled between these increases their annual HRS. Males travel around 4km to rutting stands but females' seasonal grounds are around 2km apart (Davini et al., 2004; Ciuti et al., 2003).

2.1.1.4: Muntjac (Muntiacus reevesi)

Muntjac (*Muntiacus reevesi*) are an alien species originally introduced in the home counties and known to be moving northwards, with approximately 128,000 in 2019 in the UK in 2018 (99% are in England) (The Mammal Society, 2018; Battersby, 2005). Currently, they are widely distributed across much of England to the south of Yorkshire (British Deer Society, 2020). Muntjac spend time in areas of thick woodland or scrub with rich mixed vegetation, often utilising small areas and travelling between these through routes in dense vegetation. They are unusual in that they breed all year around, with does producing fawns every 7 months (The Deer Initiative, 2008c). As such, there are no closed seasons and both sexes may be culled throughout the year. Both males and females are territorial, occupying very small home ranges; on average males HRS are 0.28km^2 and females 0.15km^2 (Chapman et al., 1993). Muntjacs have not been reported as being utilised as sentinels for TBEV surveillance, having limited distribution outside of their native range in China. However, their very small HRS gives them great potential as sentinels, aiding in narrowing down locations of TBEV foci.

2.1.1.5: Sika deer (Cervus nippon)

Sika deer *(Cervus nippon)* is also a species introduced to UK, with an estimated population of around 103,000 (The Mammal Society, 2018), is the second least abundant of UK deer species. Sika have a sparse distribution across England and Wales, the main pockets of population are on the southern coast and in the north west of England. The majority (78%) of the UK population of sika are in Scotland where they are much more widely spread, particularly in the north west area (British Deer Society, 2020; Battersby, 2005). They are a herding species, favouring woodlands and thickets close to moorland, heathland or farmland grazing. They are strongly hefted and have a relatively small HRS; females ranges are approximately 0.18-0.22km^2 and males slightly larger at 0.45-0.70km^2 (McCullough et al., 2009).

2.1.1.6: Chinese water deer (CWD) (Hydropotes inermis)

Chinese water deer (CWD) (*Hydropotes inermis)* or water deer are of very limited distribution in the UK, with an estimated population of around 3,600 (The Mammal Society, 2018). There are only small populations, all within England - mainly in central England and East Anglia (British Deer Society, 2020). CWD spend time in

woodlands, and also more open wet areas. Like muntjac, they are territorial and tend to have very small home ranges, often just 0.04km^2 (The Deer Initiative, 2008d).

2.1.1.7: Seasonality of deer culling in the UK

There are different seasons in which deer may be culled in the UK, which align with their behaviour and reproduction cycle. In England and Wales female deer of all UK species are culled between 1st November and 31st of March, with the exception of muntjac, where both the females (without dependent young) and males may be culled all year around (*Figure 2:3*). The culling periods for male deer are longer than that of females; in England and Wales red, fallow and sika having a closed season for males of just three months between May and the beginning of August. Roe bucks may be culled between 1st April and 31st October and both sexes of Chinese water deer (CWD) must be culled during the traditional 'female' culling period (Nov 1st– Mar 31st) (The Deer Initiative, 2007). The open seasons in Scotland vary from those of England and Wales, generally opening and closing a month earlier.

Table 2:1: *Average home range sizes of UK deer species*

Species	Male average home range size	Female average home range size
Muntjac (Chapman et al., 1993)	0.28 km^2	0.15 km^2
Sika (McCullough et al., 2009)	0.45-0.70 km^2	0.18-0.22 km^2
Roe (Saïd et al., 2009; Saïd and Servanty, 2005; Kjellander et al., 2004; Chapman et al., 1993)	0.45-1.10 km^2	0.20-0.70 km^2
Fallow (Borkowski and Pudełko, 2007; Davini et al., 2004; Ciuti et al., 2003)	6-10 km^2	2-5 km^2
Red (Reinecke et al., 2014; Luccarini et al., 2006; Szemethy et al., 1998)	10-67 km^2	1-26 km^2
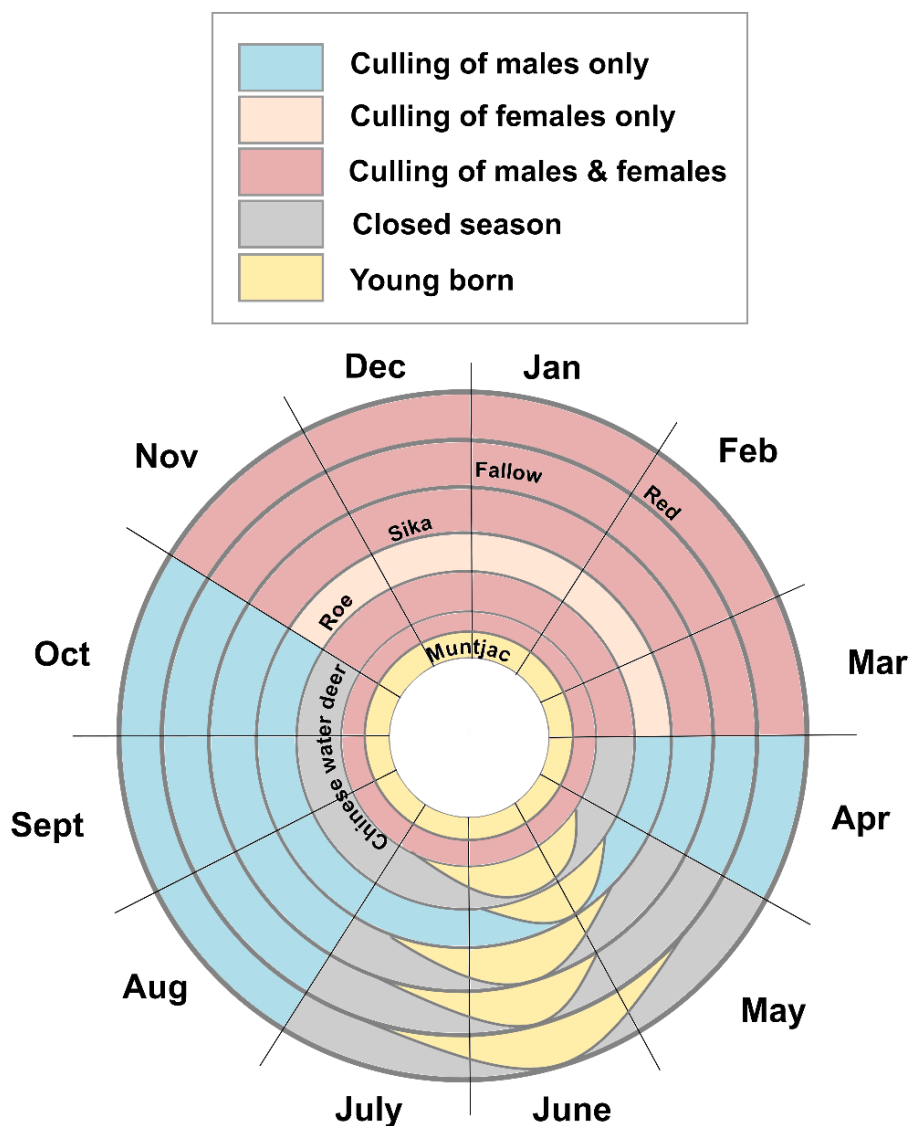
Figure 2:3: *Seasonality of UK deer management and periods in which young are born. Information from* (The Deer Initiative, 2008a, 2008e, 2008b, 2008d, 2008c)

2.1.2: Study aims and rationale

There have been no previous investigations of whether TBEV might be circulating in enzootic cycles in the UK. Serological surveillance of wildlife populations has been shown effective at monitoring TBEV distribution and potential emergence (Jahfari et al., 2017; Imhoff et al., 2015).

The aim of this study is to investigate whether there is any serological evidence of TBEV presence and to contribute to the mapping of LIV presence and prevalence across the UK. To address these aims, there were two objectives: (1) serological screening of sentinel animals (deer) to identify regions of exposure to TBEV-

serocomplex pathogens; and (2) testing of ticks collected from sentinel animals from seroprevalent sites for presence of TBEV-serocomplex virus.

Due to the relatively large HRS of deer, they will be used as sentinels to maximise the area of the UK coverage through this study. In addition, deer of all UK species will be utilised to allow for the inclusion of as wide a geographic coverage across the UK as possible. The utilisation of the wider-ranging deer species will enable more land to be covered by each sample and the more hefted species will provide a greater resolution in areas where seropositive deer are identified. Serological cross-reactivity is widely reported between TBEV and LIV due to the close homology between these two viruses (Klaus et al., 2014; Mansfield et al., 2011). Using current methods available, it will not be feasible to reliably differentiate between antibodies produced against these two viruses. To confirm this, it would be necessary to detect the virus which would not be practical in deer sera due to their short and low level of viraemia. Therefore, ticks that are collected from deer carcasses at the same time as a blood sample, from areas of identified seropositivity in deer, will be screened using molecular methods to attempt to identify the virus responsible for the local seropositivity.

2.2: Materials and methods

2.2.1: Development and implementation of a deer serosurveillance study

2.2.1.1: Protocol and study pack design

A volunteer sampling method was considered the most effective method of collecting a large number of deer serum and tick samples removed from deer, from across the UK. Due to ethical considerations, the samples were collected from culled deer, and specifically those that were already being routinely culled as part of pre-existing deer management i.e. not for the purposes of this study. Therefore, UK deer stalkers who were already culling deer were identified as the target group for this volunteer-based study.

The key aspects which were considered for the design of study protocol and study packs were cost, simplicity and speed for the volunteers collecting the samples. This approach was vital in order to maximise recruitment and ensure as many deer as possible could be sampled by each volunteer.

2.2.1.2: Volunteer pack

A volunteer pack was sent to each volunteer which provided the essential information and equipment necessary for taking part in the study. It consisted of a consent form, protocol, participant information sheet, tick information sheet, risk assessment and contents sheet (Appendix 2). Two sets of tick twisters were also provided, one for the volunteer's use in case they received a tick bite, the other for use in removing ticks from deer. Fine-tipped tweezers were also provided to avoid the need to directly handle the tick and assist in tick removal for smaller life stages.

2.2.1.3: Sampling packs

Dr Hein Sprong who had conducted a similar study (Jahfari et al., 2017) was consulted and provided advice on sampling of deer serum; his recommendation on the use of Sardstedt Luer sampling tubes was also taken. Sardstedt 9ml Luer Monovette Serum separation tubes (catalogue number 02.263) were selected for distribution in the study sampling packs for deer serum collection. These have a built-in syringe system which allows the sampling of deer serum (

*Figure 2:***4**) from a pool of blood, without the need for a needle attachment.

1. Put on a pair of disposable gloves

2. Remove lid   3. Place tube tip in the pool of blood in chest cavity   4. Slowly withdraw the plunger to the end. Pull this until it clicks

Click

5. Break off the plunger at the the tube base

6. Wipe tube tip clean with tissue and replace lid

7. Place tube in an upright position for 30 mins to allow the blood to separate

8. Remove and discard the disposable gloves together with the plunger and tissue

9. The tube is numbered with 'Deer ID number'; please fill in the corresponding Deer Information Form with matching Deer ID. Plese complete each field in the Deer Information Form.

Figure 2:4: *Diagram to illustrate deer serum sample collection provided to deer serosurveillance study volunteers*

Each sample pack contained the equipment to collect blood and tick samples from one culled deer. Each pack contained: Sardstedt Luer sampling tubes labelled with a unique DSS identifying number; a 7ml universal tube labelled also labelled with the DSS number, in which multiple ticks from the same deer could be placed. A pair of nitrile gloves were included for health and safety reasons, and also a tissue to clean the blood tube after sample collection (*Figure 2:6*).

A sampling form was also included in the sample pack to collect information about the deer from which the samples had been collected. This included the unique DSS reference number that could be cross-referenced to the serum and tick sampling tubes that had also been pre-labelled with this number. The sampling form was designed to be straight forward for the volunteers to complete, where possible using categorical variables in a 'tick box' format. Data collected on this form was the date, volunteer name, co-ordinates/location deer was shot, habitat where the deer was shot, the deer species, sex, age, category and condition (*Figure 3.5*). All the listed components for the collection of samples from one deer were contained in a pocket-sized zip seal bag for ease of transport.

Figure 2:5: *Deer record sheet included in each sample collection pack*

2.2.1.4: Deer record sheet data

The date on which the deer was shot was recorded, in addition to the volunteer's name, so that following testing, the individual deer-blood results could be returned to the volunteer. The co-ordinates/location at which the deer was shot was also logged, in order to geographically map the source of each sample. This data was used to check whether LIV had been reported in the area and subsequently, to generate 15km buffer zones around any ELISA positives. It would also inform any follow up surveys that might be required.

The volunteers were also asked to submit the species of deer and the habitat in which it was shot; these are interlinked as deer species have different habitat preferences. The sex of the deer was also recorded; this together with deer species information provides an indication of the deer's home range size, which could

indicate the possible radius/distance from the cull site, to the area where the deer may have encountered an infected tick. If the deer is found to be seropositive, this information, was sometimes used to plan follow up tick surveys. A caveat regarding the cull site habitat is that multiple habitats are often present in the locality, in which the deer are resident. Deer spend time in different habitats, which must be considered when analysing habitat location. As TBEV and LIV each have very different ecologies, this local habitat information is valuable.

The age and body condition of the deer were recorded by the hunter, based on their detailed knowledge and experience of handling deer. If the deer was juvenile, it is unlikely to have travelled far from the area in which it was culled; if seropositive, it will have been exposed to the virus within the last year. The body condition gives an indication of the health of the animal. The deer's age and body condition responses on the form were subjective decisions guided by the hunter's experience, and observations, based on information that could be determine in the field.

2.2.1.5: Return of samples

Due to the need to distribute a large number of study packs to support the collection of a large sample size, a cost-effective means of postage of was necessary. Therefore, the postage packs (*Figure 3.6*) were designed to be compatible to be posted by Royal Mail with a large letter first class stamp. Stamped-addressed A0 padded envelopes were provided with an absorbent 2+2 SpeciSafe mailing pack (Catalogue number: SH0400SS) which could contain two blood collection tubes, for sample return. Volunteers were instructed to send samples by post (via post box or post office) as soon as possible after collection.

Figure 2:6: *Full packs sent out to volunteers for sample collection, showing mailing pack for return of blood tubes on the left.*
Ethical approval

The University of Liverpool Ethics Committee (ref: VREC596) granted ethics approval for this study on February 1, 2018.

2.2.1.6: Volunteer recruitment

Deer conservation and management organisations such as the British Deer Society (BDS), the Deer Initiative and the Forestry Commission were contacted to ask for assistance in recruiting volunteers. Some organisations directly involved in managing deer, including the Forestry Commission, agreed for their Wildlife Rangers to assist in sample collection. The BDS placed articles with information about the study in their e-newsletter and also in their Deer Journal magazine, providing contact details for those interested in volunteering for the study. A snowball sampling method was utilised, with individuals volunteering in the study often passing on information about the study to their contacts.

In addition, British Deer Society events were attended, and presentations delivered to promote the study, giving opportunities to speak with potential volunteers about

taking part, and to distribute study packs. Any volunteers who said they would like to take part by email or telephone were posted the study packs, usually via courier.

2.2.2: Serological testing of deer serum samples

2.2.2.1: Sample collection

Volunteers were asked to use the disposable gloves provided for blood and tick sample collection. Shortly after culling of deer, volunteers collected a blood sample using the Sarstedt Serum Separation tube (shown in

*Figure 2:***4**) from the pools of blood that gather in the chest cavity during gralloching (disembowelling the deer). The lid was placed back on the tube tip and tube cleaned with the provided tissue. Where possible, the blood collection tube was placed in an upright position for 30 minutes to allow the blood to separate.

Volunteers removed ticks from the deer using the tick twisters provided and placed all ticks in the same 7ml universal tube provided. Tweezers were also provided to avoid the need to handle the tick whilst removing it from tick twisters or for ticks that were difficult to remove from the deer.

The serum separation tube was placed in the SpeciSafe sample packing and then into the mailing envelope together with the 7ml universal tick tube and Deer Record Sheet (as shown on arrival at Porton Down *Figure 2:7*). This was then posted via Royal Mail to Porton Down for processing.

2.2.2.2: Sample Processing and storage

Samples arrived at Porton Down daily by post and were unpacked and processed on the day of arrival with the exception of samples that arrived on a Saturday, which were processed on the following Monday. The sample postage packs were refrigerated (2-6°C) on arrival until unpacking and processing.

Following unpacking and recording of samples received, serum separation tubes (also bearing the DSS identifying number) were centrifuged at 1500 relative centrifugal force for 10 minutes. Serum was aliquoted into 1.8ml Nunc Cryovials (Cat Number: 10669071) and stored at -80°C until testing. Many of the serum samples were haemolysed to some degree (*Figure 2:7*), with some being very haemolysed and not separating at all.

Tick samples were stored at -80°C in the 7ml universal tubes, (bearing the DSS identifying number), in which they arrived, until processing.

The data from the deer sample forms were entered and recorded on an Excel database. When the necessary co-ordinates were not provided, these were obtained as accurately as possible by checking the location provided on the map. If further location information was needed, the volunteer was subsequently contacted to ask for more details.

Figure 2:7: *Top to bottom: Sample postage packs on arrival at lab. Packaging of samples on arrival. Deer serum samples following centrifugation*

2.2.2.3: TBEV enzyme linked immunosorbent assay (ELISA)

Collected sera samples were tested in batches using a commercial IgG All Species Progen FSME (TBE) ELISA (cat number: 7701075); performed according to manufacturer's instructions. All components were warmed to room temperature and one aliquot of serum from each deer serum sample was thawed for use in this analysis.

Working buffer was prepared by adding 30ml WASH 10x to 270ml distilled water in a sterile glass duran bottle and mixed thoroughly. The calibrators and control sera were reconstituted by adding 200µl working buffer, which were then vortexed for 10 seconds and left for 15 minutes.

500 µl of working buffer was added to each well of a 96 well deep well plate. 200 µl calibrators, control sera and diluted samples were added into the manufacturer prepared ELISA test strips in a 96 well plate format.

Each serum sample and positive control was tested in duplicate, 10µl of each positive control sera and samples were added to corresponding well according to a plate layout (*Figure 2:8*). The wells were covered with adhesive foil and incubated at room temperature for 60 minutes.

Conjugate working solution was prepared immediately before the sample incubation period was completed. For each 96-well ELISA plate, 240µl of conjugate (protein G) was diluted with 24ml working buffer. Wells were washed 3 times with 200µl/well working buffer. After the final wash, wells were drained, and the plate tapped on absorbent paper to remove excess buffer. 200µl of the prepared conjugate working solution was added into each well of the ELISA plate and covered with adhesive foil. The plate was incubated at room temperature for 60 minutes. Following the incubation period, the wells were washed 3 times with 200µl/well of working buffer. After the final wash, wells were drained, and plate tapped on absorbent paper to remove excess buffer. 200 µl of substrate solution was added into test wells, covered with an adhesive film and incubated at room temperature for 30 minutes. Following the incubation period, 50µl of stop solution was added into each of the test wells. The absorbance of the samples was measured

immediately at an optical density of 450 nm using a plate absorbance reader (SpectraMax M3, Molecular Devices). Samples with a reading of >127 Vienna units/mL (VIEU/ml) were considered to be positive, samples with a reading of 63-126 VIEU/ml were borderline and those <63 were negative. These values were the recommended cut-offs by the manufacturer.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Cal 1	Cal 1	Sample 1	Sample 1	Sample 2	Sample 2	Sample 3	Sample 3	Sample 4	Sample 4	Sample 5	Sample 5
B	Cal 2	Cal 2	Sample 6	Sample 6	Sample 7	Sample 7	Sample 8	Sample 8	Sample 9	Sample 9	Sample 10	Sample 10
C	Cal 3	Cal 3	Sample 11	Sample 11	Sample 12	Sample 12	Sample 13	Sample 13	Sample 14	Sample 14	Sample 15	Sample 15
D	Cal 4	Cal 4	Sample 16	Sample 16	Sample 17	Sample 17	Sample 18	Sample 18	Sample 19	Sample 19	Sample 20	Sample 20
E	Cal 5	Cal 5	Sample 21	Sample 21	Sample 22	Sample 22	Sample 23	Sample 23	Sample 24	Sample 24	Sample 25	Sample 25
F	HL	HL	Sample 26	Sample 26	Sample 27	Sample 27	Sample 28	Sample 28	Sample 29	Sample 29	Sample 30	Sample 30
G	LL	LL	Sample 31	Sample 31	Sample 32	Sample 32	Sample 33	Sample 33	Sample 34	Sample 34	Sample 35	Sample 35
H	Neg	Neg	Sample 36	Sample 36	Sample 37	Sample 37	Sample 38	Sample 38	Sample 39	Sample 39	Sample 40	Sample 40

Figure 2:8: *Plate layout for ELISA assay*

2.2.2.4: LIV hemagglutination inhibition assay (HAI)

LIV HAI tests were performed at the Moredun Research Institute according to the following methodology. 200 µl of deer serum sample was added into individual 7ml universal tubes, with LIV seropositive sheep serum being used as a positive control. Serum samples were diluted 1 in 10 with 1.8 ml of a 12.5% kaolin solution (25 g kaolin +180 ml borate saline solution). Samples were incubated at 4°C for at least 20 minutes and were occasionally mixed during this time before centrifuging at 192 xg (1000 rpm) for 10 minutes.

Diluent was prepared from 0.4% bovine serum albumin in borate saline (BABS). Standardised LIV antigen stored at -80°C was thawed and diluted to an appropriate dilution to contain between 4 and 8 haemagglutinating units in 0.4% BABS with a drop of 0.4% phenol red added. A pre-test check of antigen titration was prepared in U-bottomed plates (*Figure 3.9*). 100 µl of diluted goose red blood cells (RBCs) (0.25% in adjusting diluent) was added to relevant wells and the plate covered and left at room temperature for 20-45 minutes. The plate was monitored during this period as the time to react varies.

For testing the samples, twelve samples were tested per plate (*Figure 3.10*). Samples were diluted in a ten-fold serial dilution, with BABS. The last row, Row H, was a negative control. Diluted goose RBCs were then added, and the assay incubated as before for the pre-test of antigen.

Samples in which hemagglutination occurred at a 1:20 dilution were considered positive. Samples found to be positive by the routine LIV HAI testing were further tested for IgM/IgG discrimination. For this, 200 µl of serum sample was first heat inactivated at 64.5 °C for 30 minutes to destroy any IgM present. The sample was then tested as described above. The titre from the routine testing was compared to the IgM/IgG discrimination result with a fourfold or greater reduction in titre in the heated sample indicates that much of the antibody activity is due to IgM.

	1	2	3	4	5	6	7	8	9	10	11	12	
	Cell control	1024 HAU	512 HAU	256 HAU	128 HAU	64 HAU	32 HAU	16 HAU	8 HAU	4 HAU	2 HAU	1 HAU	
A	Control	1:10240	1:5120	1:2560	1:1280	1:640	1:320	1:160	1:80	1:40	1:20	1:10	Pre-test Ag
B	Control	1:10240	1:5120	1:2560	1:1280	1:640	1:320	1:160	1:80	1:40	1:20	1:10	Test Ag
C	Control	1:10240	1:5120	1:2560	1:1280	1:640	1:320	1:160	1:80	1:40	1:20	1:10	Pos Control
D	Control	1:10240	1:5120	1:2560	1:1280	1:640	1:320	1:160	1:80	1:40	1:20	1:10	Neg Control
E													
F													
G													
H													

50 µl 0.4% BABS

Figure2:9: *Plate layout for pre-test check of antigen titration in 96 well U bottomed plate*

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1 1:10	Sample 2 1:10	Sample 3 1:10	Sample 4 1:10	Sample 5 1:10	Sample 6 1:10	Sample 7 1:10	Sample 8 1:10	Sample 9 1:10	Sample 10 1:10	Sample 11 1:10	Sample 12 1:10
B	Sample 1 1:20	Sample 2 1:20	Sample 3 1:20	Sample 4 1:20	Sample 5 1:20	Sample 6 1:20	Sample 7 1:20	Sample 8 1:20	Sample 9 1:20	Sample 10 1:20	Sample 11 1:20	Sample 12 1:20
C	Sample 1 1:40	Sample 2 1:40	Sample 3 1:40	Sample 4 1:40	Sample 5 1:40	Sample 6 1:40	Sample 7 1:40	Sample 8 1:40	Sample 9 1:40	Sample 10 1:40	Sample 11 1:40	Sample 12 1:40
D	Sample 1 1:80	Sample 2 1:80	Sample 3 1:80	Sample 4 1:80	Sample 5 1:80	Sample 6 1:80	Sample 7 1:80	Sample 8 1:80	Sample 9 1:80	Sample 10 1:80	Sample 11 1:80	Sample 12 1:80
E	Sample 1 1:160	Sample 2 1:160	Sample 3 1:160	Sample 4 1:160	Sample 5 1:160	Sample 6 1:160	Sample 7 1:160	Sample 8 1:160	Sample 9 1:160	Sample 10 1:160	Sample 11 1:160	Sample 12 1:160
F	Sample 1 1:320	Sample 2 1:320	Sample 3 1:320	Sample 4 1:320	Sample 5 1:320	Sample 6 1:320	Sample 7 1:320	Sample 8 1:320	Sample 9 1:320	Sample 10 1:320	Sample 11 1:320	Sample 12 1:320
G	Sample 1 1:640	Sample 2 1:640	Sample 3 1:640	Sample 4 1:640	Sample 5 1:640	Sample 6 1:640	Sample 7 1:640	Sample 8 1:640	Sample 9 1:640	Sample 10 1:640	Sample 11 1:640	Sample 12 1:640
H	Cell Control	Cell Control	Cell Control	Cell Control	Cell Control	Cell Control	Cell Control	Cell Control	Cell Control	Cell Control	Cell Control	Cell Control

Figure 2:10: *Plate layout for HAI sample plates*

2.2.3: Sampling, processing, and testing of ticks removed from deer

2.2.3.1: Sampling of ticks

All of the tick samples removed from deer that were submitted with deer blood samples were logged onto a database. Following the completion of ELISA testing, all samples were mapped on ArcMap 10.5.1 software. A 15km buffer zone was added around all ELISA positive deer samples, any deer sample with ticks that were within this buffer zone were selected for testing by PCR.

2.2.3.2: Tick identification

All ticks removed from deer, that were selected for testing by PCR, were identified using a light microscope and maintained on dry ice throughout the process to avoid freeze thaw. A petri-dish was filled with dry ice and then one 7ml universal tube at a time of ticks was placed in another petri dish that was set on top of the dry ice. Nymphs and adult ticks were identified using ventral and dorsal features based on the Hillyard, (1996) and also Estrada-Peña, Mihalca and Petney (2017) keys.

The tick life stage, species and sex were recorded for each tick, prior to being placed individually into Precellys MK-28R tube for processing, which was labelled with a unique identifying code. The identifying code could be cross-referenced to the 'DSS' ID number with associated deer and location information, tick life stage, sex, species. The tick containing MK-28R tubes were frozen at -80℃ freezer until homogenisation.

2.2.3.3: Homogenisation and extraction

Within a fume hood, RLT was prepared by adding 10µl β-mercaptoethanol per 1 ml of Buffer RLT. This was stored at room temperature and used within one month of preparation. The MK-28R tubes with ticks in them were placed on ice and 300µl buffer RLT added. Samples were kept on ice until homogenisation. Samples were homogenised in an MSc I cabinet in a Bertin Precellys 24 tissue homogeniser at 5500 rpm for 5 seconds followed by a 30 second break, this was repeated 4 times (total of 20 seconds homogenisation). Samples were left at room temperature in the Precellys tissue homogeniser for 10 minutes following the completion of the homogenisation step. Samples were placed back on ice and 1 volume of 100% isopropanol was added and mixed by repeated inversion of each tube.

If samples were not immediately extracted, they were placed in a refrigerator (between 4°C and 8°C) until further processing.

All lysate was transferred on ice to QIAshredder spin column and centrifuged at full speed for two minutes. Lysate was transferred into QIAGEN S-blocks for extraction with the BioSprint 96 One-For-All Vet Kit (384) (Cat No./ID: 947057). QIAGEN MagAttract Suspension G beads were vortexed for 3 mins and 25 μl to each well. Additional S-Blocks and one microplate was prepared as in **Table 2:2** below (slots 2-5). Once prepared these were loaded along with the microplate and rod cover, as delineated in **Table 2:2** into the QIAGEN Omega Bio-tek KingFisher-BioSprint-MagMAX 96 extraction robot and extracted using the 'BS96_Vet_Blood_200_DW96 96DW' pre-set programme. Following programme completion, the elution and lysate plates were sealed with sealing tape. If the extracts were not tested within 24hrs of extraction, these were stored at -80°C until testing.

Table 2:2: *BioSprint 96 One-For-All Vet Kit extraction preparation*

Slot	Loading message	Plate Format	Buffer/item to add	Volume per well (μl)
7	Load Rod Cover	96-well microplate	Large 96-Rod Cover	-
6	Load Elution	96-well microplate	Buffer AVE	100 μl
5	Load Wash 4	S-Block	Buffer RPE	500
4	Load Wash 3	S-Block	Buffer RPE	500
3	Load Wash 2	S-Block	Buffer AW1	500
2	Load Wash 1	S-Block	Buffer AW1	700
1	Load Lysate	S-Block	Sample/buffer	665

2.2.3.4: TBEV RT-PCR

All extracts were tested using the Schwaiger and Cassinotti RT-PCR (Schwaiger and Cassinotti, 2003) in a 20 μl reaction mix of 0.4 μl nuclease free water, 1.6 μl 50mM MgSO$_4$, 1.0 μl 1 μM TM TBE FWD primer, 1.0 μl 18 μM TM TBEV REV primer, 0.2 μl 25 μM TM TBE probe, 0.8 μl Invitrogen Superscript III/Taq enzyme mix, 10 μl 2x reaction mix and 5.0 μl template. The primers are listed in **Table 2:3**. The PCR was run on a ViiA 7 RT-PCR machine using the run conditions listed in **Table 2:4**. The Schwaiger and Cassinotti RT-PCR assay detects viral RNA in the TBEV-serocomplex including LIV and TBEV, targeting a non-coding region.

Table 2:3: *Schwaiger and Cassinotti 2003 primers* (Schwaiger and Cassinotti, 2003)

TM TBE FWD	5' GGG CGG TTC TTG TTC TCC 3'
TM TBE REV	5' ACA CAT CAC CTC CTT GTC AGA CT 3'
TM TBE PROBE	5'-6FAM- TGA GCC ACC ATC ACC CAG ACA CA – BHQ -3'

(6FAM = 6-carboxyfluorescein, BHQ1 = black hole quencher 1).

Table 2:4: *Invitrogen Superscript III run conditions*

Step name	Analysis mode	Temp (°C)	Time (M:S)	Acquisition mode	Cycles	Rate (°C/sec)
RT	None	50	10:00	None	1	20
Denature	None	95	02:00	None	1	20
Amplify	Quantification	95	0:10	None	45	20
		60	0:40	Single		20
Cooling	None	40	0:30	None	1	20

2.2.3.5: LIV RT-PCR

Any samples that tested positive using the Schwaiger and Cassinotti RT-PCR assay were also tested using the Marriott RT-PCR assay, which targets the LIV E Gene and is designed to only detect LIV (Marriott et al., 2006). Therefore, those samples that test positive on the Schwaiger and Cassinotti assay and negative on the Marriott assay are unlikely to be positive for specific LIV RNA. A 20 µl reaction mix was used made up of 3.74 µl nuclease free water, 0.08 µl 100 µM LIV FWD primer, 0.18 µl 100 µM LIV REV primer, 0.2 µl 100 µM LIV probe, 0.8 µl Invitrogen Superscript III/Taq enzyme mix, 10 µl 2x reaction mix and 5.0 µl template. The primers are listed in **Table 2:5**. The PCR was run on a ViiA 7 RT-PCR machine using the run conditions listed **Table 2:4**.

Table 2:5: Marriott 2006 primers

Name	Sequence
LIV F	5' GCT GTC AAG ATG GAT GTG TAC A 3'
LIV R	5' ACT TGT TTC CCT CAA TGT GT 3'
LIV P	5'-6FAM CTG GAG TGC TGC TGA A MGB -3'

(6FAM = 6-carboxyfluorescein, MGB = minor groove binder).

2.2.3.6: 18S ribosomal RT-PCR

A random 10% subset of samples on each extraction plate were tested for tick 18S ribosomal RNA to ensure the extraction process was successfully extracting nucleic acid from tick samples. The RT-PCR assay was designed and developed by Daniel P. Carter, Genomics, Porton Down, Public Health England. A 20 µl reaction mix was used made up of 2.49 µl nuclease free water, 1.3 µl 50mM $MgSO_4$, 0.18 µl 100 µM Dret 18S FWD primer, 0.18 µl 100 µM Dret 18S REV primer, 0.05 µl 100 µM Dret 18S probe, 0.8 µl Invitrogen Superscript III/Taq enzyme mix, 10 µl 2x reaction mix and 5.0 µl template. The primers are listed in **Table 2:6**. The PCR was run on a ViiA 7 RT-PCR machine using the run conditions listed **Table 2:4**.

Table 2:6: Dret 18S primers

Name	Sequence
Dret_18S FWD	5'-TCC CAG CAC CTT ACA ACC TTC-3'
Dret_18S_REV	5'-AGA CAC GCT GCT TCC TTC AG-3'
Dret 18S_ PROBE	5'-CY5-CCG CAC GAA ACA GAG CAA TAA CA-BBQ650-3'

2.2.3.7: Genome sequencing and phylogenetic analysis

Metagenomic sequencing was attempted for samples that were positive using the Schwaiger and Cassinotti RT-PCR assay (Schwaiger and Cassinotti, 2003) and had a Ct value of below 30. Sequencing and assembly work were performed by Dan Carter, with assistance from Steve Pullan both from the PHE Genomics team. Tick samples were prepared for metagenomic RNA sequencing (Kafetzopoulou et al.,

2018) and then sequence reads were sequence assembled using SPAdes version 3.1.1 (Nurk et al., 2013). The evolutionary history was inferred by using the maximum-likelihood method based on the Tamura 3-parameter model (Tamura, 1992). A phylogenetic tree was constructed using the tree with the highest log likelihood by Roger Hewson, PHE Virology & Pathogenesis group. Initial trees for the heuristic search were automatically obtained by applying neighbor-joining and BioNJ (Gascuel, 1997) algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach and then selecting the topology with superior log likelihood value. The analysis involved 10 full-length genomic TBEV nucleotide sequences and was performed using Molecular Evolutionary Genetics Analysis version 7.0 software (Kumar et al., 2016). Data was compiled with a variety of other published TBEV genomes circulating in Europe, in addition to reference genomes from other TBEV sub- types to infer the evolutionary history.

2.2.4: Analysis

Agresti coull 95% confident intervals (95% CI), Fisher's exact P values and all data manipulation and analysis was conducted in Stata 15.1. All mapping was produced in ArcMap 10.5.1.

2.3: Results

2.3.1: Demographics and geography of sampled population of UK deer

A total of 145 deer stalker volunteers submitted samples to the study between February 2018 and January 2019, submitting a mean of 9 samples each. The median number of samples submitted by volunteers was 5, and the largest number of samples submitted by an individual was 83 and the least was 1. The majority (64.1%) of samples were submitted over two months in the spring (March and April 2018) and two months in the autumn (October and November 2018) as shown in *Figure 2:11*. The least samples were submitted in June, July and December.

In total, serum samples were submitted from 1,323 deer; of these 14 samples were excluded as they had no or insufficient location or deer species information. Samples were submitted from a large number of geographic locations across England and Scotland as shown in *Figure 2:18*. Samples were submitted from 56 English counties/Scottish council areas; no samples were submitted from Wales or

Northern Ireland. The greatest number of samples were submitted from Argyll & Bute (12.1%), followed by Hampshire (8.0%), Northumberland (7.5%), Cumbria (7.3%) and Highland (6.2%), detailed in **Table 2:9**.

Of the 1,309 deer serum samples included in the study, these were from the 5 of the 6 UK deer species and also from a hybrid. No Chinese water deer samples were submitted. The most frequently sampled deer were roe deer (*Capreolus capreolus*; 50.6%), followed by fallow deer (*Dama dama*; 18.8%), red deer (Cervus elaphus; 18.5%), muntjac (*Muntiacus reevesi*; (8.3%), sika (*Cervus nippon*; 3.7%), and red/sika hybrid (0.2%).
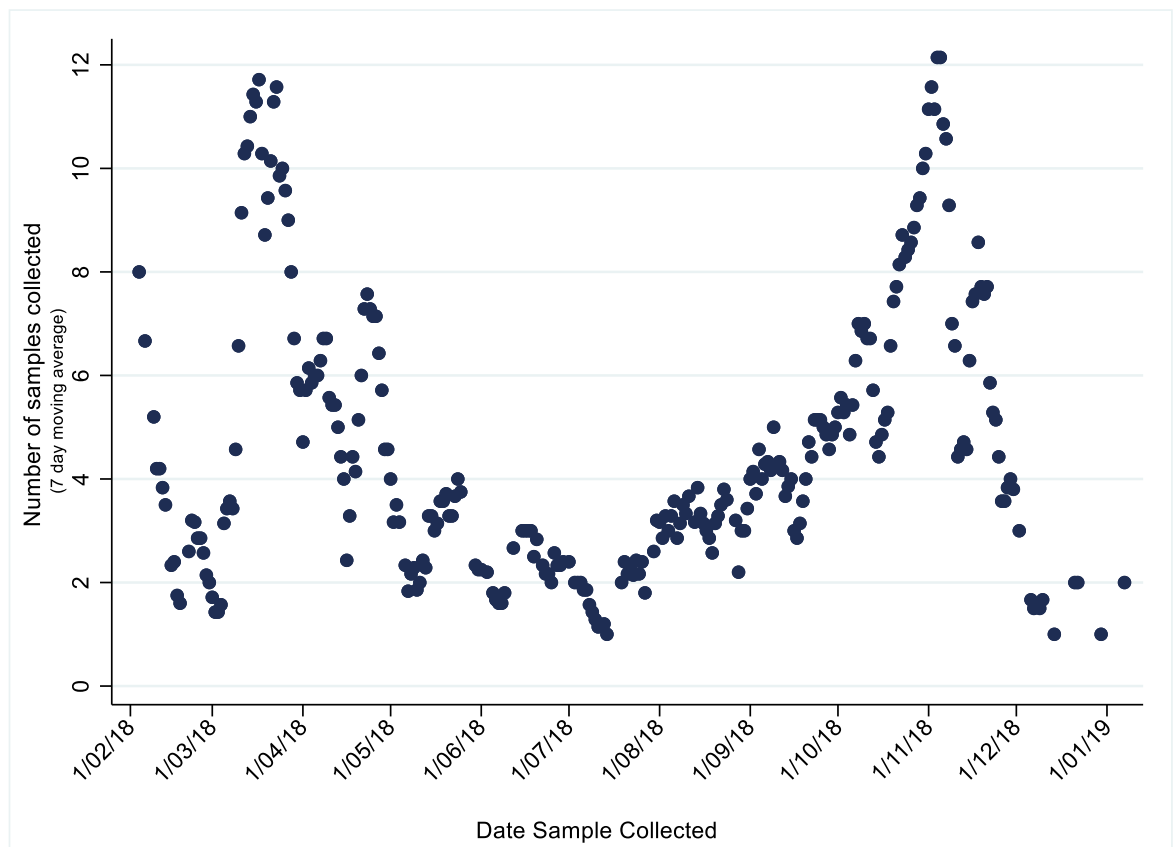
Figure 2:11: *Samples collected between February 2018 and January 2019 (7 day moving average)*

2.3.1.1: Demographics of deer from which samples were collected

Overall, the majority (61.0%) of samples were submitted from male deer, 37.9% were female; this information was not provided for the remaining 1.2%. More samples were submitted from male deer for all species, other than for red/sika hybrids for which one sample was submitted from each sex.

Most deer were adult (57.5%), followed by yearlings (24.5%), juveniles (13.0%) and least frequently, old (3.8%).

The majority of deer sampled were of good or very good condition (51.6% and 41.8% respectively), with just 4.8% being listed as in poor condition. There was variation in the scoring of body condition by species, with muntjac and fallow being more commonly scored as very good (79.6% and 54.9% respectively) and red, roe and sika were more often scored as average condition (63.6%, 57.5% and 52.1% respectively).

2.3.1.2: Habitat type from which deer were culled

Samples were submitted from deer culled in coniferous (32.9%), deciduous (30.1%) and mixed woodlands (3.2%), grassland/arable (21.0%), upland heath (7.4%) and lowland heath (4.6%) habitats. The habitat in which deer were more often culled varied by species as shown in *Table 2:7*: fallow and muntjac were more commonly culled in deciduous woodland followed by grassland/arable habitats; red were more commonly culled in coniferous woodland followed by upland heath habitats; roe deer were more commonly culled in coniferous followed by deciduous habitats; and sika deer were equally commonly culled in coniferous and grassland/arable habitats. Results suggest that there is a statistically significant relationship between habitat deer were culled in and deer species (Fisher's exact p = <0.000), demonstrating that the habitat in which deer were culled varied between species.

2.3.1.3: Geographic distribution of deer sampled by species

Roe deer provided the widest distribution of samples, with this species being sampled from across 45 counties/council areas in England and Scotland (*Figure 2:12* A). Despite being the second most sampled species in this study, just 2.0% of submitted fallow samples were culled in Scotland (across 2 counties). The majority were collected from Southern and Central England (*Figure 2:12* B). In contrast to fallow deer, 80.2% of red deer were sampled from Scotland (*Figure 2:12* C). All sampled muntjac were from within England, 33.3% were sampled from Norfolk and Suffolk (*Figure 2:13* D). Sika were sampled from just 6 county/council areas in England and Scotland, 77.1% of these were culled in Dorset (*Figure 2:13* E). Only

two samples were collected from Red/Sika hybrids, both of which were from Argyll & Bute (*Figure 2:13* F).
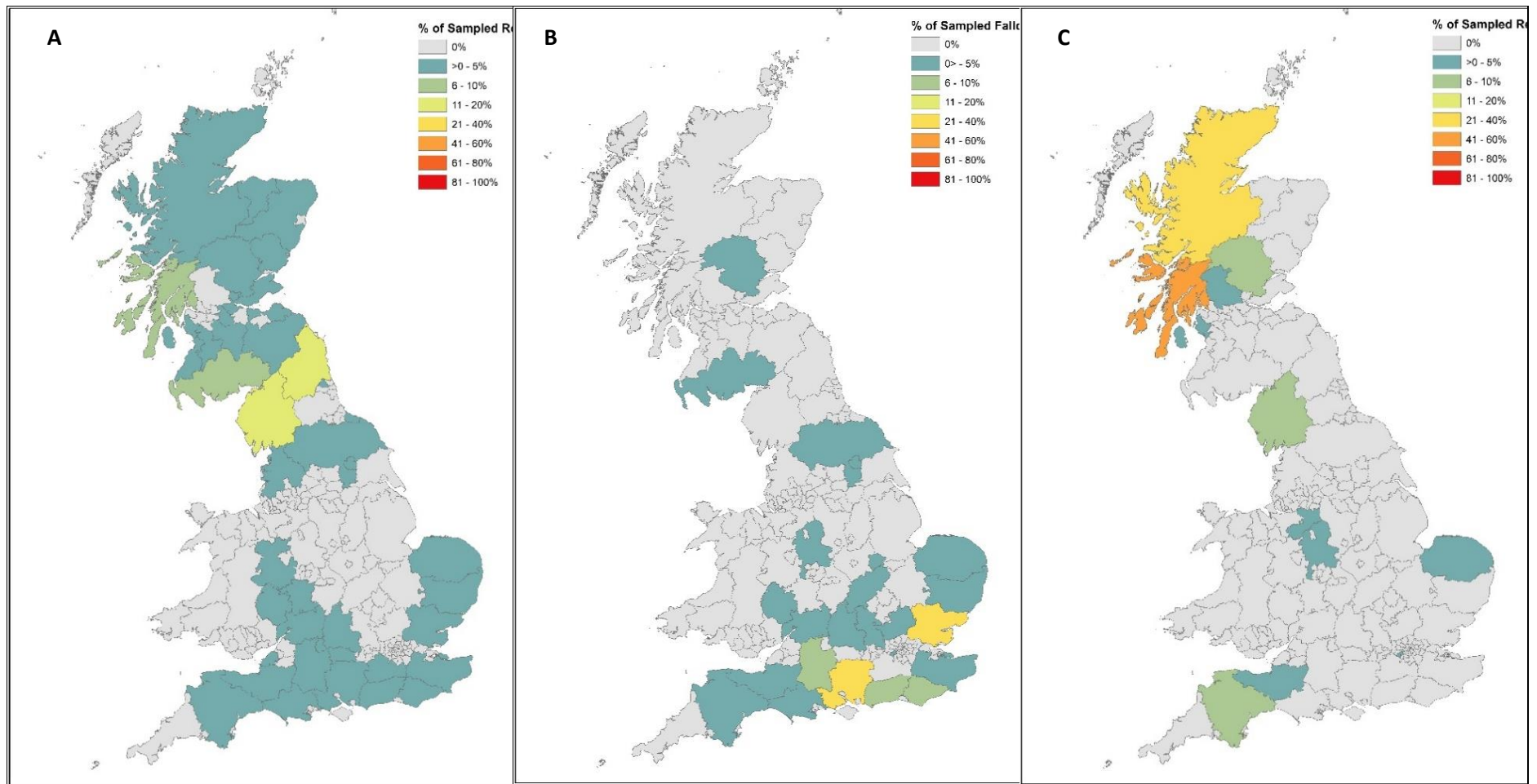
Figure 2:12: *Proportion of sampled species of deer by county/council area. A: Roe, B: Fallow and C: Red deer*
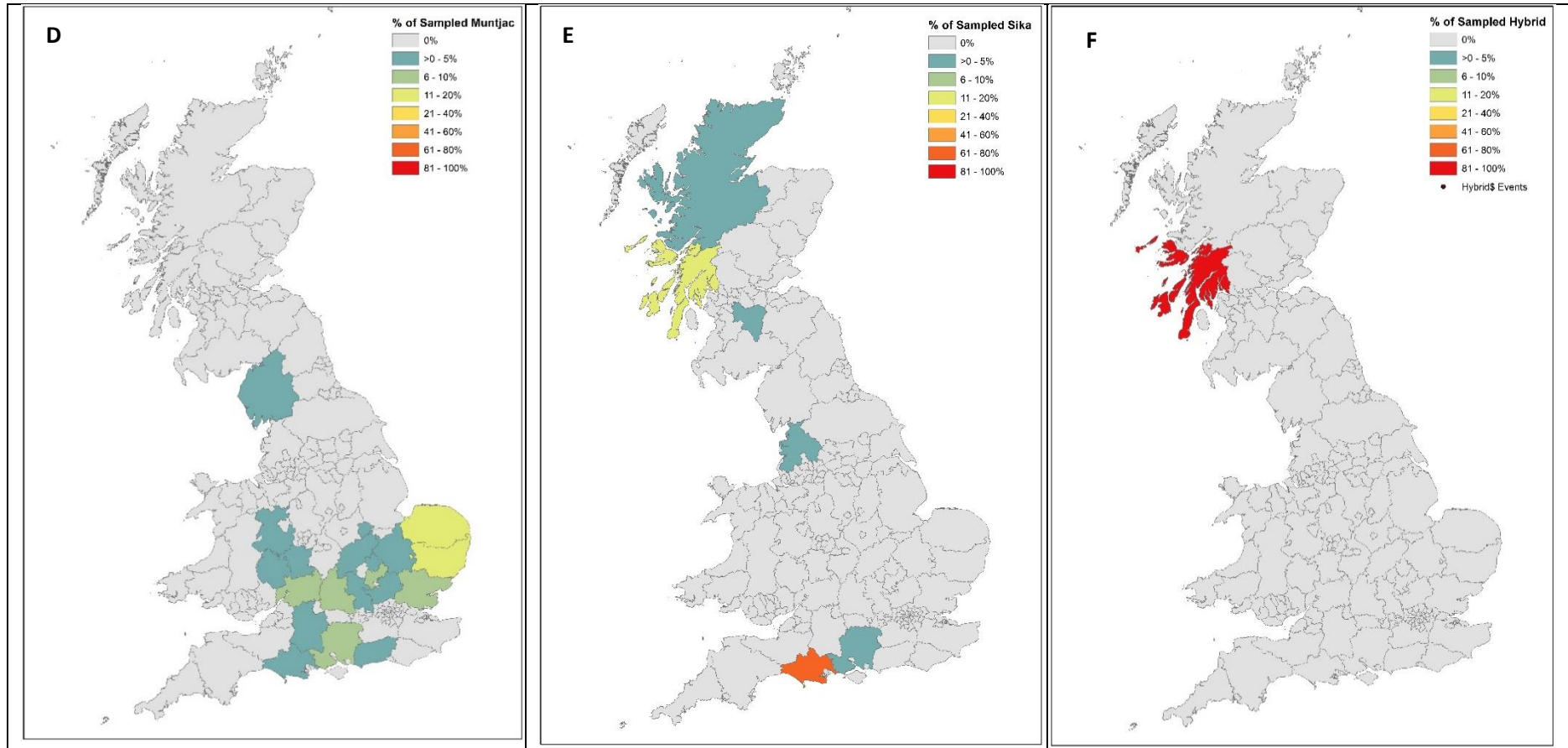
Figure 2:13: *Proportion of sampled species of deer by county/council area. D: Muntjac, E: Sika and F: Red/Sika hybrid deer*

Table 2:7: The number and percentage of each species of deer culled broken down by the habitat type in which they were culled.

Habitat	Roe n (%)	Fallow	Red	Muntjac	Sika	Other	Total
Coniferous	281 (42.38)	24 (9.76)	98 (40.50)	12 (11.11)	14 (29.17)	2 (100.00)	431 (32.93)
Deciduous	188 (28.36)	101 (41.06)	26 (3.92)	75 (69.44)	4 (8.33)	0 (0.00)	394 (30.10)
Grassland/ Grassland	132 (19.91)	90 (36.59)	25 (3.77)	14 (12.96)	14 (29.17)	0 (0.00)	275 (21.01)
Upland heath	12 (1.81)	0 (0.00)	82 (12.37)	0 (0.00)	3 (6.25)	0 (0.00)	97 (7.41)
Lowland heath	21 (3.17)	13 (5.28)	9 (1.36)	5 (4.63)	12 (25.00)	0 (0.00)	60 (4.58)
Mixed woodland	22 (3.32)	18 (7.32)	0 (0.00)	1 (0.93)	1 (2.08)	0 (0.00)	42 (3.21)
Unknown	7 (1.06)	0 (0.00)	2 (0.30)	1 (0.93)	0 (0.00)	0 (0.00)	10 (0.76)
Total	**663 (100.00)**	**246 (100.00)**	**242 (100.00)**	**108 (100.00)**	**48 (100.00)**	**2 (100.00)**	**1309 (100.00)**

2.3.2: Serological screening of deer sampled across the UK for antibodies against TBEV and LIV

Of serum samples from across the United Kingdom, 4.1% [3.1-5.3] were positive by ELISA, and 5.3% [4.2-6.7] by HAI. Thirty-eight of the samples were positive by both ELISA and HAI. Cohen's κ test indicated substantial agreement (0.61) between the methods, indicating ELISA results agreed closely with HAI test results (*Table 2:8*).

	HAI Result			
ELISA Result	Positive	Negative †	Not tested	Total
Positive	38	14	1	53
Negative ‡	31	1219	6	1225
Total	69	1233	7	1309

Table 2:8: *Variation between ELISA for TBEV and HAI for LIV**

*HAI, hemagglutination inhibition. †HAI negative, borderline, unknown. ‡ELISA negative/borderline.

ELISA tests yielded positive results in all deer species for which it was used, aside from the red/sika hybrid by ELISA for which only two samples were obtained. ELISA positives were detected in 27/663 roe, 10/246 fallow, 9/242 red deer, 6/108 muntjac, 1/48 sika, and 0/2 red/sika hybrids. HAI determined the following positives: 28/662 roe, 15/245 fallow, 18/242 red, 7/106 muntjac, 0/45 sika, and 1/2 red/sika hybrid. The seropositivity between deer species by both ELISA and HAI were very similar, with overlapping confidence intervals, as shown in *Table 2:10* and

*Table 2:***11**. Seropositivity by ELISA ranged from 2.08% [0.00%-11.91%] for Sika to 5.56% [2.33%-11.84%] for Muntjac. No seropositive Sika were detected by HAI 0% [0.00%-9.38%] and the highest seroprevalence by HAI of 7.44% [4.69-11.51] was detected in red deer.

ELISA- and HAI-positive samples were distributed in geographically specific areas (*Figure 2:15* and *Figure 2:16*). Most areas from which seropositive samples were submitted were identified through both ELISA and HAI testing. However, some

additional localities were identified by seropositive samples by HAI. Seroprevalence was high in south western Norfolk and north western Suffolk (Thetford Forest) in the east of England. Norfolk had the highest seroprevalence detected by ELISA (51.4%), followed by Hampshire (14.3%), Suffolk (10.7%), and Highland (8.6%). No seropositive samples were detected either by ELISA or HAI in 42 of the 56 counties/council areas sampled (*Table 2:9*).

Seropositivity varied by the habitat type in which the deer were culled, with grassland arable habitats having the lowest seroprevalence by both ELISA and HAI (ELISA 0.7%, 95% CI 0.0-2.8%; HAI 2.92%, 95% CI 1.39-5.75%). The remaining habitats had varying seroprevalences by both ELISA and HAI. The seroprevalence, by habitat in which shot, when tested by ELISA was mixed woodlands 11.9%, lowland heath 8.3%, upland heath 7.2%, coniferous 4.9% and deciduous 3.3. All confidence intervals overlapped and some such as mixed woodland had a small sample size (n=42), full results are shown in *Table 2:10*. There were also different levels of seropositivity across habitat types deer were culled in for HAI (

*Table 2:***11**); coniferous (5.1%), deciduous (4.6%), mixed (7.1%), lowland heath (10.2%) and upland heath (12.4%); however, there was still overlap in the confidence intervals for most of the habitats.

Deer had similar seroprevalence by sex and this was consistent across species. These results suggest that there is not a statistically significant relationship between sex and positive TBEV ELISA or LIV HAI results (Fisher's Exact ELISA p=0.565; HAI= p=0.800) (*Table 2:12* and *Table 2:13*).

There was no relationship between the condition of the deer and positivity by TBEV ELISA or LIV HAI (Fisher's exact ELISA= 0.090; HAI p=0.610). Of the deer listed of average condition, 5.2% were TBEV ELISA positive and 5.8% LIV HAI positive. Of those listed as poor condition 3.2% were TBEV ELISA positive and 6.5% LIV HAI positive. For those in very good condition, 2.7% were TBEV ELISA positive and 4.8% LIV HAI positive.

Table 2:9: *ELISA and HAI positive results for TBEV from counties with submitted serum samples. Counties with positive results by ELISA and/or HAI underlined.*

County (Country)	ELISA positive/total tested (%) [95% CI]†	HAI positive/total tested (%) [CI]†
Norfolk (England)	18/35 (51.43) [35.57–67.01]	16/35 (45.71) [30.46–61.82]
Hampshire (England)	15/105 (14.29) [8.74–22.35]	14/104 (13.46) [8.07–21.46]
Suffolk (England)	3/28 (10.71) [2.90–28.01]	2/28 (7.14) [0.90–23.73]
Highland (Scotland)	7/81 (8.64) [3.99–17.04]	8/81 (9.88) [[4.86–18.53]
Perth and Kinross (Scotland)	2/33 (6.06) [0.68–20.60]	10/33 (30.30) [17.25–47.46]
Dorset (England)	2/72 (2.78) [0.19–10.15]	0/70 (0.00) [0.00–6.23]
Cumbria (England)	2/95 (2.11) [0.12–7.81]	4/95 (4.21) [1.31–10.67]
Argyll and Bute (Scotland)	3/158 (1.90) [0.40–5.69]	5/158 (3.16) [1.16–7.39]
Wiltshire (England)	1/56 (1.79) [0.00–10.34]	5/55 (9.09) [3.53–19.99]
Stirling (Scotland)	0/2 (0.00) [0.00–70.98]	1/2 (50.00) [9.45–90.55]
Somerset (England)	0/13 (0.00) [0.00–26.59]	1/13 (7.69) [0.00–35.42]
Moray (Scotland)	0/19 (0.00) [0.00–19.79]	1/19 (5.26) [0.00–26.48]
Gloucestershire (England)	0/24 (0.00) [0.0016.31]	1/24 (4.17) [0.00–21.87]
Aberdeenshire (Scotland)	0/32 (0.00) [0.00–12.73]	1/32 (3.13) [0.00–17.11]
Northumberland (England)	0/98 (0.00) [0.00-4.53]	0/97 (0.00) [0.00-4.57]
Essex (England)	0/64 (0.00) [0.00-6.78]	0/64 (0.00) [0.00-6.78]
Dumfries & Galloway (Scotland)	0/55 (0.00) [0.00-7.80]	0/55 (0.00) [0.00-7.8]
North Yorkshire (England)	0/42 (0.00) [0.00-9.99]	0/42 (0.00) [0.00-9.99]
Devon (England)	0/34 (0.00) [0.00-12.07]	0/34 (0.00) [0.00-12.07]
West Sussex (England)	0/33 (0.00) [0.00-12.39]	0/33 (0.00) [0.00-12.39]
East Sussex (England)	0/27 (0.00) [0.00-14.76]	0/27 (0.00) [0.00-14.76]
Oxfordshire (England)	0/24 (0.00) [0.00-16.31]	0/23 (0.00) [0.00-16.91]
Falkirk (Scotland)	0/19 (0.00) [0.00-19.79]	0/19 (0.00) [0.00-19.79]
Northamptonshire (England)	0/14 (0.00) [0.00-25.15]	0/14 (0.00) [0.00-25.15]
Richmond upon Thames (England)	0/14 (0.00) [0.00-25.15]	0/14 (0.00) [0.00-25.15]
Herefordshire, County of (England)	0/11 (0.00) [0.00-30.02]	0/11 (0.00) [0.00-30.02]
Hertfordshire (England)	0/10 (0.00) [0.00-32.09]	0/10 (0.00) [0.00-32.09]
West Berkshire (England)	0/10 (0.00) [0.00-32.09]	0/10 (0.00) [0.00-32.09]
Kent (England)	0/9 (0.00) [0.00-34.46]	0/9 (0.00) [0.00-34.46]
South Ayrshire (Scotland)	0/9 (0.00) [0.00-34.46]	0/9 (0.00) [0.00-34.46]
Edinburgh, City of (Scotland)	0/8 (0.00) [0.00-37.22]	0/8 (0.00) [0.00-37.22]

Angus (Scotland)	0/7 (0.00) [0.00-40.44]	0/7 (0.00) [0.00-40.44]
Central Bedfordshire (England)	0/7 (0.00) [0.00-40.44]	0/7 (0.00) [0.00-40.44]
Clackmannanshire (Scotland)	0/5 (0.00) [0.00-48.91]	0/5 (0.00) [0.00-48.91]
Scottish Borders (Scotland)	0/5 (0.00) [0.00-48.91]	0/5 (0.00) [0.00-48.91]
Buckinghamshire (England)	0/4 (0.00) [0.00-54.60]	0/4 (0.00) [0.00-54.6]
Lancashire (England)	0/4 (0.00) [0.00-54.60]	0/4 (0.00) [0.00-54.6]
Redcar and Cleveland (England)	0/4 (0.00) [0.00-54.60]	0/4 (0.00) [0.00-54.6]
Swindon (England)	0/4 (0.00) [0.00-54.60]	0/4 (0.00) [0.00-54.6]
Cambridgeshire (England)	0/3 (0.00) [0.00-61.75]	0/3 (0.00) [0.00-61.75]
North Somerset (England)	0/3 (0.00) [0.00-61.75]	0/3 (0.00) [0.00-61.75]
Surrey (England)	0/3 (0.00) [0.00-61.75]	0/3 (0.00) [0.00-61.75]
Worcestershire (England)	0/3 (0.00) [0.00-61.75]	0/2 (0.00) [0.00-70.98]
Bedford (England)	0/2 (0.00) [0.00-70.98]	0/2 (0.00) [0.00-70.98]
East Lothian (Scotland)	0/2 (0.00) [0.00-70.98]	0/2 (0.00) [0.00-70.98]
Newcastle upon Tyne (England)	0/2 (0.00) [0.00-70.98]	0/2 (0.00) [0.00-70.98]
North Ayrshire (Scotland)	0/2 (0.00) [0.00-70.98]	0/2 (0.00) [0.00-70.98]
North Lanarkshire (Scotland)	0/2 (0.00) [0.00-70.98]	0/2 (0.00) [0.00-70.98]
Rutland (England)	0/2 (0.00) [0.00-70.98]	0/2 (0.00) [0.00-70.98]
Shropshire (England)	0/2 (0.00) [0.00-70.98]	0/2 (0.00) [0.00-70.98]
South Lanarkshire (Scotland)	0/2 (0.00) [0.00-70.98]	0/2 (0.00) [0.00-70.98]
Staffordshire (England)	0/2 (0.00) [0.00-70.98]	0/2 (0.00) [0.00-70.98]
York (England)	0/2 (0.00) [0.00-70.98]	0/2 (0.00) [0.00-70.98]
Cheshire East (England)	0/1 (0.00) [0.00-83.25]	0/1 (0.00) [0.00-83.25]
East Ayrshire (Scotland)	0/1 (0.00) [0.00-83.25]	0/1 (0.00) [0.00-83.25]
Fife (Scotland)	0/1 (0.00) [0.00-83.25]	0/1 (0.00) [0.00-83.25]
Total	53/1309 (4.05) [3.10-5.27]	69/1302 (5.30) [4.20-6.66]
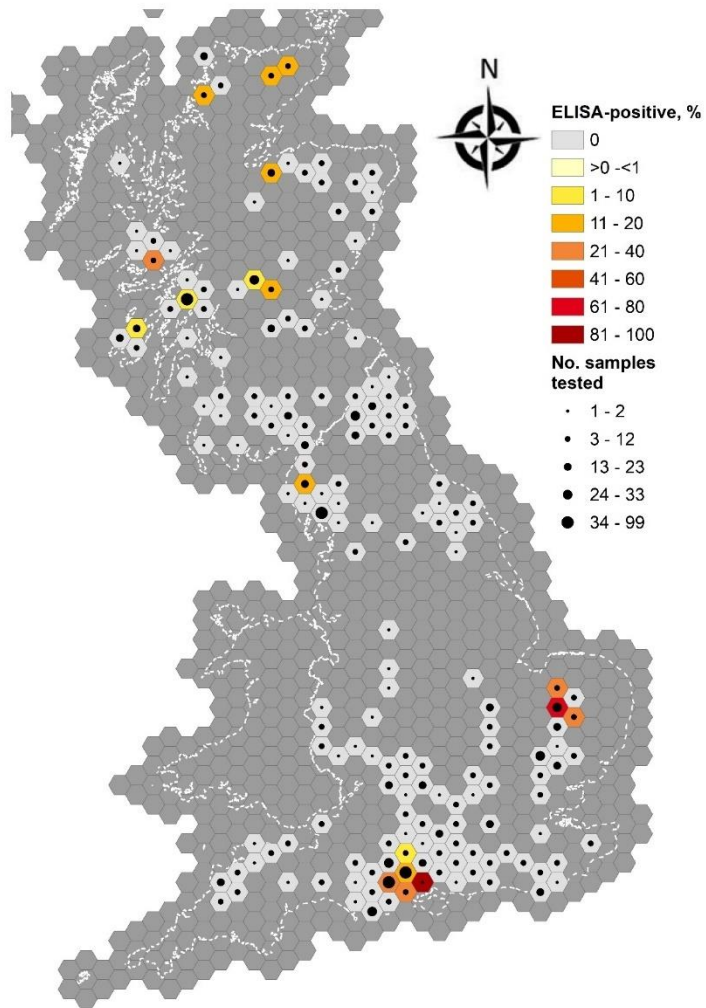
Figure 2:14: *Number of samples tested and seroprevalence of samples positive by ELISA*
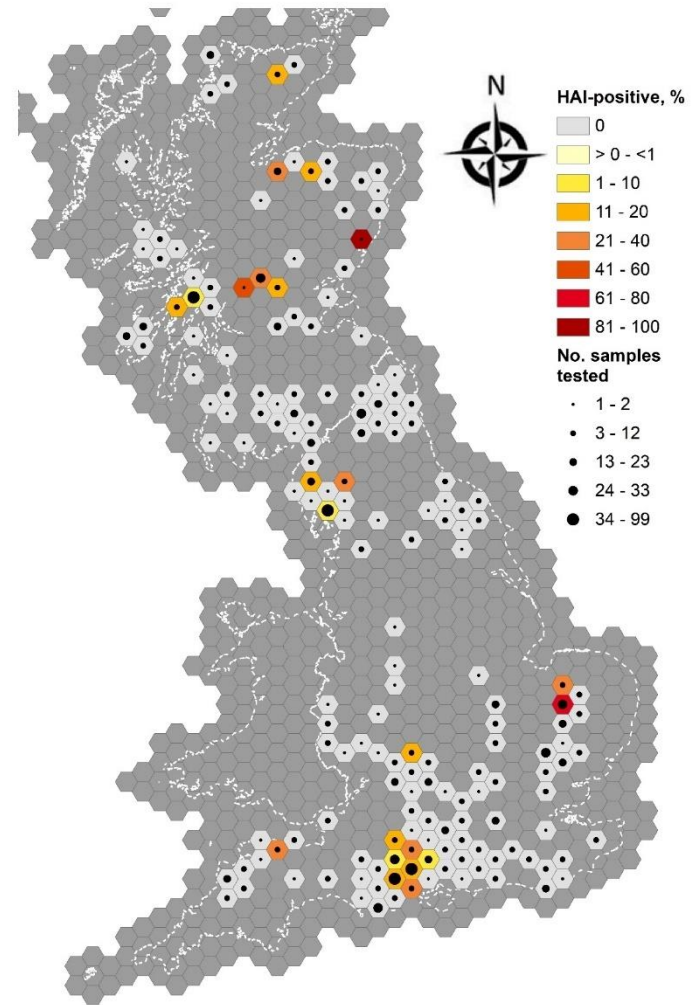
Figure 2:15:*Number of deer samples tested and seroprevalence of samples positive by HAI*

Table 2:10: ELISA positive results for TBEV from deer culled in different habitats by species

Species	Habitat ELISA positive/total tested (%) [95% CI]							
	Grassland/ Arable	*Coniferous woodland*	*Deciduous woodland*	*Mixed woodland*	*Lowland Heath*	*Upland Heath*	*Unknown*	**Total**
Fallow	2/90 (2.22) [0.13-8.23]	1/24 (4.17) [0.00-21.87]	1/101 (0.99) [0.00-5.94]	3/18 (16.67) [5.01-40.05]	3/13 (23.08) [7.5-50.94]	-	-	10/246 (4.07) [2.13-74.14]
Muntjac	0/14 (0.00) [0.00-25.15]	3/12 (25.00) [8.27-53.85]	2/75 (2.67) [0.17-9.77]	0/1 (0.00) [0.00-83.25]	1/5 (20) [2.03-64.04]	-	0/1 (0.00) [0.00-83.25]	6/108 (5.56) [2.33-11.84]
Red	0/25 (0.00) [0.00-15.76]	3/98 (3.06) [0.67-9]	1/26 (3.85) [0.00-20.45]	-	0/9 (0.00) [0.00-34.46]	5/82 (6.1) [2.3-13.82]	0/2 (0.00) [0.00-70.98]	9/242 (3.72) [1.87-7.02]
Roe	0/132 (0.00) [0.00-3.4]	14/281 (4.98) [2.92-8.26]	8/188 (4.26) [2.04-8.3]	2/22 (9.09) [1.34-29]	1/21 (4.76) [0.00-24.42]	2/12 (16.67) [3.5-46]	0/7 (0.00) [0.00-40.44]	27/663 (4.07) [2.79-5.88]
Sika	0/14 (0.00) [0.00-25.15]	0/14 (0.00) [0.00-25.15]	1/4 (25) [3.41-71.09]	0/1 (0.00) [0.00-83.25]	0/12 (0.00) [0.00-28.2]	0/3 (0.00) [0.00-61.75]	-	1/48 (2.08) [0.00-11.91]
*Hybrid**	-	0/2 (0.00) [0.00-70.98]	-	-	-	-	-	0/2 (0.00) [0.00-70.98]
Total	2/275 (0.73) [0.02-2.79]	21/431 (4.87) [3.17-7.37]	13/394 (3.30) [1.88-5.62]	5/42 (11.90) [4.73-25.46]	5/60 (8.33) [3.21-18.47]	7/97 (7.22) [3.3-14.39]	0/10 (0.00) [0.00-32.09]	53/1309 (4.05) [3.10-5.27]

Table 2:11: *HAI positive results for TBEV from deer culled in different habitats by species*

Species	Habitat HAI positive/total tested (%) [95% CI]							
	Grassland/ Arable	*Coniferous woodland*	*Deciduous woodland*	*Mixed woodland*	*Lowland Heath*	*Upland Heath*	*Unknown*	**Total**
Fallow	2/90 (2.22) [0.13-8.23]	1/24 (4.17) [0.00-21.87]	6/100 (6.00) [2.52-12.73]	3/18 (16.67) [5.01-40.05]	3/13 (23.08) [7.5-50.94]	-	-	15/245 (6.12) [3.67-9.93]
Muntjac	0/14 (0.00) [0.00-25.15]	2/12 (16.67) [3.5-46]	4/73 (5.48) [1.74-13.67]	0/1 (0.00) [0.00-83.25]	1/5 (20.00) [2.03-64.04]	-	0/1 (0.00) [0.00-83.25]	7/106 (6.6) [3.01-13.23]
Red	2/25 (8.00) [1.09-26.1]	6/98 (6.12) [2.58-12.98]	2/26 (7.69) [10.18-25.26]	-	0/9 (0.00) [0.00-34.46]	8/82 (9.76) [4.79-18.32]	0/2 (0.00) [0.00-70.98]	18/242 (7.44) [4.69-11.51]
Roe	4/132 (3.03) [0.93-7.79]	12/280 (4.29) [2.39-7.42]	6/188 (3.19) [1.31-6.94]	0/22 (0.00) [0.00-17.55]	2/21 (9.52) [1.45-30.12]	4/12 (33.33) [13.55-61.2]	0/7 (0.00) [0.00-40.44]	28/662 (4.23) [2.92-6.07]
Sika	0/13 (0.00) [0.00-26.59]	0/14 (0.00) [0.00-25.15]	0/3 (0.00) [0.00-61.75]	0/1 (0.00) [0.00-83.25]	0/11 (0.00) [0.00-30.02]	0/3 (0.00) [0.00-61.75]	-	0/45 (0) [0.00-9.38]
Hybrid*	-	1/2 (50.00) [9.45-90.55]	-	-	-	-	-	1/2 (50) [9.45-90.55]
Total	8/274 (2.92) [1.39-5.75]	22/430 (5.12) [3.37-7.66]	18/390 (4.62) [2.89-7.22]	3/42 (7.14) [1.77-19.65]	6/59 (10.17) [4.4-20.81]	12/97 (12.37) [7.07-20.54]	0/10 (0.00) [0.00-32.09]	69/1302 (5.3) [4.20-6.66]

Table 2:12: ELISA positive results for TBEV by species and sex of deer

	ELISA						
Species	*Fallow*	*Muntjac*	*Red*	*Roe*	*Sika*	*Hybrid*	**Total**
Female	4/88 (4.55) [1.42-11.47]	1/42 (2.38) [0.00-13.44]	3/96 (3.13) [0.68-9.17]	10/247 (4.05) [2.12-7.39]	0/22 (0.00) [0.00-17.55]	0/1 (0.00) [0.00-83.25]	18/496 (3.63) [2.27-5.70]
Male	6/153 (3.92) [1.62-8.48]	5/66 (3.92) [2.90-16.92]	6/139 (4.32) [1.78-9.30]	17/413 (4.12) [2.54-6.54]	1/26 (3.85) [0.00-20.45]	0/1 (0.00) [0.00-83.25]	35/798 (4.39) [3.15-6.06]
Not stated	0/5 (0.00) [0.00-48.91]		0/7 (0.00) [0.00-40.44]	0/3 (0.00) [0.00-61.75]			0/15 (0.00) [0.00-23.86]
Total	10/246 (4.07) [2.13-74.14]	6/108 (5.56) [2.33-11.84]	9/242 (3.72) [1.87-7.02]	27/663 (4.07) [2.79-5.88]	1/48 (2.08) [0.00-11.91]	0/2 (0.00) [0.00-70.98]	53/1309 (4.05) [3.10-5.27]

Table 2:13: *HAI positive results for TBEV by species and sex of deer*

Species	HAI						
	Fallow	*Muntjac*	*Red*	*Roe*	*Sika*	*Hybrid*	**Total**
Female	6/88 (6.82) [2.88-14.37]	3/42 (7.69) [1.77-19.70]	7/96 (7.29) [3.34-14.53]	11/246 (4.47) [2.43-7.92]	0/21 (0.00) [0.00-18.23535]	1/1 (100) [16.75-100.00]	27/494 (5.47) [3.75-7.86]
Male	9/152 (5.92) [2.10-11.02]	4/64 (5.92) [2.01-15.44]	10/139 (7.19) [3.81-12.88]	17/413 (4.12) [3.81-12.88]	0/24 (0.00) [0.00-16.31265]	0/1 (0.00) [0.00-83.25]	41/793 (5.17) [3.82-6.95]
Not stated	0/5 (0.00) [0.00-48.91]		1/7 (14.28) [0.53-53.35]	0/3 (0.00) [0.00-61.75]			1/15 (6.67) [0.00-31.84]
Total	15/245 (6.12) [3.67-9.93]	7/106 (6.6) [3.01-13.23]	18/242 (7.44) [4.69-11.51]	28/662 (4.23) [2.92-6.07]	0/45 (0) [0.00-9.38]	1/2 (50) [9.45-90.55]	69/1302 (5.3) [4.20-6.66]

Results indicated that there was a relationship between age and seropositivity by both serological testing methods (Fisher's exact ELISA= 0.037; HAI p=0.002). Younger deer (juvenile and yearlings) displayed a lower seropositivity by both TBEV ELISA and LIV HAI (*Figure 2:16* and *Figure* 2:17). There was variation between TBEV ELISA and LIV HAI in seropositivity by age for adult and old deer. 4.8% of adult deer were seropositive by TBEV ELISA; however, this was higher when tested by HAI at 7.3%. This relationship was the reverse for old deer, with 10.0% positive by TBEV ELISA and 4.1% by LIV HAI. However, the sample size of the old deer population was small (TBEV ELISA n=50; LIV HAI n=49), therefore they both had overlapping and wide confidence intervals (*Figure 2:16* and *Figure* 2:17).
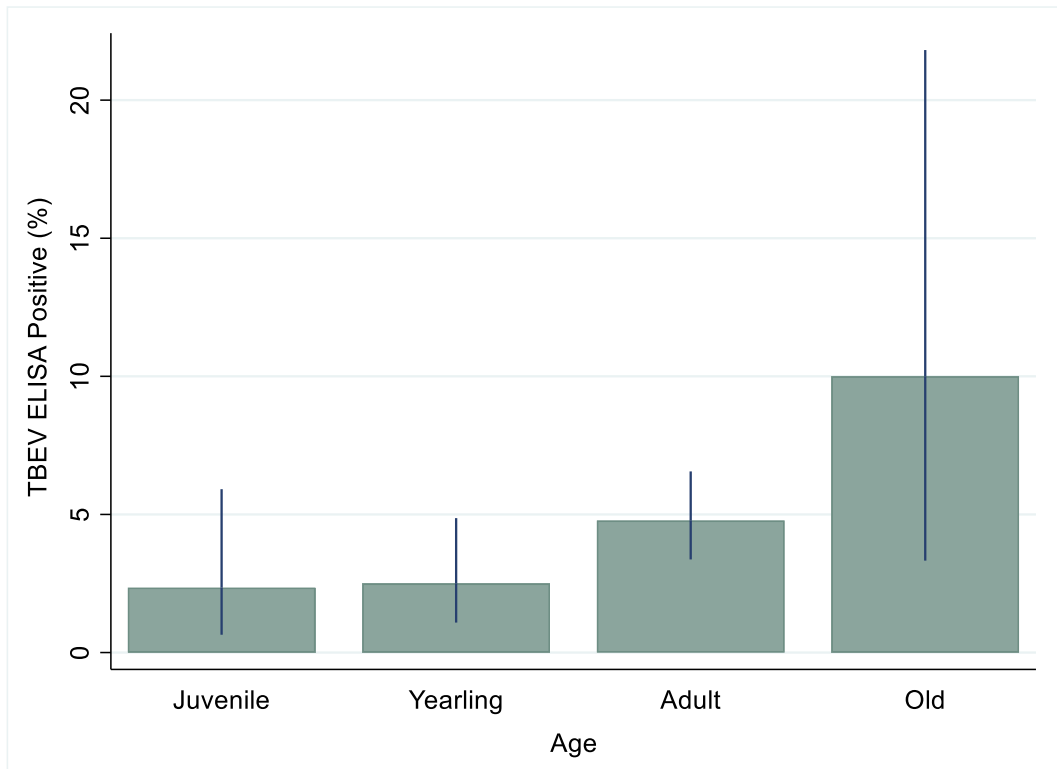
Figure 2:16: *Seroprevalence of deer by age by TBEV ELISA including 95% confidence intervals*
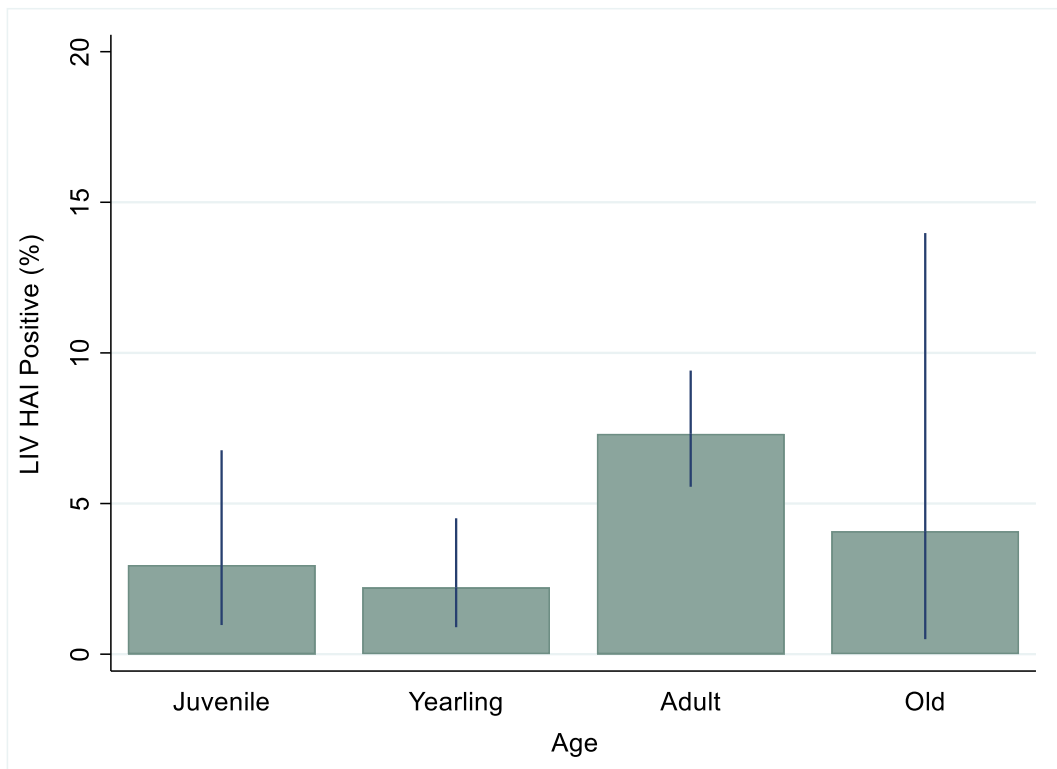
Figure 2:17: *Seroprevalence of deer by age by LIV HAI including 95% confidence intervals*

2.3.3: Molecular analysis of ticks removed from deer

Of all ticks submitted from deer carcasses, 2,041 were collected from 339 deer which were within 15 km of an ELISA-positive result. The hatched area in *Figure 2:18* shows the locations where deer samples were collected and the 15km buffer zones around the ELISA positive samples.
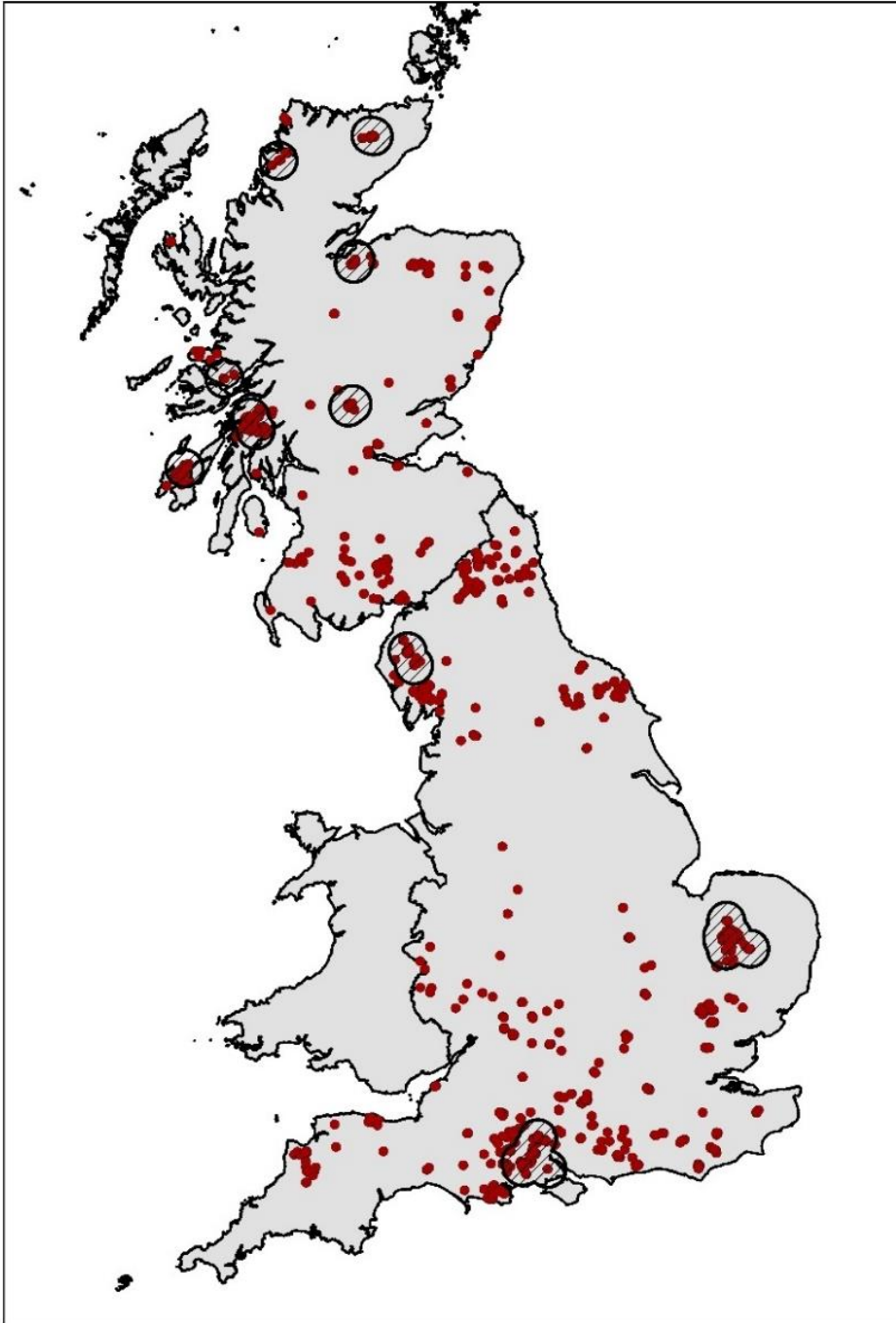
Figure 2:18*: The points show locations where deer serum samples were collected. The hatched area shows 15km buffer zones around all ELISA positive samples*

From the 339 deer, an average of 6 ticks were tested per carcass. The availability of ticks for testing by area of seropositivity varied (*Figure 2:19*). The highest number of ticks tested by geographical area were submitted from deer culled in Argyll & Bute (n=888), followed by Perth & Kinross (n=246) Hampshire (n=243), Highland (n=243 ticks), Norfolk (n=156 ticks), Wiltshire (n=119 ticks), Cumbria (n=58 ticks), Dorset (n=52 ticks) and Suffolk (n=36 ticks).

All ticks were identified as *I. ricinus*; 1,450 were adult females, 585 adult males and 6 were nymphs. Of all 2041 tested ticks by the LIV/TBEV rRT-PCR (Schwaiger and Cassinotti, 2003), 5 (4 adult males and 1 adult female) tested positive. No LIV RNA was detected in these 5 ticks when they were tested by rRT-PCR designed to detect only LIV.

All of the five positive ticks were collected from deer within the Thetford Forest area (Norfolk/Suffolk border) *(Figure 2:19)*. The 192 ticks tested from within the Norfolk/Suffolk focus resulted in a prevalence of 2.6% in this area. One tick (male) showed high levels of TBEV RNA (cycle threshold (Ct) 15.4). The Ct values of the four remaining ticks were all greater than 30.

Sequencing of the high viral RNA load tick sample revealed a full-length TBEV genome designated TBEV-UK (Gen- Bank accession no. MN128700). Phylogenetic analysis illustrates this as a TBEV-Eu subtype; it is most closely related to the Norwegian Mandal strain of TBEV isolated from ticks in 2009 (*Figure 2:20*), sharing a 99% sequence identity.
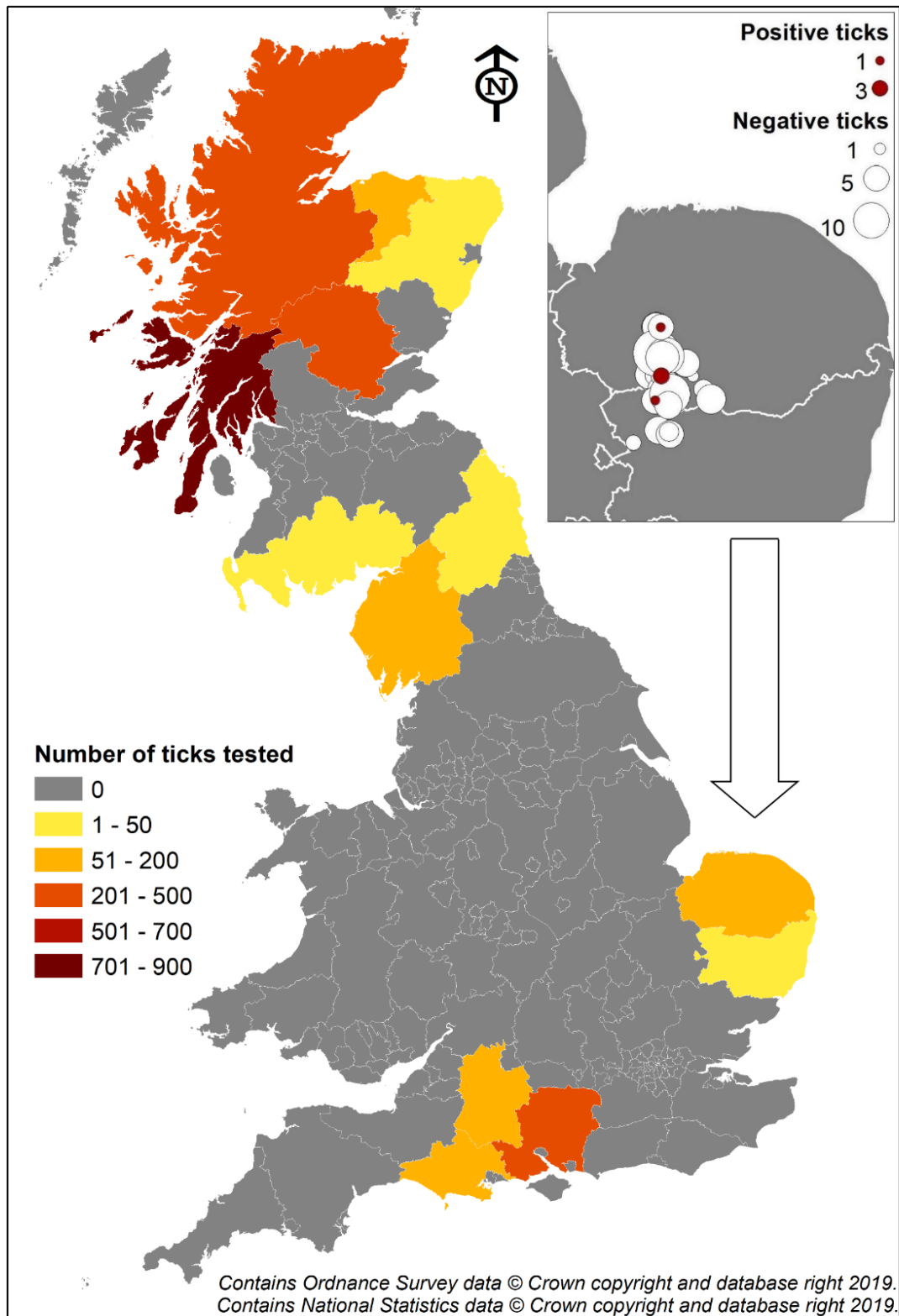
Figure 2:19: *Number of ticks tested by county; inset shows magnification of testing area with ticks positive by real-time reverse transcription PCR.*
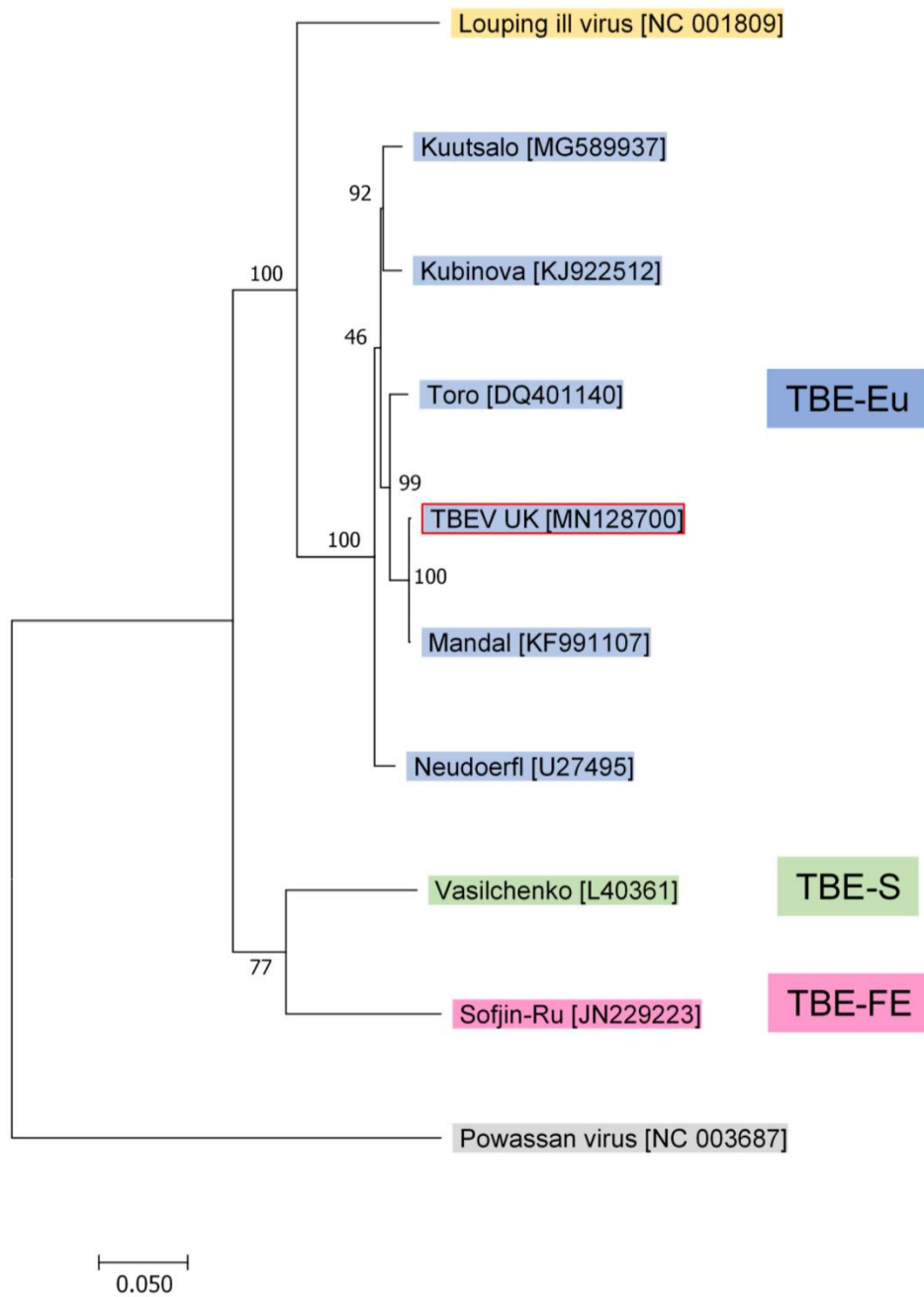
Figure 2:20: *Phylogenetic relationship between TBEV-UK from a tick in the United Kingdom and contemporary strains of TBEV. The tree was constructed with a maximum-likelihood analysis using full-length complete TBEV genomes and is rooted with the tick-borne Powassan virus. GenBank accession numbers of each sequence are provided in brackets.*

2.4: Discussion

A large-scale blood sampling of UK deer was achieved over much of England and Scotland, both in areas with previous evidence of LIV cases and in areas which have never had reports. Levels of seroprevalence matched up with areas where there were previous cases of LIV in livestock, in particular areas of Scotland, the South West of England and Northern England (Animal & Plant Health Agency; Scotland's Rural College, 2020b, 2020a; Jeffries et al., 2014). Seroprevalence by ELISA in these areas ranged from 1.79% [95% CI 0.00–10.34] in Wiltshire, an area with very few LIV reports, to 8.64% (95% CI 3.99–17.04%) in Highland, which has a higher incidence of LIV.

The seroprevalence levels observed in areas of Scotland in which LIV has been found previously, were considerably lower than reported in previous studies in 1960 and 1977 (Adam et al., 1977; Dunn, 1960). Angus (1960) (Dunn, 1960) detected a seroprevalence against LIV of 37% in red deer stags and 22% in hinds across 6 regions of Scotland. Similarly, seroprevalence found by Adam *et al* (1977) (Adam et al., 1977) found that 29.7% of 333 red deer from five council areas in Scotland were seropositive by LIV HAI. The variation in seroprevalence may be due to the studies using different sampling sites, as within each locality the presence of tight LIV geographic foci could be impacting the results. Alternatively, it is possible that prevalence has decreased since the last surveys. This may be influenced by the LIV vaccination of livestock (now unavailable), which may have contributed to the decrease in the prevalence of the virus in enzootic cycles in these areas.

There have not been any recent LIV serosurveillance studies across multiple regions in the UK, since the late 1970s, to provide a more current comparison. In one study Laurenson *et al* (Laurenson et al., 2007) found seroprevalence in sheep varied between 6% to 67% in farms in the Forest of Bowland, although a comparison with this study is not possible as no samples were obtained from this area.

This study found that the three areas with the highest seroprevalence were Norfolk 51.43% (95% CI 35.57–67.01%), Hampshire 14.29% (95% CI 8.74–22.35%) and Suffolk 10.71% (95% CI 2.90–28.01%) which have never had reports of LIV (Animal & Plant Health Agency; Scotland's Rural College, 2020b, 2020a; Jeffries et al., 2014).

The Norfolk and Suffolk seropositive results were in the Thetford Forest area, which straddles the border of the two counties. The seroprevalence in the Thetford Forest area of the two counties of 47.7% indicates there is sustained transmission of a TBEV-serocomplex virus in an enzootic cycle. This detected seroprevalence in the Thetford Forest area is among the upper levels found in TBEV endemic areas in Europe (Duscher, Leschnik, et al., 2015; Imhoff et al., 2015; Paul et al., 2015; Kiffner et al., 2012).

Within the UK, an overall seroprevalence of 4% was detected; in deer, however, caution must be used in comparisons between studies, particularly when reported nationally or over a large geographical area. Due to the focal nature of TBEV, distribution of samples within a national setting will have a substantial impact on the study specific seroprevalence detected. It is important that TBEV serocomplex serosurveillance studies report seroprevalence broken down into smaller geographies, ideally into suspected focal areas in order to allow comparison between studies.

Due to the sampling methodology and the ecological nature of the study, it was not possible to obtain an even distribution of samples across the UK. Very low or no samples were obtained from central England and Wales, one key contributory factor being low deer density in parts of central England. The utilisation of all free roaming UK deer species had a positive effect on the distribution of samples submitted, with varying proportions of species contributing to sample numbers in different parts of the country. For example, 43.8% of samples submitted from Scotland were from red deer, whereas just 5.5% of samples submitted from England were from this species. By contrast 1.1% submitted from Scotland were fallow deer whereas 27.8% of samples submitted from England were fallow. A further benefit of utilising multiple deer species is the great variation in their home range size. Species such as red deer and fallow are very useful indicators of potential exposure to infected ticks over a relatively wide area; this is particularly valuable for a national study. Species such as muntjac, roe and sika have tight home ranges, which despite being less likely to have spent time in an endemic focus, if seropositive, should aid in identifying the specific locality of the focus. A number of

seropositive deer in the Thetford Forest and Hampshire areas were roe and muntjac.

The inclusion of all UK deer species ensured a wide variation of habitats were sampled, with a highly significant (p=<0.000) relationship between species and the habitat in which they were culled. Due to the tight relationship between deer species and habitat, statistical analysis was not performed to assess the impact of these factors on seroprevalence detected. Seroprevalence detected for each species was similar, and confidence intervals were overlapping. Muntjac have not been utilised as sentinels for TBEV or LIV previously, but in the UK, seropositivity was demonstrated to be consistent with other deer species, in the same geographic areas. This study therefore highlights that they are also efficient sentinels. Muntjac have a particular utility in being territorial, tending to be hefted to a specific home range and also having the smallest home range of all UK deer species (Chapman et al., 1993); therefore they are likely to provide valuable information, in locating the focus to which a muntjac was exposed when conducting follow up questing tick surveys.

The confounding nature of relationship between deer species, habitat occupied and geographic location results in difficulties in studying the relationship between these factors and seropositivity. It must also be noted that the habitat stated is that in which the deer were culled; however they are likely to have also spent time in other habitats. For many deer there are mixed types of habitat in their home range and cull location is normally determined by deer management best practice.

As might be expected given varied ecology between LIV and TBEV, there was greater variation in seroprevalence by habitat than there was by deer species; lowland and upland heath had the greatest seroprevalence when tested by LIV HAI, which is aligned with habitats in which LIV is traditionally maintained. Mixed woodland demonstrated the highest seroprevalence by ELISA; however, for both methods, confidence intervals were overlapping across several habitats.

As the behaviours of male and female deer vary, with males of each species tending to roam further than females, one might expect the seroprevalence to vary by sex.

In this study, no significant effect of sex was detected for seroprevalence against TBEV serocomplex virus by ELISA or HAI in deer. Past studies have reported conflicting evidence of the effect of sex. A study of roe deer sampled between 1987 and 1992 in south west Germany found that males had a higher seroprevalence than females (39.0% vs 17.6%) (Gerth et al., 1995). Similarly Adam *et* al. (1977) found significantly higher seroprevalence in male red and roe deer compared to females (Adam et al., 1977). On the other hand, in Austria a study of roe deer found contrary findings; all 22 seropositive deer were female (91 males and 844 females tested) (Duscher, Wetscher, et al., 2015). Elsewhere, a study of roe deer in samples collected in Germany between 2007-2009 did not detect a significant effect of sex on seroprevalence against TBEV (Kiffner et al., 2012). Likewise, a study of roe deer in Denmark did not demonstrate any significant difference of seroprevalence between sex of deer (Skarphédinsson et al., 2005). The variation in these results indicates that there may be confounding factors that link with sex. The complex ecological factors that affect deer movement and behaviour may have an influence on this. For example, the deer density within each study area may have been responsible for the differences found across studies, as higher densities can cause more displacement among young males. In areas with high food resource and low densities, the home range tends to be smaller (Hemami et al., 2004, 2005; Kjellander et al., 2004).

Age was found to have an effect on the seroprevalence in deer, which may be explained by the older the deer, the more chances it has had to become exposed to a TBEV/LIV infected tick; particularly because the prevalence of the TBEV-serocomplex viruses in ticks is low. However, there are conflicting reports on the impact of age; Duscher et al., (2015b) suggested that age will have minimal impact on results, finding that the mean age of seropositive deer was lower than those not infected; however, Skarphédinsson et al., (2005) found the opposite with the young-adult and adult groups being significantly more likely to be seropositive than fawn-yearling groups.

Due to the close homology between LIV and TBEV it was not possible to differentiate between the two viruses with the methods used, with the majority of

positives cross-reacting, being detected as positive on both methods. This is not an uncommon issue; even in studies testing samples using gold standard PRNT methodologies, cross-reaction between LIV and TBEV is reported. In one study, four of five LIV positive sheep sera tested positive with TBEV-VNT, three of which were positive at >1/40 titre (Klaus et al., 2014). Furthermore Mansfield *et al.,*(2011) found most individuals vaccinated against TBEV were detected as positives using a LIV PRNT, finding a highly significant (P<0.001) correlation for PRNT titres against LIV and TBEV (Mansfield et al., 2011). Many sentinel studies utilise serum neutralisation tests to confirm ELISA positive results as the gold standard method (Rijks et al., 2019; Jahfari et al., 2017; Frimmel et al., 2016); unfortunately due to the containment requirements, this was not possible during this time for this study. In future, the optimisation of a plaque reduction neutralisation test (PRNT) methodology for both viruses may aid in the differentiation between them.

The serology results provide invaluable data; however, for reasons discussed above, alone it does not confirm which of the TBEV serocomplex viruses the deer in each geographical area were exposed to. In order to achieve this, it was necessary to detect the virus itself by molecular methods. The sampling method enabled testing by TBEV/LIV PCR of a large number of ticks removed from deer in areas where seropositivity was identified. This was possible due to these samples already being collected and submitted with the deer blood samples.

A limitation of the sampling method is that an even distribution of ticks for testing was not achieved across all target areas. Despite ticks only being tested when from areas within 15km of seropositive deer, and the majority (67.5%) of ticks being tested from Scotland from Council areas in which LIV is often reported, surprisingly no LIV was detected in ticks. Studies reporting LIV prevalence in ticks are limited, with a single study showing up to 15.3% LIV prevalence in ticks (Watts et al., 2009). However, this has not been confirmed by other studies. Other non-unpublished contemporary studies testing for LIV in ticks from the UK find either no LIV or extremely few RT-PCR positive ticks (personal correspondence, Moredun Research Institute, and Animal and Plant Health Agency (APHA).

Although only one of the five RT-PCR positive ticks collected in Thetford Forest had sufficient viral RNA present for full-length genome sequencing, all of these ticks were positive on the TBEV/LIV RT-PCR and were not detected on the PCR assay designed only to detect LIV. Due to the lower viral RNA levels it was not possible to sequence the remaining four PCR positive ticks' genome sequence directly without further amplification. This will be used to confirm the relationship of the virus strain in the different tick samples. The genomic sequence of TBEV-UK shows a very close sequence identify to TBEV-Mandal, first isolated from ticks in the Mandal area of Norway in 2009 (Asghar et al., 2014). This strain has not been directly reported to be linked to human disease; however, at least seven cases of human disease have been reported in this Mandal region (Dobler et al., 2019). Large numbers of migratory birds travel to and through the UK from Nordic countries each year, including Norway. Thrushes (*Turdus* spp.) such as blackbirds (*Turdus merula*) and redwings (*Turdus iliacus*) migrate to the UK from Norway each autumn; this family, due to their ground feeding behaviour, are also known to be hosts to *I. ricinus* (Hasle, 2013). Migratory birds are able to cross natural barriers, in particular for the UK, the North Sea/English Channel, but also mountains and rivers. For these reasons, migratory birds are the probable route of importation of this TBEV strain to the UK.

The prevalence detected in the ticks from the Thetford Forest area of 2.6% falls within the range of other studies in mainland Europe where ticks removed from deer were tested (Imhoff et al., 2015). The ticks from the Thetford Forest area were collected from culled deer that lived in a large forest environment, this being more aligned with a habitat in which TBEV is maintained, rather than LIV, which is more commonly found in moorland habitats due to the transmission and reservoir host availability (Jeffries et al., 2014; Lindquist and Vapalahti, 2008).

This evidence, in combination with the fact that LIV has never been reported in livestock in this area (Animal & Plant Health Agency; Scotland's Rural College, 2020a, 2020b), supports the hypothesis that the high seroprevalence in deer in the Norfolk/Suffolk area was a result of TBEV exposure rather than LIV. This high seroprevalence would suggest that TBEV is being maintained in enzootic cycles in

the Thetford Forest area. The alternative hypothesis is that it was as a result of continued importation from migratory birds. Should this be the case, one would expect more seropositive deer samples along bird migration routes rather than within such a specific focussed area. Co-feeding between larval and nymphal life stages on small mammal hosts is seen as a key mechanism for the maintenance of TBEV. Until recently it had been thought that the appropriate climatic conditions were not present in the UK for the maintenance of TBEV to occur (Randolph and Rogers, 2000). However, the hypothesis that TBEV is being maintained in Thetford Forest is also supported by recent research, which found that co-feeding on small mammals between life stages of ticks does occur in the UK (Cull et al., 2017). Further work should include small mammal studies within the Thetford Forest area to investigate the ecology of small mammals present and whether co-feeding occurs there. In addition, to conduct analysis of climate and microclimate in these locations, to assess whether suitable climatic conditions are present to drive co-feeding behaviour in ticks.

The seroprevalence of 14.3% in Hampshire, despite no detection of TBEV/LIV RNA in ticks tested in this area, warrants further research to investigate the cause of this. Similar to Thetford Forest, this is an area with no history of LIV reports in livestock (Animal & Plant Health Agency; Scotland's Rural College, 2020a, 2020b).

To conclude, deer have again proven to be very efficient sentinels; their use has led to the first detection of TBEV in the UK. When utilising deer as sentinels, researchers should consider where possible utilising all available deer species provided the variations in their ecology and range are accounted for in the analysis. The detection of TBEV in the UK has potential public health significance with further research being required to investigate the size and nature of the potential foci and whether this is being maintained.

Chapter 3: Investigating evidence of TBEV presence in natural ecological cycles in the UK

3.1: Introduction

The possible presence of TBEV in ecological cycles in the UK has never been assessed. This may be due to the previous view that TBEV becoming established in within the UK was highly unlikely based on climate change scenarios, as the mathematical modelling suggested that the UK climate would not support the specific conditions required for its maintenance (Randolph and Rogers, 2000).

Evidence presented in Chapter 2 indicates a strong possibility that TBEV may be being maintained in ecological cycles within the Thetford Forest area of the UK. Of the ticks removed from deer culled in Thetford Forest, 2.6% were positive when tested by TBEV-PCR. In addition a full genomic sequence was obtained from one of the ticks which had close sequence identity to the TBEV-Eu Mandal strain (Asghar et al., 2014). Deer serology described in Chapter 2 provides convincing evidence to suggest that the presence of TBE-positive ticks is not just a result of a small number of ticks imported by migratory birds overwintering and then feeding on deer. The serology on the local deer population showed evidence of exposure to a TBEV serocomplex virus in nearly half (47.7%) of sampled deer; there were no previous records of the only endemic flavivirus, LIV in livestock in this region (Animal & Plant Health Agency; Scotland's Rural College, 2020b, 2020a; Jeffries et al., 2014).

Similarly in Hampshire, research conducted in Chapter 2 was unable to establish the causation of 14.3% of sampled deer being seropositive against TBEV-serocomplex in an area, which also has no published reports of LIV in livestock (Animal & Plant Health Agency; Scotland's Rural College, 2020b, 2020a; Jeffries et al., 2014).

These findings suggest that the suitable ecological conditions may currently be present in the Thetford Forest area (within the Breckland biogeographic region) of the UK, and possibly in the New Forest National Park area of Hampshire for the maintenance of TBEV in enzootic cycles. These areas are geographically distinct from each other, as shown in *Figure 3:1*. Despite similarities in geology and resulting present day forest usage, they have quite different natural histories that led to their current uses; these are outlined below.

Figure 3:1: The locations in the UK of the target areas for follow up TBEV surveys

3.1.1: Thetford Forest

Thetford Forest fits within Breckland (also interchangeably known as The Brecks), which is a 1,019km^2 biogeographical region spanning North Western Suffolk and South West Norfolk (Rothera, 1998; Ratcliffe and Claridge, 1996). Breckland ranges from m -0.02 m below sea level to a maximum of 85m, with Thetford Forest mostly sitting 15-30m above sea level (Natural England, 2015a; Ratcliffe and Claridge, 1996). Breckland has a drier climate when compared to Southern England with low rainfall and sandy, free-draining and low nutrient soils; combined, these impact on land use history, wildlife and vegetation. These distinct characteristics make it a good example of the natural areas concept, with it being very distinct from the adjoining areas, despite similar topography (Natural England, 2015a; Rothera, 1998). East Anglian plains have heavier soils, East Anglian Chalk has a sharper landscape, the Fens have a flat marshy landscape with peat soils and tidal silts, and North Norfolk has a more maritime climate (Rothera, 1998).

Much of Breckland is made up of a varied mosaic of semi-natural habitats produced by naturally low fertility, free-draining soil, with internationally important lowland heath, calcareous and acid grassland, large areas of mixed plantations and also open farmland (Natural England, 2015a). Of the 18,079 ha of Breckland Forest, 13,335 ha is Breckland farmland and 4,681ha is Stanford Training Area (STANTA), which is used for military activities (Dolman et al., 2010).

Due to the unique habitat and rich biodiversity, the area has been assigned a number of conservation statuses, including four National Nature Reserves, four Special Areas of Conservation and the Breckland Special Protection Area (Natural England, 2015a; Dolman et al., 2010).

3.1.1.1: *Historic and current day land use*

Breckland was largely wooded until 4,000BC, after which much was cleared, with very little woodland present until the late 18th century. Since prehistoric times, sheep grazing has been commonplace and commercial rabbit warrens widespread from the middle ages until World War II (Rothera, 1998; Ratcliffe and Claridge, 1996). The newly formed Forestry Commission brought and leased large areas of former arable land of the Brecklands on which to create new forests, with tree

planting beginning in 1922 and 10,000 ha being planted by 1934 (Natural England, 2015a; Ratcliffe and Claridge, 1996). An area of the gorse covered sandy Breckland heathland and parkland on the east of Thetford Forest, now known as STANTA Heath, was acquired by the MoD in 1944 and is used for military training to this day (Breckland District Council, 2007).

Considering present day land use, forestry matches if not exceeds the area of arable land in Breckland. Agriculture is mostly largescale, with high value production of vegetable, salad and arable crops, outdoor pig and both indoor and outdoor poultry farms (Natural England, 2015a). In addition to being utilised for commercial forest operations, Thetford Forest and Kings Forest are popular destinations for recreation, receiving 1 million day visits each year. Visitors travel to the area for activities such as walking, horse riding and mountain biking, in addition to utilising facilities such as a tree-top adventure course, activity and visitor centre, Centre Parcs and campsites (Natural England, 2015a; Dolman et al., 2010). Due to the multiuse of the area, in 1990 Thetford Forest was designated as a Forest Park.

3.1.2: The New Forest
The New Forest is on the south coast of England, falling within south west Hampshire and a small southern section of Wiltshire. The New Forest National Park was designated in 2005 and covers an area of 57,100 ha. Its biodiversity is of exceptional importance, which has been recognised throughout the European Union; there are 20 Sites of Special Scientific Interest (SSSI), two Ramsar Convention sites and six Natural 2000 sites that include at least part of the National Park (Mainstone, 2010b, 2010a).

The New Forest is at the centre of the Hampshire basin, which is a broad, shallow geological basin (syncline) (Natural England, 2015b). There are 26 miles of coastline on the southern edge of the New Forest which includes mudflats, single spits, saltmarsh and low cliffs (New Forest National Park Authority, 2007a). The north of the New Forest area is the highest above sea level, at up to 126m; there is a general slope southward down to sea level (Natural England, 2015b).

It is made up of an extensive complex mosaic of different habitat types, thirteen of which are of European importance (New Forest National Park Authority, 2013). This exceptional landscape is made up of ancient pasture, enclosed woodlands and Open Forest, wet and dry grasslands and dry and wet heaths - which include rich mires, (areas of waterlogged deep peat) (Forestry Commission, 2018; Mainstone, 2010b).

The New Forest National Park has bordering areas of rich ecology, with the Hampshire, Wiltshire and Dorset chalk downlands all within 20km of the national park (Medcalf and Bell, 2014). The Dorset heaths sit to the west, South Hampshire Lowlands to the east and the South Downs National Park 15km away to the north east (Natural England, 2015b).

3.1.2.1: Historic and current day land use

In 1079, the traditional subsistence uses of the land were regulated and legislation was introduced to designate the land as a Royal Forest by King William I. Through medieval times the area was used as a hunting forest by the Crown, which had exclusive ownership over the deer and other game (Newton, 2011). The primary management aim was to conserve the deer for hunting by the monarch; therefore, enclosing land for small holdings were prohibited. The commons system was introduced, where small holders were given the right to graze livestock on the open, or common land (Natural England, 2015b), whilst other land was protected as "Inclosures". This ancient term for enclosures remains in use to this day in the New Forest.

Deer and commoners' free-ranging livestock continue to be a strong presence in the open areas of forest; ponies, mules, donkeys, cattle and occasional sheep graze on commons and even road verges. In the autumn, pigs are run in woodlands to eat the acorns (Forestry Commission, 2018). The historic commoning system and forestry land use has shaped and developed the area into the unique landscape that is seen today, being one of the last remaining of its kind in lowland Europe (Natural England, 2015b; New Forest National Park Authority, 2013). Between 6,000 and 7,400 ponies, cattle, donkeys, pigs and sheep still graze the common

land, with the animals owned by around 550 different commoners (Mainstone, 2010a).

The Forestry Commission took over the responsibility of managing the New Forest from the monarchy in 1923 (Newton, 2011). Today, nearly a quarter of the National Park is made up of settlements and farmland, whilst three quarters is made up of Crown Lands, managed by the Forestry Commission, which are used in a variety of ways (Newton, 2011). Sixty percent of the woodland cover in the New Forest National Character Area (NCA) is under the direct management of the Forestry Commission (Natural England, 2015b), and of the 24,000 ha of the New Forest that it manages, the two main types of use are the 'Open Forest' and 'Inclosures'. Approximately 8,500 ha are within Inclosures and the remainder in the Open Forest (Forestry Commission, 2018).

Inclosures are areas where the New Forest Act permits management of woodland areas for tree production, that are fenced against commoners' animals (Newton, 2011; Cadman, 1962). The enclosing of open land for timber production started in 1700s and over the last 300 years, the permissible area for management has been extended, most recently in the 1960s (Forestry Commission, 2018). The majority of these enclosed woodlands have been felled and regenerated during the last 240 years (Young, 1935).

During the 19th century, in the areas that were used for commercial forestry, older trees were felled and replanted as plantations, with blocks of softwood tree. These softwood blocks mostly consisted of one or two tree species, usually non-native conifers (New Forest National Park Authority, 2007b). In addition, in the 20th century more semi-natural woodlands were felled to meet wartime demand (New Forest National Park Authority, 2007b). Following the World Wars further Inclosures were developed on open land as strategic timber reserves made up of conifer plantations (Forestry Commission, 2018). The management objectives were later adjusted to prioritise conservation of the natural habitat, so in 1971 a mandate was issued ceasing conversion of broadleaf areas to conifer (Newton, 2011). There has been further change in policy in recent years, resulting in large scale harvesting and clearance of non-native plantations on ancient semi-natural

woodland sites, which are being returned to broadleaved woodlands through natural regeneration (New Forest National Park Authority, 2007b), once conifer plantations have been harvested (New Forest National Park Authority, 2007b). Some areas are also being reverted to heathland (Natural England, 2015b). Remaining plantations continue to be managed for timber production; however, this is carried out with due consideration of the landscape and conservation (New Forest National Park Authority, 2007b). Within the Inclosures the age, class and species diversity may be the greatest of any other large British commercial forest, due to historic and present-day management. Clear felling areas tend to be limited to less than a few acres, which leads to a patchwork effect of woodland of different ages. Partial felling and replanting over time of the early hardwood enclosures has even enabled some relics of woodland to remain dating back to the early eighteenth century (Newbould and Tubbs, 1970).

The unenclosed vegetation within the New Forest is known as the 'Open Forest', which makes up approximately 50% of the National Park (Mainstone, 2010a). Much of the Open Forest is also managed by the Forestry Commission, but the management objectives are different to the Inclosures, the main purpose being nature conservation and maintenance of the internationally important mosaic of habitats (Forestry Commission, 2018; New Forest National Park Authority, 2013). There are also extensive common areas bordering the Crown Land, which are still within the National Park (Mainstone, 2010a). These are made up of heathland, mire, ancient pasture woodland - including riparian (woodland adjacent to a body of water) - and bog woodland. Water bodies in this area include both temporary and permanent pools, rivers and streams (Natural England, 2015b; New Forest National Park Authority, 2013). The New Forest Open Forest is the largest area of semi-natural vegetation in lowland Britain and has been described as 'one of the richest places for wildlife in Europe and one of the best wetlands in the world' (Mainstone, 2010a, 2010b). One aspect of this rich habitat is the marked lack of monoculture arable fields. This unique habitat has been created in a large part because of the long commoning history. It is now one of lowland Europe's few

remaining extensive systems of common rights and pastoral farming (Forestry Commission, 2018; New Forest National Park Authority, 2013).

In order to support the maintenance of the heathland habitat, controlled heather burning is carried out to boost the production of new young growth. This has been carried out in a formalised manner since 1870 and was probably customary for centuries before that. The management programme is controlled by the Forestry Commission, in consultation with the commoners and Verderers, a group of unpaid officers responsible for the protection of the traditional character of the forest, and the commoners' interests (New Forest National Park Authority, 2007b).

There are a limited number of farms in the National Park, the majority of which have small acreages with 85% being less than 20 ha in 2009. The farmland is mostly used for grazing, cattle being the most numerous livestock (Natural England, 2015b).

3.1.3: Factors associated with TBEV

As highlighted in Chapter 1, there is a complex interplay between the numerous key drivers required for the maintenance of TBEV. These include the presence of reservoir hosts, the habitat, climate and seasonality, microclimate, vectors and size of tick amplification host populations, as summarised in *Table 3:1.* Understanding the dynamics of the ecology and climatic conditions in Thetford Forest and the New Forest National Park is key to the assessment of their potential suitability for the maintenance of TBEV in enzootic cycles. The complex relationship between these factors is illustrated in *Figure 3:2*., highlighting factors directly addressed in 1.4.7: Natural transmission cycles and the relationship of those that will be now be applied in this chapter.
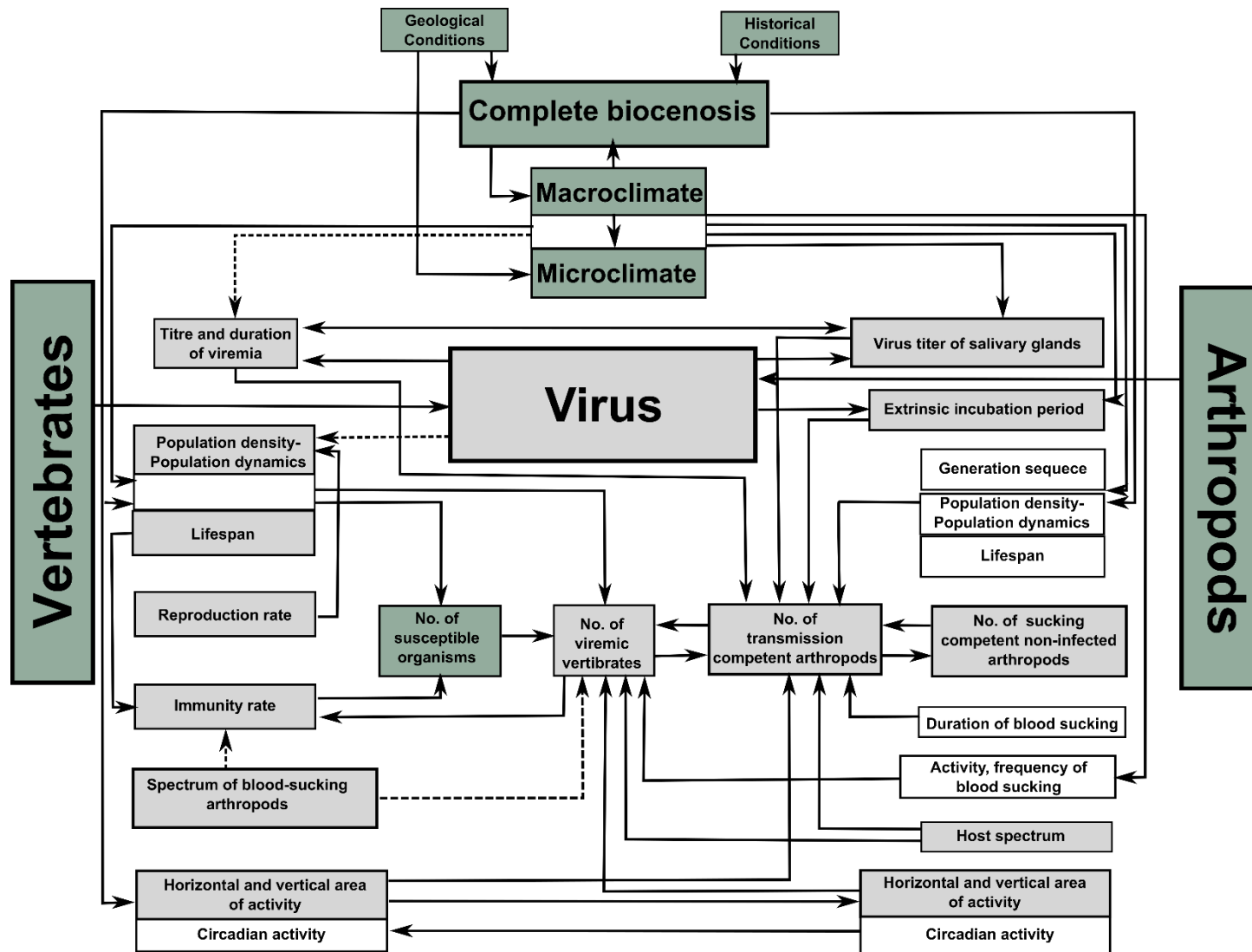
Figure 3:2: The complex interplay between these factors in arbovirus transmission. Grey boxes: Factors directly applied to TBEV in Chapter 1. Green boxes: Addressed in Chapter 1 and then applied to Thetford Forest and the New Forest in Chapter 3. Figure modified from Dobler et al.(2011), originally produced by Aspöck (1970) (Dobler et al., 2011; Aspöck, 1970).

Table 3:1: *Summary of seasonal, climatic and habitat requirements for TBEV foci, as outlined in Chapter 1.*

	Factor	Requirement	Impact
Seasonality and Climate	Autumn	Rapidly cooling temperatures. Not averaging below 5-8°C (Lindgren and Gustafson, 2001).	Prompts tick questing to cease and larvae and nymphs enter behavioural diapause. Not too cold to permit tick survival (Lindgren and Gustafson, 2001).
	Winter	Cool winter temperatures. Optimum mean January land-surface temperature between -2 and 3 ^{o}C (TBEV foci also found within -4 and 6°C range) (Andreassen et al., 2012; Randolph and Sumilo, 2007).	Allows sufficient temperature gradient for rapid spring rise to initiate co-feeding (Andreassen et al., 2012; Randolph and Sumilo, 2007).
	Spring	Rapid rise of spring temperatures to ≥10 ^{o}C. Particularly once warmer than 5-7°C, where nymphs become active, but not larvae (Andreassen et al., 2012; Jaenson et al., 2012; Burri et al., 2011; Lindgren and Gustafson, 2001)	Stimulate larvae and nymphs to simultaneously start questing to allow for co-feeding (Andreassen et al., 2012; Jaenson et al., 2012; Burri et al., 2011; Lindgren and Gustafson, 2001).
	Summer	Maximum summer temperatures of above 20°C may be beneficial (Marsh et al., 2001).	This has been found to be favourable for yellow-necked mice (Marsh et al., 2001), arguably the most efficient reservoir host (Labuda and Randolph, 1999; Labuda et al., 1997; M. Labuda et al., 1993).
Microclimate	Relative Humidity (RH) / Saturation Deficit (SD)/other Microclimatic Variables	RH >80% but with raised SD. Possibly 82-89% RH. (Kiffner et al., 2011; Boyard et al., 2008). Regular dew during dry summer periods can be of benefit. Soil type, vegetation cover affects local humidity and also local temperature. Vegetation cover and leaf litter coverage insulates soil and shields producing a more stable microclimate. Ground flora, tree cover including canopy density and foliage can all impact this. Small scale (<100m) temperature difference can vary up to 5°C and landscape level can vary as much as 20°C. Topography, slope exposition affecting radiation balance (Rotach and Calanca, 2003) (Burmeier et al., 2010).	A RH of >80% allows tick questing, though drier conditions above this promote lower nymph questing, increasing small mammal encounters and co-feeding. RH of 82-89% have shown increase in larval burden on small mammals (Kiffner et al., 2011; Boyard et al., 2008; Randolph and Rogers, 2000; Randolph and Storey, 1999). However Norwegian studies have shown higher RH produces highest TBEV prevalence (Esser et al., 2019; Andreassen et al., 2012). Localised temperature variations will affect the localised climatic conditions experienced described in the seasonality and climate section.

Habitat	Areas associated with TBEV presence	Large forest patches • High proportions of natural forest regeneration • High mean shape index • Open areas within forest ecotones • Broadleaf, mixed forest, particularly well connected oak, birch, beech or pine forests • High landscape diversity • Tree height variation of ≥5m (Rubel et al., 2020; Zeimes et al., 2014; Cagnacci et al., 2012; Vanwambeke et al., 2010; Pugliese and Rosà, 2008; Minár, 1992)	All have been shown to significantly correlate with areas of TBEV presence. These are likely to be factors that impact the reservoir host, tick population and activity, for example the high shape index produces larger ecotonal area which is optimal for tick abundance (Rubel et al., 2020; Zeimes et al., 2014; Cagnacci et al., 2012; Vanwambeke et al., 2010; Pugliese and Rosà, 2008; Minár, 1992).
	Yellow-necked mice	Mature deciduous woodland >50-100 years old. Coppiced woodland favoured. Good canopy cover. Less often found in conifer forests. Good shrub understory particularly with *Corylus* and high tree diversity. (*Corylus* spp., *Fagus* spp. and oak spp.) (Flowerdew and Ellwood, 2001; Marsh et al., 2001).	Yellow-necked mouse is arguably the most efficient TBEV reservoir host. TBEV has a limited reservoir host range, therefore presence and sufficient numbers are necessary for foci maintenance (Labuda and Randolph, 1999; Labuda et al., 1997; M. Labuda et al., 1993).
	Bank voles	Prefer mature broadleaved and mixed woodland, also inhabiting conifer plantations, hedgerows and field margins. More open canopy and understory with good ground cover (Amori et al., 2015; Flowerdew and Ellwood, 2001).	Bank vole is also an important TBEV reservoir host (Labuda et al., 1997). It inhabits a wider variety of habitats than yellow-necked mice (Amori et al., 2015; Flowerdew and Ellwood, 2001) .
Reservoir hosts	Factors affecting population of yellow-necked mice and bank voles	Fruitification of *Corylus* spp., *Fagus* spp. and oak spp. Mast years causing peaks in rodent population the following year and then it to crash the year after. There is stronger relationship between a good mast year and increased reproduction of yellow-necked mice than with bank voles (Reil et al., 2015; Flowerdew and Ellwood, 2001).	A small mammal population peak occurs one year after mast seeding, resulting in increased densities of larval *I. ricinus*. Followed by peak in nymph density the following year as those larvae feed and moult (Id et al., 2021). This second year the population of small mammals crash resulting in a high aggregation of ticks feeding on the reduced population (Brugger et al., 2018). There is a complex relationship between dilution effect by high rodent densities and large and also increases in tick population (Bournez et al., 2020). However, TBE cases increase the second year after a mast year (Rubel et al., 2020).

Dilution Hosts	**Deer**	Moderate deer numbers ≥0.03 - ≤0.14 roe deer/ha and ~ 0.04-0.1 may be optimal (Cagnacci et al., 2012; Pugliese and Rosà, 2008).	Very low deer density, can limit hosts for adult life stage, can result in fewer adults completing their lifecycle and reproduce. High deer density can divert immature stages of ticks from feeding on deer instead of TBEV competent reservoir hosts (Cagnacci et al., 2012; Pugliese and Rosà, 2008).
Vector	***Ixodes ricinus***	*Ixodes ricinus* (Randolph, 2002) *Dermacentor reticulatus* (Földvári et al., 2016)	*Ixodes ricinus* is the primary competent vector present in the UK (Medlock and Leach, 2015). *Dermacentor reticulatus* is a secondary vector but are less important due to their shorter lifecycle resulting in reduced opportunities for co-feeding occurrences (Földvári et al., 2016).

3.1.3.1: Habitat

As outlined in *Table 3:1*, forest habitats are key areas for TBEV presence, particularly large forest patches with a high mean shape index and open areas, within the ecotonal areas of high landscape diversity. Forests composed of broadleaved or mixed species, particularly oak, birch and spruce are associated with TBEV presence in addition to those with a high proportion of natural forest regeneration and tree height variation (Zeimes et al., 2014; Vanwambeke et al., 2010).

3.1.3.1.1   Thetford Forest

Breckland has a rich biodiversity that is unique in the UK, with areas of characteristic "steppe" type flora and containing 28% of all UK rare species (Natural England, 2015a; Rothera, 1998). Within the Thetford Forest area of Breckland, where most seropositive deer serum samples were collected, is an area where conifers predominate which is the UK's largest lowland conifer forest (Dolman et al., 2010; Breckland District Council, 2007). There are also areas of mixed/broadleaved plantations, particularly stands of beech, with other species of deciduous tree (Dolman et al., 2010). There are few open spaces, with some small areas of farmed heath and grassland used for sheep grazing interspersed between the forest blocks (Breckland District Council, 2007). The main habitat types within the Thetford Forest area are shown in *Figure 3:3*.

The acidic soils are reflected in the flora within the forested area, with pine dominating (Breckland District Council, 2007). Coniferous trees, predominantly Corsican pine, Scots pine, larch and fir, dominate within the Breckland area, with 16,586 ha of coniferous, 9,281 ha broadleaved, 329 ha mixed and 2,802 ha other forest (Natural England, 2015a; Breckland District Council, 2007).

Scots pine was the main crop species originally planted in geometric blocks by the Forestry Commission, which reached the end of the first rotation in the late 1990s (Ratcliffe and Claridge, 1996). These produced a mature ecosystem similar to mature continental conifer forests. During replanting for the second rotation of the forest, Scots pine are being replaced with Corsican pine, with just 10% of forest to remain planted with Scots pine (Rothera, 1998). Scots pine has a lighter open crown

compared to Corsican pine; the former allowing abundant ground flora. Corsican pine are planted in a high stocking rate, have a denser crown and produce a dense needle litter, smothering plants - factors which reduce biodiversity due to restriction on the establishment of ground flora (Rothera, 1998; Ratcliffe and Claridge, 1996). Corsican pine also have a shorter cropping period and combined with the denser stocking rate with narrow rides, prevent development of shrubby understory that was developed in the first Scots pine rotation (Rothera, 1998). Douglas fir, which requires specific conditions, has established and thrived on deeper soils and is able to naturally regenerate (Ratcliffe and Claridge, 1996).

Within Thetford Forest, just 12% of tree are broadleaved; semi-natural ancient woodland is very limited, covering just 0.2% of the Breckland NCA (Natural England, 2015a; Rothera, 1998). As part of management strategies to increase the proportion of the open mosaic of forest and heath, the proportion of broadleaved trees species have been increased (Natural England, 2015a). These species correspond with the sandy soil type where oak, birch and hazel are most common whereas on the wetter and clay soils, ash, field maple, hornbeam and wild cherry are found (Natural England, 2015a; Rothera, 1998). Beech were planted with Corsican Pine and larch as nurse crops; however, they fare poorly, in part due to the Breckland's dry and frosty conditions. Despite this, beech have remained a constituent part of Thetford Forest (Ratcliffe and Claridge, 1996). The few areas of ancient woodland have mostly grown out into high canopied, multi-stemmed trees due to previous coppicing for fuel. Over recent decades the practice of coppicing has been reintroduced in some of these woodlands (Rothera, 1998).
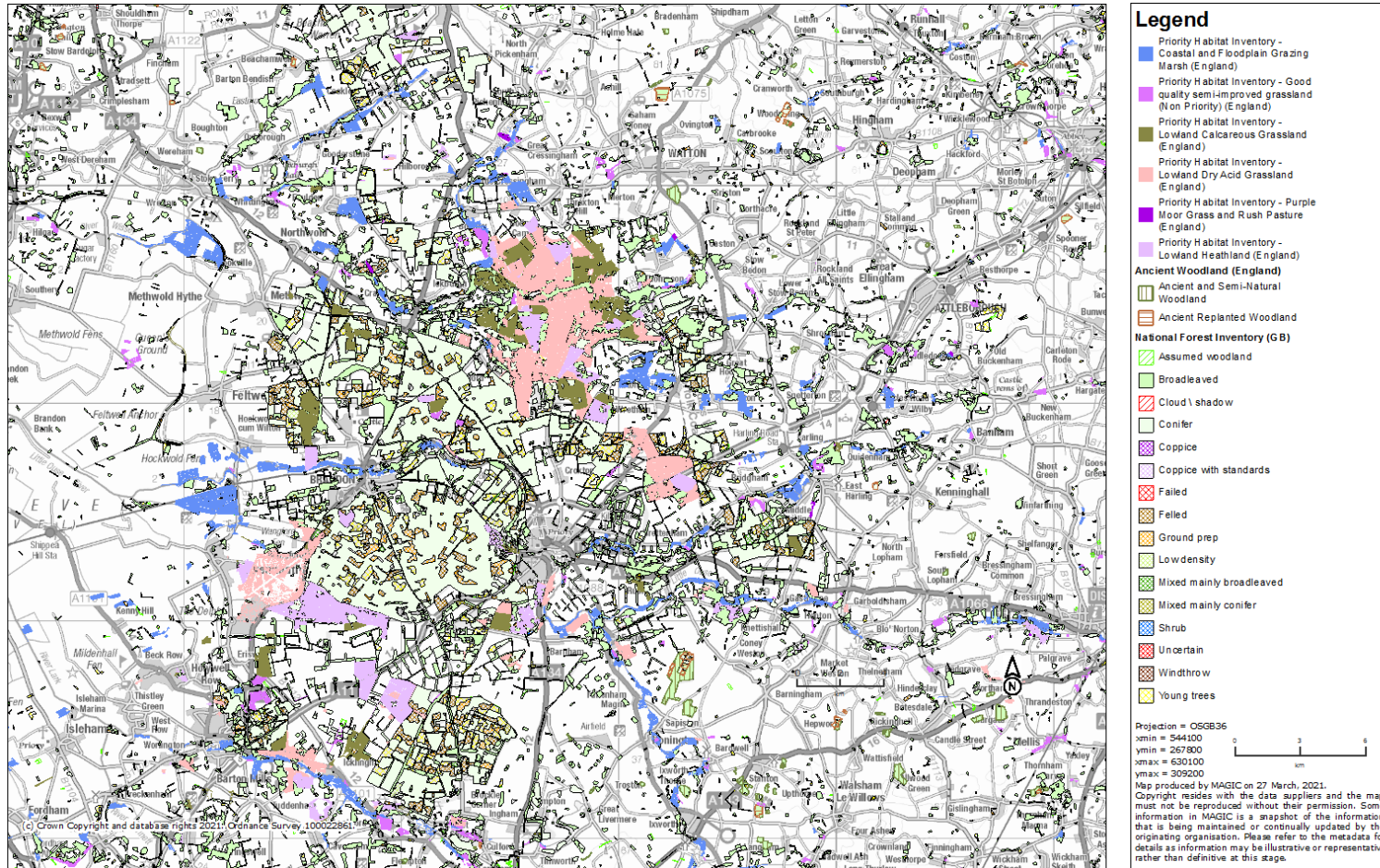
Figure 3:3: *Main habitat types present in Thetford Forest. Produced on https://magic.defra.gov.uk/*

3.1.3.1.2   New Forest

The special character of the New Forest National Park, with its complex mosaic of ecologically important habitat types, which create microhabitats within the Open Forest landscape, is due to several factors. These contributing factors are the geology, climate, location, sustained management practices - such as heathland cutting and burning - and continuous history of pastoralism, in addition to natural processes such as flooding (New Forest National Park Authority, 2013).

The New Forest National Park is lowland Europe's largest area of unsown vegetation, which includes the ancient pasture woodland, lowland heath and fen habitats (New Forest National Park Authority, 2013). As discussed earlier, the New Forest is made up of a rich mosaic of many habitat types; however, this study will focus upon the woodland habitats which are considered to be the most favourable locations for TBEV foci. The variety of the main habitat types is shown in *Figure 3:4*.

Within the NCA 31% of the land-cover is made up of woodland, identified as broadleaved 21% (15,495 ha), followed by coniferous at 8% (6,054 ha), mixed at 1% (796 ha) and 'other' at 1% (780 ha). In terms of age, 32% (7,405 ha) of the NCA woodland is ancient semi-natural and 10% (2,345 ha) is ancient re-planted woodland (Natural England, 2015b).

Records of the tree types and species within the New Forest National Park woodlands shows how much of the habitat is made up of broadleaf tree cover, within which the oaks are predominant, followed by beech, as broken down in Table 3:2.

In the New Forest, the majority (58%) of broadleaved tree species are over 80 years and the majority (57%) of conifer trees are 41-80 years old, as shown in *Table 3:3* (Forestry Commission, 2015). These figures summarise both the Inclosed and Open Forest areas and both Forestry Commission managed Crown Land and private forest. The unenclosed and enclosed woodland vary greatly in age, species and origin. This is in part due to management strategies and goals, with Inclosures having a production aspect and not being open to livestock grazing in Crown owned Inclosures (Cantarello et al., 2010).

Table 3:2: Woodland by type and tree species in stocked areas of the New Forest National Park

Tree type		Species	
Broadleaf:	71%	Oak	31%
		Beech	17%
		Other broadleaf	23%
Coniferous	29%	Scots pine	12%
		Corsican pine	7%
		Douglas fir	5%
		Other coniferous	5%

Table 3:3: *Age class of trees by stocked area in the New Forest at 31 March 2012- Data extracted from* (Forestry Commission, 2015)

Age	FC area (ha)	Private land area (ha)	Total (ha)
Conifer (all)			
0-40 years	900	600	1,500
41-80 years	2,200	1,000	3,200
80+ years	700	100	8,000
Broadleaf (all)			
0-40 years	300	3,200	3,400
41-80 years	1,000	1,200	2,200
80+ years	6,200	1,800	7,900
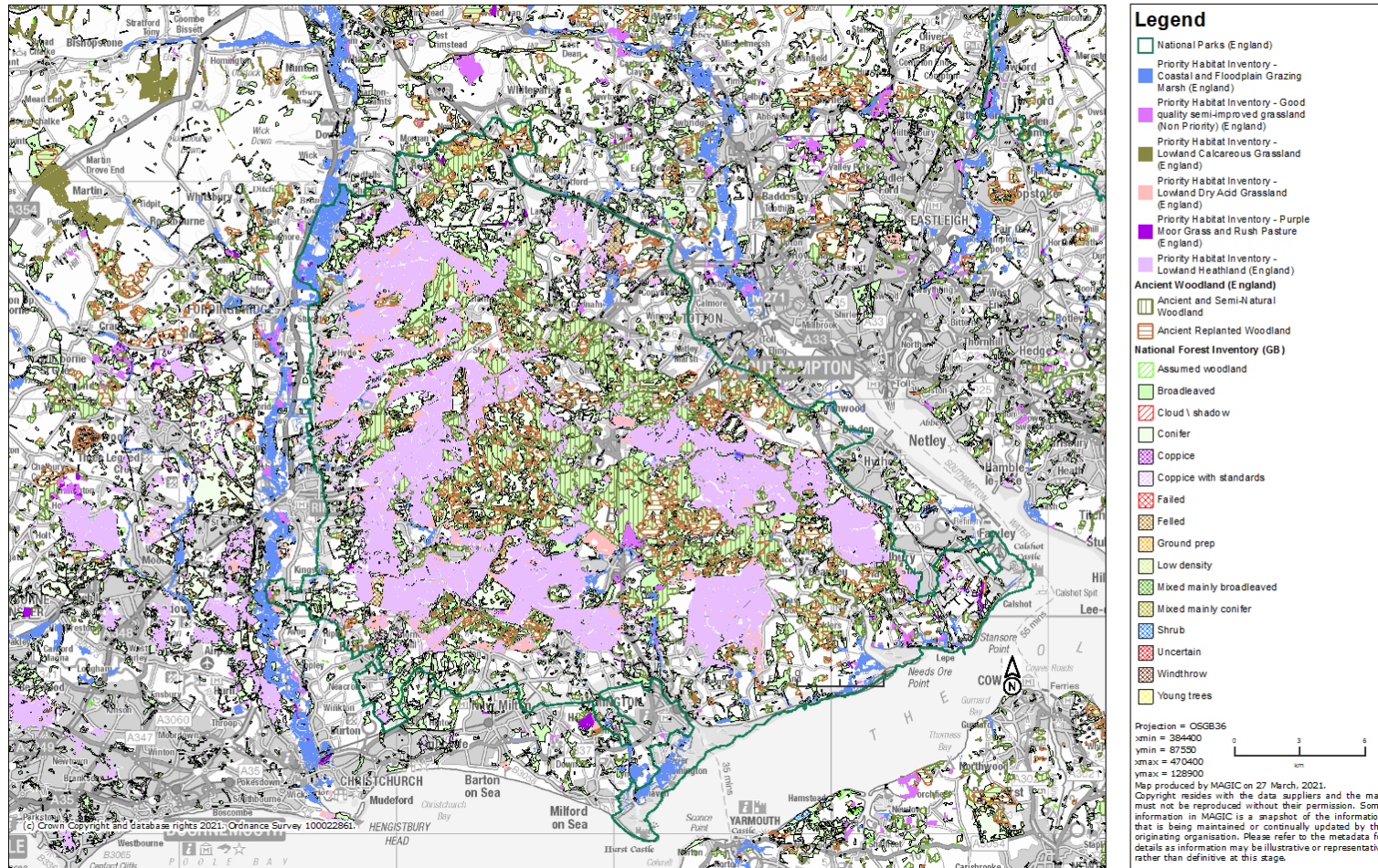
Figure 3:4: Main habitat types present in the New Forest. Produced on https://magic.defra.gov.uk/

Open forest

There are almost 20,000 ha of unenclosed woodland in the Open Forest, which have been so since at least 1700 AD; many have never actually been enclosed (Mainstone, 2010a; Young, 1935). Half of the woods are clumped along a central ridge running roughly west north west to east south east which is approximately nine miles long by three miles wide (Young, 1935). Within the unenclosed Open Forest landscape, the ancient pasture woodlands are largely unmanaged, with trees left to live their natural lifespan; those that die are left to decay and allow for regeneration to occur (New Forest National Park Authority, 2007a).

The forests are largely made up of a diverse age structure, although biologically mature trees are most common in many of these woodlands (Newbould and Tubbs, 1970). Most unenclosed woodlands consist of at least two to three generations, the first - designated "A" generation' - dating back to between 1650 and 1750. "A" generation' is mostly made up of widely spaced beech and oak often in monospecific stands, with a holly understory; whereas the two following generations have a greater species diversity (Newbould and Tubbs, 1970).

Holly, hawthorn and oak are successful species across the New Forest, able to colonise all but the poorest nutrient deficient soils (Newbould and Tubbs, 1970). Within the unenclosed Crown Land, 3,700 ha are made up of oak, beech and holly woodland (Mainstone, 2010a). Oak has maintained its dominance with both sessile and pedunculate present, the former being most successful (Cadman, 1962).

Beech has increased in numbers to become the most dominant tree species largely as a result of human disturbances of the woodland ecosystem with abundant regeneration and extensive planting (Grant and Edwards, 2008; Cadman, 1962). The considerable area of mature semi-natural beech woodland in the New Forest is the largest in Britain. It is also the most southerly Atlantic acidophilous beech woodland in the UK, the species usually being more suited to the western seaboard of Europe due to climatic factors (Grant and Edwards, 2008).

Holly is abundant in the understory; however, this species also occurs as almost pure stands. They can be found as central cores of mixed woods particularly in the northern part of the forest. The herb layer is limited and species present are nondiverse with the dominant species being bracken (*Pteridium aquilinum*) and bramble (Carpenter et al., 2012; Newbould and Tubbs, 1970). This limited ground flora is likely to be a result of the long history of livestock and deer grazing (Newbould and Tubbs, 1970). The forest edge, some with ancient coppice and veteran trees, often border unimproved grassland, hedges and ponds, and are rich in biodiversity (New Forest National Park Authority, 2013).

There are a number of ecologically important woodland habitat types within the Open Forest and Inclosure woodlands that are part of the New Forest Special Areas of Conservation plan (*Table 3:4*); these include woodlands with tree species that are relevant to TBEV ecology.

Table 3:4: Ecologically important Habitats Directive woodland types that are part of the New Forest Special Areas of Conservation (SAC) plan (Cantarello et al., 2010)

SAC Management plan	National Vegetation Classification (NVC)	Habitats Directive
Pasture woodland and Inclosure woodland	*W15, W14*	Atlantic acidophilous beech forests with *Ilex* and sometimes *Taxus* in the shrub layer
	W16, W10a/W11	Old acidophilous oak woods with English oak on sandy plains
	W14, W8b	*Asperulo-fagetum* beech forests
	W10b/W11 (oak-birch woodland with bluebell)	*No equivalent*

Ancient native pasture woodlands in the New Forest are described as 'Ancient and Ornamental' (A&O) woods which are largely unenclosed. The majority of the remaining A&O woodlands are found in a broad belt of near continuous woodland around Lyndhurst, although other outlying A&O woodlands are also found in the western and southern districts (Newton et al., n.d.). Like the rest of the Open Forest, within the A&O woodlands beech and oak (pedunculate and sessile) dominate the canopy and holly is dominant in the understory. Birch (silver and downy) are found on the periphery of main A&O woodland blocks (Newton et al., n.d.). These woodlands are highly valued and have developed as a result of the

combination of climatic conditions of the last one or two millennia on the ecology and also due to human activities (Grant and Edwards, 2008). They have been described by Tubbs (2001) as 'collectively the finest remnants of comparatively undisturbed deciduous forest in the lowlands of Europe' (Newton et al., n.d.). Approximately 3,671 ha remain of these woodlands, previously having been incorporated in Silvicultural (woodland management) Inclosures and replaced by plantations (Newton et al., n.d.).

Inclosures

Total Inclosure woodland cover is c. 8,500 ha, with many of the Crown Land Inclosures being made up of relatively recent plantations on ancient woodland stands (AWS) or former heathland (Forestry Commission, 2018; Cantarello et al., 2010). Over a third (c. 3,237 ha) of the enclosure woodland is deciduous and resembles natural woodland (Newbould and Tubbs, 1970). The locations and land use of the Inclosures are shown in *Figure 3:5*.

Within the Inclosures, species diversity is lower than in the Open Forest due to the commercial forestry management; the canopy is also more dense and less deadwood is present (New Forest National Park Authority, 2007b). Scots pine is the most common conifer species in the New Forest, having also colonised many Open Forests. It was native up until the Roman times during which it became extinct, then reintroduced in 1766. Corsican pine is the next most common New Forest conifer species, having been planted quite widely on the plateau gravels; it can regenerate, but to a more limited extent. Douglas fir was first introduced to the area in around 1860, it is nearly as abundant as Corsican pine, thriving in the New Forest. It is ideally suited to the climate which allows it to successfully naturally regenerate. Norway (*Picea abies*) and Sitka spruce (*Picea sitchensis*) also regenerate freely there, although the former tolerates the hot summers and spring frosts better than Sitka spruce. Yew (*Taxus baccata*)is also present in many areas (Forestry Commission, 2015; Cadman, 1962).

The conifer plantations that have been cleared have been replaced with mainly pedunculate oak but also sessile oak plantations. Many of these plantations also have self-sown beech, Scots pine (*Pinus sylvestris*) and silver birch. Similar to the

Open Forest, holly is dominant in the understory (Carpenter et al., 2012). Within the plantations there is less diversity in the herb layer than the Open Forest, with the young conifer plantation floors and stands of dense beech having almost no vegetation (Carpenter et al., 2012; Young, 1935).
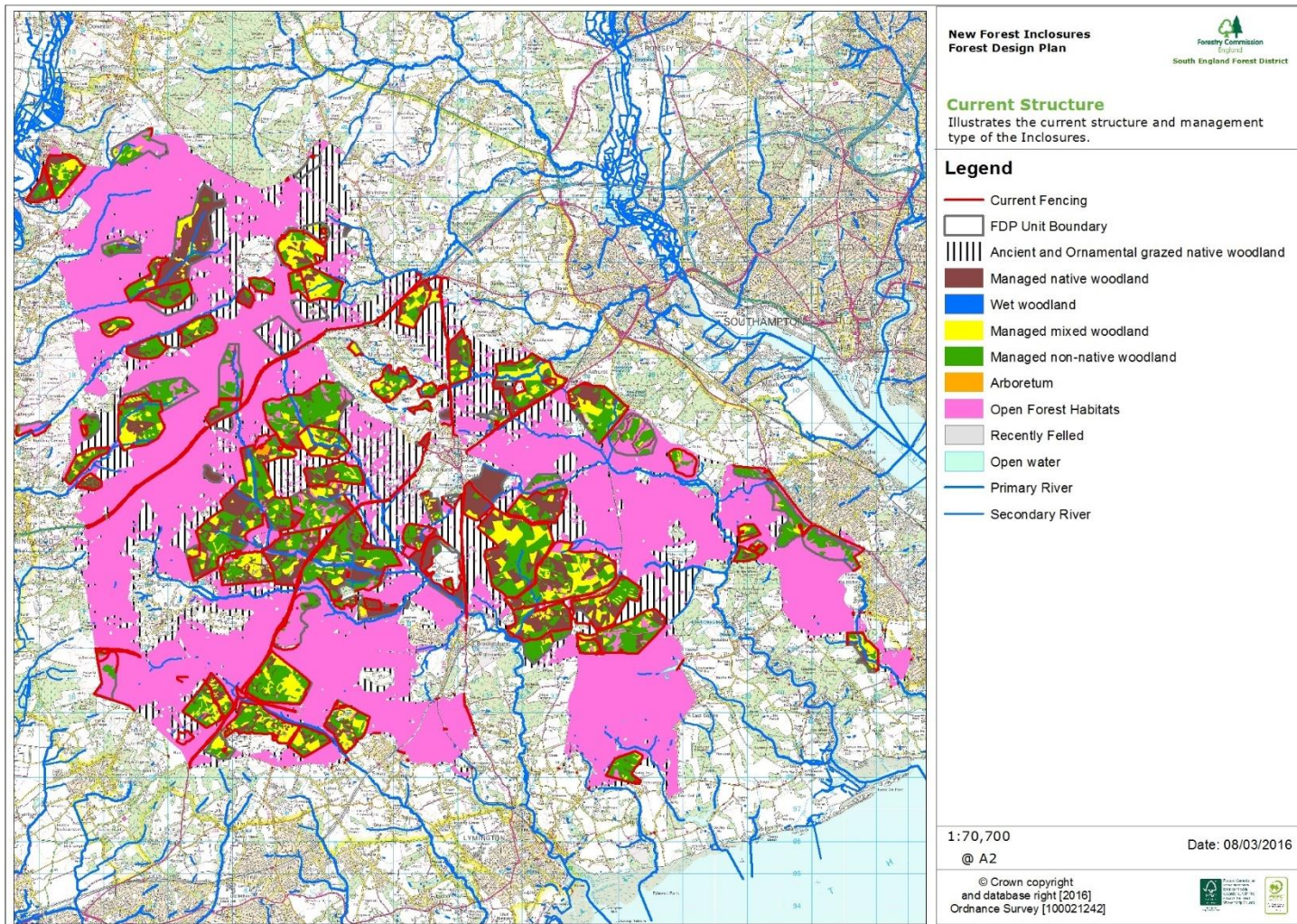
Figure 3:5: *Map illustrates the locations, current structure and management type of the New Forest Inclosures. Map extracted from Forestry Commission England (2015)* (Forestry Commission England, 2015)

3.1.3.2: Reservoir hosts

As discussed in Chapter 1, the habitat requirements of the main reservoir hosts for TBEV differ, detailed in *Table 3:1.* Yellow-necked mice have more specific habitat requirements than bank voles. Yellow-necked mice favour mature and ancient deciduous woodland of high tree diversity, particularly oak, beech and hazel that is over at least 50 years old, preferably older. They require a good canopy cover with shrub understory, particularly made up of hazel (Flowerdew and Ellwood, 2001; Marsh et al., 2001). Bank voles also prefer mature broadleaved and mixed woodlands with the same species favoured as yellow-necked mice; however, conifer plantations, hedgerows and field margins are also inhabited. They differ from yellow-necked mice in their preference for a more open canopy and understory, with good ground cover (Amori et al., 2015; Flowerdew and Ellwood, 2001).

3.1.3.2.1   Thetford Forest

The large number of coniferous blocks of Thetford Forest are likely not to be suited to yellow-necked mice. The areas of mature deciduous woodland that remain from the first plantings around 1930 may be suited, particularly as hazel, beech and oak which are favoured by yellow-necked mice, are among the most common of the broadleaved species present in the area. The coppiced areas and those with a good understory, particularly with hazel, would be most suited. Bank voles are much more tolerant of different habitats; despite preferring broadleaved woodlands, they also inhabit coniferous woodlands, therefore are more likely to thrive across the forest. Bank voles would be more likely to be found in the forest blocks with a more open canopy and understory with good ground cover.

There has been limited research on the distribution of both bank voles and yellow-necked mice in Thetford Forest. Within Thetford Forest, the population of bank voles found in coniferous woodland has been shown to be higher than is usually found in coniferous woods. Mature coniferous woods within Thetford Forest produced the same densities of bank voles as in deciduous woodlands, but a lower density was observed in young conifer plantations. Densities of just 4 bank voles per ha were found in young plantations compared to 27 per ha in old plantations (Ratcliffe and Claridge, 1996). No direct research information is available on yellow-

necked mice in Thetford Forest; however, reports to NBN Atlas (an open source platform in which ecological records can be submitted) indicates that yellow-necked mice may be present in Thetford Forest (NBN Atlas, 2021). This data shows Thetford Forest is towards the northern-most part of consistent yellow-necked mice presence in England (*Figure 3:6*). Bank voles are recorded across much of England, including the Norfolk and Suffolk areas that Thetford Forest falls within (NBN Atlas, 2021).

3.1.3.2.2   The New Forest

The New Forest area has habitat types more suited for yellow-necked mice in particular, but also to some extent bank voles, than Thetford Forest. The greater suitability is due to the large proportion (71%) of the tree species being broadleaved and 58% of these being over 80 years old; in addition, 32% (7,405 ha) of the woodland in the NCA is ancient semi-natural which is highly suited for both species (Forestry Commission, 2015; Natural England, 2015b). The older conifer plantations may also be suitable, as 63% of the conifer trees are over 80 years old and the older conifer plantations in Thetford Forest were found to support bank voles as well as broadleaved woodland (Forestry Commission, 2015; Ratcliffe and Claridge, 1996).

There is limited recent research on the presence of small mammals in the New Forest woodlands; however, there were 63 records of yellow-necked mice and 248 of bank voles in Hampshire that were submitted to Hampshire Wildlife Trust between 2006-2016 (Spall, 2017). The NBN atlas data, shown in *Figure 3:6,* indicates the presence of both yellow-necked mice and bank voles in the New Forest. Studies conducted during the 1980s found that all ungrazed woodlands in the New Forest supported substantial populations of bank voles, and yellow-necked mice at lower densities; however, they were rare or absent from grazed woodlands (Putman, 1996). This is likely to be due to the effect of grazing on the vegetation types and structure of the woodland with only scant cover from predation (Putman, 1996). Therefore, both species are more likely to be found in enclosed areas.

Figure 3:6: *NBN Atlas reports of bank voles and yellow-necked mice presence in UK [46], the New Forest National Park boundary and the Breckland National Character Area are marked in light green. The New Forest SSSI and Breckland Forest SSSI are marked in dark green*

3.1.3.3: Seasonality and climate comparisons

Providing autumn temperature does not average below 5-8°C to maximise tick survival, rapidly cooling temperatures promote larvae and nymphs to simultaneously enter behavioural diapause, [23].

Cool winter temperatures will ensure nymphs remain dormant (below 5-7°C) and allow for a sufficient temperature gradient for rapid temperature increase in spring, with a rapid rise required between 5-7°C and 10°C when larvae become active, to allow for the maximum co-feeding period between larvae and nymphs (Andreassen et al., 2012; Jaenson et al., 2012; Burri et al., 2011; Randolph and Sumilo, 2007; Lindgren and Gustafson, 2001).

3.1.3.3.1   Thetford Forest

Breckland is known for its semi-continental climate, experiencing extremes in temperatures and being one of the warmest and driest parts of the UK (Ratcliffe and Claridge, 1996). Its dryness is a result of a combination of its geology - with free-draining drought prone sandy soils, which have a high soil moisture deficit in summer - low rainfall and relatively hot summers (Natural England, 2015a; Rothera, 1998). East Anglia, which Thetford Forest falls within, is the driest region of the British Isles, with Breckland experiencing more extremes in weather than surrounding areas. A greater extreme between minimum and maximum daily temperature in Thetford Forest is shown through all seasons when compared to the average for south-eastern and central southern England (see *Figure 3*:7). Breckland has cold, dry winters with both more frequent frosts and lower minimum temperatures in all seasons, when compared to East Anglia and Central Southern England (Natural England, 2015a; Rothera, 1998). As clearly illustrated in *Figure 3*:7, Breckland experiences a long frost season , with late spring frosts being common; it regularly records the lowest monthly extreme temperature, with the coldest meteorological station below 300m in the British Isles (Rothera, 1998).

When compared to mid last century records, the weather in Breckland has become milder and less continental, with fewer frosts; spring and summer nights are milder in addition to milder winters (Dolman et al., 2010). The mean daily minimum air temperature has steadily increased for all seasons since at least 1950 (Dolman et

al., 2010). There is more rainfall in spring, autumn and winter; anecdotal evidence indicates that this in combination with fewer frosts and cold winters, has resulted in greater sward encroachment by perennial grasses on bare ground. Climate change predictions suggest these changes will continue and that Breckland will experience hotter and drier summers (Dolman et al., 2010).

Ratcliffe and Claridge (1996) noted that the proximity of Breckland to the east coast, and therefore mainland Europe, combined with its continental climate might make it suited for species arriving and establishing from the continent (Ratcliffe and Claridge, 1996). As shown in *Figure 3:8*, the relatively new Sallandse Heuvelrug focus, which has replaced Alsace as the western most TBEV foci, has a similar climate to Thetford Forest, with the exception of its having lower maximum daily temperatures over the winter. Mandal (Norway), the TBEV focus the TBEV-UK Thetford virus has most sequence similarity to, has a much colder climate than Thetford Forest.

3.1.3.3.2 The New Forest

The southern edge of the New Forest is coastal, resulting in a mild coastal climate; with very little snow and mild winters. There are relatively hot summers but also spring frosts; rainfall is distributed across the year. Dorset heathlands to the west are even milder, but also have more rainfall (Newbould and Tubbs, 1970; Cadman, 1962; Young, 1935).

The New Forest NCA identified climate change as the single factor likely to influence the area in the medium to long term. These changes are likely to be increased average temperature rises, precipitation changes and longer summer drought periods, particularly affecting the woodland and heath areas (Natural England, 2015b).

Of particularly note, the New Forest area and Thetford Forest area both have more frost days later in the spring and earlier in the autumn than the average for both the south of England and also the England average. Both experience greater monthly temperature variation than either the south of England or England averages. Both the New Forest and Thetford Forest have higher average maximum monthly temperatures, and Thetford Forest also experiences lower minimum

average daily temperatures than the south of England average. Thetford experiences has more rapid spring warming and autumnal cooling than the New Forest, south of England and England averages.

Both Thetford Forest and the New Forest have similar average minimum daily temperatures during the autumn to spring period, as do the European areas where TBEV foci are found, including: Sallandse Heuvelrug (Netherlands); Mandal (Norway); and Alsace (France). However maximum average temperatures vary greatly by month, with both Thetford Forest and the New Forest experiencing warmer average maximum temperatures from autumn to the spring than the TBEV endemic areas listed above. Sallandse Heuvelrug, Netherlands, the previous most western TBEV focus, is very similar in temperature profile to both UK areas between March and October.
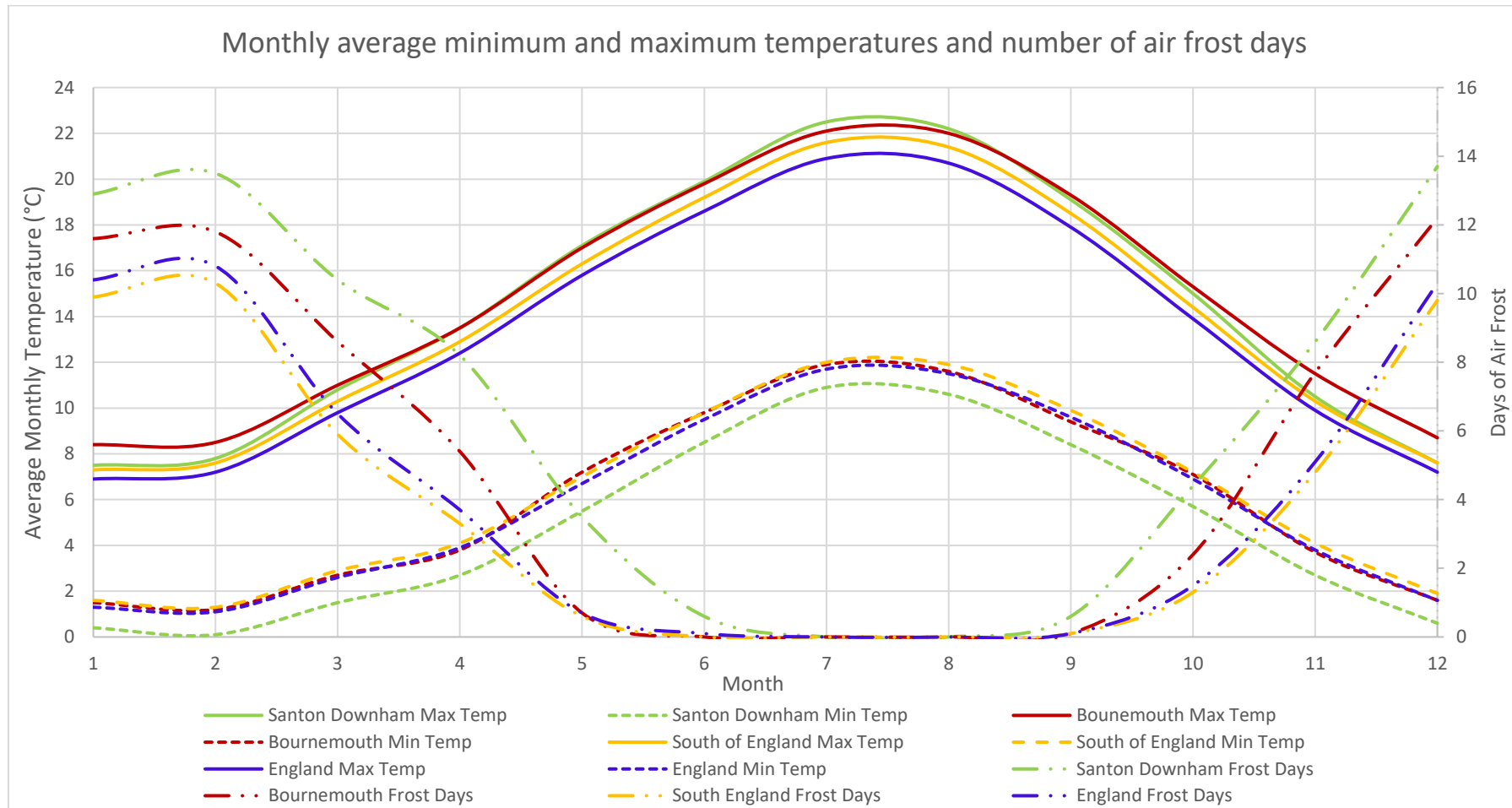
Figure 3:7: *Seasonal climate of Thetford Forest area (Santon Downham) and the New Forest area (Bournemouth Airport nearest weather station) in comparison with South of England and England averages. Data sources: Met Office*
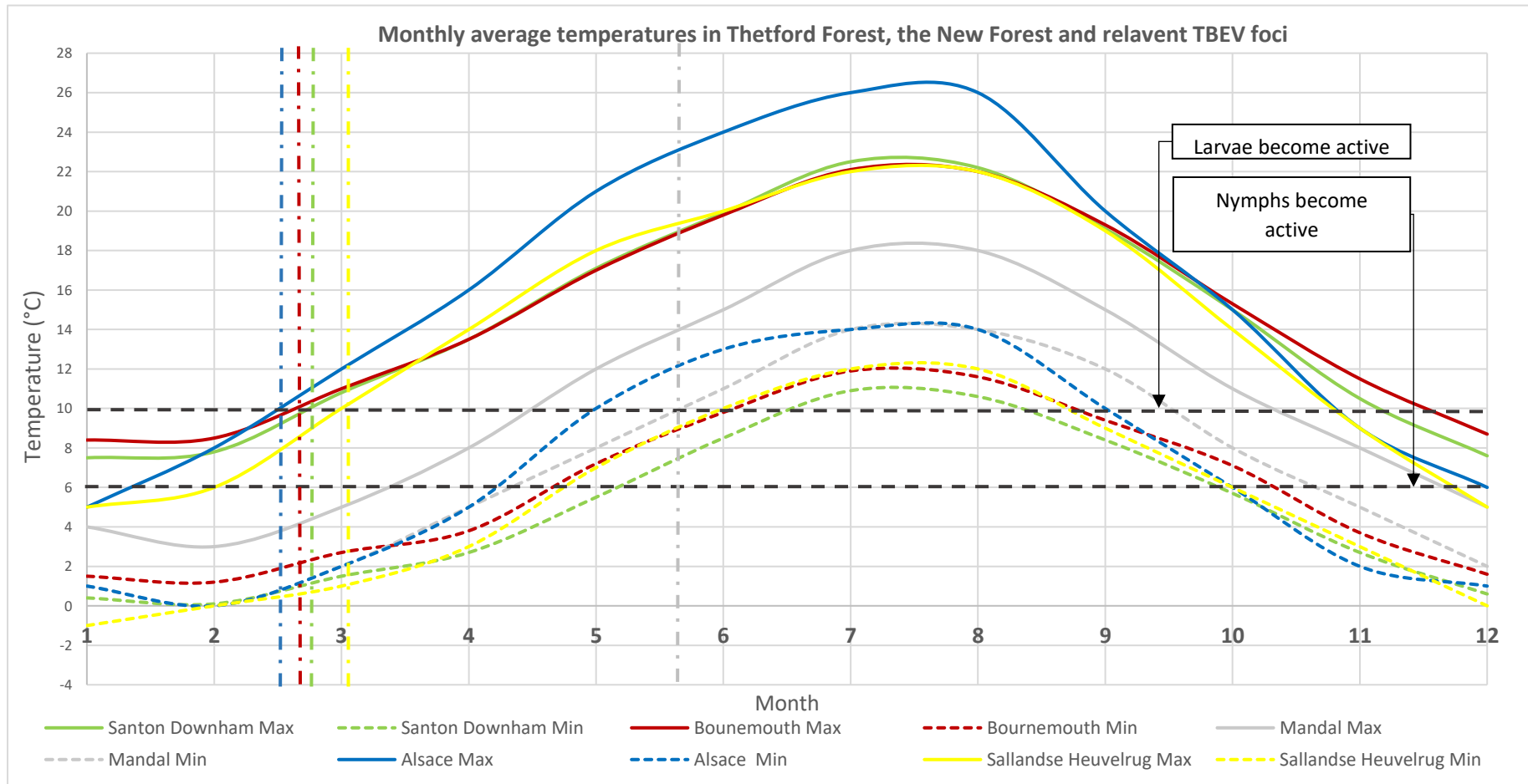
Figure 3:8: Seasonal climate of Thetford Forest area (Santon Downham weather station) and the New Forest area (Bournemouth Airport weather station nearest) in comparison with western-most TBEV foci Sallandse Heuvelrug, Netherlands and Alsace, France. Also, Mandal, Norway (a TBEV focus with which TBEV-UK Thetford shares closest similarity). Lower black dashed line indicates when nymphs become active, and higher one, larvae. Dotted horizontal lines indicate the time both larvae and nymphs will start being active simultaneously. Data sources: Met Office and National Centres for Environmental Information

3.1.3.4: Microclimate

Climate is experienced by fauna and flora at a localised microclimatic scale, which can vary considerably from macroclimatic conditions (Bramer et al., 2018). Global factors, such as incoming solar radiation, set the range that microclimatic variables fall within; however, surface properties directly define the local microclimatic conditions (Rotach and Calanca, 2003). Microclimate is measured by many variables including temperature, precipitation, solar radiation, cloud cover, wind speed and direction, humidity, evaporation and water availability. These are influenced by small-scale biotic and abiotic factors including soil type, land cover (particularly vegetation), topography and proximity to the coast (Bramer et al., 2018).

Key components affect different aspects of microclimate; for example, soil and surface cover type particularly affect the moisture status; the radiation balance is affected by factors such as slope and exposition. More dynamic variables also influence microclimate, such as diurnal changes in soil moisture conditions, large scale wind fields, seasonal changes in flora and the presence of snow (Rotach and Calanca, 2003).

Within the forest canopy, the microclimate is influenced by a combination of environmental conditions and canopy architecture. Branches, foliage and soil within the forest environment absorb solar energy, with water evaporation also occurring from each element (Landsberg and Gower, 1997). Microclimate does not just vary laterally, but also vertically, where conditions nearer the ground level are impacted by soil moisture and rate of soil evaporation with higher areas more impacted by influence of taller features such as trees and buildings (Landsberg and Gower, 1997).

Temperatures at small-scale resolution can vary from the macroclimatic conditions by as much as 5°C and variations in landscapes can produce a remarkable variation of by as much as 20 °C at the same time-point (Bramer et al., 2018). Soil type can also impact temperature; for example, an experimental study demonstrated that within the same locality average daily temperatures are higher in clay than sandy soil when measured between November and June (Burmeier et al., 2010).

Bramer *et al.*, 2018 outlined that microclimate varies over a small spatial resolution and fluctuates in a short timeframe. Thus it should be measured in areas of <100m and is specific to within few meters of the vegetation canopy. This is measured at hourly intervals or smaller temporal scales(Bramer et al., 2018). Topoclimate are changes in climate that vary at a larger resolution than microclimate; these are influenced by topogeographical features such as aspect, slope and elevation (Bramer et al., 2018).

Microclimates should be considered in many applications including vector-borne disease ecology (Bramer et al., 2018). The soil type and vegetation cover, which affects local humidity and local temperature, are key microclimatic variables that are of importance for TBEV foci development and maintenance. The vegetation cover has been discussed in the habitat section - therefore this section will focus on soil types across the two areas of interest (Thetford Forest and the New Forest). The soil of the area has both a direct and indirect impact on microclimate, directly influencing ground moisture status and ground temperature, indirectly impacting vegetation and ground cover.

3.1.3.4.1   Thetford Forest

The climate and soil are the two factors which define Breckland (Rothera, 1998). The Breckland lies within a depression in Cretaceous chalk, which is part of a spine which runs from north west Norfolk to the Chilterns. The depression that Breckland falls within runs from Newmarket to Swaffham where the low-lying plateau varies between 15-30m above sea level, occasionally exceeding 50m (Rothera, 1998; Ratcliffe and Claridge, 1996).

The depression has been infilled by glacial debris, producing a subsoil consisting of chalk rubble, gravel, sand, loam and chalky boulder clay - the dominance of which varies across different areas of Breckland. A common feature for the whole of Breckland is that sand covers these deposits across the area, varying in thickness from 1-2cm to 5m. Breckland is well known for these sandy, light free-draining soils, which create dry surface conditions and which to a considerable extent, determines its unique ecology (Ratcliffe and Claridge, 1996).

The Thetford Forest area is a gently undulating landscape where deposits of sand and gravel are thin overlying the chalk (as shown in *Figure 3:9*), resulting in the barren sandy soils that have given rise to Thetford Forest and heaths.

Complex variation in soil with mixes of chalk, sand, silt, clay and flint occur across Breckland soils which can change across small distances, and may have marked changes in soil pH within an area. These variations have an important impact on natural vegetation and land cover (Dolman et al., 2010). Alternating acidic and alkaline soils are present in Thetford Forest in stripes and polygon-like shapes, which result from periglacial action. For example, in this area acid heathland and chalk grassland occur in a 'stripe' formation (Breckland District Council, 2007).

Soilscapes is a classification system that is used to describe the soils of England and Wales. The soil types discussed fit within the classifications that are described in *Table 3:5.* The main two soil types in the Thetford Forest area of Breckland are sandy, free draining and of low or low to lime rich fertility; both are rare in England. The most widespread type in Thetford Forest is 'Freely draining sandy Breckland soils' and covers just 0.3% of England. The second most common soil in Thetford Forest is 'Freely draining slightly acid sandy soils', these cover just 2.8% of England.

3.1.3.4.2   New Forest

The geology of the New Forest is complex, being formed over several geological periods; sands, clays and silt sit (shown in *Figure 3:10*) upon a Cretaceous chalk bedrock in a syncline basin (Natural England, 2015b; Medcalf and Bell, 2014). The area is made up of gently rolling hills and plateaus joined by shallow valleys (Young, 1935). The plateaus are mostly topped with quaternary flint gravel of various depths, some brickearth, a deposit of homogenous silt/loam, and other shallow deposits. Erosion of the plateaus across the forest exposes underlying strata of several types of tertiary clay and sands. The north of the forest, which has the highest plateaus with wide valleys and hollows between them, have been subject to the greatest erosion resulting in the greatest fragmentation. The lower plateaus in the south of the Forest are less eroded and fragmented (Natural England, 2015b). This has resulted in complex mosaics of variable soils with differing drainage properties. The soils are mainly poor in nutrients and acidic, with slow permeability

(Forestry Commission, 2018). Bracklesham and Barton (Bagshot) sands predominate, but clays of these bed types also are widespread. Soils vary from pure sand, sands and gravels with a layer of peat, sandy loams, loamy clay and stiff valley clays. The plateaus and hills tend to be gravel capped and particularly acidic, arid in summer but become saturated in extended wet weather. Valley bottoms usually consist of a clay-type soil varying in consistency. Most woods (Open and Inclosed) are on Barton beds, with small sections on Brecklesham beds and plateau gravels (Young, 1935). The landform and vegetation are closely linked to the underlying soil type, with the poorest most acidic soils being heath and richer soils being used for farmland and enclosures. Where the geology has resulted in the formation of poor acidic soils which are of limited agricultural value, this has been a key factor in the formation and maintenance of this ancient uncultivated landscape. (Natural England, 2015b). Scots pine and birch, in addition to heather and gorse commonly occur on the plateau gravels, whereas lighter better soils have the best natural oaks, some hazel, willow, alder buckthorn, bracken, bramble and many shrubs, particularly holly (Cadman, 1962).

Much of the vegetation communities in the New Forest are defined by the soil base, nutrient level and moisture content in addition to human influences of burning and grazing (Newton et al., n.d.). In woodlands, particularly sandy soils suit certain tree species more, altering the ground cover from a mix of bracken, heather and bilberry to more sparse bracken and denser cover of bilberry (Young, 1935).

There is a wider variation in Soilscapes soils classifications in the New Forest than Thetford Forest. The two most common 'Slowly permeable seasonally wet slightly acid but base-rich loamy and clayey soils', followed by 'Naturally wet very acid sandy and loamy soils' have very different characteristics. The former is more common in England covering 19.9% of the country; it is loamy and clayey with impeded drainage and moderate fertility. The latter is described as sandy and loamy, naturally wet and of very low fertility. Similar to Thetford Forest Soilscape characteristics, but less widespread. 'Freely draining very acid sandy and loamy soils': this covers just 1% of England, free draining, sandy with some loam and of very low fertility. Naturally these different characteristics have led to different land

uses and habitats in these different strata. Full details of the key soil classifications can be found in *Table 3:6.*
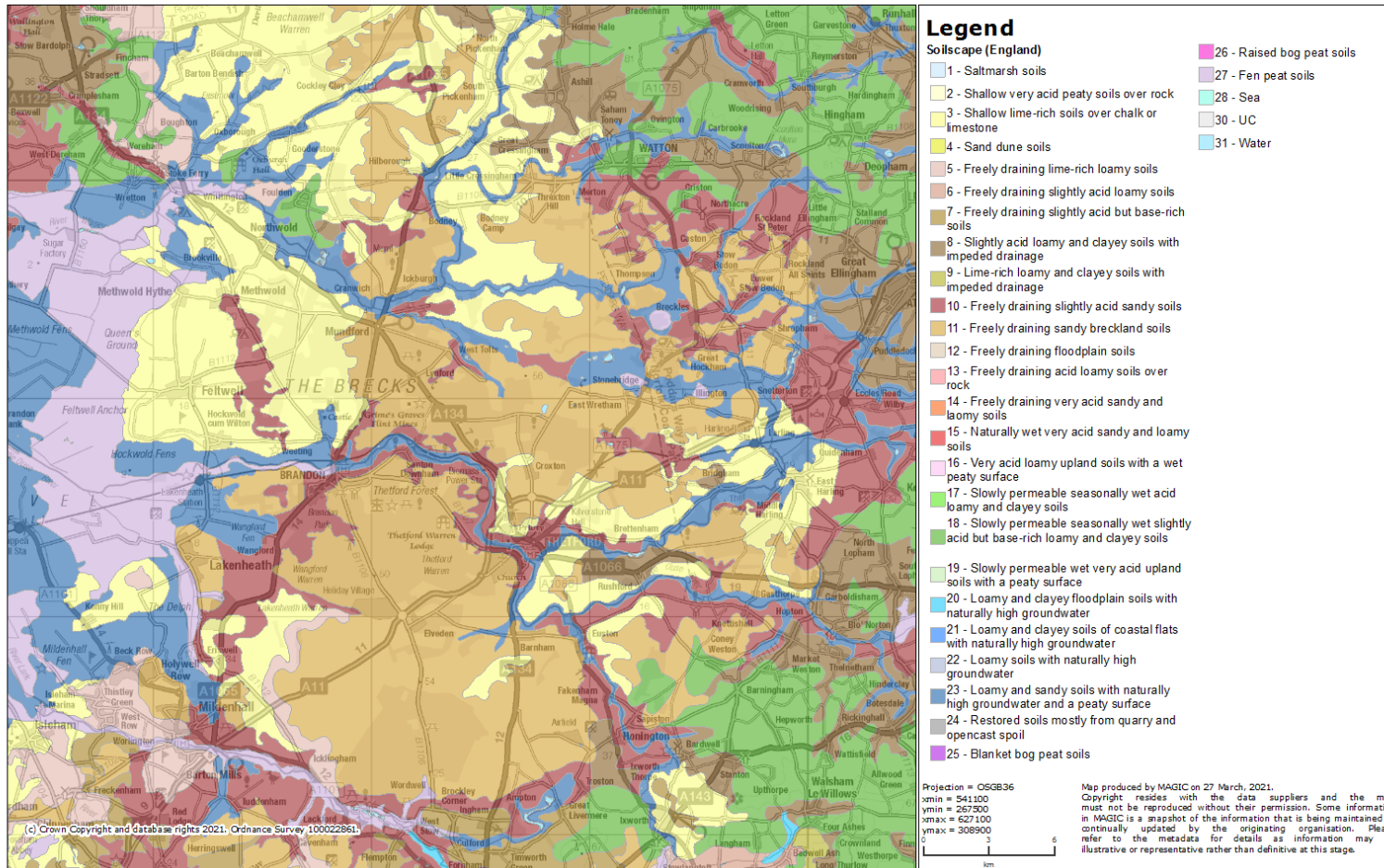
Figure 3:9: *Map of the geology and soil types of Thetford Forest. Produced on https://magic.defra.gov.uk/*

Table 3:5: Characteristics of main Soilscape types within Thetford Forest. Table reproduced from Land Information System, Cranfield University, 2021 (Cranfield University, 2021).

	Freely draining sandy Breckland soils (11)	**Freely draining slightly acid sandy soils (10)**	**Shallow lime-rich soils over chalk or limestone (3)**
England coverage:	0.3%	2.8%	7%
Texture:	Sandy	Sandy	Loamy
Drainage:	Freely draining	Freely draining	Freely draining
Fertility:	Mixed, low to lime-rich	Low	Lime-rich
Land Cover:	Arable forestry and heath	Arable	Arable and grassland
Habitats:	Characteristic Breckland heathland communities	Acid dry pastures; acid deciduous and coniferous woodland; potential for lowland heath	Herb-rich downland and limestone pastures; limestone pavements in the uplands; beech hangers and other lime-rich woodlands
Topsoil Carbon:	Low	Low	Low/Medium
Mostly Drains To:	Groundwater	Groundwater	Chalk or limestone groundwater
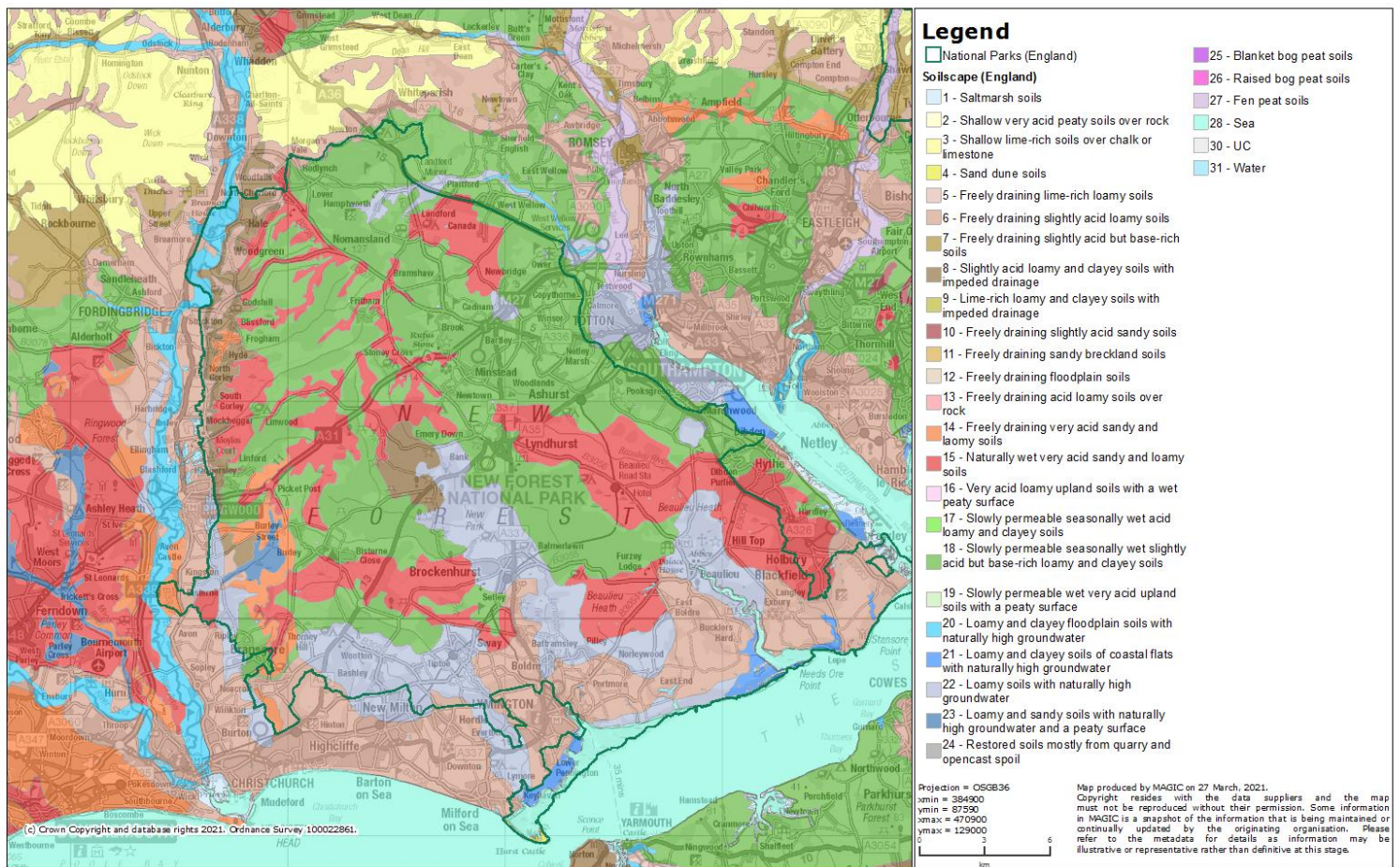
Figure 3:10: *Map of the geology and soil types of the New Forest. Produced on https://magic.defra.gov.uk/*

Table 3:6: Characteristics of main Soilscape types in the New Forest and surrounding area. Table reproduced from Land Information System, Cranfield University, 2021 (Cranfield University, 2021).

	Naturally wet very acid sandy and loamy soils (15)	Slowly permeable seasonally wet slightly acid but base-rich loamy and clayey soils (18)	Freely draining slightly acid loamy soils (6)	Freely draining very acid sandy and loamy soils (14)	Slightly acid loamy and clayey soils with impeded drainage (8)
England Coverage:	1.9%	19.9%	15.5%	1%	10.6%
Texture:	Sandy and loamy	Loamy and clayey	Loamy	Sandy, some loamy	Loamy some clayey
Drainage:	Naturally wet	Impeded drainage	Freely draining	Freely draining	Slightly impeded drainage
Fertility:	Very low	Moderate	Low	Very low	Moderate to high
Land Cover:	Arable and horticulture some wet lowland heath	Grassland and arable some woodland	Arable and grassland	Heath and forestry	Arable and grassland
Habitats:	Mixed dry and wet lowland heath communities	Seasonally wet pastures and woodlands	Neutral and acid pastures and deciduous woodlands; acid communities such as bracken and gorse in the uplands	Mostly lowland dry heath communities	Wide range of pasture and woodland types
Topsoil Carbon:	Medium	Low	Low	Medium	Low
Mostly Drains To:	Shallow groundwater	Stream network	Local groundwater and rivers	Groundwater	Stream network

3.1.3.5: Vectors

Chapter 1 highlighted that *I. ricinus* are present in both Thetford Forest and also the New Forest areas.

3.1.3.5.1   Thetford Forest

A study in 1990 found that 43% of Forestry Commission workers in Thetford Forest are often bitten by *I. ricinus* (Craine et al., 1997). Thetford Forest remains an important area of *I. ricinus* distribution (Medlock et al., 2018). Recent studies on tick activity in Thetford Forest have not been published; however, a study conducted in the forest in 1992 recorded tick activity from February to November through blanket dragging. Questing nymphs increased from February to a June peak, then remained at a fairly constant level until October, then rapidly declined in November. Questing adult tick activity also increased from February, to a peak in September and declining after that; there was also a slight decline in August. Questing larvae were observed through blanket dragging from June to September, with one larval peak demonstrated in July (Craine et al., 1997).

It was demonstrated blanket dragging in woodland vegetation under-sampled larval numbers, as they were found to be active through much more of the year on host species. From June to September both larvae and nymphs (at much lower numbers) were observed feeding on wood mice and bank voles; although numbers feeding on the latter were much lower (Craine et al., 1997).

3.1.3.5.2   New Forest

Unpublished data from private correspondence from a five-year study from 2013-2017 at four sites in the north of the New Forest indicates a consistently high abundance of *I. ricinus* in this area. No seasonal abundance studies in the New Forest have been published.

3.1.4: Rationale and study aims

The detection of TBEV RNA in 5 ticks removed from deer in Thetford Forest, in addition to widespread evidence of TBEV-serocomplex seropositivity in both Thetford Forest and the New Forest and bordering areas, warrants further investigation. No LIV cases in livestock or ecological evidence have been reported in

these areas and LIV reservoir hosts are not widespread either. Therefore, the aim of this study is to investigate whether TBEV RNA can be detected in questing ticks in both Thetford Forest and the New Forest and bordering areas; giving evidence that it is circulating in enzootic cycles. The following objectives will be addressed: Objective 1: detection of viral RNA in questing ticks. Objective 2: explore associations with different habitats and soil types.

3.2: Materials and Methods

3.2.1: 2018 Questing tick collection

3.2.1.1: 2018 Site identification

Deer serum samples submitted to the 2018 deer serosurveillance study were tested for TBEV-serocomplex antibodies by ELISA whilst the study was still ongoing. This enabled the identification of specific geographical locations from which infected culled deer had been taken, thus providing initial evidence of areas in which targeted tick surveys should be completed. Areas of seropositivity identified through the deer serosurveillance study were compared to reports of LIV in livestock for the area. For this, three separate sources of LIV data reporting cases in livestock were examined; these were Jefferies *et al*, 2014 (Jeffries et al., 2014) providing data on livestock between 1974 and 2013; the Animal and Plant Health Agency (APHA) Sheep dashboard; and the APHA Cattle dashboards which provided data from 2012 to present (Animal & Plant Health Agency; Scotland's Rural College, 2020b, 2020a). Areas in which there was evidence of TBEV-serocomplex exposure, but no evidence of LIV in livestock, were focused upon for follow up questing tick surveys during summer of 2018. Hampshire and Thetford Forest were highlighted as areas of seropositivity in deer by TBEV ELISA. As there were no previous records of LIV in livestock reported (Animal & Plant Health Agency; Scotland's Rural College, 2020a, 2020b; Jeffries et al., 2014) in those areas, these were areas identified for further detailed investigation.

For each sample submitted to the deer serosurveillance study, location or co-ordinates were provided for sites where deer were shot. In areas where there were seropositive samples and no previous records of LIV infection in livestock, each locality in which a seropositive deer was shot was identified as a site for follow up tick surveying. Areas which had multiple seropositive deer shot in close proximity, were identified as one site, as tick surveys would overlap between these locations.

Once sites were selected through the above method, Ordnance Survey (OS) 1:25K maps were then examined to identify suitable surrounding locations for tick surveying. Specific locations were determined through identifying the closest blocks

of woodland to the co-ordinates where the deer were shot, if not already within a block.

3.2.1.2: 2018 Tick collection

As a result of the deer seroprevalence analysis, a total of 16 sites were selected for tick collections within the Thetford Forest area and 4 sites in the Hampshire and bordering areas in 2018 (**Table 3:7** and **Table 3:8**) Most tick collections were conducted during July, towards the end of the peak of the questing tick season. This was because it was the first available time-point to collect ticks once sufficient deer serum had been collected and tested. Two sites were not visited for collection until September 2018, as this was military land and the earliest date that access could be arranged.

Ticks were collected through flagging, dragging a $1m^2$ cotton flag attached to a wooden dowel, in 10m transects over vegetation. Ticks were collected after each transect and placed into a 7ml universal tube labelled with the site name and date for follow up testing. Transects were conducted in areas of suitable tick habitat surrounding the location at which the deer were culled. Areas from which ticks were collected from for each site are shown in Appendix 1. Transects were predominately conducted along the edges of paths and within adjacent woodland. Most detailed surveys were focused on areas of each site which were considered suitable tick habitat. Tick density was not measured for these surveys, a minimum of 3 hours was spent collecting ticks in each locality, with more time spent at those sites which had higher tick densities or high numbers of seropositive deer culled there.

A small amount of vegetation was added into each universal of collected ticks, in order to maintain sufficient humidity to ensure ticks were kept alive prior to storage at -80°C until further processing.

Table 3:7: *New Forest and bordering areas 2018 sites, dates surveyed and the main habitat type*

Site ID	Date(s) Surveyed	Main habitat
SGP	26/6/18, 27/7/18	Conifer
EW	2/7/18	Broadleaved (ancient and semi natural)
WB	2/7/18	Broadleaved (ancient and semi natural)
PC	4/7/18	Broadleaved (ancient replanted woodland)

Table 3:8: *Thetford Forest area 2018 sites, dates surveyed and the main habitat type*

Site ID	Date(s) Surveyed	Main habitat
CH	6/7/18	Conifer
SHC1	8/7/18	Mixed mainly conifer
SHC2	7/7/18	Mixed mainly conifer
SW	6/7/18	Conifer
SF1	9/7/18	Conifer
SCC	5/7/18	Conifer
SD	5/7/18	Broadleaved
TAP	8/7/18	Broadleaved
DRMW	7/7/18, 8/7/18	Conifer
DRME	7/7/18	Conifer
DRMH	13/7/18	Conifer
QC	9/7/18	Conifer
SCD	5//7/18	Mainly mixed broadleaved
WW	4/7/18	Conifer
STFH	11/9/18	Conifer
STMH	11/9/18	Broadleaved

3.2.2: 2019 questing tick collections and surveys

3.2.2.1: Site identification

Sites were identified following the completion of testing of all of the deer serosurveillance study samples for tick-borne viruses, and completion of testing of most of the 2018 questing ticks. Site selection followed the same criterion

explained for the 2018 sampling; however, due to limited time available, sampling effort was focused on areas that had higher numbers of seropositive deer blood samples and those where TBEV had been detected in the previous year's questing tick sampling or in ticks removed from deer from a site. A total of 12 sites across the Thetford Forest area (**Table 3:10**) and 4 sites in Hampshire and bordering areas (**Table 3:9**) were surveyed.

For the Thetford Forest surveys the same localities were surveyed as the 2018 sites SCC, SCB, WW, SD and SW. The SHC1 and SHC2 sites were split into smaller survey areas for the 2019 surveys, these were SHC3, SHC4 and SHC5. SF2 surveyed in 2019 is very near (1 to 1.5km) from SF1 surveyed in 2018. Due to time constraints, access issues or low tick numbers TAP, STFH, STMH, CH, QC and DRMH were not surveyed in 2019.

3.2.2.2: Tick Surveys

In 2019, tick surveys were conducted in addition to pure tick collection. Flagging and tick collection were conducted as described for the 2018 questing tick surveys. Approximately 70 to 100 ticks were collected in each tube before starting a new one. In addition to the site and date, the tube number and surveyor was also recorded on the tube. Each transect was recorded on the ArcGIS Collector app, with GPS location, whether larvae were present, number of nymphs, adult males and adult females collected and any specific notes were recorded in order to aid identification of specific areas and habitats where TBEV foci may be located. The sites were broken down into 'subsites' defined by the area where the surveyor started and finished each tube. This system enabled the narrowing of location of any TBEV positive tick pool following testing. The purpose to enabling recording of the more specific location of TBEV positive ticks collected is to help to target future tick surveys and identify specific focus locations. Additionally, a photo was taken each time there was a change in habitat, at the start of a new tube or start of a new subsite section, which was linked to that transect via the Arc GIS Collector app. On some occasions colleagues also assisted in tick collections at some sites. This was to increase sample size, so they did not record numbers of ticks collected by transect and transect locations on the ArcGIS Collector app. The same overall area was

covered by the additional surveyors and the localities at which tubes were changed were recorded. All tick density analysis was based on records from the Collector app and did not include collections from the additional surveyors.

The ArcGIS Collector app survey data was merged with Forestry Commission Forest Sub compartment data for analysis of dominant tree species within each site.

Table 3:9: *New Forest and bordering areas 2019 sites, dates surveyed and the main habitat type*

Site ID	Date(s) Surveyed	Main habitat
SA	17/6/19	Conifer
SGP	2/7/19	Conifer
SMH	17/6/19	Conifer and Broadleaved
WG	8/8/19 23/8/19	Broadleaved

Table 3:10: *Thetford Forest 2019 sites, dates surveyed and the main habitat type*

Site ID	Date(s) Surveyed	Main habitat
SF2	28/6/19, 29/6/19	Mixed mainly conifer
SD	28/6/19	Broadleaved
WTH	26/6/19	Conifer
WW	29/6/19	Conifer
DRME	27/6/19	Conifer
DRMW	28/6/19	Conifer
SHC3	24/6/19	Conifer
SHC4	25/6/19	Broadleaved
SHC5	24/6/19	Conifer
SW	26/6/19	Conifer
SCD	26/6/19	Mixed mainly broadleaved
SCC	27/6/19 29/6/19	Conifer

3.2.3: Tick processing and testing

3.2.3.1: Tick identification and pooling

Due to the probability of the questing ticks collected being *I. ricinus* and the volume

collected, just 10% of ticks from each site were morphologically identified using a

light microscope as described in *2.2.3.2: Tick identification*. Questing ticks were

pooled by site into groups of 10 nymphs or 5 adult males or 5 adult females. These

were placed into Precellys MK-28R homogenisation tubes. When there were

insufficient ticks to form a complete tick pool, they were stored for possible future

testing.

3.2.3.2: Homogenisation and extraction

Samples were homogenised and then nucleic acid extracted as described in *2.2.3.3:*

Homogenisation and extraction.

3.2.3.3: Testing samples by RT-PCR

Samples were tested for presence of TBEV/LIV RNA using the Schwaiger and

Cassinotti (2003) RT-PCR (Schwaiger and Cassinotti, 2003) as described in *2.2.3.4:*

TBEV RT-PCR. Any samples that were positive on this PCR were then tested with the

Marriott (2006) RT-PCR (Marriott et al., 2006) designed to only detect LIV as

described in *2.2.3.5: LIV RT-PCR*. Ten percent of samples from each extraction plate

were selected at random and tested for 18S ribosomal RNA using the Carter RT-PCR

(unpublished) as described in *2.2.3.6: 18S ribosomal RT-PCR*.

3.2.3.4: Genome sequencing and phylogenetic analysis

Genome sequencing and phylogenetic analysis was conducted on PCR positive

samples as described in *2.2.3.7: Genome sequencing and phylogenetic analysis*.

3.2.4: Data analysis

The general habitat type by site was determined by mapping site locations to the

Priority Habitat Inventory (England) dataset produced by Natural England (Natural

England, 2020). Each site was also mapped to the Soilscapes dataset produced by

Cranfield University, to provide information on the geology/soil type in each site

(Cranfield University, 2013).

Each transect from the tick density surveys carried out in Thetford Forest in 2019

was recorded in a database including number of each life stage collected and the

spatial location (coordinate) of the transect. This was produced through inputting each transect on the Arc GIS Collector app when carrying out the field work.

Subsequently the spatial location of each transect was then joined with the National Forest Estate Sub-compartments England 2019 dataset produced by the Forestry Commission using ArcMap 10.5.1. This dataset gives high resolution detail of the dominant tree species of small forest blocks termed 'sub-compartments' within the areas surveyed. Due to the high resolution of the National Forest Estate Sub-compartments England 2019 dataset, transects from each site crossed a number of 'sub-compartments'. Data was analysed by both site and subsite. The minimum infection rate (MIR), was calculated by dividing the number of PCR positive pools by the total number of ticks tested. For the 2019 Thetford tick density surveys, the mean number nymphs/ $10m^2$ transect, mean number of adult males/ $10m^2$ transect and mean number adult females/ $10m^2$ transect were calculated for each site. The DIN/$100m^2$ was calculated using the following formula: (number of PCR positive nymph pools/number of nymphs tested) x density of nymphs/$100m^2$. 95% confidence intervals (95% CI) were calculated in Stata 15.1 and graphs were produced in R studio version 4.0.3.

3.3:   Results

In total 10,290 ticks were collected and tested by TBEV/LIV rRT-PCR from study sites in Thetford Forest and the New Forest and surrounding areas during 2018 and 2019. During 2018, 2,980 ticks were collected and tested from 18 sites in Thetford Forest, and 915 ticks from four sites in the New Forest and bordering areas. During 2019, 4,105 ticks were collected and tested from 12 sites in Thetford Forest and 2,290 ticks from six sites in the New Forest and bordering areas. Of the 10% of ticks collected in 2018 and 2019 that were morphologically identified to species level, all were *I. ricinus*.

3.3.1: 2018 tick collection and testing

During the 2018 questing tick collections, 3,130 ticks were collected and tested from 16 sites in the Thetford Forest area; 83.1% (770) were nymphs, 7.2% (225) adult males and 9.7% (305) adult females. The main habitat of ten of these sites were coniferous woodland, three broadleaved, two mixed mainly broadleaved, and

one mixed mainly conifer. The sites were on three soil types, freely draining sandy Breckland soils (FDSB), freely draining slightly acid sandy soils (FDSAS) and shallow lime-rich soils over chalk or limestone (SLOCL).

TBEV/LIV rRT-PCR positive questing tick pools were detected in two of the 16 sites, DRMH and SHC2; both of these were coniferous areas, as detailed in *Table 3:11*. SHC2 was FDSB soil type and DRMH was on the border of two soil types, FDSB and FDSAS.

Three of 8 nymph pools tested (80 nymphs) at DRMH were TBEV/LIV rRT-PCR positive giving a nymphal minimum infection rate (MIR) of 3.75% (95% CI 0.84-10.90) and one pool of adult females of just one pool tested (5 adult females), producing an adult female MIR of 20.00% (95% CI 2.03-64.04). The second site with TBEV/LIV rRT-PCR positive pools was SHC2 with lower MIR than DRMH. Two pools were TBEV/LIV rRT-PCR positive, one nymph pool of 51 tested (510 nymphs) with a nymphal MIR of 0.20% (95% CI 0.00-1.22) and one adult male pool of four tested (20 adult males) producing an adult male MIR of 5.00% (95% CI 0.00-25.41).

During 2018, ticks were also collected and tested from four sites in the New Forest and its surrounding areas where a total of 915 ticks were collected; 84.2% (770) were nymphs, 6.0% (55) adult males and 9.8% (90) adult females. The breakdown of the number of ticks tested by site is displayed in *Table 3:12*. The main habitat of three of the sites were broadleaved woodland sites and one coniferous. No TBEV/LIV rRT-PCR PCR positive questing tick pools were detected in the New Forest and the surrounding areas from the 2018 questing tick collections.

No LIV RNA was detected in any of the TBEV/LIV rRT-PCR, when they were tested by rRT-PCR designed to detect only LIV.

Table 3:11: *Questing ticks tested and prevalence (MIR-minimum infection rate) of by TBEV/LIV rRT-PCR from sites in Thetford Forest in 2018. Main habitat type: B= Broadleaved, C= Conifer; MMB= Mixed mainly broadleaved; MMC= Mixed mainly conifer. Soil type: FDSB= Freely draining sandy Breckland soils. FDSAS: Freely draining slightly acid sandy soils. SLOCL=Shallow lime-rich soils over chalk or limestone. Sites positive for TBEV RNA are underlined.*

Code	Main Habitat *	Soil**	Nymphs positive/tested (pools)	Nymph MIR % (95% CI)	AM positive/tested (pools)	AM MIR % (95% CI)	AF positive/tested (pools)	AF MIR % (95% CI)	Total positive/tested (pools)	Total MIR % (95% CI)
TAP	B	SLOCL & FDSB	0/80 (80)	0.00 (0.00-5.49)	0/15 (3)	0.00 (0.00-23.86)	0/30 (6)	0.00 (0.00-13.47)	0/125 (17)	0.00 (0.00-35.83)
SCC	C	FDSB	0/420 (42)	0.00 (0.00-1.09)	0/15 (3)	0.00 (0.00-23.86)	0/20 (4)	0.00 (0.00-18.98)	0/455 (49)	0.00 (0.00-1.01)
SCB	MMB	FDSB & FDSAS	0/230 (23)	0.00 (0.00-1.98)	0/15 (3)	0.00 (0.00-23.86)	0/10 (2)	0.00 (0.00-32.09)	0/255 (28)	0.00 (0.00-1.79)
WW	C	FDSB & FDSAS	0/40 (4)	0.00 (0.00-10.44)	0/0 (0)	0.00	0/5 (1)	0.00 (0.00-48.91)	0/45 (5)	0.00 (0.00-9.38)
SD	B	FDSB & FDSAS	0/170 (17)	0.00 (0.00-21.63)	0/10 (2)	0.00 (0.00-32.09)	0/20 (4)	0.00 (0.00-18.98)	0/200 (23)	0.00 (0.00-2.27)
STFH	C	FDSB	0/70 (7)	0.00 (0.00-6.23)	0/35 (7)	0.00 (0.00-11.76)	0/35 (7)	0.00 (0.00-11.76)	0/140 (21)	0.00 (0.00-3.21)
STMH	B	SLOCL	0/90 (9)	0.00 (0.00-4.91)	0/20 (4)	0.00 (0.00-18.98)	0/10 (2)	0.00 (0.00-32.09)	0/120 (15)	0.00 (0.00-3.73)
SHC1	MMC	SLOCL	0/140 (14)	0.00 (0.00-3.21)	0/10 (2)	0.00 (0.00-32.09)	0/15 (3)	0.00 (0.00-23.86)	0/165 (19)	0.00 (0.00-2.74)
SHC2	*C*	*FDSB*	*1/510 (51)*	*0.20 (0.00-1.22)*	*1/20 (4)*	*5.00 (0.00-25.41)*	*0/30 (6)*	*0.00 (0.00-13.47)*	*2/560 (61)*	*0.36 (0.01-1.38)*
SW	C	FDSB	0/280 (28)	0.00 (0.00-1.63)	0/55 (11)	0.00 (0.00-7.80)	0/75 (15)	0.00 (0.00-5.84)	0/410 (54)	0.00 (0.00-1.12)
CH	C	SLOCL	0/130 (13)	0.00 (0.00-3.45)	0/0 (0)	N/A	0/0 (0)	0.00	0/130 (13)	0.00 (0.00-3.45)
DRME	C	FDSB	0/80 (8)	0.00 (0.00-5.49)	0/5 (0)	0.00 (0.00-48.91)	0/10 (2)	0.00 (0.00-32.09)	0/95 (10)	0.00 (0.00-4.66)
DRMW	C	FDSB & FDSAS	0/10 (1)	0.00 (0.00-32.09)	0/0 (0)	N/A	0/0 (0)	0.00	0/10 (1)	0.00 (0.00-32.09)
DRMH	*C*	*FDSB & FDSAS*	*3/80 (8)*	*3.75 (0.84-10.90)*	*0/5 (0)*	*(0.00-48.91)*	*1/5 (1)*	*20.00 (2.03-64.04)*	*4/90 (9)*	*4.44 (1.39-11.23)*
QC	C	FDSAS	0/90 (9)	0.00 (0.00-4.91)	0/5 (0)	(0.00-48.91)	0/15 (3)	0.00 (0.00-23.86)	0/110 (12)	0.00 (0.00-4.05)
SF1	C	SLSOCL & FDSB	0/60 (6)	0.00 (0.00-7.20)	0/0 (0)	(0.00-23.86)	0/10 (2)	0.00 (0.00-32.09)	0/70 (8)	0.00 (0.00-6.23)

Table 3:12: *Questing ticks tested and prevalence (MIR) of TBEV/LIV rRT-PCR from sites in Hampshire and bordering areas in 2018. Main habitat type: B= Broadleaved, C= Conifer; MMB= Mixed mainly broadleaved; MMC= Mixed mainly conifer. Soil type: WVASL= Naturally wet very acid sandy and loamy soils, ABLC = Slowly permeable seasonally wet slightly acid but base-rich loamy and clayey soils, FDSAL=Freely draining slightly acid loamy soils*

Code	Habitat*	Soil**	Nymphs positive/tested (pools)	Nymph MIR % (95% CI)	AM positive/tested (pools)	AM MIR % (95% CI)	AF positive/tested (pools)	AF MIR % (95% CI)	Total positive/tested (pools)	Total MIR % (95% CI)
SGP	C	WVASL	0/420 (42)	0.00 (0.00-1.09)	0/25 (5)	0.00 (0.00-15.76)	0/35 (7)	0.00 (0.00-11.76)	0/480 (54)	0.00 (0.00-0.96)
EW	B	ABLC & WVASL	0/160 (16)	0.00 (0.00-2.82)	0/10 (2)	0.00 (0.00-32.09)	0/30 (6)	0.00 (0.00-13.47)	0/200 (24)	0.00 (0.00-2.27)
WF	B	ABLC	0/100 (10)	0.00 (0.00-4.44)	0/15 (3)	0.00 (0.00-23.86)	0/20 (4)	0.00 (0.00-18.98)	0/135 (17)	0.00 (0.00-3.33)
PC	B	ABLC & FDSAL	0/90 (9)	0.00 (0.00-4.91)	0/5 (1)	0.00 (0.00-48.91)	0/5 (1)	0.00 (0.00-48.91)	0/100 (11)	0.00 (0.00-4.41)

3.3.2: 2019 Thetford Forest tick density surveys

During the 2019 questing tick surveys, 4,105 ticks were collected and tested from 12 sites in the Thetford Forest area; 82.1% (3,370) were nymphs, 8.5% (350) adult males and 9.4% (385) adult females. Two of the sites were broadleaved, three mixed mainly broadleaved, six conifer and one mixed mainly conifer.

There was a large variation in tick densities across the sites, shown in *Table 3:13*. The highest density of nymphs (DON) was at site SHC3, a mixed mainly broadleaved woodland at 5.04 nymphs/$10m^2$ (95% CI 3.23-6.85) and the lowest was DRMW, a coniferous woodland at 0.24 nymphs/$10m^2$ (95% CI 0.14-0.34). The main woodland type of the site, as classified by the National Forest Inventory, did not seem to correlate with DON. For example, for coniferous woodland DON varied from 0.24/$10m^2$ at site DRMW to the second highest DON detected at SHC5 4.4/$10m^2$.

Broken down into the dominant tree type in the forest sub-compartment of each transect, there is some interesting variation in the DON within sites between the different dominant tree species (Figure 3:11). The highest DON was detected in site SHC3, an area classified as mixed mainly broadleaved. However, the highest number of transects within this woodland, which is classified as mixed mainly broadleaved, were actually conducted in sub-compartments of conifer species. The most transects were conducted in Corsican pine sub-compartments, followed by beech and also Scots pine.

The variation between sub-compartment species is more prominent for some sites, as similar to SHC3, Corsican pine also had the highest DON at SHC5, followed by Douglas fir and Scots pine. However, some sites surveyed with Corsican pine sub-compartments had low DON for this species, such as SW and SCC. Interestingly, none of the sites had particularly high DON in broadleaved sub-compartments, and overall, the coniferous tree species DON was higher than that of the broadleaved species such as beech.

When split into subsites, there are similar but more noticeable patterns, with the highest 5 subsite DON densities being Corsican pine (Figure 3:12).

Table 3:13: *Thetford Forest 2019 questing tick survey density by site and main habitat type. Main habitat type: B= Broadleaved, C= Conifer; MMB= Mixed mainly broadleaved; MMC= Mixed mainly conifer. Soil type: FDBS= Freely draining sandy Breckland soils. FDSAS: Freely draining slightly acid sandy soils. SLOCL=Shallow lime-rich soils over chalk or limestone*

Site	Main Habitat*	Soil**	Mean nymphs/ 10m² transect (95% CI)	Mean AF/ 10m² transect (95% CI)	Mean AM/ 10m² transect (95% CI)	Total ticks/ 10m² transect (95% CI)	Number of Transects
SCC	Conifer	FDSB	1.82 (1.51-2.14)	0.08 (0.03-0.14)	0.16 (0.08-0.24)	2.07 (1.74-2.40)	142
SCB	MMB	FDSB & FDSAS	1.04 (0.72-1.35)	0.08 (0.02-0.14)	0.05 (0.01-0.10)	1.17 (0.85-1.50)	110
WW	Conifer	FDSB & FDSAS	0.55 (0.21-0.89)	0.06 (0.00-0.16)	0.03 (0.00-0.1)	0.65 (0.28-1.01)	31
SD	Broadleaved	FDSB & FDSAS	1.11 (0.76-1.47)	0.13 (0.04-0.21)	0.13 (0.04-0.21)	1.36 (0.97-1.75)	80
WTH	Conifer	FDSB	3.31 (2.64-3.98)	0.10 (0.03-0.16)	0.14 (0.06-0.23)	3.55 (2.88-4.21)	84
SHC3	MMB	FDSB & SLOCL	5.04 (3.23-6.85)	0.13 (0.05-0.21)	0.16 (0.03-0.3)	5.34 (3.46-7.22)	68
SHC4	Broadleaved	SLOCL	1.29 (0.98-1.59)	0.2 (0.12-0.28)	0.09 (0.03-0.14)	1.57 (1.25-1.90)	105
SHC5	Conifer	FDSB	4.4 (3.46-5.34)	0.42 (0.27-0.57)	0.28 (0.17-0.39)	5.10 (4.11-6.10)	97
SW	Conifer	FDSB	1.18 (0.89-1.47)	0.36 (0.21-0.52)	0.42 (0.26-0.57)	1.96 (1.61-2.31)	72
DRME	Conifer	FDBS	0.31 (0.15-0.47)	0.04 (0.00-0.09)	0.06 (0.00-0.12)	0.40 (0.24-0.57)	52
DRMW	Conifer	FDSAS & FDSB	0.24 (0.14-0.34)	0.04 (0.00-0.08)	0.38 (0.00-0.08)	0.32 (0.19-0.44)	79
SF2	MMC	FDBS & SLOCL	1.69 (1.43-1.96)	0.34 (0.24-0.43)	0.34 (0.24-0.44)	2.37 (2.07-2.67)	188
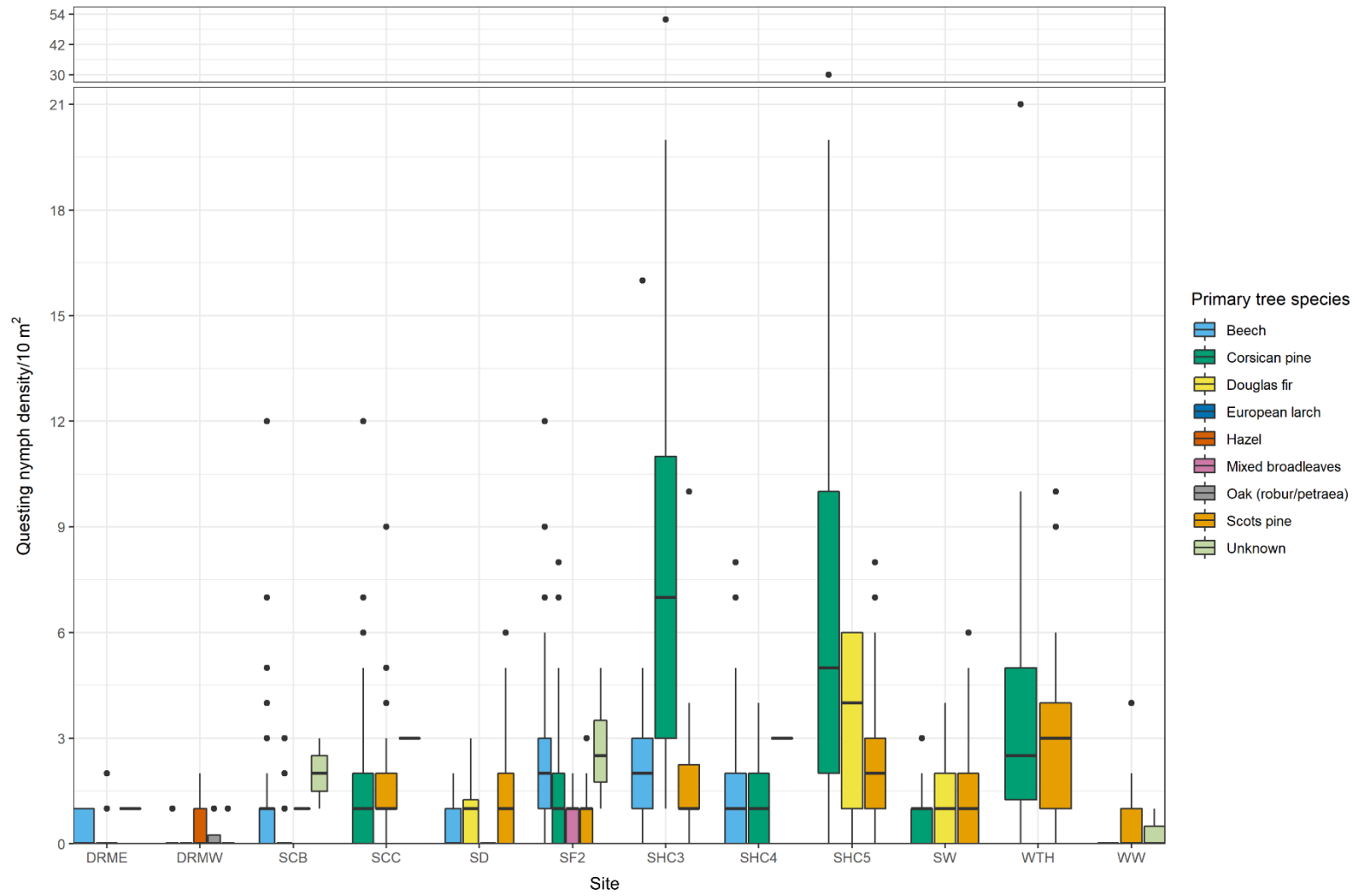
Figure 3:11: *Density of nymphs by site and dominant tree type (Primary tree species) in the forest sub-compartment of each transect in Thetford Forest (2019)*
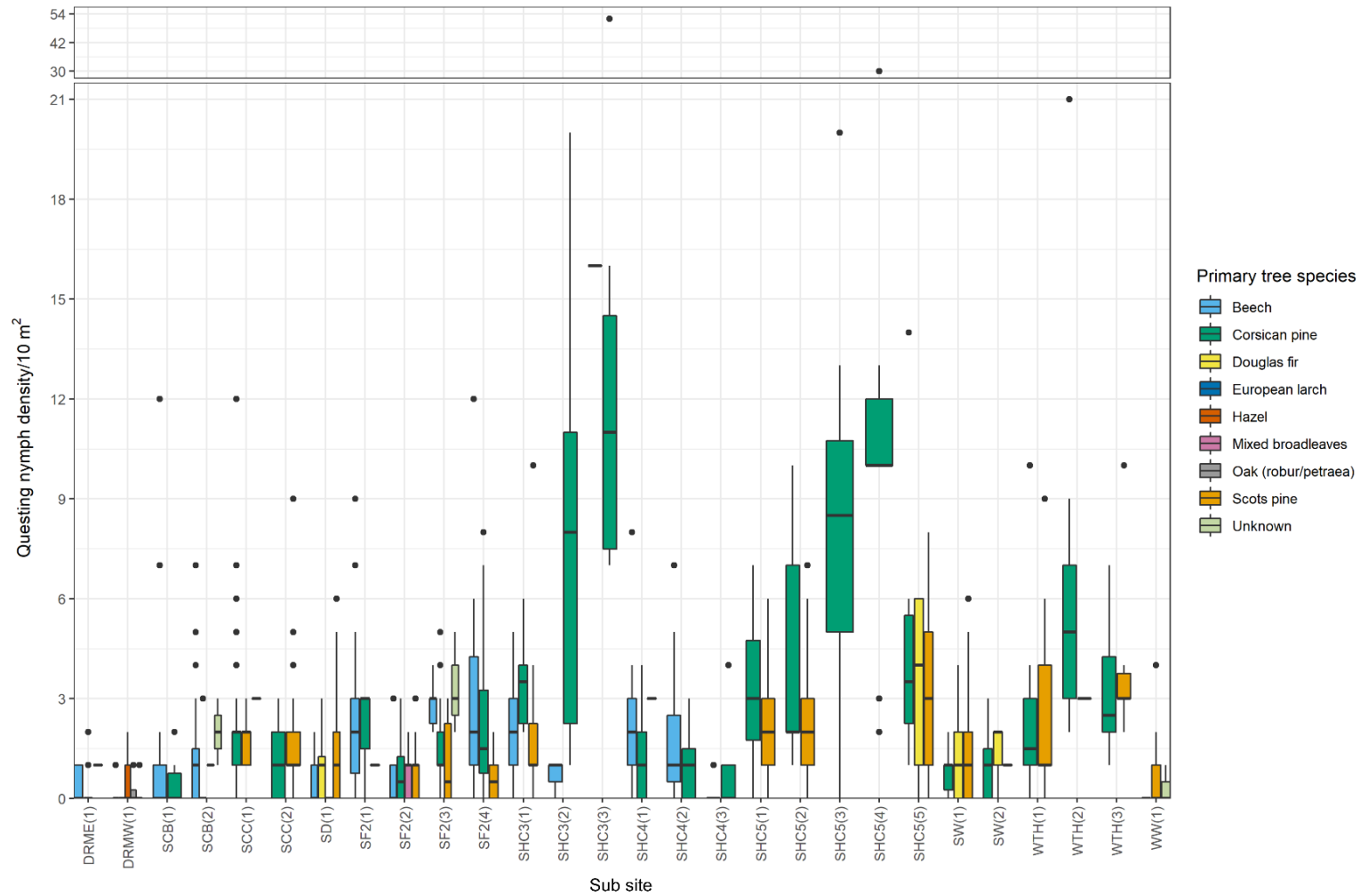
Figure 3:12: *Density of nymphs by subsite and dominant tree type (Primary tree species) in the forest sub-compartment of each transect in Thetford Forest (2019). Subsites SCC(1), SHC5(1), SW(1), WTH(1) and WTH(2) had TBEV/LIV rRT-PCR positive pool(s).*

3.3.3: 2019 Thetford Forest tick testing

TBEV/LIV rRT-PCR positive tick pools were detected in four of the 12 sites in the Thetford Forest area. All four were in sites where conifer is dominant, from the total of seven conifer dominant sites. Five sites surveyed had the soil type of only FDSB and interestingly four of these were the sites where TBEV/LIV rRT-PCR positive pools were detected. Six other sites were on the borders of two soil types, four were FDSB and FDSAS borders, and two FDSB and SLOCL borders. One was just on SLOCL (*Table 3:14*).

Of the 2019 Thetford sites, SCC had the highest overall site MIR of 0.84% (95% CI 0.25-2.23) and a total of 475 ticks were tested. The TBEV/LIV rRT-PCR positive pools were all nymph pools, with four of 42 (420) positive, producing a nymph MIR of 0.95% (95% CI 0.28-2.51). The second highest MIR was SW, with a just slightly lower site MIR of 0.83% (95% CI 0.27-1.98) of 605 ticks tested. A total of five pools were TBEV/LIV rRT-PCR positive; four were positive nymph pools of 380 nymphs tested (38 pools) producing a nymph MIR of 1.05% (95% CI 0.31-2.78). One adult male pool of 105 adult males tested (21 pools) was positive. The adult male MIR was 0.95% (95% CI 0.00-5.72). The third highest site WTH, was a new site for 2019. The overall site MIR was quite a lot lower than SCC and SW at 0.29% (95% CI 0.06-0.88) with a total of 3 TBEV/LIV rRT-PCR positive pools of 1050 ticks tested. There was one positive nymph pool (of 940 nymphs [94 pools]) producing a MIR of 0.11% (95% CI 0.00-0.66). There were also two positive adult male pools of 50 males (10 pools) tested producing a MIR of 4.00% (95% CI 0.34-14.22). Finally, the site with the lowest site MIR was from SHC5. SHC5 is within the same but more restricted area as the 2018 site SHC2. Overall, 510 ticks were tested from this site; the site MIR was 0.20% (95% CI 0.00-1.22). Just one pool was TBEV/LIV rRT-PCR positive, this was a nymph pool; out of 43 pools (430 nymphs) tested, the nymph MIR was 0.24% (95% CI 0.00-1.44). The results of all sites for 2019 Thetford Forest tick testing are detailed in *Table 3:14*.

None of the samples that were positive on the Schwaiger and Cassinotti (2003) TBEV/LIV rRT-PCR assay, were positive on the Marriott et al. (2006) rRT-PCR designed to detect only LIV.

Table 3:14: *Questing ticks tested and prevalence (MIR) of TBEV from sites in Thetford Forest in 2019. Main habitat type: B= Broadleaved, C= Conifer; MMB= Mixed mainly broadleaved; MMC= Mixed mainly conifer. Soil type: FDBS= Freely draining sandy Breckland soils. FDSAS: Freely draining slightly acid sandy soils. SLOCL=Shallow lime-rich soils over chalk or limestone*

Code	Main Habitat *	Soil**	Nymphs pools positive/ N tested (pools)	Nymph MIR % (95% CI)	AM pools positive/ N tested (pools)	AM MIR % (95% CI)	AF pools positive/ N tested (pools)	AF MIR % (95% CI)	Total pools positive/N tested (pools)	Total MIR % (95% CI)
SCC	C	FDSB	4/420 (42)	0.95 (0.28-2.51)	0/35 (7)	0.00 (0.00-11.76)	0/20 (4)	0.00 (0.00-18.98)	4/475 (53)	0.84 (0.25-2.23)
SCB	MMB	FDSB & FDSAS	0/150 (15)	0.00 (0.00-3.00)	0/10 (2)	0.00 (0.00-32.09)	0/15 (3)	0.00 (0.00-23.86)	0/175 (20)	0.00 (0.00-2.58)
WW	C	FDSB & FDSAS	0/10 (1)	0.00 (0.00-32.09)	0/0 (0)	0.00 N/A	0/0 (0)	0.00 N/A	0/10 (1)	0.00 (0.00-32.09)
SD	B	FDSB & FDSAS	0/80 (8)	0.00 (0.00-5.49)	0/5 (0)	0.00 (0.00-48.91)	0/10 (0)	0.00 (0.00-32.09)	0/95 (8)	0.00 (0.00-4.66)
WTH	C	FDSB	1/940 (94)	0.11 (0.00-0.66)	2/50 (10)	4.00 (0.34-14.22)	0/60 (12)	0.00 (0.00-7.20)	3/1050 (116)	0.29 (0.06-0.88)
SHC3	MMB	FDSB & SLOCL	0/340 (34)	0.00 (0.00-1.35)	0/15 (3)	0.00 (0.00-23.86)	0/10 (2)	0.00 (0.00-32.09)	0/365 (39)	0.00 (0.00-1.26)
SHC4	B	SLOCL	0/140 (14)	0.00 (0.00-3.21)	0/5 (1)	0.00 (0.00-48.91)	0/20 (4)	0.00 (0.00-18.98)	0/165 (19)	0.00 (0.00-2.74)
SHC5	C	FDSB	1/430 (43)	0.23 (0.00-1.44)	0/35 (7)	0.00 (0.00-11.76)	0/45 (9)	0.00 (0.00-9.38)	1/510 (59)	0.20 (0.00-1.22)
SW	C	FDSB	4/380 (38)	1.05 (0.31-2.78)	1/105 (21)	0.95 (0.00-5.72)	0/120 (24)	0.00 (0.00-3.73)	5/605 (83)	0.83 (0.27-1.98)
DRME	C	FDBS	0/40 (4)	0.00 (0.00-10.44)	0/15 (3)	0.00 (0.00-23.86)	0/15 (3)	0.00 (0.00-23.86)	0/70 (10)	0.00 (0.00-6.23)
DRMW	C	FDSB & FDSAS	0/20 (2)	0.00 (0.00-18.98)	0/0 (0)	0.00 N/A	0/0 (0)	0.00 N/A	0/20 (2)	0.00 (0.00-18.98)
SF2	MMC	FDBS & SLOCL	0/420 (42)	0.00 (0.00-1.09)	0/75 (15)	0.00 (0.00-5.84)	0/70 (14)	0.00 (0.00-6.23)	0/565 (71)	0.00 (0.00-0.81)

The area with the highest detected infection risk was site SCC with a density of infected nymphs (DIN) per of 0.17/100m^2. SW was the next highest at 0.12/100m^2, followed by SHC5 at 0.10/100m^2 and finally WTH at 0.04/100m^2

The DON in the subsites with TBEV/LIV rRT-PCR positive pools were fairly comparable to the other Thetford Forest subsites surveyed in 2019, apart from a small number of subsites which did not have any positive pools but had higher DON such as SHC(3), SHC5(3) and SHC5(4) (Figure 3:13).

Transects were most commonly conducted in Corsican pine dominant sub-compartments (38.9%), followed by Scots pine (30.4%), beech (21.5%), Douglas fir (4.4%), oak (1.8%), hazel (1.2%), mixed broadleaves (0.5%) and European larch (0.4%).

Due to subsites covering multiple sub-compartments of different tree species, it is not possible to know from which tree species habitat a positive tick was collected. However, when analysed by numbers of ticks collected by primary tree species habitats, and then split by the positivity of each subsite (either designated 'No positive pools' or at least one 'Positive pool'), an interesting variation by tree species was found (*Figure* 3:14).

Despite not confirming which tree species habitats positive ticks were collected from, this does confirm which tree species habitats had no TBEV/LIV rRT-PCR positive ticks collected from them. None of the ticks collected in beech dominant woodlands were from a TBEV/LIV rRT-PCR positive subsite, whereas 27.2% of those collected in Scots pine habitats were from a TBEV/LIV rRT-PCR positive subsite and 26.8% for Corsican pine. Interestingly, 46.2% of the limited number of ticks collected in Douglas fir habitats were from a TBEV/LIV rRT-PCR positive subsite; this was SW(1). Within this subsite 5 TBEV/LIV rRT-PCR positive pools were collected, 49.5% of ticks were collected in Scots pine, 39.5% in Douglas fir and 11.0% in Corsican pine.
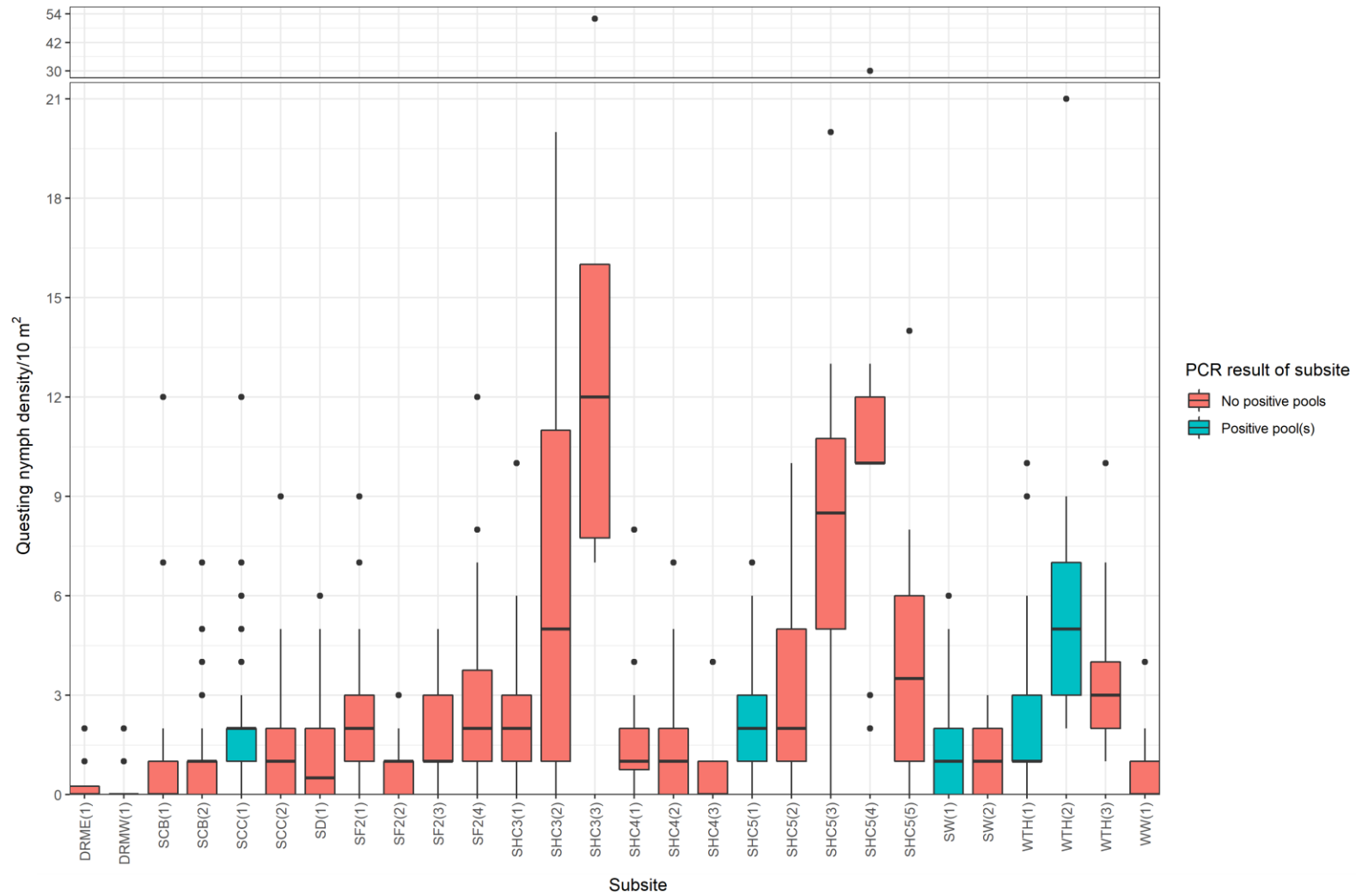
Figure 3:13: *Thetford Forest questing nymph density by subsite and PCR positive pools*
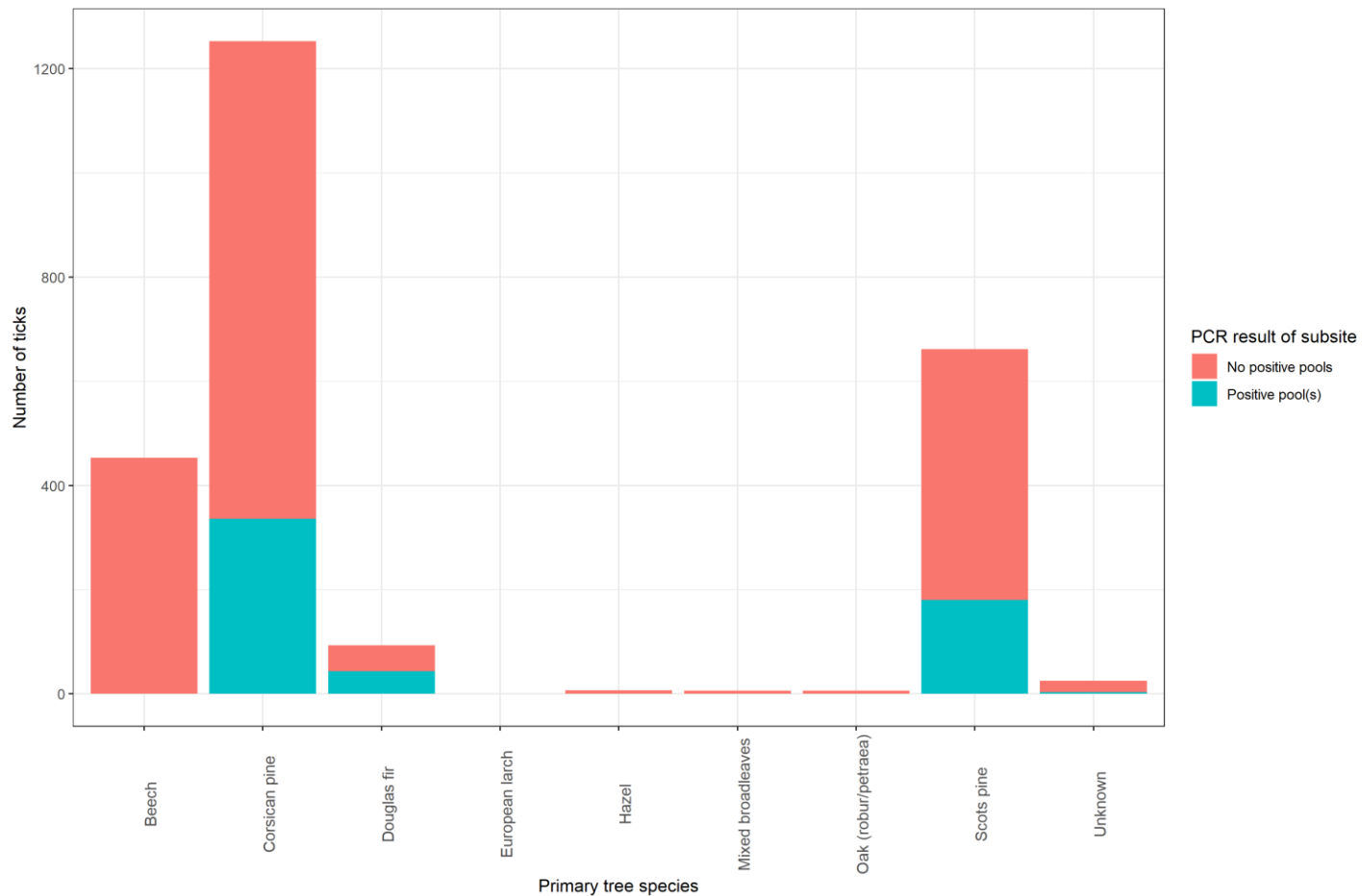
Figure 3:14: *Number of ticks collected by primary tree species of habitats surveyed in Thetford Forest (2019). The number of ticks surveyed per tree species is split by the positivity of each subsite, either designated as 'No positive pools' or at least one 'Positive pool(s)'. Subsites included multiple sub-compartments of different tree species, therefore it cannot be confirmed which primary tree species the positive pool(s) originated from. However, this does confirm which primary tree species habitats had no TBEV positive ticks collected from them.*

3.3.4: 2019 New Forest area tick testing

A total of 2,290 ticks were collected and tested from four sites in the New Forest and its surrounding areas; 74.7% (1,710) were nymphs, 11.6% (265) adult males and 13.8% (315) adult females. Of the ticks tested from four sites in the New Forest and surrounding areas, one TBEV/LIV rRT-PCR positive pool was detected from site SMH which is on the Hampshire/Dorset border, just outside of the New Forest National Park boundary. The positive pool was an adult female pool out of 16 pools tested; the detected adult female MIR was 1.25% and whole site MIR was 0.17% (*Table 3:15)*. SA, SGP and SMH were all within a 5km radius. This site spanned across both coniferous broadleaved areas of woodland. Of the three sites where no TBEV/LIV rRT-PCR positive pools were detected, two were coniferous and one was broadleaved.

Although the New Forest does have some sandy type soils, the soil type at these sites were different to those in the Thetford Forest area. The TBEV/LIV rRT-PCR positive site was on freely draining very acid sandy and loamy soils (FDVASL). The two conifer sites where no positive pools were detected were on naturally wet very acid sandy and loamy soils (WVASL), and the broadleaved site where also no positive pools were detected was across a border of FDVASL, WVASL and slightly acid loamy and clayey soils with impeded drainage (SALCID).

3.3.5: Genomic sequencing

Of the six TBEV/LIV rRT-PCR positive pools collected in 2018 from Thetford Forest, three sequences were obtained. In addition, six sequences were obtained from the 13 TBEV/LIV rRT-PCR positive tick pools collected in Thetford Forest in 2019 (*Table 3:14*). All of these nine sequences from Thetford Forest pools were found to have close identity to TBEV-UK-Thetford.

Sequences with 100% coverage were obtained from 2018 samples at the SHC2 site in two samples (*Table 3:11*); one of which was an adult male pool and the other a nymph pool. In addition, a sequence was obtained from an adult female tick pool collected from site DRMH, from which a 99.56% sequence coverage was obtained (

*Table 3:*16).

Of the six sequences obtained from the 2019 Thetford sites, all but one was over 90% coverage (*Table 4:13)*. Sequences were obtained from all three positive pools from the WTH site and the one pool from the SHC5 site.

The positive pool on the Hampshire/Dorset border was sequenced metagenomically and a consensus sequence was obtained (TBEV-UK Hampshire, GenBank accession number MN661145). *Figure 3:15* shows the phylogenetic relationship and indicates that TBEV-UK-Hampshire is most closely related to TBEV-NL (LC171402.1), which is within the European TBEV subtype that was detected in ticks from the Netherlands in 2017 [3]. When compared with the TBEV-NL strain, TBEV-UK Hampshire contains 49 single nt polymorphisms leading to 12 amino acid substitutions within the coding sequence.

Table 3:15: Questing ticks tested and prevalence (MIR) of TBEV from sites in Hampshire and bordering areas in 2019. Main habitat type: B= Broadleaved, C= Conifer; MMB= Mixed mainly broadleaved; MMC= Mixed mainly conifer. Soil type: FDVASL= Freely draining very acid sandy and loamy soils, WVASL=Naturally wet very acid sandy and loamy soils, SALCID=Slightly acid loamy and clayey soils with impeded drainage, FDSAL=Freely draining slightly acid loamy soils. Sites positive for TBEV RNA are underlined.

Code	Habitat		Nymphs positive/tested (pools)	Nymph MIR % (95% CI)	AM positive/tested (pools)	AM MIR % (95% CI)	AF positive/tested (pools)	AF MIR % (95% CI)	Total positive/tested (pools)	Total MIR % (95% CI)
SA	C	WVASL	0/340(34)	0.00 (0.00-5.84)	0/75(15)	0.00 (0.00-5.84)	0/85(17)	0.00 (0.00-5.19)	0/500	0.00 (0.00-0.92)
SGP	C	WVASL	0/870(87)	0.00 (0.00-4.44)	0/100(20)	0.00 (0.00-4.44)	0/110(22)	0.00 (0.00-4.05)	0/1080	0.00 (0.00-0.43)
SMH	*C & B*	*FDVASL*	*0/430(43)*	*0.00 (0.00-1.44)*	*0/65(13)*	*0.00 (0.00-6.68)*	*1/80(16)*	*1.25 (0.00-5.49)*	*1/575*	*0.17 (0.00-1.08)*
WG	B	WVASL & SALCID & FDSAL	0/70(7)	0.00 (0.00-6.23)	0/25(5)	0.00 (0.00-15.76)	0/40(8)	0.00 (0.00-10.44)	0/135	0.00 (0.00-3.33)

Table 3:16: Questing tick pools in which sequence coverage obtained and methodology used

Area	Year Collected	Site	Subsite	Positive Pool	Sequencing Method	Sequence Coverage
Thetford Forest	2018	SHC2	-	AM	MinION	100%
Thetford Forest	2018	SHC2	-	N	MinION	100%
Thetford Forest	2018	DRMH	-	AF	MinION	99.56%
Thetford Forest	2019	WTH	WTH(1)	N	MinION	92.13%
Thetford Forest	2019	WTH	WTH(2)	AM	MinION	99.96%
Thetford Forest	2019	WTH	WTH(1)	AM	Illumina	99.46%
Thetford Forest	2019	SHC5	SHC5(1)	N	MinION	96.32%
Thetford Forest	2019	SW	SW(1)	AM	Illumina	99.26%
Thetford Forest	2019	SW	SW(1)	N	Illumina	81.93%
Hampshire/Dorset Border	2019	SMH	-	AF	MinION	97.38%

Figure 3:15: *The two sequences outlined in red highlights the sequenced pool collected on the Hampshire/Dorset border in 2019 in addition to the TBEV-UK Thetford sequence. The tree was constructed with a maximum-likelihood analysis of full-length genomes and is rooted with the tick-borne Powassan virus. European TBEV strains are in blue, Siberian TBEV in green, and Far eastern in red*
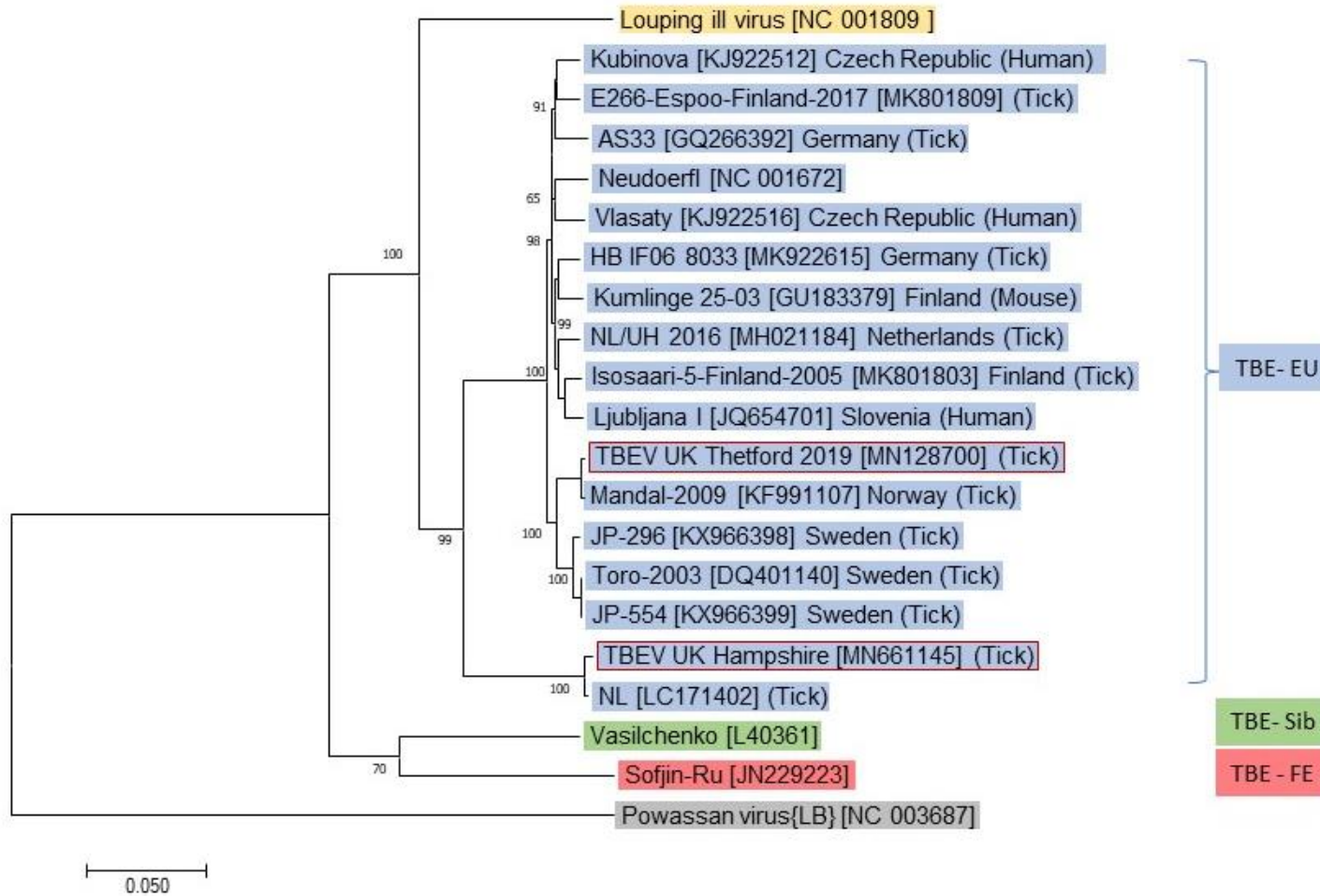
3.4: Discussion

It has always been thought that TBEV was absent from the UK due to unfavourable conditions, with climate models indicating conditions incompatible with establishment of the virus following any introduction (Randolph and Rogers, 2000). However, the combined findings of Chapter 2 and 3 indicate that TBEV foci may indeed be established and maintained in the UK. This is indicated by the high TBEV-serocomplex seroprevalence in deer in Thetford Forest, an area where LIV is thought to be absent. This is in addition to the detection of TBEV in ticks collected from deer in spatially distinct locations in the same area.

The hypothesis that Thetford Forest was a TBEV focus would be strengthened by the detection of TBEV in questing ticks in multiple locations across the forest. Therefore, a questing tick study was conducted to investigate this. Sites where seropositive deer have been culled were selected to maximise the chances of detecting locations of foci. The seropositive deer culled at a number of these sites were species with small home ranges (muntjac and roe), which increased confidence that there was a nearby focus present. The detection of TBEV in questing ticks over two consecutive years in 2018 and 2019, in multiple locations across the forest, indicates there is an established enzootic cycle maintained by wildlife hosts in Thetford Forest. In addition, the distance of over 14km between the furthest sites (SCC and DRMH) where TBEV positive questing ticks have been collected, indicates that foci may be distributed across this forest area. Both SW and SCC were also localities where TBEV positive ticks were removed from deer, supporting the value of also testing fed ticks to assist in detecting possible foci, particularly considering the much higher prevalence detected.

It was not possible to obtain viral genomes from all TBEV PCR positive questing tick pools due to low viral RNA levels. Those that were detected were the same TBEV-UK-Thetford strain as was sequenced from the tick removed from a Thetford Forest deer in Chapter 2. Further work is required to optimise primer-amplification sequencing approaches to enable sequencing of more of the TBEV PCR positive samples detected through this study.

In addition to the aim of detecting TBEV in questing ticks in Thetford Forest, a second aim was to identify which virus might be causing the 14% TBEV-serocomplex seropositivity in deer in the New Forest and surrounding areas through questing tick collection and testing. A TBEV-positive pool was detected on the Hampshire/Dorset border which is just outside of the New Forest. This finding indicates that TBEV in ticks is not limited to the Thetford Forest area, but also that it is present on the Hampshire/Dorset border. Despite the more limited number of sites in the New Forest and surrounding areas, serological evidence is strong that a TBEV-serocomplex virus is also circulating there. This detection indicates the 14% seropositivity detected across this area may be a result of TBEV rather than LIV, particularly in the absence of any reports of LIV in the livestock there. The viral genome detected on the Hampshire/Dorset border is most similar to TBEV-NL-Salland, which was identified in the Netherlands in 2017 (Jahfari et al., 2017).

A study within the focus in which the Norwegian Mandal 2009 strain was detected, which is the strain that TBEV-UK-Thetford shares closest homology to, found a nymphal MIR of 0.68% (Andreassen et al., 2012). Interestingly, the nymphal MIR of the combined positive Thetford sites for 2018 and 2019 where TBEV-UK-Thetford was detected, was lower but comparable at 0.51%. The overall MIR detected at TBEV positive sites in Thetford Forest ranged from 0.20% and 4.44%, with the latter being the only site with a detected total MIR over 1%. The only positive site in the New Forest area (Hampshire/Dorset border) was lower at 0.17%. As is commonly found in other studies, the detected MIR was higher in adults than in nymphs for most sites. The SW site in Thetford Forest which had a 0.1% lower MIR in adult males than nymphs, however due to the lower sample size of adult ticks the confidence intervals were much wider. The MIR in TBEV-positive sites, was in line with those in European TBEV foci, where the MIR is often found to be below 1% (Imhoff et al., 2015; Pettersson et al., 2014; Rieille et al., 2014; Stefanoff et al., 2013; Andreassen et al., 2012). Due to this being an initial exploratory study aiming to identify microfoci, the precise and defined boundaries are not yet known. Therefore, sampling may have occurred outside of these boundaries, which may be

an explanation for some of the lower MIR compared to some European TBEV foci (Stefanoff et al., 2013).

In order to establish the boundaries of the foci, follow up and more detailed surveys should be conducted at each of the TBEV positive sites. A similar approach could be used to that conducted in Thetford Forest in 2019 as part of this study, to pinpoint the areas from which positive ticks were obtained. However, a limitation of this current study was that at points when additional surveyors assisted, they only collected ticks and did not record transect locations and tick densities on the Collector App. In future surveyors should record all transects through this method to increase the quality and consistency of data.

An inherent problem in questing tick surveys for TBEV is the very low prevalence, leading to the requirement for testing a very large number of ticks, which is expensive; ticks are difficult to collect in sufficiently high numbers (Stefanoff et al., 2013). For this current study, despite 10,290 ticks being tested overall, in some sites it was difficult to collect sufficient ticks due to a very low tick density. Therefore, it cannot be ruled out that TBEV was not circulating in the sites where it was undetected. In most of the sites with low tick numbers, TBEV was not detected - such as DRME, DRMW, WW and SD in Thetford Forest and EW and WG in the New Forest area.

DRMH is a prime example of the importance of seeking to collect as many ticks as is possible. This site had very low tick numbers, and therefore a small sample size (n=90); however, interestingly it had the highest detected prevalence of TBEV throughout the study. This finding in itself warrants further investigation into why the tick numbers might be so low but TBEV prevalence high. A hypothesis might be that in combination with suitable climatic conditions, deer numbers were relatively low, coincident with low, but sufficient, numbers of small mammals, resulting in a high number of the limited population of ticks co-feeding.

A limitation of the study in the New Forest and surrounding areas was that only a small number of sites were surveyed. Therefore, to fully investigate the extent of

TBEV infection in this area, additional sites where TBEV seropositive deer were found should be surveyed for ticks.

Despite TBEV not being detected within the boundary of the New Forest National Park during this study, the site of detection on the Hampshire/Dorset border is adjacent to it. This site was on the western edge of the Hampshire cluster of the TBEV-serocomplex positive deer detected in Chapter 2, with the majority of the cluster being encompassed within the New Forest National Park. Therefore, guided by these factors, the ecology of this area should be considered for characteristics associated with TBEV foci.

There are some striking similarities, but also differences, between Thetford Forest and the New Forest as TBEV emergence zones. These factors should be investigated in combination with European TBEV foci characteristics, to seek to understand why these appear suitable areas and where else TBEV might establish in the UK.

Both Breckland and the New Forest are medium to large internationally important landscapes, made up of a mosaic of habitats including lowland heathland, grassland and forest, with a larger area of open farmland in the former (Natural England, 2015b, 2015a). Both have large areas of mosaics of forest, with 28,998ha in Breckland and 22,329ha in the New Forest NCA (Natural England, 2015a, 2015b; Breckland District Council, 2007). Breckland and the New Forest area share various factors of landscape composition that have been associated with TBEV presence, such as high landscape diversity, large forest patch size, high mean shape index and open ecotonal areas within forest patches (Vanwambeke et al., 2010). The New Forest has the greater degree of natural regeneration of the two forests and also has over one and half times more broadleaved and mixed forest than Breckland (16,291 ha (Natural England, 2015b) vs 9,610 ha (Natural England, 2015a; Breckland District Council, 2007), respectively), which both are also favourable factors for TBEV presence (Zeimes et al., 2014; Vanwambeke et al., 2010).

Rather unexpectedly, all six of the TBEV-positive sites in Thetford Forest are coniferous forest areas. This was surprising, as this is not a particularly suitable habitat type for yellow-necked mice, a key reservoir of TBEV (Marsh et al., 2001). A

review study compiling and mapping TBEV detections in Europe found over twice the number of locations with TBEV infected ticks in broadleaved forest when compared to coniferous, with detections considerably higher in both forest types than in agricultural areas (Walter et al., 2020). Interestingly, mathematical modelling has also found varying effects of conifer and broadleaved forests; a different study showed the impact of coniferous cover had a greater effect than that of mixed or broadleaved woodland on the probability of TBEV infection in Germany (Kiffner et al., 2010). A consideration when assessing habitat suitability for TBEV foci based on TBE cases is that bias towards human land use of the forest type may be a confounding factor.

Due to the subsites crossing different forest sub-compartments with different dominant tree species, it is not possible to confirm through this data the specific habitat that TBEV is circulating in within the UK. Future surveys should subdivide the tick testing and analysis sites into sub-compartments by tree species. However, given that 18% of ticks were collected from beech sub-compartments, which are good small mammal habitats due to the beech nut provision, it is interesting that no TBEV-positive ticks were collected from these areas. TBEV-positive ticks were detected in subsites where only Corsican pine, Scots Pine and Douglas fir sub-compartments were surveyed. There is a limited amount of research focusing on tree species and their impact on TBEV foci, which are likely to have an influence due to their varying resource of provision of food for reservoir hosts, influencing ground cover and resulting microclimates. Further research in this area may assist in identifying specific locations of foci when sentinels indicate presence in a locality, in addition to forecasting localities that may be suitable for emergence.

Due to lack of published research, the densities of the TBEV reservoir hosts (yellow-necked mice and bank voles) in the New Forest is not clear. NBN Atlas data indicates both species are present in the area and research conducted in the 1980s found that all ungrazed woodlands supported substantial bank vole populations, and to a lesser extent, yellow-necked mice (NBN Atlas, 2021; Putman, 1996). In addition, Hampshire Wildlife Trust data from 2006 to 2016 found four times more records of bank voles than yellow-necked mice in the county indicating that bank

voles may be particularly important for potential maintenance of TBEV in the New Forest (Spall, 2017). The absence or low numbers of both species from grazed New Forest woodlands is likely due to limited ground cover in these areas (Putman, 1996). The greater adaptability of bank voles to coniferous forest, which is more widespread in enclosure areas, may be an explanation for the differences in population. Therefore, TBEV foci may be more likely to occur within New Forest Inclosures than the Open Forest due to livestock grazing, and further research should be conducted to investigate this potential.

Similar to the New Forest, limited research on small mammals in Thetford Forest means that the current densities of TBEV reservoir hosts is also unknown. A 1990's study found that bank voles fares particularly well in the numerous mature coniferous woodlands (27/ha) of Thetford Forest, producing the same densities as deciduous woodlands (Ratcliffe and Claridge, 1996). NBN atlas data indicates that there has been a report of yellow-necked mice in the Thetford Forest area; however, this is towards the north- eastern most range of yellow-necked mice in the UK. Given that there is just 198ha of ancient semi natural woodland in Breckland, the densities of yellow-necked mice may not be high if present.

These factors, combined with the dominance of coniferous woodland in Thetford Forest, and the fact that all TBEV positive sites were in coniferous woodland, indicates that bank voles may be the primary host maintaining TBEV in enzootic cycles in these foci. Small mammal surveys in both the New Forest area including the Hampshire/Dorset border, and Thetford Forest, would assist in establishing the degree of their roles in enzootic cycles for the maintenance of TBEV in the UK.

In addition to both Thetford Forest and the New Forest being large mosaics of forests and other habitats such as heathland and calcareous grassland, there are close similarities in their geology (Newbould and Tubbs, 1970). Both are in geological basins of calcareous chalk, infilled with sand, gravel, clay and silt deposits (Natural England, 2015b, 2015a). Despite having different soil types, both have large areas of sandy soils, particularly across the Thetford Forest area of Breckland. The largely nutrient-poor soils, climate and land-use history have been defining factors in the resulting unique ecology of these areas. Due to being of insufficient

quality for many agricultural uses, this has resulted in large scale forestation of both areas, albeit at quite different periods of time in history (Newton, 2011; Hemami et al., 2005; Rothera, 1998). Soils have an important influence on temperature, soil moisture, ground level humidity and vegetation cover - all important microclimatic factors (Burmeier et al., 2010). The light sandy free-draining soils may be a defining factor in the creation of a suitable microclimate in these areas. All TBEV sites in Thetford Forest -bar one - were solely on the freely draining sandy Breckland soils. The one site that differed was on the boundary between this soil type and freely draining slightly acid sandy soils. The light free-draining sandy soil type has an important influence on the temperature fluctuations and dry environment that creates the 'semi-continental climate' for which Breckland is known.

Interestingly, the positive site detected on the Hampshire/Dorset border was also on a free-draining sandy soil type (Freely draining very acid sandy and loamy soil). The influence of soil type on TBEV foci presence or absence may assist in understanding areas that are suitable to support foci and further work should be conducted to investigate this.

Value could be added to the data produced in this chapter and also future studies by conducting a more detailed statistical analysis to identify possible relationships between the ecological variables and TBEV presence. Generalised linear mixed models (GLMMs) would be an appropriate approach to take for analysis of data of this kind. GLMMs are particularly suited to analysing non-normal data when random effects are present, characteristics common of ecological datasets (Bolker et al., 2009). For example, Cagnacci et al., (2012) used a GLMM with binormal error distribution when investigating the possible relationship between TBEV occurrence and deer density and co-feeding ticks on rodents. Using this model, they were able to identify a strong positive correlation between the occurrence of TBEV with co-feeding ticks and a negative correlation with deer density[2]. The utility of GLMM for studies on tick-borne pathogen prevalence has also been illustrated by Millins et al., (2016) using a GLMM to investigate the effect of growing degree-day and mean nymph abundance on the density of *B.burgdorferi (s.l.)*. They found a positive effect of growing degree days and a negative effect of nymph abundance on *B.*

burgdorferi (s.l.) prevalence. A similar approach could be used to investigate any possible relationship of the effect of habitat and soil type and also microclimatic variables such as humidity and temperature on occurrence of TBEV focus locations.

Both Thetford Forest and the New Forest have greater seasonal temperature extremes than both the England and southern England average. They each experience more frost days than average; Thetford experiences the more extreme conditions, with colder winters, a more rapid increase in spring temperatures and marginally hotter summers than the New Forest. The comparison of climate with other relevant TBEV foci indicates that the TBEV foci in Sallandse Heuvelrug, Netherlands, has the more similar climate to the UK areas. The above average number of frosts in both the New Forest and Thetford Forest, in addition to above average summer temperatures, may be contributory factors in the development of TBEV foci. With the New Forest, followed by Thetford Forest, now being the most westerly points of TBEV presence, comparisons between TBEV foci in Europe modelled alongside analysis of more detailed climatological data and microclimate data measured at each site may contribute to understanding the factors that have enabled the westerly spread of TBEV. Comparisons with climatic predictions from Randolph *et al*. 2000 forecasting that up until 2050 TBEV would not emerge in the UK could support strengthening of future models seeking to better understand spread of the virus (Randolph and Rogers, 2000).

To conclude, TBEV has been detected in two areas in the England in questing ticks. This combined with the serological evidence in deer gives a strong case that TBEV foci are present in both the Thetford Forest area and the Hampshire/Dorset border just outside of the New Forest. Further evidence on geological, ecological and climatic conditions should be assessed to understand the enabling factors for the emergence of these foci.

Chapter 4: Discussion

Building on findings that TBEV is increasing in range in Europe (Andersen et al., 2019; Petersen et al., 2019; Jahfari et al., 2017), and the lack of UK research investigating possible presence, this project was initiated to investigate the following hypothesis: "There is ecological and epidemiological evidence of TBEV in the UK causing foci that present a risk to public health".

Through an extensive deer serosurveillance study, strong serological evidence of a TBEV-complex virus circulating in Thetford Forest (47.7%) and Hampshire (14.3%) was detected. Of note, there are no recent or historic reports of LIV in livestock in either of these areas. In addition, through this deer study TBEV was detected in 2.6% of ticks removed from deer in the Thetford Forest area. Targeted questing tick studies in these areas of seroprevalence provided further evidence of circulation of TBEV in Thetford Forest. Widespread tick collections and testing of 7,085 questing ticks in Thetford Forest resulted in TBEV being detected in 6 sites out of 24 surveyed over 2018 and 2019. In addition, collection and testing of 3,205 ticks from 7 sites over 2018 and 2019 in the New Forest and bordering areas resulted in the detection of TBEV in one site on the Hampshire/Dorset border.

Prior to this study, it was thought that TBEV was absent from the UK, and that climatic modelling highlighted no likely threat of importation in the coming decades (Randolph and Rogers, 2000). Thus, the detection of TBEV is highly significant, both in terms of public health and because it challenges previous assumptions regarding spread and distribution of TBEV, as well as highlighting the need for empirical field research on emerging vector-borne diseases.

These findings highlight the value of periodic ecological surveillance in regions where TBEV is thought to be absent, rather than solely relying on clinical cases indicating presence of emergence - as these may go undiagnosed. As TBEV was thought to be absent from the UK until these research findings presented were obtained, it is unlikely that TBE would have been considered as a differential diagnosis in an encephalitic patient - even with history of tick bite - if there was no

recent travel to an endemic country (Kennedy et al., 2017; Public Health England, 2013). Therefore, as up to 60% of encephalitis cases in the UK have unknown causes (Kennedy et al., 2017), it is feasible that TBEV could have previously caused undetected autochthonous human disease.

4.1:   Public health implications in the UK

The one-health approach adopted in this PhD has important implications for public health and will guide new research. The outcomes have already informed both a national government and local public health response; in addition, the implications of these findings for public health are also guiding future research activities. Therefore, it is important that a one-health approach is embraced, and the human implications of the detection on TBEV are considered alongside emerging ecological data.

Following the first detection of TBEV in UK ticks during May 2019, there have been two probable cases of autochthonous TBE; both were within Hampshire. One was an infant from Germany who had holidayed with their family and received a tick bite whilst in the New Forest during July 2019 (Kreusch et al., 2019). The second was diagnosed in a Hampshire resident in July 2020 (Human Animal Infections and Risk Surveillance (HAIRS) group, 2021; Public Health England, 2020). These findings corroborate the hypothesis that was developed through this PhD research that TBEV is present across the wider New Forest and bordering areas as well as in the detected focus on the Hampshire/Dorset border.

As a result of the outcomes of this research, Public Health England (PHE) shared information about the findings locally in Thetford Forest and the New Forest in addition to nationally (Joint Committee on Vaccination and Immunisation, 2019). Both local Health Protection teams and healthcare workers in clinical settings were informed of the potential risk of TBEV infection. It is important to identify any new cases, both for clinical purposes but also to inform public health policy makers of the risk of TBEV to the UK population. Therefore, in order to screen for potential undiagnosed cases the PHE Rare and Imported Pathogens Laboratory (RIPL) initiated a testing programme for TBEV exposure in cases of unidentified acute

encephalitis in these areas (Joint Committee on Vaccination and Immunisation, 2019).

LIV is endemic in areas of the UK and cross reacts on TBEV serological tests; thus, it is not possible for suspected UK TBE patients with the absence of TBE viral nucleic acid or viable virus for isolation to have the diagnosis of TBE confirmed. This is due to the European Case definition criteria for serological testing requiring TBEV-specific antibodies, and the current assays in use are not able to discriminate between LIV and TBEV specifically. Until this point, it is likely most infections of TBEV in the UK will result in a 'probable' case diagnosis in line with the European case definition (Commission Implementing Decision 2018/945, 2018). It is imperative to develop discriminatory assays for use in countries where both TBEV and LIV are present.

A high proportion of individuals do not experience clinical disease following infection with TBEV (70-98%) (Bogovic and Strle, 2015), creating the possibility that infections may have occurred in individuals but were either asymptomatic or did not develop severe disease. To assist in informing health professionals and policy makers of the potential exposure to humans of TBEV - and therefore assessment of whether vaccination recommendations should be updated, serosurveillance studies in local populations should be established (Joint Committee on Vaccination and Immunisation, 2019). Exposure is likely to be relatively low, therefore a serosurveillance study targeting individuals in high risk occupational groups, and those who undertake recreational activities which give a higher risk of tick bites in the identified foci, would assist in this assessment.

It is interesting that despite the higher detected seroprevalence in deer from Thetford Forest compared to the New Forest area, the two probable TBE cases were reported from the New Forest/Hampshire area and that to date there have been no reports from the Thetford Forest area (Human Animal Infections and Risk Surveillance (HAIRS) group, 2021; Kreusch et al., 2019). This variation in reported cases may be associated with various factors, such as the New Forest receiving more visitors compared to Thetford Forest, or perhaps differences in clinical awareness of TBEV presence. Alternatively, there may be differences in

pathogenicity and virulence of the different TBEV strains present in these areas; differences in pathogenicity have been found even within TBEV subtypes. For example, in Germany, a Bavarian focus with relatively low detected TBEV prevalence in questing ticks (overall MIR of 0.23%), reported a cluster of nine clinical cases over a five-year period in a tight geographic area. All patients had unusually severe disease and three resulted in fatality (Kupča et al., 2010).

The most common route of TBEV infection is from the bite of an infected tick; therefore the highest risk will be to those individuals who spend considerable time in suitable tick habitats in the TBEV foci (Bogovic and Strle, 2015). Both Thetford Forest and the New Forest National Park attract a high volume of visitors to the natural area each year. Thetford Forest receives 1.5 million visitors a year (Suffolk Local Access Forum, 2011; Ratcliffe and Claridge, 1996) and the New Forest 13.5 million visitor days a year (Natural England, 2015b). Direct comparative data is not available; however, this highlights the high footfall in each area. With the sheer volume of visitors and locals accessing the area for recreation, this does increase the potential for human exposure. Cases in visitors as well as locals may occur; such as was the circumstance of the first autochthonous probable TBE case (Kreusch et al., 2019). The fact that many visitors are from outside the area necessitates the urgency of national awareness for infectious disease clinicians , of the presence of TBEV in the UK, which is why results from this work have been published in the key medical journal *The Lancet* (Holding et al., 2020).

Among those at highest risk of infection, are individuals who spend the majority of their working days within the forest environment. Due to the high volume of commercial forestry in both areas, many staff are employed in associated occupations working within the forest environments; therefore, cases may occur as a result of occupational as well as recreational exposure (Natural England, 2015b, 2015a).

 Alimentary infection through ingestion of raw milk or cheese is an additional route of exposure, but this only accounts for ~1% of TBEV infections (Bogovic and Strle, 2015). There are ~4 registered producers of raw milk in the identified UK TBEV foci areas, and the UK Human Animal Infections and Risk Surveillance (HAIRS) group

have assessed the risk of raw milk consumption from these areas to be 'low to very low' (Human Animal Infections and Risk Surveillance (HAIRS) group, 2021).

Despite the detection of TBEV in the UK being of public health significance, the disease risk as a result of tick bite is likely to be much greater for *Borrelia*, with an estimated 1.95 Lyme disease cases per 100,000 population in the UK in 2016 (Tulloch et al., 2019). This compares to case numbers in TBE-endemic countries with a notification rate of 0.6 cases per 100,000 population during 2018 in EU/EAA countries (ECDC, 2019). Therefore clear, informed and proportional messaging is important to avoid unnecessary alarm for the public to be informed of the risk. Messaging should aim to detail the research findings and the symptoms, focusing upon general tick awareness and highlighting that the risk of acquiring Lyme disease from a tick-bite remains much higher than TBE - even in the newly identified TBEV foci. In addition to government press releases (Public Health England, 2020; Gov.uk, 2019), information sharing to the public has already been implemented with relevant sources of information, both nationally and locally, being updated. This includes websites such as the UK National Health Service (NHS) tick-borne encephalitis page (National Health Service, 2019) and New Forest National Park "Staying Safe" page (New Forest National Park Authority, n.d.).

4.2: Future of TBEV in the UK

The distinct TBEV-Eu genomes found in Thetford Forest and on the Hampshire/Dorset border provide convincing evidence that there have been at least two separate importation events to the UK that have enabled TBEV to establish in geographically distinct locations in England.

The findings of this study support the hypothesis that TBEV infected ticks imported on migratory birds may have been the route that has enabled TBEV to arrive in the UK. Ticks can be transported large distances by birds during their migration. During the autumn there is a large influx of birds to the UK from Northern and Central Europe, including blackbirds (*Turdus merula*) and redwings (*Turdus iliacus*) (Sparagano et al., 2015), which are known to transport ticks over wide distances, including from TBEV endemic areas (Klaus et al., 2016; Geller et al., 2013; Hasle, 2013; Waldenström et al., 2007).

Thrushes (*Turdus spp.*) take various routes to different parts of the UK depending on the country from which they are flying. Those flying from Northern European countries, such as Norway, arrive on the east coast of England and Scotland. Those travelling from central and north-western European countries, such as the Netherlands, often take a more lateral route, sometimes arriving on the south coast of England (Flegg, 2004). There is close similarity between TBEV-UK-Thetford and the Norwegian TBEV-Mandal strain. This information, combined with the location of the TBEV-UK-Thetford focus in east England, on the migration flyway from Nordic countries, suggests it is likely that Norway was the origin of the TBEV-UK-Thetford.

The TBEV-UK Hampshire genome detection in southern England is of close homology to the TBEV-Netherlands-Sallandse Heuvelrug genome, which are both quite distinct from other TBEV-Eu strains. With these two localities being linked by autumn bird migration routes, it is probable TBEV-UK-Hampshire originated in the Netherlands.

Investigating the species of birds arriving in the UK with the highest burden of *I. ricinus*, the tick life stages present and the countries they are migrating from would assist investigation and modelling of any further emergence of TBEV in the UK. In addition, mapping the key migration routes and destinations of highly infested species, and whether suitable conditions for enzootic maintenance are present, would support predictions.

As previously highlighted, TBEV and LIV antibodies cross-react on available serological tests. LIV endemicity across large areas of the UK is an inherent challenge in seeking to identify any other emerging TBEV foci. If co-circulation of both viruses were to occur in any areas, this may be difficult to identify (Jeffries et al., 2014). The development of specific neutralisation tests would allow differentiation between infections in areas in which LIV is endemic. Direct testing of ticks is an alternative methodology which could distinguish between TBEV and LIV; however, due to the low prevalence and focal nature of both viruses it can be a very expensive and ineffective method. Even in known endemic areas, being unable to detect the presence of TBEV in questing tick studies is relatively common (Stefanoff et al., 2013). This lack of suitability of questing tick testing as the first

screen is highlighted by a large-scale study assessing data from testing of 65,000 ticks in Poland and Germany. The study concluded that direct testing for TBEV in ticks with no prior evidence of presence is not a sensitive indicator for TBE risk in humans, and that systematic testing of wild or domestic animals was a more effective approach (Stefanoff et al., 2013).

In addition, despite the challenge of cross-reactivity on serological tests, this study has demonstrated that it is possible to detect new foci through sentinel serological surveillance of deer; with the two separate hitherto unknown localities of UK TBEV foci being detected using this method. Therefore, evidence suggests that this methodology remains the most cost-effective and efficient tool for identification of areas that warrant more detailed molecular investigation of ticks.

Despite the deer serosurveillance study being extensive in sample number and distribution, there are localities in the UK where few or no deer samples were collected due to the scale of the geographic area covered. Therefore, to increase coverage across the UK, a follow-up sentinel deer serosurveillance survey should be conducted. This should seek to detect any possible TBEV foci unidentified through the 2018 study or any that may have emerged since, despite the current limitations of cross-reactivity with LIV.

The results in this project provide evidence that targeting sites of sample collection from seropositive deer for questing tick surveys and testing is effective; providing evidence of widespread TBEV circulation in Thetford Forest and resulting in the detection of the new TBEV focus on the Hampshire/Dorset border.

Analysis of data indicated that TBEV may be predominantly circulating in enzootic cycles in coniferous habitats in Thetford Forest, possibly in Corsican pine, Scots pine and Douglas fir. Now that foci have been identified, these can be studied with more specific questing tick surveys and comparing reservoir host populations, splitting sites by forest sub-compartments. This would assist in establishing any relationship between these tree species and the maintenance of the foci.

Establishing the size of the foci, their borders and microfoci characteristics within them will increase understanding of the areas where the public may be at increased

risk of exposure and which habitats within them pose greatest risk. This could be achieved by carrying out a gridded longitudinal study similar to that conducted by Zöldi et al. (2015). In selected known TBEV foci, a multi-year longitudinal small mammal trapping and questing tick study could be carried out via a grid format. Surveys would be monthly during the main tick season from April to October and sensors measuring temperature and humidity placed at each site. Serological and molecular testing of small mammal samples to indicate present and past TBEV infection, and collection and testing of ticks to monitor for co-feeding would provide a breadth of data. The mechanism of the enzootic cycle could be established including the main reservoir(s) responsible for the maintenance. The study would also provide data on seasonal variation in TBEV prevalence in ticks and risk periods - identified from when seropositivity in small mammals increases.

The reported drop in TBEV prevalence over winter, with two temporal studies reporting no detection of TBEV-infected questing ticks before May (Zöldi et al., 2015; Perez-Eid et al., 1992), is interesting. This, combined with the identification of a temperature-sensitive riboswitch in TBEV, with the breakpoint temperature varying by strain - warrants further consideration (Elväng et al., 2011). Elväng *et al.* 2011 hypothesised that the climate in which the virus was isolated may be responsible, due to observed correlations between breakpoint temperature and the climate of where the TBEV strain originated (Elväng et al., 2011). Based on this data, it is possible that the variation in temperatures at which virus replication commences may impact when TBEV foci become 'active' each year, depending on strain present. For example, in areas like Thetford Forest with a 'semi-continental climate', whereas TBEV-Mandal of closest homology to TBEV-UK-Thetford originated in the much cooler climate of Norway.

The viruses that TBEV-UK-Thetford and TBEV-UK- Hampshire share closest homology with originate from quite different climates. This provides an interesting opportunity for characterisation of the breakpoint temperature of each virus, then used to predict the temperature at which the foci would become active. The characterisation of the breakpoint data could be analysed alongside the field studies, monitoring the period at which point TBEV can be first detected each year,

to test the impact. This may provide useful insights on the impacts of different strains on seasonal foci characteristics and advance a new area for public health intervention.

4.3: Potential impact on understanding TBEV distribution and spread

The Hampshire/Dorset border and Thetford Forest are now the most westerly TBEV foci, having replaced Utrechtse Heuvelrug and Sallandse Heuvelrug, first identified in the Netherlands in 2016, following serological evidence in deer serum collected during 2010 (Dekker et al., 2019; Jahfari et al., 2017). For many years prior to TBEV emergence in the Netherlands, the Alsace region in France was the most westerly established foci, which was recognised as being weak but stable (Randolph, 2008; Randolph et al., 1999; Perez-Eid et al., 1992). The foci in the Netherlands compared to Alsace, France are only a small distance further west in longitude (longitude approximately 5.3 vs 6.1, respectively); however, both Thetford Forest and the Hampshire/Dorset border are quite substantial distances apart. This seemingly rapid spread westward raises the question as to whether TBEV might also be present in other areas in between, as yet unidentified.

It is quite remarkable that TBEV is so focal to very specific areas, despite its wide geographic range over considerable variations in climate. Considering the identified importance of climate and microclimate in foci development and maintenance, the spread of TBEV foci from the relatively cold climates in Sweden and Norway (Jaenson et al., 2018; Csángó et al., 2004) to the warm Mediterranean climate in Italy (Rosà et al., 2019; Rizzoli et al., 2007) is of interest. TBEV has even recently been identified in a pool of *I. ricinus* in Tunisia and also a separate study identifying antibodies against TBEV in sheep in a similar area (Fares et al., 2021; Khamassi Khbou et al., 2020).

It seems likely that depending on the climate of an area, different biotic and abiotic factors may have greater importance in the development of specific conditions that TBEV foci require. Stratified modelling of TBEV foci based on areas with similar macroclimates may enable identification of further factors that assist in explaining the very focal nature of the virus. It will be important to establish what the key factors are that have led to the suitable conditions for the development of the two

UK foci 200 miles (320 km) apart - and seemingly none in-between - despite a relatively similar climate covering the south of England, and presence of other large woodland areas. This may be supported by establishing models stratified by grouping European TBEV foci with similar climates.

As previously mentioned, the climate of the TBEV focus Sallandse Heuvelrug in the Netherlands is similar to Thetford Forest and the New Forest. Interestingly, there are also a number of factors that are of similarity across these areas. Breckland/Thetford Forest and the New Forest are lowland heathlands with vast forested areas of ecological importance with considerable large areas of sandy soils (Natural England, 2015a, 2015b). These soils have great influence on topoclimate in Thetford Forest, as do the ecological characteristics and land use history of both of these areas. Similarly, Sallandse Heuvelrug National Park is a lowland area of vast forest and dry heathland on a sandy glacial ridge with sprawling coniferous and mixed deciduous woodlands (Ministry of Agriculture, 2005). Thetford Forest was previously mostly heathland of low agricultural value with soil made up of drifting sands caused by overgrazing from sheep and rabbits, with large commercial rabbit warrens and very little forest (Rothera, 1998). Similarly, in the early 1900s Sallandse Heuvelrug was almost solely a heather heathland with a history of sheep and goat grazing, with the sandy soils drifting, resulting from overgrazing (de Boer and Bressers, 2013; Latham et al., 1999). Both Thetford Forest and Sallandse Heuvelrug have been largely afforested in the last century. Development of conifer plantations in Thetford Forest commenced in 1924 which transformed the ecology of the area (Natural England, 2015a) and large scale afforestation with conifer commenced in Sallandse Heuvelrug around the 1930s (de Boer and Bressers, 2013; Latham et al., 1999). Similarities, such as the sandy soil type may be of particular relevance to areas with a similar climate to the UK and the Netherlands; this may be important in allowing sufficient spring and autumn temperature gradients to generate the conditions required for co-feeding larvae and nymphs to support TBEV transmission. The abiotic and biotic factors required to produce similar conditions, in a different climate, are likely to be quite different. For example, factors important in generating the conditions required for TBEV transmission in areas with

cold climates, such as Sweden, may generate unsuitable conditions for transmission in the warmer climates, such as Italy and Tunisia. Therefore, stratified analysis by climate may assist in understanding factors that foci have in common and key factors for development. This could narrow down general localities in which TBEV foci may develop, and well recognised factors such as altitude, aspect and slope may be utilised to identify possible foci within these localities.

4.4:   Conclusion

The main hypothesis that there is ecological and epidemiological evidence of TBEV in the UK causing foci that present a risk to public health, has been confirmed. TBEV has been detected in the UK for the first time, in two geographically distinct foci that are 200 miles apart. This is likely to have been a result of two separate importation events evidenced by separate localities of the foci and the two different genomic sequences of the TBEV-Eu viruses detected. The importation of infected ticks by migratory birds is a convincing argument for their potential importation route. This is supported by the overlap between migratory bird routes and the locations of the UK foci and the European locations of TBEV viruses, with close sequence identities fitting the autumn migration origins and destinations.

The high seropositivity in deer in both Thetford Forest and the Hampshire area of the New Forest, in addition to detection in questing ticks in numerous locations across both 2018 and 2019, indicate that TBEV is being maintained in enzootic cycles in these areas. Although no confirmed TBE cases have been diagnosed, there have been two probable cases of TBE in the UK in the Hampshire area. Despite not being possible to confirm these, primary diagnosis being based on serology, the lack of reports of LIV in this area indicates that both are likely to be from TBEV infection. In addition, both TBEV-UK-Thetford and TBEV-UK-Hampshire have close homology to pathogenic TBEV strains. Therefore, these detections should be considered a potential public health risk to those living in, and visiting, Thetford Forest, the New Forest and surrounding areas. In addition, there should be awareness of the possibility that additional foci may be undetected, therefore TBE cases could yet occur in separate areas to those identified. The risk to the general population is currently assessed to be very low, and only low for those in high risk groups (such

as those living, working or visiting affected areas, as determined by duration of time spent outside) (Human Animal Infections and Risk Surveillance (HAIRS) group, 2021).

If presented with a patient with meningoencephalitis, UK clinicians should consult the European case definition of TBE (Commission Implementing Decision 2018/945, 2018). TBE should be included in a differential diagnosis in patients with relevant symptoms, particularly if they have recent history of a tick bite, even if they don't have recent travel history. The confirmatory diagnosis of TBE in UK patients is complicated by the circulation of LIV which is cross-reactive in standard serological tests.

Serosurveillance studies are required to continue providing evidence for the risk of TBEV to the UK population. In addition, further work is required to monitor and characterise current TBEV foci. The wide distribution of the natural vector in the UK supports the need to carry out mathematical modelling and further surveillance to identify the potential for geographic spread with new emerging foci.

References

Adam, K. M. G., Beasley, S. J. and Blewett, D. A. (1977) 'The occurrence of antibody to Babesia and to the virus of louping-ill in deer in Scotland', *Research in Veterinary Science*, W.B. Saunders, vol. 23, no. 2, pp. 133–138 [Online]. DOI: 10.1016/S0034-5288(18)33144-8 (Accessed 5 December 2018).

Amori, G., Castigliani, V., Locasciulli, O. and Luiselli, L. (2015) 'Long-term density fluctuations and microhabitat use of sympatric *Apodemus flavicollis* and *Myodes glareolus* in central Italy', *Community Ecology*, vol. 16, no. 2, pp. 196–205 [Online]. DOI: 10.1556/168.2015.16.2.7.

Andersen, N. S., Larsen, S. L., Olesen, C. R., Stiasny, K., Kolmos, H. J., Jensen, P. M. and Skarphédinsson, S. (2019) 'Continued expansion of tick-borne pathogens: Tick-borne encephalitis virus complex and *Anaplasma phagocytophilum,* in Denmark', *Ticks and Tick-borne Diseases*, Elsevier, vol. 10, no. 1, pp. 115–123 [Online]. DOI: 10.1016/j.ttbdis.2018.09.007.

Andreassen, A., Jore, S., Cuber, P., Dudman, S., Tengs, T., Isaksen, K., Olav Hygen, H., Viljugrein, H., Ånestad, G., Ottesen, P., Vainio, K., Hygen, H. O., Viljugrein, H., Ånestad, G., Ottesen, P., Vainio, K., Anestad, G., Ottesen, P. and Vainio, K. (2012) 'Prevalence of tick borne encephalitis virus in tick nymphs in relation to climatic factors on the southern coast of Norway.', *Parasites & Vectors*, BioMed Central, vol. 1, no. 1, p. 1 [Online]. DOI: 10.1186/1756-3305-5-177 (Accessed 30 April 2019).

Animal & Plant Health Agency; Scotland's Rural College (2020a) *GB Sheep Disease Surveillance Dashboard* [Online]. Available at https://public.tableau.com/profile/siu.apha#!/vizhome/SheepDashboard_/Overview (Accessed 25 November 2020).

Animal & Plant Health Agency; Scotland's Rural College (2020b) *GB Cattle Disease Surveillance Dashboard* [Online]. Available at https://public.tableau.com/profile/siu.apha#!/vizhome/CattleDashboard/Overview (Accessed 25 November 2020).

Anon (n.d.) 'Louping Ill Virus (LIV) in the Far East: A Perspective', [Online]. Available

at file:///C:/Users/maya.holding/Downloads/Louping_Ill_Virus_(LIV)_in_the (1).pdf (Accessed 10 October 2018).

Asghar, N., Lindblom, P., Melik, W., Lindqvist, R. and Haglund, M. (2014) 'Tick-Borne Encephalitis Virus Sequenced Directly from Questing and Blood-Feeding Ticks Reveals Quasispecies Variance', *PLoS ONE*, vol. 9, no. 7, p. 103264 [Online]. DOI: 10.1371/journal.pone.0103264 (Accessed 27 April 2019).

Aspöck, H. (1970) 'Das synökologische Beziehungsgefüge bei Arboviren und seine Beeinflussung durch den Menschen', *Zbl Bakt I Orig*, vol. 213, pp. 434–454.

Balling, A., Plessow, U., Beer, M. and Pfeffer, M. (2014) 'Prevalence of antibodies against tick-borne encephalitis virus in wild game from Saxony, Germany', *Ticks and Tick-borne Diseases*, vol. 5, no. 6, pp. 805–809 [Online]. DOI: 10.1016/j.ttbdis.2014.06.007.

Balseiro, A., Royo, L. J., Pérez, C., Copano, M. F., Rubio, T., Höfle, U., Prieto, M. and García Marín, J. F. (2013) 'Louping Ill in Goats in Northern Spain: More Than a Forgotten Disease', *Journal of Comparative Pathology*, W.B. Saunders, vol. 148, no. 1, p. 51 [Online]. DOI: 10.1016/j.jcpa.2012.11.025 (Accessed 27 November 2017).

Baneth, G. (2014) 'Tick-borne infections of animals and humans: a common ground.', *International journal for parasitology*, vol. 44, no. 9, pp. 591–6 [Online]. DOI: 10.1016/j.ijpara.2014.03.011 (Accessed 3 February 2017).

Barrett, D., Collins, D. M., McGrath, G. and O Muireagain, C. (2012) 'Seroprevalence of Louping Ill virus (LIV) antibodies in sheep submitted for post mortem examination in the North West of Ireland in 2011.', *Irish veterinary journal*, BioMed Central, vol. 65, no. 1, p. 20 [Online]. DOI: 10.1186/2046-0481-65-20 (Accessed 20 May 2019).

Battersby, J. (2005) 'UK mammals: Species status and population trends', *JNCC/Tracking Mammals Partnership*, p. 116 [Online]. Available at file:///Users/alicelaguardia/Documents/Papers/2005/Battersby/JNCCTracking Mammals Partnership.(3 November 2006 2005 Battersby.pdf%5Cnpapers://7175c388-3326-430d-b62e-9bcfb485bac2/Paper/p36.

Belova, O. A., Burenkova, L. A. and Karganova, G. G. (2012) 'Different tick-borne encephalitis virus (TBEV) prevalences in unfed versus partially engorged Ixodid ticks - Evidence of virus replication and changes in tick behavior', *Ticks and Tick-borne Diseases*, Elsevier GmbH., vol. 3, no. 4, pp. 240–246 [Online]. DOI: 10.1016/j.ttbdis.2012.05.005 (Accessed 27 June 2017).

de Boer, C. and Bressers, H. (2013) 'Complex and Dynamic Implementation Processes: the renaturalization of the Dutch Regge River', *University of Twente and the Dutch Water Governance Centre*, vol. 53, no. 9, pp. 1–244.

Bogovic, P. and Strle, F. (2015) 'Tick-borne encephalitis: A review of epidemiology, clinical characteristics, and management.', *World Journal of Clinical Cases*, Baishideng Publishing Group Inc, vol. 3, no. 5, pp. 430–41 [Online]. DOI: 10.12998/wjcc.v3.i5.430 (Accessed 26 July 2016).

Bolker, B. M., Brooks, M. E., Clark, C. J., Geange, S. W., Poulsen, J. R., Stevens, M. H. H. and White, J. S. S. (2009) 'Generalized linear mixed models: a practical guide for ecology and evolution', *Trends in Ecology and Evolution*, vol. 24, no. 3, pp. 127–135 [Online]. DOI: 10.1016/j.tree.2008.10.008.

Borkowski, J. and Pudełko, M. (2007) 'Forest habitat use and home-range size in radio-collared fallow deer', *Annales Zoologici Fennici*, vol. 44, no. 2, pp. 107–114.

Bournez, L., Umhang, G., Moinet, M., Richomme, C., Demerson, J.-M., Caillot, C., Devillers, E., Boucher, J.-M., Hansmann, Y., Boué, F. and Moutailler, S. (2020) 'Tick-Borne Encephalitis Virus: Seasonal and Annual Variation of Epidemiological Parameters Related to Nymph-to-Larva Transmission and Exposure of Small Mammals', *Pathogens*, MDPI AG, vol. 9, no. 7, p. 518 [Online]. DOI: 10.3390/pathogens9070518 (Accessed 31 August 2020).

Boyard, C., Vourc'h, G. and Barnouin, J. (2008) 'The relationships between *Ixodes ricinus* and small mammal species at the woodland-pasture interface', *Experimental and Applied Acarology*, vol. 44, no. 1, pp. 61–76 [Online]. DOI: 10.1007/s10493-008-9132-3.

Brackney, D. E. and Armstrong, P. M. (2016) 'Transmission and evolution of tick-

borne viruses.', *Current opinion in virology*, Elsevier B.V., vol. 21, no. Table 1, pp. 67–74 [Online]. DOI: 10.1016/j.coviro.2016.08.005 (Accessed 3 February 2017).

Bramer, I., Anderson, B. J., Bennie, J., Bladon, A. J., De Frenne, P., Hemming, D., Hill, R. A., Kearney, M. R., Körner, C., Korstjens, A. H., Lenoir, J., Maclean, I. M. D., Marsh, C. D., Morecroft, M. D., Ohlemüller, R., Slater, H. D., Suggitt, A. J., Zellweger, F. and Gillingham, P. K. (2018) 'Advances in Monitoring and Modelling Climate at Ecologically Relevant Scales', *Advances in Ecological Research*, vol. 58, pp. 101–161 [Online]. DOI: 10.1016/bs.aecr.2017.12.005.

Breckland District Council (2007) *Breckland District Landscape Character Assessment*, no. May [Online]. Available at https://www.breckland.gov.uk/media/2069/Landscape-Character-Assessment/pdf/Landscape_Character_Assessment_-_May_2007_Final2.pdf.

British Deer Society (2020) *Deer Distribution Survey* [Online]. Available at https://www.bds.org.uk/index.php/research/deer-distribution-survey (Accessed 11 February 2020).

Brugger, K., Walter, M., Chitimia-Dobler, L., Dobler, G. and Rubel, F. (2018) 'Forecasting next season's *Ixodes ricinus* nymphal density: the example of southern Germany 2018', *Experimental and Applied Acarology*, Springer International Publishing, vol. 75, no. 3, pp. 281–288 [Online]. DOI: 10.1007/s10493-018-0267-6.

Burmeier, S., Donath, T. W., Otte, A. and Eckstein, R. L. (2010) 'Rapid burial has differential effects on germination and emergence of small- and large-seeded herbaceous plant species', *Seed Science Research*, vol. 20, no. 3, pp. 189–200 [Online]. DOI: 10.1017/S0960258510000127.

Burri, C., Bastic, V., Maeder, G., Patalas, E. and Gern, L. (2011) 'Microclimate and the zoonotic cycle of tick-borne encephalitis virus in Switzerland', *Journal of Medical Entomology*, vol. 48, no. 3, pp. 615–627 [Online]. DOI: 10.1603/ME10180 (Accessed 27 April 2019).

Bush, E. R., Buesching, C. D., Slade, E. M. and Macdonald, D. W. (2012) 'Woodland recovery after suppression of deer: Cascade effects for small mammals, wood mice

(*Apodemus sylvaticus*) and Bank Voles (*Myodes glareolus*)', Amaral, L. A. N. (ed), *PLoS ONE*, Public Library of Science, vol. 7, no. 2, p. e31404 [Online]. DOI: 10.1371/journal.pone.0031404 (Accessed 28 September 2020).

Buxton, D. and Reid, H. W. W. (2017) '120 years of Louping-ill Research: an Historical Perspective from the Archive of the Journal of Comparative Pathology', *Journal of Comparative Pathology*, W.B. Saunders, vol. 157, no. 4, pp. 270–275 [Online]. DOI: 10.1016/J.JCPA.2017.09.002 (Accessed 27 November 2017).

Cadman, W. A. (1962) 'The New Forest', *Forestry*, vol. 35, no. 1, pp. 27–34 [Online]. DOI: https://doi.org/10.1093/forestry/35.1.27.

Cagnacci, F., Bolzoni, L., Rosà, R., Carpi, G., Hauffe, H. C., Valent, M., Tagliapietra, V., Kazimirova, M., Koci, J., Stanko, M., Lukan, M., Henttonen, H. and Rizzoli, A. (2012) 'Effects of deer density on tick infestation of rodents and the hazard of tick-borne encephalitis. I: Empirical assessment', *International Journal for Parasitology*, Pergamon, vol. 42, no. 4, pp. 365–372 [Online]. DOI: 10.1016/j.ijpara.2012.02.012 (Accessed 3 May 2018).

Cantarello, E., Green, R. and Westerhoff, D. (2010) 'The condition of The New Forest habitats: an overview', pp. 124–131 [Online]. Available at http://eprints.bournemouth.ac.uk/13926/1/Newton_Biodiversity_in_the_New_Forest_2010_Chapter13.pdf.

Carne, P. (2000) *Deer of Britain and Ireland: Their Origins and Distribution*, Shrewsbury, Swan Hill Press.

Carpenter, D., Hammond, P. M., Sherlock, E., Lidgett, A., Leigh, K. and Eggleton, P. (2012) 'Biodiversity of soil macrofauna in the New Forest: A benchmark study across a national park landscape', *Biodiversity and Conservation*, vol. 21, no. 13, pp. 3385–3410 [Online]. DOI: 10.1007/s10531-012-0369-0.

Casula, P., Luiselli, L. and Amori, G. (2019) 'Which population density affects home ranges of co-occurring rodents?', *Basic and Applied Ecology*, vol. 34, pp. 46–54 [Online]. DOI: 10.1016/j.baae.2018.11.002 (Accessed 24 September 2020).

Čepelka, L., Šipoš, J., Suchomel, J. and Heroldová, M. (2020) 'Can we detect

response differences among dominant rodent species to climate and acorn crop in a Central European forest environment?', *European Journal of Forest Research*, Springer Berlin Heidelberg, vol. 139, no. 4, pp. 539–548 [Online]. DOI: 10.1007/s10342-020-01267-7.

Chapman, N. G., Claydon, K., Claydon, M., Forde, P. G. and Harris, S. (1993) 'Sympatric populations of muntjac (Muntiacus reevesi) and roe deer (Capreolus capreolus): a comparative analysis of their ranging behaviour, social organization and activity', *Journal of Zoology*, vol. 229, no. 4, pp. 623–640 [Online]. DOI: 10.1111/j.1469-7998.1993.tb02660.x.

Chitimia-Dobler, L., Mackenstedt, U., Kahl, O. and Petney, T. N. (2019) 'Transmission / Natural Cycle', *The TBE Book*, pp. 62–86 [Online]. DOI: 10.33442/978-981-14-1_3.

Chitimia-Dobler, L., Schaper, S., Rieß, R., Bitterwolf, K., Frangoulidis, D., Bestehorn, M., Springer, A., Oehme, R., Drehmann, M., Lindau, A., Mackenstedt, U., Strube, C. and Dobler, G. (2019) 'Imported Hyalomma ticks in Germany in 2018', *Parasites and Vectors*, BioMed Central, vol. 12, no. 1, pp. 1–9 [Online]. DOI: 10.1186/s13071-019-3380-4.

Cisak, E., Wójcik-Fatla, A., Sroka, J., Zajac, V., Bilska-Zajc, E., Chmurzyńska, E., Dutkiewicz, J., Zając, V., Bilska-Zając, E., Chmurzyńska, E. and Dutkiewicz, J. (2012) 'Prevalence of tick-borne encephalitis virus antibodies in domestic and game animals from Eastern Poland', *Bulletin of the Veterinary Institute in Pulawy*, vol. 56, no. 3, pp. 275–278 [Online]. DOI: 10.2478/v10213-012-0049-6 (Accessed 13 September 2017).

Cisak, E., Wójcik-Fatla, A., Zajac, V., Sroka, J., Buczek, A. and Dutkiewicz, J. (2010) 'Prevalence of tick-borne encephalitis virus (TBEV) in samples of raw milk taken randomly from cows, goats and sheep in Eastern Poland', *Annals of Agricultural and Environmental Medicine*, vol. 17, no. 2, pp. 283–286 [Online]. DOI: 17283 [pii] (Accessed 21 September 2017).

Ciuti, S., Davini, S., Luccarini, S. and Apollonio, M. (2003) 'Variation in home range

size of female fallow deer inhabiting a sub-Mediterranean habitat', *Revue d'Ecologie (La Terre et la Vie)*, vol. 58, no. 4, pp. 381–395.

Clark, J. J., Gilray, J., Orton, R. J., Baird, M., Wilkie, G., Filipe, A. da S., Johnson, N., McInnes, C. J., Kohl, A. and Biek, R. (2020) 'Population genomics of louping ill virus provide new insights into the evolution of tick-borne flaviviruses', *PLoS Neglected Tropical Diseases*, vol. 14, no. 9, pp. 1–25 [Online]. DOI: 10.1371/journal.pntd.0008133.

Commission Implementing Decision 2018/945 (2018) 'On the communicable diseases and related special health issues to be covered by epidemiological surveillance as well as relevant case definitions', *Official Journal of the European Union*, no. 2119, pp. 1–74 [Online]. Available at https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32018D0945&from=EN#page=45 (Accessed 20 May 2019).

Craine, N. G., Nuttall, P. A., Marriott, A. C. and Randolph, S. E. (1997) 'Role of grey squirrels and pheasants in the transmission of *Borrelia burgdorferi sensu lato*, the Lyme disease spirochaete, in the U.K.', *Folia Parasitologica*, vol. 44, no. 2, pp. 155–160.

Cranfield University (2013) *Soilscapes* [Online]. Available at https://data.gov.uk/dataset/26d61739-e05b-420d-8fd0-d11edffa8b27/soilscapes (Accessed 27 March 2021).

Cranfield University (2021) *Soilscapes in England and Wales* [Online]. Available at http://www.landis.org.uk/services/soilsguide/soilscapes_list.cfm (Accessed 27 March 2021).

Csángó, P. A., Blakstad, E., Kirtz, G. C., Pedersen, J. E. and Czettel, B. (2004) 'Tick-borne encephalitis in southern Norway.', *Emerging infectious diseases*, Centers for Disease Control and Prevention, vol. 10, no. 3, pp. 533–4 [Online]. DOI: 10.3201/eid1003.020734 (Accessed 4 May 2019).

Cull, B., Vaux, A. G. C., Ottowell, L. J., Gillingham, E. L. and Medlock, J. M. (2017) 'Tick infestation of small mammals in an English woodland', *Journal of Vector*

Ecology, vol. 42, no. 1, pp. 74–83 [Online]. DOI: 10.1111/jvec.12241 (Accessed 27 November 2018).

Dai, X., Shang, G., Lu, S., Yang, J. and Xu, J. (2018) 'A new subtype of eastern tick-borne encephalitis virus discovered in Qinghai-Tibet Plateau, China article', *Emerging Microbes and Infections*, Springer US, vol. 7, no. 1, p. 74 [Online]. DOI: 10.1038/s41426-018-0081-6 (Accessed 30 September 2019).

Danielová, V., Holubová, J., Pejčoch, M. and Daniel, M. (2002) 'Potential significance of transovarial transmission in the circulation of tick-borne encephalitis virus', *Folia Parasitologica*, vol. 49, no. 4, pp. 323–325 [Online]. DOI: 10.14411/fp.2002.060.

Dantas-Torres, F., Chomel, B. B. and Otranto, D. (2012) 'Ticks and tick-borne diseases: a One Health perspective', *Trends in Parasitology*, Elsevier Current Trends, vol. 28, no. 10, pp. 437–446 [Online]. DOI: 10.1016/j.pt.2012.07.003 (Accessed 21 June 2016).

Davidson, M. M., Williams, H. and Macleod, J. A. J. (1991) 'Louping ill in man: A forgotten disease', *Journal of Infection*, vol. 23, no. 3, pp. 241–249 [Online]. DOI: 10.1016/0163-4453(91)92756-U.

Davini, S., Ciuti, S., Luccarini, S. and Apollonio, M. (2004) 'Home range patterns of male fallow deer Dama dama in a sub-Mediterranean habitat', *Acta Theriologica*, vol. 49, no. 3, pp. 393–404.

Davison, G. and Neubauer, C. (1948) 'Meningo-encephalitis in man due to the louping-ill virus', *The Lancet*, Elsevier, vol. 252, no. 6525, pp. 453–457 [Online]. DOI: 10.1016/S0140-6736(48)91034-4 (Accessed 27 November 2017).

Dekker, M., Laverman, G. D., de Vries, A., Reimerink, J. and Geeraedts, F. (2019) 'Emergence of tick-borne encephalitis (TBE) in the Netherlands', *Ticks and Tick-borne Diseases*, Elsevier, vol. 10, no. 1, pp. 176–179 [Online]. DOI: 10.1016/j.ttbdis.2018.10.008.

Diller, R., Schrader, C., Wohanka, N., Abel, U., Suss, J., Klaus, C., Diller, R., Schrader, C., Wohanka, N., Abel, U., Sus, J., Klaus, C., Diller, R., Schrader, C., Wohanka, N. and Abel, U. (2006) 'TBE incidence versus virus prevalence and increased prevalence of

the TBE virus in Ixodes ricinus removed from humans', *International Journal of Medical Microbiology*, Urban & Fischer, vol. 296, no. 1, pp. 63–68 [Online]. DOI: 10.1016/j.ijmm.2005.12.005 (Accessed 27 June 2017).

Dobler, G. (2010) *Zoonotic tick-borne flaviviruses*, *Veterinary Microbiology*, Elsevier, vol. 140, no. 3–4 [Online]. DOI: 10.1016/j.vetmic.2009.08.024 (Accessed 14 September 2017).

Dobler, G., Erber, Wilhelm, Bröker, Michael and Schmitt, H. J. (2019) 'TBE by country', in Dobler, G., Erber, W., Bröker, M., and Schmitt, H.-J. (eds), *The TBE Book*, Singapore, Global Health Press Pte Ltd, pp. 234–426.

Dobler, G., Hufert, F., Pfeffer, M. and Essbauer, S. (2011) 'Tick-borne encephalitis: From microfocus to human disease', in Mehlhorn, H. (ed), *Progress in Parasitology*, London, Springer, pp. 232–339 [Online]. DOI: 10.1007/978-3-642-21396-0.

Dolman, P., Panter, C. and Mossman, H. (2010) *Securing Biodiversity in Breckland*, *First Report of the Breckland Biodiversity Audit. ...*, School of Environmental Sciences, University of East Anglia, Norwich [Online]. Available at https://scholar.google.pt/scholar?hl=pt-PT&q=Securing+Biodiversity+in+Breckland.+Guidance+for+Conservation+and+Research.+First+Report+of+the+Breckland+Biodiversity+Audit.+University+of+East+Anglia,+Norwich.&btnG=&lr=#0 (Accessed 27 September 2020).

Dunn, A. M. (1960) 'Louping-ill : The red deer (*Cervus Elaphus*) as an alternative host of the virus in Scotland', *British Veterinary Journal*, Elsevier Masson SAS, vol. 116, no. 8, pp. 284–287 [Online]. DOI: 10.1016/s0007-1935(17)44105-4 (Accessed 21 April 2018).

Duscher, G. G., Hodžić, A., Hufnagl, P., Wille-Piazzai, W., Schötta, A. M., Markowicz, M. A., Estrada-Peña, A., Stanek, G. and Allerberger, F. (2018) 'Adult *Hyalomma marginatum* tick positive for *Rickettsia aeschlimannii* in Austria, October 2018', *Eurosurveillance*, European Centre for Disease Control and Prevention (ECDC), vol. 23, no. 48, pp. 1–3 [Online]. DOI: 10.2807/1560-7917.ES.2018.23.48.1800595.

Duscher, G. G., Leschnik, M., Fuehrer, H. P. and Joachim, A. (2015) 'Wildlife

reservoirs for vector-borne canine, feline and zoonotic infections in Austria', *International Journal for Parasitology: Parasites and Wildlife*, vol. 4, no. 1, pp. 88–96 [Online]. DOI: 10.1016/j.ijppaw.2014.12.001.

Duscher, G. G., Wetscher, M., Baumgartner, R. and Walder, G. (2015) 'Roe deer sera used for TBE surveillance in Austria', *Ticks and Tick-borne Diseases*, Elsevier GmbH., vol. 6, no. 4, pp. 489–493 [Online]. DOI: 10.1016/j.ttbdis.2015.03.018 (Accessed 11 September 2017).

ECDC (2019) 'Tick-borne encephalitis Annual Epidemiological Report for 2018 Key facts', *European Center for Disease Prevention and Control*, no. December [Online]. Available at https://www.ecdc.europa.eu/sites/default/files/documents/TBE-annual-epidemiological-report-2018.pdf.

Elväng, A., Melik, W., Bertrand, Y., Lönn, M. and Johansson, M. (2011) 'Sequencing of a tick-borne encephalitis virus from *Ixodes ricinus* reveals a thermosensitive RNA switch significant for virus propagation in ectothermic arthropods', *Vector-Borne and Zoonotic Diseases*, vol. 11, no. 6, pp. 649–658 [Online]. DOI: 10.1089/vbz.2010.0105.

Esser, H. J., Mögling, R., Cleton, N. B., Van Der Jeugd, H., Sprong, H., Stroo, A., Koopmans, M. P. G., De Boer, W. F. and Reusken, C. B. E. M. (2019) 'Risk factors associated with sustained circulation of six zoonotic arboviruses: A systematic review for selection of surveillance sites in non-endemic areas', *Parasites and Vectors*, vol. 12, no. 1 [Online]. DOI: 10.1186/s13071-019-3515-7 (Accessed 5 October 2020).

Estrada-Peña, A., Bouattour, A., Camicas, J. L. and Walker, A. R. (2004) 'Ticks of domestic animals in the Mediterranean Region: a guide to identification of species', no. January 2014, p. 131 [Online]. Available at http://hufind.huji.ac.il/Record/HUJ001396958.

Estrada-Peña, A. and de la Fuente, J. (2014) 'The ecology of ticks and epidemiology of tick-borne viral diseases', *Antiviral Research*, Elsevier, vol. 108, pp. 104–128 [Online]. DOI: 10.1016/j.antiviral.2014.05.016 (Accessed 9 April 2016).

Estrada-Peña, A., Mihalca, A. D. and Petney, T. (eds.) (2017) *Ticks of Europe and North Africa*, Cham, Switzerland, Springer International Publishing [Online]. DOI: 10.1007/978-3-319-63760-0.

European Centre for Disease Prevention and Control (2012) *Epidemiological situation of tick-borne encephalitis in the European Union and European Free Trade Association countries*, *ECDC Technical Report* [Online]. DOI: 10.2900/62311 (Accessed 27 April 2019).

Fares, W., Dachraoui, K., Cherni, S., Barhoumi, W., Slimane, T. Ben, Younsi, H. and Zhioua, E. (2021) 'Tick-borne encephalitis virus in *Ixodes ricinus* (Acari: Ixodidae) ticks, Tunisia', *Ticks and Tick-borne Diseases*, Elsevier GmbH, vol. 12, no. 1, pp. 2019–2022 [Online]. DOI: 10.1016/j.ttbdis.2020.101606.

Flegg, J. (2004) *Time to Fly, Exploring Bird Migration*, Thetford, The British Trust for Ornithology.

Flick, R. (2007) *Crimean Congo Hemorrhagic Fever: A Global Perspective*, *Book*, Springer Netherlands [Online]. DOI: https://www.springer.com/gp/book/9781402061059.

Flowerdew, J. R. and Ellwood, S. A. (2001) 'Impacts of woodland deer on small mammal ecology', *Forestry*, Corbet and Harris, vol. 74, no. 3, pp. 277–287 [Online]. DOI: 10.1093/forestry/74.3.277.

Földvári, G., Široký, P., Szekeres, S., Majoros, G. and Sprong, H. (2016) '*Dermacentor reticulatus*: a vector on the rise.', *Parasites & vectors*, BioMed Central, vol. 9, no. 1, p. 314 [Online]. DOI: 10.1186/s13071-016-1599-x (Accessed 16 August 2017).

Forestry Commission (2015) *NFI provisional estimates for woodland in the New Forest National Park*, no. May [Online]. Available at https://www.forestresearch.gov.uk/documents/3122/New_Forest_NP_Report_new_pooled_FINAL.pdf.

Forestry Commission (2018) *New Forest Inclosures Open Habitat Proposals 2018 Environmental Statement*, [Online]. Available at https://www.forestryengland.uk/sites/default/files/documents/Environmental

Statement 30-07-18 %282%29.pdf.

Forestry Commission England (2015) *New Forest Inclosures Forest Plan Draft South England Forest District*, [Online]. Available at https://consult.forestryengland.uk/forest-districts/new-forest-inclosures-forest-design-plan-2016/supporting_documents/Introduction.pdf.

Frimmel, S., Leister, M., Löbermann, M., Feldhusen, F., Seelmann, M., Süss, J. and Reisinger, E. C. (2016) 'Seroprevalence of tick-borne-encephalitis virus in wild game in Mecklenburg-Western Pomerania (north-eastern Germany)', *Ticks and Tick-borne Diseases*, Urban & Fischer, vol. 7, no. 6, pp. 1151–1154 [Online]. DOI: 10.1016/J.TTBDIS.2016.08.004 (Accessed 10 June 2019).

Gaff, H. D. and Gross, L. J. (2007) 'Modeling Tick-Borne Disease: A Metapopulation Model', *Bulletin of Mathematical Biology*, Springer-Verlag, vol. 69, no. 1, pp. 265–288 [Online]. DOI: 10.1007/s11538-006-9125-5 (Accessed 21 April 2017).

Garcia, S., Billecocq, A., Crance, J.-M., Munderloh, U., Garin, D. and Bouloy, M. (2005) 'Nairovirus RNA Sequences Expressed by a Semliki Forest Virus Replicon Induce RNA Interference in Tick Cells', *Journal of Virology*, vol. 79, no. 14, pp. 8942–8947 [Online]. DOI: 10.1128/jvi.79.14.8942-8947.2005.

Gascuel, O. (1997) 'BIONJ: An improved version of the NJ algorithm based on a simple model of sequence data', *Molecular Biology and Evolution*, vol. 14, no. 7, pp. 685–695 [Online]. DOI: 10.1093/oxfordjournals.molbev.a025808.

Gaumann, R., Muhlemann, K., Strasser, M. and Beuret, C. M. (2010) 'High-throughput procedure for tick surveys of tick-borne encephalitis virus and its application in a national surveillance study in Switzerland', *Applied and Environmental Microbiology*, vol. 76, no. 13, pp. 4241–4249 [Online]. DOI: 10.1128/AEM.00391-10.

Geller, J., Nazarova, L., Katargina, O., Leivits, A., Järvekülg, L. and Golovljova, I. (2013) 'Tick-borne pathogens in ticks feeding on migratory passerines in western part of Estonia', *Vector-Borne and Zoonotic Diseases*, vol. 13, no. 7, pp. 443–448 [Online]. DOI: 10.1089/vbz.2012.1054.

Gerth, H.-J., Grimshandl, D., Stage1, B., Doller 'and, G. and Kunz2, C. (1995) 'Roe deer as sentinels for endemicity of tick-borne encephalitis virus', *Epidemiol. Infect*, vol. 115, pp. 355–365 [Online]. Available at https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2271401/pdf/epidinfect00053-0136.pdf (Accessed 11 September 2017).

Gilbert, L. (2016) 'Louping ill virus in the UK: a review of the hosts, transmission and ecological consequences of control.', *Experimental & applied acarology*, Springer International Publishing, vol. 68, no. 3, pp. 363–374 [Online]. DOI: 10.1007/s10493-015-9952-x.

Gilbert, L., Brülisauer, F., Willoughby, K. and Cousens, C. (2020) 'Identifying Environmental Risk Factors for Louping Ill Virus Seroprevalence in Sheep and the Potential to Inform Wildlife Management Policy', *Frontiers in Veterinary Science*, Frontiers Media S.A., vol. 7, p. 377 [Online]. DOI: 10.3389/fvets.2020.00377 (Accessed 23 October 2020).

Gilbert, L., Jones, L. D., Hudson, P. J., Gould, E. A. and Reid, H. W. (2000) 'Role of small mammals in the persistence of Louping-ill virus: Field survey and tick co-feeding studies', *Medical and Veterinary Entomology*, vol. 14, no. 3, pp. 277–282 [Online]. DOI: 10.1046/j.1365-2915.2000.00236.x.

Gilbert, L., Jones, L. D., Laurenson, M. K., Gould, E. A., Reid, H. W. and Hudson, P. J. (2004) 'Ticks need not bite their red grouse hosts to infect them with louping ill virus', *Proceedings of the Royal Society B: Biological Sciences*, vol. 271, no. SUPPL. 4, pp. 202–205 [Online]. DOI: 10.1098/rsbl.2003.0147.

Gilbert, L., Norman, R., Laurenson, K. M., Reid, H. W. and Hudson, P. J. (2001) 'Disease persistence and apparent competition in a three-host community: An empirical and analytical study of large-scale, wild populations', *Journal of Animal Ecology*, vol. 70, no. 6, pp. 1053–1061 [Online]. DOI: 10.1046/j.0021-8790.2001.00558.x.

Gould, E. A., Higgs, S., Buckley, A. and Gritsun, T. S. (2006) *Potential arbovirus emergence and implications for the United Kingdom*, *Emerging Infectious Diseases*,

vol. 12, no. 4 [Online]. DOI: 10.3201/eid1204.051010 (Accessed 7 October 2016).

Gov.uk (2019) *Tick-borne encephalitis virus detected in ticks in the UK* [Online]. Available at https://www.gov.uk/government/news/tick-borne-encephalitis-virus-detected-in-ticks-in-the-uk (Accessed 22 March 2021).

Grant, M. J. and Edwards, M. E. (2008) 'Conserving idealized landscapes: Past history, public perception and future management in the New Forest (UK)', *Vegetation History and Archaeobotany*, vol. 17, no. 5, pp. 551–562 [Online]. DOI: 10.1007/s00334-007-0100-3.

Gray, J. S., Dautel, H., Estrada-Peña, A., Kahl, O. and Lindgren, E. (2009) 'Effects of Climate Change on Ticks and Tick-Borne Diseases in Europe', *Interdisciplinary Perspectives on Infectious Diseases*, vol. 2009, pp. 1–12 [Online]. DOI: 10.1155/2009/593232.

Greig, J. R., Brownlee, A., Wilson, D. R. and Gordon, W. S. (1931) 'The nature of louping ill', *Veterinary Record*, pp. 325–333.

Hansford, K. M., Carter, D., Gillingham, E. L., Hernandez-Triana, L. M., Chamberlain, J., Cull, B., McGinley, L., Paul Phipps, L. and Medlock, J. M. (2019) 'Hyalomma rufipes on an untraveled horse: Is this the first evidence of Hyalomma nymphs successfully moulting in the United Kingdom?', *Ticks and Tick-borne Diseases*, vol. 10, no. 3, pp. 704–708 [Online]. DOI: 10.1016/j.ttbdis.2019.03.003.

Hansford, K. M., Pietzsch, M. E., Cull, B., Gillingham, E. L. and Medlock, J. M. (2018) 'Potential risk posed by the importation of ticks into the UK on animals: records from the Tick Surveillance Scheme.', *The Veterinary record*, British Medical Journal Publishing Group, vol. 182, no. 4, p. 107 [Online]. DOI: 10.1136/vr.104263 (Accessed 16 May 2018).

Hansford, K. M., Pietzsch, M. E., Cull, B. and Medlock, J. M. (2014) 'Tickborne diseases: Importation of *R sanguineus* into the UK via dogs', *Veterinary Record*, vol. 175, no. 15, pp. 385–386 [Online]. DOI: 10.1136/vr.g6226.

Harmer, R. (1995) 'Natural regeneration of broadleaved trees in Britain: III. Germination and establishment', *Forestry*, vol. 68, no. 1, pp. 1–9 [Online]. DOI:

10.1093/forestry/68.1.1-b.

Harrison, A., Newey, S., Gilbert, L., Haydon, D. T. and Thirgood, S. (2010) 'Culling wildlife hosts to control disease: Mountain hares, red grouse and louping ill virus', *Journal of Applied Ecology*, Wiley/Blackwell (10.1111), vol. 47, no. 4, pp. 926–930 [Online]. DOI: 10.1111/j.1365-2664.2010.01834.x (Accessed 11 June 2018).

Hasle, G. (2013) 'Transport of Ixodid ticks and tick-borne pathogens by migratory birds', *Frontiers in Cellular and Infection Microbiology*, vol. 4, no. SEP, p. 48 [Online]. DOI: 10.3389/fcimb.2013.00048.

Hasle, G., Bjune, G., Edvardsen, E., Jakobsen, C., Linnehol, B., Røer, J. E., Mehl, R., Røed, K. H., Pedersen, J. and Leinaas, H. P. (2009) 'Transport of ticks by migratory passerine birds to Norway.', *The Journal of parasitology*, vol. 95, no. 6, pp. 1342–51 [Online]. DOI: 10.1645/GE-2146.1.

Health and Safety Executive. (2013) 'The Approved List of biological agents: Advisory Committee on Dangerous Pathogens.', *Health and Safety Executive*, no. 3, pp. 1–35 [Online]. Available at https://www.hse.gov.uk/pubns/misc208.pdf.

Hemami, M. R., Watkinson, A. R. and Dolman, P. M. (2004) 'Habitat selection by sympatric muntjac (*Muntiacus reevesi*) and roe deer (*Capreolus capreolus*) in a lowland commercial pine forest', *Forest Ecology and Management*, vol. 194, no. 1–3, pp. 49–60 [Online]. DOI: 10.1016/j.foreco.2004.01.049.

Hemami, M. R., Watkinson, A. R. and Dolman, P. M. (2005) 'Population densities and habitat associations of introduced muntjac *Muntiacus reevesi* and native roe deer *Capreolus capreolus* in a lowland pine forest', *Forest Ecology and Management*, vol. 215, no. 1–3, pp. 224–238 [Online]. DOI: 10.1016/j.foreco.2005.05.013.

Herrmann, C. and Gern, L. (2015) 'Search for blood or water is influenced by *Borrelia burgdorferi* in *Ixodes ricinus*', *Parasites and Vectors*, vol. 8, no. 1 [Online]. DOI: 10.1186/s13071-014-0526-2.

Heyman, P., Cochez, C., Hofhuis, A., van der Giessen, J., Sprong, H., Porter, S. R., Losson, B., Saegerman, C., Donoso-Mantke, O., Niedrig, M. and Papa, A. (2010) 'A

clear and present danger: tick-borne diseases in Europe', *Expert Review of Anti-infective Therapy*, vol. 8, no. 1744–8336 (Electronic), pp. 33–50 [Online]. DOI: 10.1586/eri.09.118.

Hillyard, P. D. (1996) *Ticks of North-West Europe*, Crothers, J.H. and Banks, R. S. K. (ed), Field Studies Council Publications for The Linnean Society of London y [Online]. DOI: 10.1111/j.1365-2915.1997.tb00296.x.

Hofmeester, T. R., Jansen, P. A., Wijnen, H. J., Coipan, E. C., Fonville, M., Prins, H. H. T., Sprong, H. and Van Wieren, S. E. (2017) 'Cascading effects of predator activity on tick-borne disease risk', *Proceedings of the Royal Society B: Biological Sciences*, vol. 284, no. 1859, pp. 0–7 [Online]. DOI: 10.1098/rspb.2017.0453.

Holding, M., Dowall, S. and Hewson, R. (2020) 'Detection of tick-borne encephalitis virus in the UK', *The Lancet*, vol. 395, p. 411 [Online]. DOI: 10.1016/S0140-6736(20)30040-4.

Horváth, G. and Wagner, Z. (2003) *Effect of densities of two coexistent small mammal populations on the survival of* Apodemus flavicollis *in a forest habitat*, *TISCIA*, vol. 34 [Online]. Available at https://expbio.bio.u-szeged.hu/ecology/tiscia/t34/t_34_6.pdf (Accessed 17 September 2020).

Hudson, P., Gould, E., Laurenson, K., Gaunt, M., Reid, H., Jones, L., Norman, R., MacGuire, K. and Newborn, D. (1997) 'The epidemiology of louping-ill, a tick borne infection of red grouse (*Lagopus lagopus scoticus*).', *Parassitologia*, vol. 39, no. 4, pp. 319–23 [Online]. Available at http://www.ncbi.nlm.nih.gov/pubmed/9802086 (Accessed 27 November 2017).

Human Animal Infections and Risk Surveillance (HAIRS) group (2021) 'Qualitative assessment of the risk that tickborne encephalitis (TBE) virus presents to the UK population', no. March, pp. 1–25 [Online]. Available at https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/540204/Zika_Virus_Risk_Assessment.pdf.

Hvidsten, D., Frafjord, K., Gray, J. S., Henningsson, A. J., Jenkins, A., Kristiansen, B. E., Lager, M., Rognerud, B., Slåtsve, A. M., Stordal, F., Stuen, S. and Wilhelmsson, P.

(2020) 'The distribution limit of the common tick, *Ixodes ricinus* and some associated pathogens in north-western Europe', *Ticks and Tick-borne Diseases*, Elsevier, vol. 11, no. 4, p. 101388 [Online]. DOI: 10.1016/j.ttbdis.2020.101388.

Id, J. P. B., Kaier, K., Hehn, P., Matzarakis, A., Frey, S., Bestehorn, M., Dobler, G. and Chitimia-dobler, L. (2021) 'The complex interplay of climate , TBEV vector dynamics and TBEV infection rates in ticks — Monitoring a natural TBEV focus in Germany , 2009 – 2018', vol. 530, no. 2009, pp. 1–16 [Online]. DOI: 10.1371/journal.pone.0244668.

Imhoff, M., Hagedorn, P., Schulze, Y., Hellenbrand, W., Pfeffer, M. and Niedrig, M. (2015) 'Review: Sentinels of tick-borne encephalitis risk', *Ticks and Tick-borne Diseases*, vol. 6, no. 5, pp. 592–600 [Online]. DOI: 10.1016/j.ttbdis.2015.05.001.

Jääskeläinen, A. E., Tikkakoski, T., Uzcátegui, N. Y., Alekseev, A. N., Vaheri, A. and Vapalahti, O. (2006) 'Siberian subtype tickborne encephalitis virus, Finland', *Emerging Infectious Diseases*, vol. 12, no. 10, pp. 1568–1571 [Online]. DOI: 10.3201/eid1210.060320.

Jaenson, T. G. T. G. T., Hjertqvist, M., Bergström, T., Lundkvist, Å., Lundkvist, A. and Lundkvist, Å. (2012) 'Why is tick-borne encephalitis increasing? A review of the key factors causing the increasing incidence of human TBE in Sweden', *Parasites & vectors*, BioMed Central, vol. 5, no. 1, p. 184 [Online]. DOI: 10.1186/1756-3305-5-184 (Accessed 16 August 2017).

Jaenson, T. G. T., Petersson, E. H., Jaenson, D. G. E., Kindberg, J., Pettersson, J. H. O., Hjertqvist, M., Medlock, J. M. and Bengtsson, H. (2018) 'The importance of wildlife in the ecology and epidemiology of the TBE virus in Sweden: Incidence of human TBE correlates with abundance of deer and hares', *Parasites and Vectors*, Parasites & Vectors, vol. 11, no. 1 [Online]. DOI: 10.1186/s13071-018-3057-4.

Jahfari, S., De Vries, A., Rijks, J. M., Van Gucht, S., Vennema, H., Sprong, H. and Rockx, B. (2017) 'Tick-Borne Encephalitis Virus in Ticks and Roe Deer, the Netherlands', *Emerging Infectious Diseases*, vol. 23, no. 6, pp. 1028–1030 [Online]. DOI: 10.3201/eid2306.161247 (Accessed 21 April 2017).

Jameson, L. J., Morgan, P. J., Medlock, J. M., Watola, G. and Vaux, A. G. C. (2012) 'Importation of *Hyalomma marginatum*, vector of Crimean-Congo haemorrhagic fever virus, into the United Kingdom by migratory birds', *Ticks and Tick-borne Diseases*, Elsevier GmbH., vol. 3, no. 2, pp. 95–99 [Online]. DOI: 10.1016/j.ttbdis.2011.12.002.

Jeffries, C. L., Mansfield, K. L., Phipps, L. P., Wakeley, P. R., Mearns, R., Schock, A., Bell, S., Breed, A. C., Fooks, A. R. and Johnson, N. (2014) 'Louping ill virus: An endemic tick-borne disease of Great Britain', *Journal of General Virology*, vol. 95, no. PART 5, pp. 1005–1014 [Online]. DOI: 10.1099/vir.0.062356-0.

Jensen, T. S. (1985) 'Seed-seed predator interactions of European beech, <i>Fagus silvatica and forest rodents, Clethrionomys glareolus and Apodemus flavicollis', *Oikos*, vol. 44, no. 1, p. 149 [Online]. DOI: 10.2307/3544056 (Accessed 28 September 2020).

Joint Committee on Vaccination and Immunisation (2019) *Joint Committee on Vaccination and Immunisation October 2019 Minutes*, no. October [Online]. Available at https://www.gov.uk/government/groups/joint-committee-on-vaccination-and-immunisation.

Jones, L. D., Davies, C. R., Steele, G. M. and Nuttall, P. A. (1987) 'A novel mode of arbovirus transmission involving a nonviremic host', *Science*, vol. 237, no. 4816, pp. 775–777 [Online]. DOI: 10.1126/science.3616608.

Jones, L. D., Gaunt, M., Hails, R. S., Laurenson, K., Hudson, P. J., Reid, H., Henbest, P. and Gould, E. a (1997) 'Transmission of louping ill virus between infected and uninfected ticks co-feeding on mountain hares.', *Medical and veterinary entomology*, vol. 11, no. 2, pp. 172–176 [Online]. DOI: 10.1111/j.1365-2915.1997.tb00309.x.

Jongejan, F. and Uilenberg, G. (2004) 'The global importance of ticks', *Parasitology*, vol. 129, pp. 3–14 [Online]. DOI: 10.1017/S0031182004005967.

Kafetzopoulou, L. E., Efthymiadis, K., Lewandowski, K., Crook, A., Carter, D., Osborne, J., Aarons, E., Hewson, R., Hiscox, J. A., Carroll, M. W., Vipond, R. and

Pullan, S. T. (2018) 'Assessment of metagenomic Nanopore and Illumina sequencing for recovering whole genome sequences of chikungunya and dengue viruses directly from clinical samples', *Eurosurveillance*, European Centre for Disease Prevention and Control, vol. 23, no. 50, p. 1800228 [Online]. DOI: 10.2807/1560-7917.ES.2018.23.50.1800228 (Accessed 9 July 2019).

Kaiser, R. (1999) 'The clinical and epidemiological profile of tick-borne encephalitis in southern Germany 1994-98. A prospective study of 656 patients', *Brain*, vol. 122, no. 11, pp. 2067–2078 [Online]. DOI: 10.1093/brain/122.11.2067.

Kazarina, A., Japiņa, K., Keišs, O., Salmane, I., Bandere, D., Capligina, V. and Ranka, R. (2015) *Detection of tick-borne encephalitis virus in* I. ricinus *ticks collected from autumn migratory birds in Latvia*, *Ticks and Tick-borne Diseases*, vol. 6, no. 2 [Online]. DOI: 10.1016/j.ttbdis.2014.11.011.

Kazimírová, M., Thangamani, S., Bartíková, P., Hermance, M., Holíková, V., Štibrániová, I. and Nuttall, P. A. (2017) 'Tick-borne viruses and biological processes at the tick-host-virus interface', *Frontiers in Cellular and Infection Microbiology*, Frontiers Media S.A., vol. 7, no. JUL [Online]. DOI: 10.3389/fcimb.2017.00339 (Accessed 24 August 2020).

Kearney, M. (2018) 'Microclimate Ecology', *Oxford Bibliographies* [Online]. DOI: DOI: 10.1093/OBO/9780199830060-0194.

Kellman, E. M., Offerdahl, D. K., Melik, W., Bloom, M. E., Kellman, E. M., Offerdahl, D. K., Melik, W. and Bloom, M. E. (2018) *Viral Determinants of Virulence in Tick-Borne Flaviviruses*, *Viruses*, vol. 10, no. 6 [Online]. DOI: 10.3390/v10060329 (Accessed 31 March 2019).

Kennedy, P. G. E., Quan, P.-L. and Lipkin, W. I. (2017) 'Viral Encephalitis of Unknown Cause: Current Perspective and Recent Advances.', *Viruses*, vol. 9, no. 6, p. 138 [Online]. DOI: 10.3390/v9060138 (Accessed 17 October 2017).

Khamassi Khbou, M., Romdhane, R., Foughali, A. A., Sassi, L., Suin, V., Rekik, M. and Benzarti, M. (2020) 'Presence of antibodies against tick-borne encephalitis virus in sheep in Tunisia, North Africa', *BMC Veterinary Research*, BMC Veterinary Research,

vol. 16, no. 1, pp. 1–8 [Online]. DOI: 10.1186/s12917-020-02651-6.

Kiffner, C., Vor, T., Hagedorn, P., Niedrig, M. and Rühe, F. (2011) 'Factors affecting patterns of tick parasitism on forest rodents in tick-borne encephalitis risk areas, Germany', *Parasitology Research*, vol. 108, no. 2, pp. 323–335 [Online]. DOI: 10.1007/s00436-010-2065-x.

Kiffner, C., Vor, T., Hagedorn, P., Niedrig, M. and Rühe, F. (2012) 'Determinants of tick-borne encepinhalitis virus antibody presence roe deer (*Capreolus capreolus*) sera', *Medical and Veterinary Entomology*, vol. 26, no. 1, pp. 18–25 [Online]. DOI: 10.1111/j.1365-2915.2011.00961.x (Accessed 11 September 2017).

Kiffner, C., Zucchini, W., Schomaker, P., Vor, T., Hagedorn, P., Niedrig, M. and Rühe, F. (2010) 'Determinants of tick-borne encephalitis in counties of southern Germany, 2001-2008', *International Journal of Health Geographics*, vol. 9, pp. 1–10 [Online]. DOI: 10.1186/1476-072X-9-42.

Kjellander, P., Hewison, A. J. M., Liberg, O., Angibault, J. M., Bideau, E. and Cargnelutti, B. (2004) 'Experimental evidence for density-dependence of home-range size in roe deer (<i>Capreolus capreolus L.</i>): A comparison of two long-term studies', *Oecologia*, vol. 139, no. 3, pp. 478–485 [Online]. DOI: 10.1007/s00442-004-1529-z.

Klaus, C., Gethmann, J., Hoffmann, B., Ziegler, U., Heller, M. and Beer, M. (2016) 'Tick infestation in birds and prevalence of pathogens in ticks collected from different places in Germany', *Parasitology Research* [Online]. DOI: 10.1007/s00436-016-5022-5.

Klaus, C., Ziegler, U., Kalthoff, D., Hoffmann, B. and Beer, M. (2014) 'Tick-borne encephalitis virus (TBEV) – findings on cross reactivity and longevity of TBEV antibodies in animal sera', *BMC veterinary research*, BioMed Central, vol. 10, p. 78 [Online]. DOI: 10.1186/1746-6148-10-78 (Accessed 16 August 2017).

Kreusch, T. M., Holding, M., Hewson, R., Harder, T., Medlock, J. M., Hansford, K. M., Dowall, S., Semper, A., Brooks, T., Walsh, A., Russell, K. and Wichmann, O. (2019) 'A probable case of tick-borne encephalitis (TBE) acquired in England, July 2019',

Eurosurveillance, vol. 24, no. 47, pp. 1–5 [Online]. DOI: 10.2807/1560-7917.ES.2019.24.47.1900679.

Kumar, S., Stecher, G. and Tamura, K. (2016) 'MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets', *Molecular Biology and Evolution*, vol. 33, no. 7, pp. 1870–1874 [Online]. DOI: 10.1093/molbev/msw054 (Accessed 13 June 2019).

Kupča, A. M., Essbauer, S., Zoeller, G., de Mendonça, P. G., Brey, R., Rinder, M., Pfister, K., Spiegel, M., Doerrbecker, B., Pfeffer, M. and Dobler, G. (2010) 'Isolation and molecular characterization of a tick-borne encephalitis virus strain from a new tick-borne encephalitis focus with severe cases in Bavaria, Germany', *Ticks and Tick-borne Diseases*, vol. 1, no. 1, pp. 44–51 [Online]. DOI: 10.1016/j.ttbdis.2009.11.002.

Labuda, M., Elečková, E., Ličková, M. and Sabó, A. (2002) 'Tick-borne encephalitis virus foci in Slovakia', *International Journal of Medical Microbiology*, vol. 291, pp. 43–47 [Online]. DOI: 10.1016/S1438-4221(02)80008-X.

Labuda, Milan, Jones, L. D., Williams, T., Danielova, V. and Nuttall, P. A. (1993) 'Efficient transmission of tick-borne encephalitis virus between cofeeding ticks.', *Journal of medical entomology*, vol. 30, no. 1, pp. 295–299 [Online]. DOI: 10.1093/jmedent/30.1.295 (Accessed 1 October 2019).

Labuda, M., Kozuch, O., Zuffová, E., Elecková, E., Hails, R. S. and Nuttall, P. A. (1997) 'Tick-borne encephalitis virus transmission between ticks cofeeding on specific immune natural rodent hosts', *Virology*, vol. 235, no. 1, pp. 138–143 [Online]. DOI: 10.1006/viro.1997.8622.

Labuda, M. and Nuttall, P. A. (2004) 'Tick-borne viruses', *Parasitology*, vol. 129, no. 7, pp. S221–S245 [Online]. DOI: 10.1017/S0031182004005220.

Labuda, M., Nuttall, P. A., Kožuch, O., Elečková, E., Williams, T., Žuffová, E. and Sabó, A. (1993) 'Non-viraemic transmission of tick-borne encephalitis virus: a mechanism for arbovirus survival in nature', *Experientia*, vol. 49, no. 9, pp. 802–805 [Online]. DOI: 10.1007/BF01923553.

Labuda, M. and Nuttall, Patricia A (2008) *Viruses transmitted by ticks*, Bowman, A. .

and Nuttall, P. . (eds), *Ticks: Biology, disease and control.*, Cambridge University Press [Online]. DOI: 10.1017/CBO9780511551802.013.

Labuda, M. and Randolph, S. E. (1999) *Survival Strategy of Tick-borne Encephalitis Virus: Cellular Basis and Environmental Determinants*, *Zent.bl. Bakteriol*, vol. 289 [Online]. Available at http://www.urbanfischer.de/joumalslzblbakteriol (Accessed 31 August 2020).

Landsberg, J. J. and Gower, S. T. (1997) 'Canopy Architecture and Microclimate', *Applications of Physiological Ecology to Forest Management*, pp. 51–88 [Online]. DOI: 10.1016/b978-012435955-0/50003-0.

Latham, J., Staines, B. W. and Gorman, M. L. (1999) 'Comparative feeding ecology of red (*Cervus elaphus*) and roe deer (*Capreolus capreolus*) in Scottish plantation forests', *Journal of Zoology*, vol. 247, no. 3, pp. 409–418 [Online]. DOI: 10.1017/S095283699900312X.

Laurenson, M. K., McKendrick, I. J., Reid, H. W., Challenor, R., Mathewson, G. K. and Švec, P. (2007) 'Prevalence, spatial distribution and the effect of control measures on louping-ill virus in the Forest of Bowland, Lancashire.', *Epidemiology and infection*, vol. 135, no. 6, pp. 963–973 [Online]. DOI: 10.1017/S0950268806007692.

Laurenson, M. K., Norman, R. A., Gilbert, L., Reid, H. W. and Hudson, P. J. (2003) 'Identifying disease reservoirs in complex systems: Mountain hares as reservoirs of ticks and louping-ill virus, pathogens of red grouse', *Journal of Animal Ecology*, vol. 72, no. 1, pp. 177–185 [Online]. DOI: 10.1046/j.1365-2656.2003.00688.x.

Leiper, R. T., Gordon, W. S. and Pool, W. A. (1933) *Discussion on Louping Ill-Proceedings of the Royal Society of Medicine*, *Section of Comparative Medicine* [Online]. Available at http://journals.sagepub.com/doi/pdf/10.1177/003591573402700642 (Accessed 10 October 2018).

Lindgren, E. and Gustafson, R. (2001) 'Tick-borne encephalitis in Sweden and climate change', *Lancet*, vol. 358, no. 9275, pp. 16–18 [Online]. DOI: 10.1016/S0140-6736(00)05250-8 (Accessed 23 October 2020).

Lindquist, L. and Vapalahti, O. (2008) 'Tick-borne encephalitis.', *Lancet (London, England)*, Elsevier, vol. 371, no. 9627, pp. 1861–1871 [Online]. DOI: 10.1016/S0140-6736(08)60800-4 (Accessed 23 June 2016).

Luccarini, S., Mauri, L., Ciuti, S., Lamberti, P. and Apollonio, M. (2006) 'Red deer (*Cervus elaphus*) spatial use in the Italian alps: Home range patterns, seasonal migrations, and effects of snow and winter feeding', *Ethology Ecology and Evolution*, vol. 18, no. 2, pp. 127–145 [Online]. DOI: 10.1080/08927014.2006.9522718.

Macaldowie, C., Patterson, I. A. P., Nettleton, P. F., Low, H. and Buxton, D. (2005) 'Louping ill in llamas (*Lama glama*) in the Hebrides', *The Veterinary Record* [Online]. DOI: 10.1136/vr.156.13.420 (Accessed 14 June 2018).

Mainstone, C. (2010a) 'Biodiversity in the New Forest: Preface', in Newton, A. C. (ed), *Biodiversity in the New Forest*, Bournemouth University, pp. vii–x [Online]. Available at https://core.ac.uk/download/pdf/20442855.pdf.

Mainstone, C. (2010b) '20 Synthesis: status and trends of biodiversity in the New Forest', in *Biodiversity in the New Forest*, School of Conservation Sciences, Bournemouth University [Online]. Available at http://eprints.bournemouth.ac.uk/13928/1/Newton_Biodiversity_in_the_New_Forest_2010_Chapter1.pdf.

Mansfield, K. L., Horton, D. L., Johnson, N., Li, L., Barrett, A. D. T., Smith, D. J., Galbraith, S. E., Solomon, T. and Fooks, A. R. (2011) 'Flavivirus-induced antibody cross-reactivity', *Journal of General Virology*, vol. 92, no. 12, pp. 2821–2829 [Online]. DOI: 10.1099/vir.0.031641-0 (Accessed 27 November 2017).

Marriott, L., Willoughby, K., Chianini, F., Dagleish, M. P., Scholes, S., Robinson, A. C., Gould, E. A. and Nettleton, P. F. (2006) 'Detection of Louping ill virus in clinical specimens from mammals and birds using TaqMan RT-PCR', *Journal of Virological Methods*, Elsevier, vol. 137, no. 1, pp. 21–28 [Online]. DOI: 10.1016/j.jviromet.2006.05.025.

Marsh, A. (1999) 'The National Yellow-Necked Mouse Survey', *Society*, no. 2, pp. 0–

16 [Online]. Available at file:///C:/Users/maya.holding/Downloads/Yellow-necked-Mouse-Report.pdf (Accessed 11 October 2019).

Marsh, A. C. W. and Harris, S. (n.d.) *Partitioning of woodland habitat resources by two sympatric species of Apodemus: lessons for the conservation of the yellow-necked mouse (*A. Flavicollis*) in Britain*, [Online]. Available at www.elsevier.com/locate/biocon (Accessed 6 September 2020).

Marsh, A. C. W., Poulton, S. and Harris, S. (2001) 'The yellow-necked mouse *Apodemus flavicollis,* in Britain: Status and analysis of factors affecting distribution', *Mammal Review*, vol. 31, no. 3–4, pp. 203–227 [Online]. DOI: 10.1046/j.1365-2907.2001.00089.x.

McCullough, D. R., Takatsuki, S. and Kaji, K. (2009) *Sika deer: Biology and management of native and introduced populations*, McCullough, D. R., Takatsuki, S., and Kaji, K. (eds), 1st edn, Springer Japan [Online]. DOI: 10.1007/978-4-431-09429-6.

McGinley, L., Hansford, K. M., Cull, B., Gillingham, E. L., Carter, D. P., Chamberlain, J. F., Hernandez-Triana, L. M., Phipps, L. P. and Medlock, J. M. (2021) 'First report of human exposure to *Hyalomma marginatum* in England: Further evidence of a *Hyalomma* moulting event in north-western Europe?', *Ticks and Tick-borne Diseases*, Elsevier, vol. 12, no. 1, p. 101541 [Online]. DOI: 10.1016/j.ttbdis.2020.101541.

Medcalf, K. and Bell, G. (2014) *New Forest National Park and 20km surrounding Area Ecosystem Assessment – Final Report Environment Systems Ltd*, [Online]. Available at https://www.envsys.co.uk/wp-content/uploads/2015/02/NFNPA_Project_Report_Final.pdf.

Medlock, J., Hansford, K., Vaux, A., Cull, B., Gillingham, E. and Leach, S. (2018) 'Assessment of the public health threats posed by vector-borne disease in the United Kingdom (UK)', *International Journal of Environmental Research and Public Health*, Multidisciplinary Digital Publishing Institute, vol. 15, no. 10, p. 2145 [Online]. DOI: 10.3390/ijerph15102145 (Accessed 21 May 2019).

Medlock, J. M., Hansford, K. M., Bormane, A., Derdakova, M., Estrada-peña, A., George, J., Golovljova, I., Jaenson, T. G. T., Jensen, J.-K., Jensen, P. M., Kazimirova, M., Oteo, J. A., Papa, A., Pfister, K., Plantard, O., Randolph, S. E., Rizzoli, A., Santos-Silva, M. M., Sprong, H., Vial, L., Hendrickx, G., Zeller, H., Van Bortel, W., Glass, G., Schwartz, B., Morgan, J., Johnson, D., Noy, P. and Israel, E. (2013) 'Driving forces for changes in geographical distribution of Ixodes ricinus ticks in Europe', *Parasites & Vectors*, BioMed Central, vol. 6, no. 1, pp. 1–11 [Online]. DOI: 10.1186/1756-3305-6-1 (Accessed 13 September 2017).

Medlock, J. M. and Leach, S. A. (2015) 'Review Effect of climate change on vector-borne disease risk in the UK', *The Lancet Infectious Diseases*, vol. 3099, no. 15, pp. 721–730 [Online]. DOI: 10.1016/S1473-3099(15)70091-5.

Michelitsch, A., Wernike, K., Klaus, C., Dobler, G. and Beer, M. (2019) 'Exploring the Reservoir Hosts of Tick-Borne Encephalitis Virus', *Viruses*, vol. 11, no. 7, p. 669 [Online]. DOI: 10.3390/v11070669 (Accessed 24 August 2020).

Mickiené, A., Laisškonis, A., Guünther, G., Vene, S., Lundkvist, Å. and Lindquist, L. (2002) 'Tickborne encephalitis in an area of high endemicity in Lithuania: Disease severity and long-term prognosis', *Clinical Infectious Diseases*, vol. 35, no. 6, pp. 650–658 [Online]. DOI: 10.1086/342059.

Millins, C., Gilbert, L., Johnson, P., James, M., Kilbride, E., Birtles, R. and Biek, R. (2016) 'Heterogeneity in the abundance and distribution of Ixodes ricinus and Borrelia burgdorferi (sensu lato) in Scotland: implications for risk prediction', *Parasites and Vectors*, vol. 9, no. 1, pp. 1–12 [Online]. DOI: 10.1186/s13071-016-1875-9.

Minár, J. (1992) 'Natural foci of tick-borne encephalitis in the Slovak Republic and its relation to the natural ecosystem', *Ceskoslovenská epidemiologie, mikrobiologie, imunologie* [Online]. Available at https://pubmed.ncbi.nlm.nih.gov/1291117/.

Ministry of Agriculture, N. and F. Q. (2005) *National Parks in the Netherlands*, *Ministry of Agriculture, Nature and Food Quality*.

Mishaeva, N. P. and Erofeeva, N. . (1979) 'The effect of diapause of the tick *Ixodes*

ricinus (Ixodidae) upon the multiplication of the tick encephalitis virus in them', *Parazitologiya*, pp. 218–222.

Montgomery, W. I. (1978) 'Studies on the distributions of *Apodemus sylvaticus* (L.) and *A. flavicollis* (Melchior) in Britain', *Mammal Review*, vol. 8, no. 4, pp. 177–184 [Online]. DOI: 10.1111/j.1365-2907.1978.tb00226.x.

Moredun Research Institute (2020) *PRESS RELEASE: Research gets a huge boost towards a new generation louping ill vaccine* [Online]. Available at https://www.moredun.org.uk/news/press-release-research-gets-huge-boost-towards-new-generation-louping-ill-vaccine (Accessed 30 November 2020).

Moseley, M. H., Dukes, J., Marriott, L., Nettleton, P., Irvine, R. J., Mougeot, F. and Cinegéticos, R. (2007) 'Use of real-time RT-PCR to determine the prevalence of louping ill virus in live red grouse chicks', [Online]. Available at http://veterinaryrecord.bmj.com/content/vetrec/161/19/660.full.pdf (Accessed 21 September 2017).

National Health Service (2019) *Tick-borne encephalitis (TBE)* [Online]. Available at https://www.nhs.uk/conditions/tick-borne-encephalitis/ (Accessed 22 March 2021).

Natural England (2015a) *The Brecks National Character Area Profile.*, Natural England [Online]. Available at http://publications.naturalengland.org.uk/publication/4282581.

Natural England (2015b) *131 : New Forest Area profile :*, Natural England [Online]. Available at http://publications.naturalengland.org.uk/publication/5545755456569344?category=587130.

Natural England (2020) *Priority habitat inventory (England)* [Online]. Available at https://data.gov.uk/dataset/4b6ddab7-6c0f-4407-946e-d6499f19fcde/priority-habitat-inventory-england (Accessed 27 March 2021).

NBN Atlas (2021) *NBN Atlas Apodemus flavicollis and Myodes glareolus Occurrance Download* [Online]. Available at http://nbnatlas.org (Accessed 13 January 2020).

New Forest National Park Authority (2007a) *Wildlife of the New Forest National Park: 1 Habitats of the National Park*, [Online]. Available at https://www.newforestnpa.gov.uk/app/uploads/2018/03/wildlife1_habitats.pdf.

New Forest National Park Authority (2007b) *Wildlife of the New Forest National Park: 2 Ecology and Management*, [Online]. Available at https://www.newforestnpa.gov.uk/app/uploads/2018/03/wildlife2_ecology.pdf.

New Forest National Park Authority (2013) 'Nature in the New Forest: Action for Biodiversity', *Nature in the New Forest: action of biodiversity*, no. June, pp. 1–56 [Online]. Available at https://www.newforestnpa.gov.uk/conservation/protecting-nature/biodiversity-action-plan/.

New Forest National Park Authority (n.d.) *Staying safe* [Online]. Available at https://www.newforestnpa.gov.uk/visiting/visitor-information/staying-safe/ (Accessed 22 March 2021).

Newbould, P. J. and Tubbs, C. R. (1970) 'The New Forest, an Ecological History.', *The Journal of Ecology*, vol. 58, no. 2, p. 566 [Online]. DOI: 10.2307/2258302.

Newton, A. C. (2011) 'Social-ecological resilience and biodiversity conservation in a 900-yearold protected area', *Ecology and Society*, vol. 16, no. 4 [Online]. DOI: 10.5751/ES-04308-160413.

Newton, A. C., Cantarello, E., Myers, G., Douglas, S. and Tejedor, N. (n.d.) '13 The condition and dynamics of New Forest woodlands Adrian', in Newton, A. C. (ed), Pisces Publications [Online]. Available at http://eprints.bournemouth.ac.uk/13928/1/Newton_Biodiversity_in_the_New_Forest_2010_Chapter1.pdf.

Nosek, J. and Blaškovič, D. (1973) 'Ticks as Vectors of Tick-Borne Encephalitis (TBE) Virus in Europe', in *Proceedings of the 3rd International Congress of Acarology*, pp. 589–591 [Online]. DOI: 10.1007/978-94-010-2709-0_114.

Nurk, S., Bankevich, A., Antipov, D., Gurevich, A., Korobeynikov, A., Lapidus, A., Prjibelsky, A., Pyshkin, A., Sirotkin, A., Sirotkin, Y., Stepanauskas, R., McLean, J., Lasken, R., Clingenpeel, S. R., Woyke, T., Tesler, G., Alekseyev, M. A. and Pevzner, P.

A. (2013) 'Assembling genomes and mini-metagenomes from highly chimeric reads', in Springer, Berlin, Heidelberg, pp. 158–170 [Online]. DOI: 10.1007/978-3-642-37195-0_13 (Accessed 9 July 2019).

Nussbaumer, A., Waldner, P., Etzold, S., Gessler, A., Benham, S., Thomsen, I. M., Jørgensen, B. B., Timmermann, V., Verstraeten, A., Sioen, G., Rautio, P., Ukonmaanaho, L., Skudnik, M., Apuhtin, V., Braun, S. and Wauer, A. (2016) 'Patterns of mast fruiting of common beech, sessile and common oak, Norway spruce and Scots pine in Central and Northern Europe', *Forest Ecology and Management*, Elsevier B.V., vol. 363, pp. 237–251 [Online]. DOI: 10.1016/j.foreco.2015.12.033.

Nutall, P. (2014) 'Tick-Borne Viruses', in Sonenshine, D. . and Roe, M. (eds), *Biology of Ticks*, Oxford University Press, vol. 2, pp. 180–204.

Nutall, P. and Labuda, M. (2008) 'Saliva-assisted transmission of tick-borne pathogens', in Bowman, A. and Nutall, P. (eds), *Ticks: Biology, Disease and Control*, Cambridge, Cambridge University Press, pp. 205–215.

Nuttall, P. A. and Labuda, M. (2003) 'Dynamics of infection in tick vectors and at the tick-host interface', *Advances in Virus Research*, vol. 60, pp. 233–272 [Online]. DOI: 10.1016/S0065-3527(03)60007-2 (Accessed 21 September 2017).

Paul, T., Sys, S. U., De Clercq, K., De Leeuw, I., Caij, A. B., De Baere, M., De Regge, N., Fretin, D., Roupie, V., Govaerts, M., Heyman, P., Vanrompay, D., Yin, L., Kalmar, I., Suin, V., Brochier, B., Dobly, A., De Craeye, S., Roelandt, S., Goossens, E., Roels, S. and Çelikbaş, A. (2015) 'Serologic screening for 13 infectious agents in roe deer (Capreolus capreolus) in Flanders.', *Infection ecology & epidemiology*, Taylor & Francis, vol. 5, p. 29862 [Online]. DOI: 10.3402/iee.v5.29862 (Accessed 13 August 2017).

Perez-Eid, C., Hannoun, C. and Rodhain, F. (1992) 'The Alsatian tick-borne encephalitis focus: Presence of the virus among ticks and small mammals', *European Journal of Epidemiology*, vol. 8, no. 2, pp. 178–186 [Online]. DOI: 10.1007/BF00144797.

Petersen, A., Worsøe Rosenstierne, M., Rasmussen, M., Fuursted, K., Nielsen, H. V., O'brien Andersen, L., Bødker, R. and Fomsgaard, A. (2019) 'Field samplings of Ixodes ricinus ticks from a tick-borne encephalitis virus micro-focus in Northern Zealand, Denmark', *Ticks and Tick-borne Diseases* [Online]. DOI: 10.1016/j.ttbdis.2019.05.005 (Accessed 10 June 2019).

Pettersson, J. H. O. H.-O., Golovljova, I., Vene, S. and Jaenson, T. G. T. T. (2014) 'Prevalence of tick-borne encephalitis virus in *Ixodes ricinus* ticks in northern Europe with particular reference to Southern Sweden.', *Parasites & vectors*, BioMed Central Ltd., vol. 7, no. March 2014, p. 102 [Online]. DOI: 10.1186/1756-3305-7-102 (Accessed 28 August 2020).

Pfäffle, M., Littwin, N., Muders, S. V. and Petney, T. N. (2013) 'The ecology of tick-borne diseases', *International Journal for Parasitology*, Australian Society for Parasitology Inc., vol. 43, no. 12–13, pp. 1059–1077 [Online]. DOI: 10.1016/j.ijpara.2013.06.009.

Pietzsch, M. E., Mitchell, R., Jameson, L. J., Morgan, C., Medlock, J. M., Collins, D., Chamberlain, J. C., Gould, E. A., Hewson, R., Taylor, M. A. and Leach, S. (2008) 'Preliminary evaluation of exotic tick species and exotic pathogens imported on migratory birds into the British Isles', *Veterinary Parasitology*, vol. 155, no. 3–4, pp. 328–332 [Online]. DOI: 10.1016/j.vetpar.2008.05.006 (Accessed 11 August 2016).

Pöllabauer, E. M. and Kollaritsch, H. (2019) 'Prevention : vaccines and immunoglobulins', in Dobler, G., Erber, W., Bröker, M., and Schmitt, H. J. (eds), *TBE the book*, 3rd edn, Singapore, Global Health Press [Online]. DOI: 10.33442/26613980_14-3.

Pool, A., Brownlee, A. and Wilson, D. R. (1930) 'The etiology of "Louping-Ill"', [Online]. DOI: 10.1016/S0368-1742(30)80026-2 (Accessed 27 November 2017).

Public Health England (2013) 'Immunisation Against Infectious Disease: Chapter 31, Tick Borne Encephalitis', in *The Green Book*, UK Government Publications, pp. 385–390 [Online]. Available at https://www.gov.uk/government/publications/tick-borne-encephalitis-the-green-book-chapter-31.

Public Health England (2020) *Rare tick-borne infections diagnosed in England* [Online]. Available at https://www.gov.uk/government/news/rare-tick-borne-infections-diagnosed-in-england.

Pugliese, A. and Rosà, R. (2008) 'Effect of host populations on the intensity of ticks and the prevalence of tick-borne pathogens: How to interpret the results of deer exclosure experiments', *Parasitology*, vol. 135, no. 13, pp. 1531–1544 [Online]. DOI: 10.1017/S003118200800036X.

Putman, R. J. (1986) 'Ecology and behaviour of the forest deer', *Grazing in Temperate Ecosystems Large Herbivores and the Ecology of the New Forest*, no. Plate 2, pp. 88–115 [Online]. DOI: 10.1007/978-94-011-6081-0_5.

Putman, R. J. (1996) 'The New Forest and its larger herbivores', *Competition and resource partitioning in temperate ungulate assemblies*, pp. 11–29 [Online]. DOI: 10.1007/978-94-009-1517-6_2.

Rafferty, J. P. (n.d.) *Microclimate* [Online]. Available at https://www.britannica.com/science/microclimate (Accessed 6 November 2020).

Randolph, S. (2002) 'Predicting the risk of tick-borne diseases', *International Journal of Medical Microbiology*, Urban & Fischer Verlag, vol. 291, no. SUPPL. 33, pp. 6–10 [Online]. DOI: 10.1016/S1438-4221(02)80002-9 (Accessed 28 August 2020).

Randolph, S. E. (2001) 'The shifting landscape of tick-borne zoonoses: tick-borne encephalitis and Lyme borreliosis in Europe.', Woolhouse, M. E. J. and Dye, C. (eds), *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, The Royal Society, vol. 356, no. 1411, pp. 1045–1056 [Online]. DOI: 10.1098/rstb.2001.0893 (Accessed 27 April 2019).

Randolph, S.E. (2004) 'IJNM Mini-Review: Evidence that climate change has caused "emergence" of tick-borne diseases in Europe? Introduction -the undeniable impact of climate on tick-borne diseases', *Int. J. Med. Microbiol*, vol. 293, no. 37, pp. 5–15.

Randolph, S. E. (2004) 'Tick ecology: Processes and patterns behind the epidemiological risk posed by ixodid ticks as vectors', *Parasitology*, vol. 129, no. SUPPL. [Online]. DOI: 10.1017/S0031182004004925.

Randolph, S. E. (2008) 'Tick-borne encephalitis virus, ticks and humans: short-term and long-term dynamics.', *Current opinion in infectious diseases*, vol. 21, no. 5, pp. 462–7 [Online]. DOI: 10.1097/QCO.0b013e32830ce74b (Accessed 21 March 2019).

Randolph, S. E. (2010) *Human activities predominate in determining changing incidence of tick-borne encephalitis in Europe*, *Euro surveillance*, no. 27 [Online]. Available at www.eurosurveillance.org:pii=19606.Availableonline:http://www.eurosurveillance. org/ViewArticle.aspx?ArticleId=19606 (Accessed 27 April 2019).

Randolph, S. E., Miklisová, D., Lysy, J., Rogers, D. J. and Labuda, M. (1999) 'Incidence from coincidence: Patterns of tick infestations on rodents facilitate transmission of tick-borne encephalitis virus', *Parasitology*, vol. 118, no. 2, pp. 177–186 [Online]. DOI: 10.1017/S0031182098003643.

Randolph, S. E. and Rogers, D. J. (2000) 'Fragile transmission cycles of tick-borne encephalitis virus may be disrupted by predicted climate change', *Proceedings of the Royal Society B: Biological Sciences*, vol. 267, no. 1454, pp. 1741–1744 [Online]. DOI: 10.1098/rspb.2000.1204 (Accessed 16 May 2018).

Randolph, S. E. and Rogers, D. J. (2006) 'Tick-borne Disease Systems: Mapping Geographic and Phylogenetic Space', *Advances in Parasitology*, vol. 62, no. 05, pp. 263–291 [Online]. DOI: 10.1016/S0065-308X(05)62008-8.

Randolph, S. E. and Storey, K. (1999) 'Impact of microclimate on immature tick-rodent host interactions (Acari: Ixodidae): Implications for parasite transmission', *Journal of Medical Entomology*, vol. 36, no. 6, pp. 741–748 [Online]. DOI: 10.1093/jmedent/36.6.741.

Randolph, S. E. and Sumilo, D. (2007) 'Tick-borne encephalitis in Europe: dynamics of changing risk', *Emerging Pests and Vector-borne Diseases in Europe*, no. February, pp. 187–206 [Online]. Available at http://www.wageningenacademic.com/ECVD-01.

Ratcliffe, P. and Claridge, J. (1996) 'Thetford Forest Park: the ecology of a pine forest', *Forestry Commission Technical Paper*, vol. 13 [Online]. Available at

https://www.forestry.gov.uk/PDF/FCTP013.pdf/$FILE/FCTP013.pdf (Accessed 21 March 2019).

Reid, H. W., Buxton, D., Gardiner, A. C., Pow, I., Finlayson, J. and MacLean, M. J. (1982) 'Immunosuppression in toxoplasmosis: Studies in lambs and sheep infected with louping-ill virus', *Journal of Comparative Pathology*, W.B. Saunders, vol. 92, no. 2, pp. 181–190 [Online]. DOI: 10.1016/0021-9975(82)90076-7 (Accessed 27 November 2017).

Reil, D., Imholt, C., Eccard, J. A. and Jacob, J. (2015) 'Beech Fructification and Bank Vole Population Dynamics - Combined Analyses of Promoters of Human Puumala Virus Infections in Germany', Waldenström, J. (ed), *PLOS ONE*, Public Library of Science, vol. 10, no. 7, p. e0134124 [Online]. DOI: 10.1371/journal.pone.0134124 (Accessed 24 September 2020).

Reinecke, H., Leinen, L., Thißen, I., Meißner, M., Herzog, S., Schütz, S. and Kiffner, C. (2014) 'Home range size estimates of red deer in Germany: Environmental, individual and methodological correlates', *European Journal of Wildlife Research*, vol. 60, no. 2, pp. 237–247 [Online]. DOI: 10.1007/s10344-013-0772-1.

Rieille, N., Bressanelli, S., Freire, C. C. C. M., Arcioni, S., Gern, L., Péter, O. and Voordouw, M. J. (2014) 'Prevalence and phylogenetic analysis of tick-borne encephalitis virus (TBEV) in field-collected ticks (Ixodes ricinus) in southern Switzerland', *Parasites & Vectors*, BioMed Central, vol. 7, no. 1, p. 443 [Online]. DOI: 10.1186/1756-3305-7-443 (Accessed 12 July 2016).

Rijks, J. M., Montizaan, M. G. E., Bakker, N., de Vries, A., Van Gucht, S., Swaan, C., van den Broek, J., Gröne, A. and Sprong, H. (2019) 'Tick-borne encephalitis virus antibodies in roe deer, The Netherlands', *Emerging Infectious Diseases*, Centers for Disease Control and Prevention (CDC), vol. 25, no. 2, pp. 342–345 [Online]. DOI: 10.3201/eid2502.181386 (Accessed 27 April 2019).

RIVM (2019) *Tick found in Drenthe is a Hyalomma tick* [Online]. Available at https://www.rivm.nl/en/news/tick-found-in-drenthe-is-hyalomma-tick (Accessed 13 December 2020).

Rizzoli, A., Neteler, M., Rosà, R., Versini, W., Cristofolini, A., Bregoli, M., Buckley, A. and Gould, E. A. (2007) 'Early detection of tick-borne encephalitis virus spatial distribution and activity in the province of Trento, northern Italy.', *Geospatial health*, vol. 1, no. 2, pp. 169–176 [Online]. DOI: 10.4081/gh.2007.265.

Rizzoli, A., Silaghi, C., Obiegala, A., Rudolf, I., Hubálek, Z., Földvári, G., Plantard, O., Vayssier-Taussat, M., Bonnet, S., Špitalská, E. and Kazimírová, M. (2014) '*Ixodes ricinus* and its transmitted pathogens in urban and peri-urban areas in Europe: new hazards and relevance for public health', *Frontiers in Public Health*, vol. 2, no. December, p. 251 [Online]. DOI: 10.3389/fpubh.2014.00251 (Accessed 16 August 2017).

Rosà, R., Tagliapietra, V., Manica, M., Arnoldi, D., Hauffe, H. C., Rossi, C., Rosso, F., Henttonen, H. and Rizzoli, A. (2019) 'Changes in host densities and co-feeding pattern efficiently predict tick-borne encephalitis hazard in an endemic focus in northern Italy', *International Journal for Parasitology*, vol. 49, no. 10, pp. 779–787 [Online]. DOI: 10.1016/j.ijpara.2019.05.006.

Rotach, M. W. and Calanca, P. (2003) 'Microclimate', in Holton, J. R. (ed), *Encyclopedia of Atmospheric Sciences*, Academic Press, pp. 1301–1307 [Online]. DOI: 10.1016/B0-12-227090-8/00225-6.

Rothera, S. (1998) *Breckland - The future vision*,.

Rubel, F. and Brugger, K. (2020) 'Tick-borne encephalitis incidence forecasts for Austria, Germany, and Switzerland', *Ticks and Tick-borne Diseases*, Elsevier, vol. 11, no. 5, p. 101437 [Online]. DOI: 10.1016/j.ttbdis.2020.101437.

Rubel, F., Walter, M., Vogelgesang, J. R. and Brugger, K. (2020) 'Tick-borne encephalitis (TBE) cases are not random: explaining trend, low- and high-frequency oscillations based on the Austrian TBE time series', *BMC infectious diseases*, BMC Infectious Diseases, vol. 20, no. 1, p. 448 [Online]. DOI: 10.1186/s12879-020-05156-7 (Accessed 23 October 2020).

Ruzek, D., Yoshii, K., Bloom, M. and Services, H. (2017) 'Chapter 2a- Virology in "Tick-borne encephalitis virus"-', in Global Health Press, no. October [Online].

Available at https://www.researchgate.net/publication/320310502%0ATick-borne.

Saïd, S., Gaillard, J. M., Widmer, O., Débias, F., Bourgoin, G., Delorme, D. and Roux, C. (2009) 'What shapes intra-specific variation in home range size? A case study of female roe deer', *Oikos*, vol. 118, no. 9, pp. 1299–1306 [Online]. DOI: 10.1111/j.1600-0706.2009.17346.x.

Saïd, S. and Servanty, S. (2005) 'The influence of landscape structure on female roe deer home-range size', *Landscape Ecology*, vol. 20, no. 8, pp. 1003–1012 [Online]. DOI: 10.1007/s10980-005-7518-8.

Saunders, W. B. (1948) 'Studies in Louping-ill: IV. Passive Immunity', *Journal of Comparative Pathology and Therapeutics*, vol. 58, pp. 210–226 [Online]. DOI: 10.1016/S0368-1742(48)80021-4 (Accessed 27 November 2017).

Schlinkert, H., Ludwig, M., Batáry, P., Holzschuh, A., Kovács-Hostyánszki, A., Tscharntke, T. and Fischer, C. (2016) 'Forest specialist and generalist small mammals in forest edges and hedges', *Wildlife Biology*, Nordic Council for Wildlife Research, vol. 22, no. 3, pp. 86–94 [Online]. DOI: 10.2981/wlb.00176 (Accessed 24 September 2020).

Schönbächler, K., Hatt, J.-M., Silaghi, C., Merz, N., Fraefel, C. and Bachofen, C. (2019) 'Originalarbeiten | Original contributions 23 Frühsommer-Meningoenzephalitis-Virus Nachweis beim Europäischen Igel (Erinaceus europaeus)', *Band*, vol. 1, pp. 23–31 [Online]. DOI: 10.17236/sat00191.

Schönbächler, K., Hatt, J. M., Silaghi, C., Merz, N., Fraefel, C. and Bachofen, C. (2018) 'Confirmation of tick-borne encephalitis virus in an European hedgehog (*Erinaceus europaeus*)', *Schweizer Archiv fur Tierheilkunde*, Gesellschaft Schweizer Tierärztinnen und Tierärzte, vol. 161, no. 1, pp. 23–31 [Online]. DOI: 10.17236/sat00191 (Accessed 11 September 2020).

Schwaiger, M. and Cassinotti, P. (2003) 'Development of a quantitative real-time RT-PCR assay with internal control for the laboratory detection of tick borne encephalitis virus (TBEV) RNA', *Journal of Clinical Virology*, vol. 27, no. 2, pp. 136–145 [Online]. DOI: 10.1016/S1386-6532(02)00168-3.

Skarphédinsson, S., Jensen, P. M. and Kristiansen, K. (2005) 'Survey of tickborne infections in Denmark', *Emerging Infectious Diseases*, Centers for Disease Control and Prevention, vol. 11, no. 7, pp. 1055–1061 [Online]. DOI: 10.3201/eid1107.041265 (Accessed 30 November 2017).

Slovák, M., Kazimírová, M., Siebenstichová, M., Ustaníková, K., Klempa, B., Gritsun, T., Gould, E. A. and Nuttall, P. A. (2014) 'Survival dynamics of tick-borne encephalitis virus in *Ixodes ricinus* ticks', *Ticks and Tick-borne Diseases*, Elsevier GmbH., vol. 5, no. 6, pp. 962–969 [Online]. DOI: 10.1016/j.ttbdis.2014.07.019.

Smith, C. E., Varma, M. G. and McMahon, D. (1964) 'Isolation of louping-ill virus from small mammals in Ayrshire, Scotland', *Nature*, vol. 203, no. 4948, p. 993 [Online]. DOI: 10.1038/203993a0.

Sorvillo, T. E., Rodriguez, S. E., Hudson, P., Carey, M., Rodriguez, L. L., Spiropoulou, C. F., Bird, B. H., Spengler, J. R. and Bente, D. A. (2020) 'Towards a sustainable one health approach to Crimean-Congo hemorrhagic fever prevention: Focus areas and gaps in knowledge', *Tropical Medicine and Infectious Disease*, vol. 5, no. 3, pp. 1–28 [Online]. DOI: 10.3390/tropicalmed5030113.

Spall, I. (2017) *Hampshire Mammal Group: Mammal Report 2006 to 2016*, no. October [Online]. Available at https://www.hiwwt.org.uk/sites/default/files/2018-07/HMG_Hampshire_Mammal_Report_2017.pdf.

Sparagano, O., George, D., Giangaspero, A. and Špitalská, E. (2015) 'Arthropods and associated arthropod-borne diseases transmitted by migrating birds. The case of ticks and tick-borne pathogens', *Veterinary Parasitology*, Elsevier, vol. 213, no. 1, pp. 61–66 [Online]. DOI: 10.1016/j.vetpar.2015.08.028 (Accessed 5 August 2016).

Springer, A., Glass, A., Topp, A. K. and Strube, C. (2020) 'Zoonotic Tick-Borne Pathogens in Temperate and Cold Regions of Europe—A Review on the Prevalence in Domestic Animals', *Frontiers in Veterinary Science*, vol. 7, no. December, pp. 1–21 [Online]. DOI: 10.3389/fvets.2020.604910.

Stefanoff, P., Pfeffer, M., Hellenbrand, W., Rogalska, J., Ruhe, F., Makowka, A., Michalik, J., Wodecka, B., Rymaszewska, A., Kiewra, D., Baumann-Popczyk, A. and

Dobler, G. (2013) 'Virus Detection in Questing Ticks is not a Sensitive Indicator for Risk Assessment of Tick-Borne Encephalitis in Humans', *Zoonoses and Public Health*, Blackwell Publishing Ltd, vol. 60, no. 3, pp. 215–226 [Online]. DOI: 10.1111/j.1863-2378.2012.01517.x (Accessed 12 July 2016).

Suffolk Local Access Forum (2011) *Providing independent advice on access to the countryside in Suffolk*, [Online]. Available at http://www.suffolk.gov.uk/Environment/Public RightsOfWay/SuffolkLocalAccessForum%0AYour.

Summers, A. (2018) *An Assynt Pine Marten and Louping Ill* [Online]. Available at https://www.assyntwildlife.org.uk/2018/09/pine-marten-louping-ill/ (Accessed 25 November 2020).

Suss, J. (2008) 'Tick-borne encephalitis in Europe and beyond--the epidemiological situation as of 2007', *Euro surveillance : bulletin Européen sur les maladies transmissibles = European communicable disease bulletin*, vol. 13, no. 26 [Online]. DOI: 10.2807/ese.13.26.18916-en.

Süss, J. (2003) 'Epidemiology and ecology of TBE relevant to the production of effective vaccines', *Vaccine*, vol. 21, no. SUPPL. 1, pp. S19–S35 [Online]. DOI: 10.1016/S0264-410X(02)00812-5.

Süss, J. (2011) 'Tick-borne encephalitis 2010: Epidemiology, risk areas, and virus strains in Europe and Asia—An overview', *Ticks and Tick-borne Diseases*, vol. 2, no. 1, pp. 2–15 [Online]. DOI: 10.1016/j.ttbdis.2010.10.007.

Szemethy, L., Heltai, M., Matrai, K. and Peto, Z. (1998) 'Home ranges and habitat selection of red deer (*Cervus elaphus*) on a lowland area', *Gibier Fane Sauvage - Game and Wildlife, Vol 15 (Special Number Pts 2 and 3) 1998*, no. January, p. 607–615\r1040 [Online]. Available at https://www.researchgate.net/publication/236000961.

Tamura, K. (1992) 'Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases.', *Molecular Biology and Evolution*, vol. 9, no. 4, pp. 678–87 [Online]. DOI:

10.1093/oxfordjournals.molbev.a040752 (Accessed 13 June 2019).

The British Trust for Ornithology (n.d.) *Bank Vole | BTO - British Trust for Ornithology* [Online]. Available at https://www.bto.org/our-science/projects/gbw/gardens-wildlife/garden-mammals/a-z-mammals/bank-vole (Accessed 27 September 2020).

The Deer Initiative (2007) *Deer Legislation*, *England & Wales Best Practice Guides* [Online]. Available at https://www.thedeerinitiative.co.uk/uploads/guides/89.pdf.

The Deer Initiative (2008a) 'Red Deer Species Ecology -', *England & Wales Best Practice Guides* [Online]. Available at www.thedeerinitiative.co.uk.

The Deer Initiative (2008b) 'Species ecology: Roe deer', *England & Wales Best Practice Guides* [Online]. Available at www.thedeerinitiative.co.uk.

The Deer Initiative (2008c) 'Species ecology: Muntjac', *England & Wales Best Practice Guides*, pp. 8–11 [Online]. Available at www.thedeerinitiative.co.uk.

The Deer Initiative (2008d) 'Species ecology: Chinese water deer', *England & Wales Best Practice Guides* [Online]. Available at www.thedeerinitiative.co.uk.

The Deer Initiative (2008e) 'Species Ecology: Fallow deer', *England & Wales Best Practice Guides*, pp. 1–5 [Online]. Available at www.thedeerinitiative.co.uk.

The Mammal Society (2018) *Britain's Mammals: The Mammal Society's Guide to their Population and Conservation Status*, Matthews, F., Coomber, F., Wright, J., and Kendall, T. (eds) [Online]. Available at https://www.mammal.org.uk/science-research/mammal-review-2018-technical-summary/?hilite=British+Mammals+2018.

The Mammal Society (2020) *Species Factsheet : Yellow-necked Mouse (Apodemus flavicollis)*, [Online]. Available at https://www.mammal.org.uk/?s=Yellow+necked+mouse.

The Mammal Society (n.d.) *Species Factsheet – Bank Vole – The Mammal Society* [Online]. Available at https://www.mammal.org.uk/species-hub/full-species-hub/discover-mammals/species-bank-vole/?hilite=%27bank%27%2C%27vole%27

(Accessed 27 September 2020).

The Wildlife Trusts (n.d.) *Bank vole | The Wildlife Trusts* [Online]. Available at https://www.wildlifetrusts.org/wildlife-explorer/mammals/bank-vole (Accessed 27 September 2020).

Tulloch, J. S. P., Semper, A. E., Brooks, T. J. G., Russell, K., Halsby, K. D., Christley, R. M., Radford, A. D., Vivancos, R. and Warner, J. C. (2019) 'The demographics and geographic distribution of laboratory-confirmed Lyme disease cases in England and Wales (2013-2016): An ecological study', *BMJ Open*, vol. 9, no. 8, pp. 1–8 [Online]. DOI: 10.1136/bmjopen-2018-028064.

Valarcher, J. R. F., Hägglund, S., Juremalm, M., Blomqvist, G., Renström, L., Zohari, S., Leijon, M. and Chirico, J. (2015) 'Tick-borne encephalitis', *OIE Revue Scientifique et Technique*, vol. 34, no. 2, pp. 453–466 [Online]. DOI: 10.20506/rst.34.2.2371 (Accessed 13 September 2017).

Vanwambeke, S. O., Šumilo, D., Bormane, A., Lambin, E. F. and Randolph, S. E. (2010) 'Landscape predictors of tick-borne encephalitis in Latvia: Land cover, land use, and land ownership', *Vector-Borne and Zoonotic Diseases*, vol. 10, no. 5, pp. 497–506 [Online]. DOI: 10.1089/vbz.2009.0116.

Vukićević-Radić, Kataranovski, D. and Stamenković, S. (2006) *Spatial organisazation and home range of* Apodemus flavicollis *and* A. agrarius *on Mt. Avala, Serbia*, *Acta Zoologica Academiae Scientiarum Hungaricae*, vol. 52, no. 1.

Waldenström, J., Lundkvist, A., Falk, K. I., Garpmo, U., Bergström, S., Lindegren, G., Sjöstedt, A., Mejlon, H., Fransson, T., Haemig, P. D. and Olsen, B. (2007) 'Migrating birds and tickborne encephalitis virus.', *Emerging infectious diseases*, vol. 13, no. 8, pp. 1215–8 [Online]. DOI: 10.3201/eid1308.061416.

Walkington, J., Hulgur, M., Yates, D. and Eynon, C. A. (2013) 'A case of refractory seizures caused by an unusual zoonosis', *Media, War and Conflict*, vol. 14, no. 1, pp. 65–67 [Online]. DOI: 10.1177/175114371301400113 (Accessed 3 August 2017).

Walter, M., Vogelgesang, J. R., Rubel, F. and Brugger, K. (2020) 'Tick-borne encephalitis virus and its European distribution in ticks and endothermic mammals',

Microorganisms, vol. 8, no. 7, pp. 1–12 [Online]. DOI: 10.3390/microorganisms8071065.

Watts, E. J., Palmer, S. C. F., Bowman, A. S., Irvine, R. J., Smith, A. and Travis, J. M. J. (2009) 'The effect of host movement on viral transmission dynamics in a vector-borne disease system.', *Parasitology*, vol. 136, no. 10, pp. 1221–1234 [Online]. DOI: 10.1017/S0031182009990424.

WHO (2004) 'The Vector Borne-human Infections of Europe. Their Distribution and Burden in Public Health', pp. 27–29 [Online]. Available at http://www.euro.who.int/__data/assets/pdf_file/0008/98765/e82481.pdf (Accessed 28 April 2019).

Wójcik-Fatla, A., Cisak, E., Zajac, V., Zwoliński, J. and Dutkiewicz, J. (2011) 'Prevalence of tick-borne encephalitis virus in *Ixodes ricinus* and *Dermacentor reticulatus* ticks collected from the Lublin region (eastern Poland)', *Ticks and Tick-borne Diseases*, vol. 2, no. 1, pp. 16–19 [Online]. DOI: 10.1016/j.ttbdis.2010.10.001.

Young, D. W. (1935) 'The New Forest', *Forestry*, vol. 9, no. 1, pp. 1–16 [Online]. DOI: 10.1093/oxfordjournals.forestry.a063405.

Ytrehus, B., Vainio, K., Dudman, S. G., Gilray, J. and Willoughby, K. (2013) 'Tick-borne encephalitis virus and Louping-ill virus may co-circulate in southern Norway', *Vector-Borne and Zoonotic Diseases*, vol. 13, no. 10, pp. 762–768 [Online]. DOI: 10.1089/vbz.2012.1023 (Accessed 27 November 2017).

Zeimes, C. B., Olsson, G. E., Hjertqvist, M. and Vanwambeke, S. O. (2014) 'Shaping zoonosis risk: Landscape ecology vs. landscape attractiveness for people, the case of tick-borne encephalitis in Sweden', *Parasites and Vectors*, BioMed Central Ltd., vol. 7, no. 1, pp. 1–10 [Online]. DOI: 10.1186/1756-3305-7-370 (Accessed 24 October 2020).

Zeman, P. and Januška, J. (1999) 'Epizootiologic background of dissimilar distribution of human cases of Lyme borreliosis and tick-borne encephalitis in a joint endemic area', *Comparative Immunology, Microbiology and Infectious Diseases*, vol. 22, no. 4, pp. 247–260 [Online]. DOI: 10.1016/S0147-9571(99)00015-

6.

Zintl, A., Moutailler, S., Stuart, P., Paredis, L., Dutraive, J., Gonzalez, E., O 'connor, J., Devillers, E., Good, B., Omuireagain, C., De Waal, T., Morris, F. and Gray, J. (2017) 'Ticks and Tick-borne diseases in Ireland', *Irish Veterinary Journal*, vol. 70, no. 1, p. 4 [Online]. DOI: 10.1186/s13620-017-0084-y.

Zöldi, V., Papp, T., Rigó, K., Farkas, J. and Egyed, L. (2015) 'A 4-Year study of a natural tick-borne encephalitis virus focus in Hungary, 2010–2013', *EcoHealth*, vol. 12, no. 1, pp. 174–182 [Online]. DOI: 10.1007/s10393-014-0969-0.

Appendices

Appendix 1: List of associated papers

Holding M, Dowall S, Hewson R. Detection of tick-borne encephalitis virus in the UK. Lancet 2020;395:411. doi:10.1016/S0140-6736(20)30040-4.

Holding M, Schmitt HJ, Ellsbury G. TBE in United Kingdom. Chapter 12b. In: Dobler G, Erber W, Bröker M, Schmitt HJ, eds. The TBE Book. 3rd ed. Global Health Press;2020. doi:10.33442/26613980_12b35-3

Holding M, Dowall SD, Medlock JM, Carter DP, Pullan ST, Lewis J, Vipond R, Rocchi MS, Baylis M, Hewson R. Tick-Borne Encephalitis Virus, United Kingdom. Emerg Infect Dis 2020;26:90–6. doi:10.3201/eid2601.191085.

Holding M, Dowall SD, Medlock JM, Carter DP, McGinley L, Curran-French M, Pullan ST, Chamberlain J, Hansford KM, Baylis M, Vipond R, Hewson R. Detection of new endemic focus of tick-borne encephalitis virus (TBEV), Hampshire/Dorset border, England, September 2019. Eurosurveillance 2019;24:1–5. doi:10.2807/1560-7917.ES.2019.24.47.1900658.

Kreusch TM, **Holding M**, Hewson R, Harder T, Medlock JM, Hansford KM, Dowall S, Semper A, Brooks T, Walsh A, Russell K, Wichmann O. A probable case of tick-borne encephalitis (TBE) acquired in England, July 2019. Eurosurveillance 2019;24:1–5. doi:10.2807/1560-7917.ES.2019.24.47.1900679.

Appendix 2: Chapter 2 Deer Serosurveillance Study volunteer documents

UNIVERSITY OF LIVERPOOL

Committee on Research Ethics

PARTICIPANT CONSENT FORM

Title of Research Project: Investigating evidence of tick borne pathogens in deer and UK wildlife

Researcher(s): Maya Holding, Professor Roger Hewson, Dr Jolyon Medlock, Professor Matthew Baylis and Dr Stuart Dowell

1. I confirm that I have read and have understood the four documents provided: Survey Participation and Information Sheet v2.1, Study Protocol v2.0, Tick Information Sheet v2.0 and Risk Assessment for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

2. I understand that my participation in this study is voluntary and that I am free to withdraw my participation at any time without giving any reason, without my rights being affected.

3. I understand that, under Data Protection Act, I can at any time ask for access to the information I provide and I can also request the destruction of that information if I wish.

4. I agree for the samples collected to be tested for evidence of other infectious diseases in future research.

5. I confirm that any samples provided will be obtained from legally culled deer

6. I agree to take part in the above study.

_____   _____   _____
Participant Name   Date   Signature

_____   _____   _____
Researcher   Date   Signature

Principal Investigator:
Name: Matthew Baylis
Work Address: Leahurst Campus, Chester High Road, Neston, Cheshire CH64 7TE
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Name: Maya Holding
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Work Email: Maya.Holding@phe.gov.uk

V2.1 30/05/18

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Version 2.1
June 2013

Ticks on UK Deer and Wildlife Survey Participation and Information Sheet

The project

The NIHR Health Protection Research Unit in Emerging and Zoonotic Infections is a research collaboration between University of Liverpool (UoL), Liverpool School of Tropical Medicine (LSTM) and Public Health England (PHE). This project seeks to **investigate evidence of infections spread by ticks in the UK deer and wildlife population.** Deer are important hosts for the maintenance of large tick populations as they are able to support considerable tick infestations. Ticks can spread a number of tick-borne infectious agents in the UK that can cause disease in humans and animals, such as the infectious agents responsible for Lyme disease, and louping ill.

Deer can be an important resource to carry out surveillance for the study of certain tick-borne pathogens on a national scale due to their wide distribution, large population and ability to carry large number of ticks. However due to being wild animals, opportunities for collection of samples are limited in live animals. Culling, as part of the sustainable management of deer provides is a valuable opportunity to collect samples from euthanised deer, to contribute to scientific knowledge affecting public and animal health. Both ticks and blood can be collected from deer carcasses, these can then be tested for pathogens. Blood samples can also be tested for the presence of antibodies to a pathogen. Screening deer for antibody presence to tick-borne pathogens is also a valuable means of surveillance to monitor for any new emerging viruses entering the UK.

The blood samples will be tested for antibodies to two tick-borne viruses, louping ill virus (LIV), which is endemic in the UK and tick-borne encephalitis virus (TBEV). Current data on the prevalence and distribution of LIV is limited, based on voluntary submissions from livestock with signs indicative of LIV infection. This work aims to contribute to mapping the evidence of LIV distribution in the UK. Tick-borne encephalitis virus (TBEV) has not been detected in the UK, but is present in a number of European countries including Sweden and German, and has recently been detected for the first time in the Netherlands. The recent discovery of TBEV in ticks in the Netherlands was aided by testing deer blood that was collected across the Netherlands. Deer are not thought to be implicated in amplifying these viruses; however they are a valuable resource to look for evidence of these viruses. Submitted ticks will be morphologically identified to species level, where possible. Ticks will be tested for these viruses when sampled from deer in areas in which the antibody testing from the blood samples has provided evidence of virus presence.

This project aims to **assess the geographical distribution and extent of any evidence of infection of tick-borne viruses in UK deer, and the ticks collected from them.** This work will help to aid future surveillance and guide targeted sampling of field collected ticks in risk areas.

What will the field survey entail?

The project seeks to recruit, as volunteers, gamekeepers, deer-stalkers or farmers who are involved in culling deer as part of their routine deer management activities.

Volunteers with the project will be asked to collect a sample of blood, during the process of exsanguination or dressing the animal, when euthanizing deer as part of your routine culling activities.

You will be provided with specialised blood collection tubes for the purpose. You will be asked to record basic information about the animal such as sex, approximate age, date and location culled. You will also be asked to remove any ticks with a tick removal tool that will be provided; ticks will

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need to be placed in a separate tick tube, which will be numbered to match the blood sample. Stamped address envelopes will be provided in order to post the samples to the research team.

When will the study take place?

You are asked to take part in the study during the open seasons for culling deer and other wildlife where applicable up until the end of February 2019.

Why take part

The samples you collect are vital for the success of this project. This will assist in monitoring for the presence of certain tick-borne pathogens, both currently known to be present in the UK and ones that are not yet thought to be present, on a national scale. This will provide useful information to build a picture on the risk of these pathogens in different geographical regions, and also to give guidance on areas in which more research needs to be conducted. Your valuable contribution to facilitating this research, which will help us to answer these important questions is very much appreciated. You will be given feedback on the analysed data from the samples collected once testing has been completed.

Equipment and costs

There will be no financial cost to you. All required equipment will be provided. If you do need any additional equipment, please contact the Project Coordinator. Postage will be pre-arranged and paid for by the research team.

Risks

There should be only minimal additional risks, to those incurred in hunting activities when carrying out this research providing the following guidance is followed.

Precautions that you should take to reduce any risks associated with ticks:

- **Protect any skin abrasions**

  Ensure any cuts or scratches are covered with an adhesive dressing, prior to the handling of the deer.

- **Protect yourself against ticks and check for ticks regularly**

  There is an inherent increased risk of exposure to ticks when deer stalking; please ensure that you are aware of and follow necessary precautions. Public Health England recommend the below actions to reduce the risk of tick bites:

  - Walk on clearly defined paths to avoid brushing against vegetation
  - Wear light-coloured clothes so ticks can be spotted and brushed off
  - Use repellents such as DEET
  - Carry out a tick check – make it a habit to check your clothes and body regularly for ticks when outdoors and again when you get home. Ticks prefer warm, moist places on your body, such as the groin, waist, arm pits, behind the knee and hair lines, so look out for anything as tiny as a freckle or a speck of dirt

  More information about tick awareness can be found at:
  https://www.gov.uk/government/publications/tick-bite-risks-and-prevention-of-lyme-disease

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- **If bitten by a tick remove with tick removal tool immediately**

  If you are bitten by a tick, remove it immediately with the second tick removal tool pack provided. Follow the instructions provided in the Tick Twister pack and apply antiseptic to the bite area or wash with soap and water. Report the bite to the Project Coordinator and monitor for any changes or symptoms for several weeks. Ensure you are familiar with possible symptoms and information to be aware of following a tick bite. If you do notice any symptoms or feel unwell after a tick bite, contact your GP and inform them you were bitten by a tick.

  Information on tick bite awareness and removal can be found at:
  https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/552740/Ticksandyourhealthinfoabouttickbites.pdf

Lyme disease

Lyme disease is a bacterial infection that can be spread to humans if they are bitten by an infected tick. It's estimated there are 2,000 to 3,000 new confirmed cases of Lyme disease in England and Wales each year, with some of these cases being acquired while people are abroad. The disease can be treated effectively if it's detected early on, however if it's not treated or treatment is delayed, there's a risk you could develop long-lasting symptoms. Around two thirds of people with Lyme disease report seeing a distinctive circular rash at the site of the tick bite. The rash is commonly described as looking like the bulls eye at the centre of a dart board. In addition to or instead of a rash people with Lyme disease may experience one or more flu like symptoms such as muscle pain, joint pains, headaches, tiredness, high temperature, chills and neck stiffness. Other symptoms that may be experienced are paralysis of the facial muscles, usually just on one side of the face and/or nerve pain.

If Lyme disease is left untreated early on, other more serious may develop from several weeks to even years later. These can include meningitis, inflammatory arthritis, problems affecting the nervous system and heart problems, such as inflammation of the heart muscle or sac surrounding the heart, heart block or heart failure.

Further information on Lyme disease symptoms, diagnosis and treatment can be found at:

https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/463701/LymeDisease_SignsAndSymptoms.pdf

https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/399239/Lyme_disease_referral_pathway_Jan_2015.pdf

Louping ill

Louping ill is a tick-borne virus, which very rarely causes disease in humans. Fewer than 50 documented human cases of louping ill related disease have ever recorded in the UK, mostly occurring in abattoir workers. Most patients present with no symptoms or display mild influenza like febrile illness. A two phase illness has been reported, with an initial febrile illness followed by a short period of improvement; followed by an encephalitic phase with fever, severe headache, vomiting, drowsiness, neck stiffness and tremor of head and limbs.

Tick-borne encephalitis (TBE)

TBE virus has never been detected in the UK in any host and there have been no human cases of TBE that have been acquired in the UK. In humans, TBE usually has two phases of illness. The initial phase presenting with headache, moderate fever, body pain, fatigue, anorexia and nausea.

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The second phase occurs in approximately half of those with disease. This typically results in severe fever, headache, vomiting and sometimes aversion to light and vertigo.

If you feel unwell with any of the above symptoms

You should see your GP if you feel unwell with any of the symptoms described above after being bitten by a tick, or if you think you may have been bitten. Remember to let your GP know if you've spent time in areas where ticks may live.

Tick removal and sampling of blood

- Ensure the deer has deceased before taking any samples (it is a legal requirement that all samples are collected after death).

- Disposable gloves provided should be worn when sampling blood and ticks from animals.

- Ensure good hygiene practice is used when in contact with blood and ticks, washing hands with soap and water after contact with any blood and ticks.

Removing ticks

Using the equipment provided and following the instructions for the correct use of the Tick Twister to remove the tick, should avoid any need to touch the tick directly. However, if a tick is dropped, please use the tweezers provided, rather than fingers to pick it up.

Sampling blood

Only sample blood when already in contact with the blood through your routine activities.

What if I want to or have to leave the survey team?

There is no obligation to be part of the survey team, and you are free to cease participation at any point. If you do decide to leave the team, we would appreciate it if you could let the Project Coordinator know that you will no longer be taking part in the research.

If you have any questions or issues during this project

You can contact the Project Coordinator at any time, who will be happy to answer any questions relating to the project or arrange any training required to take part in this work. If there is an issue that you feel needs to be addressed by the Research Governance Officer, then please contact them at: ethics@liv.ac.uk – provide the project name or description and the name of the Project Coordinator (in this case the student researcher named below).

Confidentiality

The data that you collect as part of this research project will be analysed and shared with the public. Volunteer anonymity and confidentiality will be fully protected, names and any identifying information will be kept on a secure system which will not be shared or accessed by any other parties.

Contact details- Project Coordinator/student researcher:

For any queries relating to the project please contact maya.holding@phe.gov.uk

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Study Protocol

Please ensure that anyone working on the research project, and collecting samples has read and understood this Study Protocol, Participant Information Sheet and the Tick Information Sheet and signed a copy of the Consent Form

This research study is dependent on data gathered by volunteers handling deer in the course of their work or recreation. We really appreciate your participation and any contribution that you can make to the study which will be acknowledged in the research.

Please ensure the deer has deceased before taking any samples; it is a legal requirement that all samples are collected after death.

Disposable gloves provided should be worn when sampling blood and ticks from animals.

Ensure good hygiene practice is used when in contact with blood and ticks, washing hands with soap and water after contact with any blood and ticks.
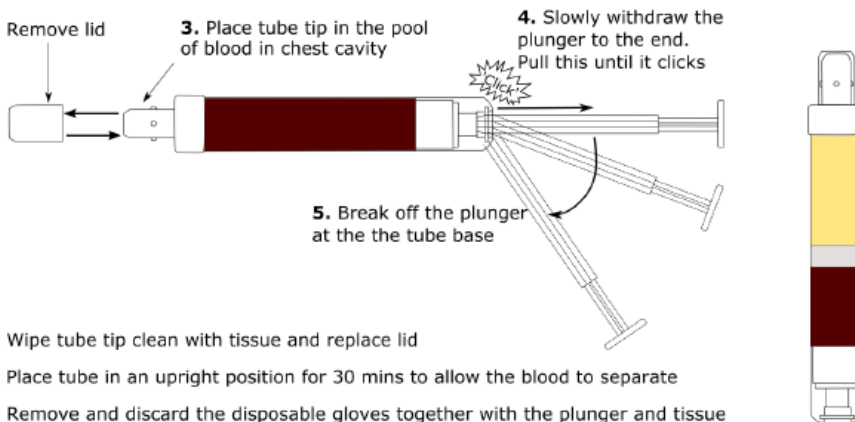
Taking a blood sample from a deer

Please take a blood sample from every deer that you euthanise, if this is practically possible.

Please sample as soon as possible after confirming the deer has deceased, prior to blood coagulation. The blood sample can be taken during dressing the deer or during the process of bleeding.

1. Put on a pair of disposable gloves

2. Remove lid 3. Place tube tip in the pool of blood in chest cavity 4. Slowly withdraw the plunger to the end. Pull this until it clicks

CLICK

5. Break off the plunger at the the tube base

6. Wipe tube tip clean with tissue and replace lid

7. Place tube in an upright position for 30 mins to allow the blood to separate

8. Remove and discard the disposable gloves together with the plunger and tissue

9. The tube is numbered with 'Deer ID number'; please fill in the corresponding Deer Information Form with matching Deer ID. Plese complete each field in the Deer Information Form.

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Removing a tick

1. Put on a pair of the provided disposable gloves.
2. Inspect the tick to see which Tick Twister would be most suitable to use for the size of the tick. For the smaller life stages, use the smaller twister, for adult or very well fed ticks use the larger Tick Twister.
3. Select the empty small tube labelled with the matching deer ID number to the tube used for blood.
4. To use the Tick Twister, slide the hook along the deer's skin to hold the base of the tick and gently twist the hook in the same direction which will result in the tick detaching itself within a few rotations. For more information on ticks and their removal, please see the Tick Information Sheet.
5. After the tick has detached (by releasing its mouth parts from the skin), it will remain in the Tick Twister removal tool. Remove the tick from the Tick Twister by sliding it off of the base of the tick remover using the neck of the new storage tube that you have prepared.
6. This procedure should avoid the need for the volunteer to personally have any contact with the tick.
7. Should a circumstance arise where it is necessary to pick up a dropped tick, please use the tweezers provided.
8. Remove and discard of the disposable gloves.

Recording data

Please complete the fields in the Deer Record Sheet

Please record the date, your name, coordinates where deer was shot and approximate location, habitat deer was shot in, deer species, sex, age category and condition.

Packing and Postage of samples

Please post samples as soon as possible, ideally on the day of shoot, to avoid spoiling of samples.

Please ensure the lids of the tubes are tightly closed. Inset the blood tube in the moulded plastic protective packing wallet, inserting the tip of the tube first. Close the packing wallet firmly, place this together with the tube of ticks and Deer Record Sheet, in the mailing bag, and seal the bag. If samples from two deer have been collected on the same day, these can be posted in the same protective packing wallet and mailing bag. The postage for the mailing bag is pre-paid and addressed; please post this as soon as possible in your nearest post box or post office.

If you have any questions or need any clarification on the methodology, please contact the Project Coordinator: maya.holding@phe.gov.uk

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Example of Deer Record Sheet completion:

Deer Record Sheet

Deer ID:	001		Date:	15/11/2017
Your Name:	John Smith	Coordinates deer was shot / approximate location		OS Grid Ref: SS 98461 40980 Longcombe Hill, Exmoor
Habitat shot in (please tick)	☐ Deciduous forest ☒ Coniferous forest	☐ Lowland heath ☐ Upland heath		☐ Grassland

Culled Deer Information

Species:	☒ Red	☐ Roe	☐ Fallow
	☐ Sika	☐ Muntjac	☐ Chinese water
	☐ Other	(please state)	....
Sex:	☐ Male	☒ Female	
Age category:	☐ Juvenile	☐ Yearling ☒ Adult ☐ Old	
Condition:	☐ Poor	☒ Average ☐ Very Good	

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Tick Information Sheet

What do ticks look like?

Ticks are ectoparasites that have three blood feeding life stages; these are larvae, nymphs and adults. Figure 1 below shows examples of each of these unfed life stages. Their appearance changes as they feed on their host, expanding to many times bigger than their original size.

Figure 1: Tick life stages from left to right, larvae, nymph, adult male, adult female (all unfed)

Photo credit: Medical Entomology and Zoonoses Ecology (PHE)

How to remove ticks

Inspect the tick to see which Tick Twister would be most suitable to use for the size of the tick. For the smaller life stages, use the smaller twister, for adult or very well fed ticks use the larger Tick Twister. To use the Tick Twister slide the hook along the skin to hold the base of the tick as shown in figure 2, gently twist the hook in the same direction, without pulling, which will result in the tick detaching itself within a few rotations.

Figure 2: Diagram illustrating the removal of ticks using the O'Tom Tick Twister

1. Choose the most suitable hook according to the size of the tick.

2. Engage the hook by approaching the tick from the side until it is held.

3. Lift the hook very lightly and **turn it**. The tick detaches by itself after 2 or 3 rotations.

Diagram credit: © O'Tom Tick Twister .

The official O'Tom Tick Twister video demonstrating tick removal using this tool can be viewed by using the following link

http://www.dailymotion.com/video/xe1ip9_tick-removal-with-o-tom-tick-twiste_animals

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