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Badgers (*Meles meles*) as reservoirs of  
vector-borne infections in the UK

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## Table of contents

<b>Abstract</b> .....	1
<b>Chapter 1: Introduction</b> .....	2
1.1 Vector-borne diseases .....	2
1.2 Tick-borne diseases and their ecology .....	2
1.3 Ixodid ticks .....	4
1.3.1 Pathogen transmission .....	5
1.3.2 <i>Ixodes ricinus</i> .....	6
1.3.3 <i>Ixodes hexagonus</i> .....	8
1.3.4 <i>Ixodes canisuga</i> .....	10
1.4 Tick-borne pathogens and their known reservoirs .....	11
1.4.1 <i>Borrelia burgdorferi</i> s. l. ....	11
1.4.2 <i>Babesia</i> spp. ....	14
1.4.3 <i>Anaplasma phagocytophilum</i> .....	16
1.4.4 <i>Rickettsia</i> spp. ....	18
1.5 Eurasian badgers as reservoirs of vector-borne pathogens .....	19
1.6 Aims and objectives .....	22
<b>Chapter 2: Materials and methods</b> .....	23
2.1. Study areas and sample specifications .....	23
2.2 Aseptic dissection of ticks and the maintenance of tick cell cultures .....	25
2.3 DNA extraction .....	27
2.4 RLB for <i>Babesia</i> spp., <i>A. phagocytophilum</i> , and <i>Rickettsia</i> spp. ....	27
2.4.1 PCR amplification .....	28
2.4.2 The RLB protocol .....	29
2.5 Additional polymerase chain reaction assays .....	30
2.5.1 RT-PCR for <i>A. phagocytophilum</i> , <i>Rickettsia</i> and <i>Borrelia</i> spp. ....	33
2.5.2 End-point PCR for <i>Babesia</i> , <i>Rickettsia</i> and <i>Trypanosoma</i> spp. ....	33

2.6 RLB for genotyping of <i>B. burgdorferi</i> s. l. ....	35
2.6.1 PCR amplification .....	35
2.6.2 Membrane probing and RLB analysis .....	36
2.7 DNA sequencing and phylogenetic analysis .....	36
2.8 Statistical analysis .....	37
<b>Chapter 3: Results</b> .....	<b>37</b>
3.1 Badgers from Gloucestershire .....	37
3.2 Ticks from Gloucestershire .....	39
3.3 Badgers from Northeast England .....	40
3.4 Ectoparasites from Northeast England .....	41
3.5 DNA sequencing and phylogenetic analysis .....	43
<b>Chapter 4: Discussion</b> .....	<b>46</b>
<b>References</b> .....	<b>54</b>

#### List of tables

Table 1: Commonly encountered species of Ixodid ticks in the UK .....	5
Table 2: List of PCR primers, probes and their targets .....	30
Table 3: List of RLB probes and their targets .....	32
Table 4: Infections detected in badgers and questing ticks from Gloucestershire .....	39
Table 5: Infections detected in badgers and ticks from Northeast England .....	41

#### List of figures

Figure 1: Life stages of <i>I. ricinus</i> .....	8
Figure 2: <i>I. hexagonus</i> and <i>I. canisuga</i> .....	10
Figure 3: Ordnance survey map of Woodchester Park, Gloucestershire .....	24
Figure 4: Ordnance survey map of Northeast England .....	24
Figure 5: Infestation rates of captured badgers .....	42

Figure 6: Phylogenetic tree based on aligned sequences of the 18S rRNA gene of <i>Babesia</i> spp. ....	44
Figure 7: Phylogenetic tree based on aligned sequences of the $\beta$ -tubulin gene of <i>Babesia</i> spp. ....	45
Appendix 1: Reverse line blot solutions .....	90

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## Abstract

In recent years, there has been an increased incidence and changing distribution of a number of vector-borne diseases, and the temperate regions are not an exception. Whilst Eurasian badgers (*Meles meles*) are known to host a wide range of pathogens, information on haemoparasites of badgers, their role as reservoirs of vector-borne infections, and whether ticks parasitizing these badgers pose any risk to animal or human health is limited. Whole badger blood samples collected at Woodchester Park in Gloucestershire, Southwest England and from around Northeast England, and ticks parasitizing these badgers, were analysed with a battery of assays targeting the DNA of *Babesia* spp., *Anaplasma phagocytophilum*, *Rickettsia*, *Borrelia* and *Trypanosoma* spp. While badgers were found to be heavily infected with *Babesia* spp. (98.9% of blood samples from Gloucestershire and 100% from NE England), no *A. phagocytophilum* or *Rickettsia* spp. were detected. Whereas rates of infections with *Trypanosoma* spp. in badgers from Gloucestershire have been previously determined in another study, no Trypanosomes were found in badgers from Northeast England. As for ticks, the DNA of *Babesia* spp., *A. phagocytophilum*, *Rickettsia helvetica*, *Borrelia garinii*, *Borrelia valaisiana*, and *Borrelia afzelii* were detected in questing ticks at Woodchester Park; only *Babesia* spp. were found in ticks removed from badgers in NE England. All Babesiae found in badgers were identical to each other and to those found in *I. canisuga* from NE England and questing *I. ricinus* from Gloucestershire, and closely related to *Babesia annae* (*Babesia vulpes*) associated with foxes. Furthermore, there was an evidence of chronic *Babesia* infections among badgers, and possibly of vertical transmission. Phylogenetic trees based on the analysis of two genetic markers, namely the 18S rRNA and  $\beta$ -tubulin genes, demonstrate the relatedness of *Babesia* spp. detected in this study to other known species of *Babesia*.

## Chapter 1: Introduction

### 1.1 Vector-borne diseases

In recent years, there has been a re-emergence of a number of vector-borne diseases, changes in their distribution and their increasing incidence, and even emergence of new vector-borne diseases worldwide (Beugnet & Marié, 2009; Bitam *et al.*, 2010; Medlock & Leach, 2015; Schiffman *et al.*, 2016; Semenza & Suk, 2018; Shragai *et al.*, 2017; Sutherst, 2004). Mosquitoes, ticks and fleas are vectors of numerous such diseases. For instance, mosquito-borne dengue, chikungunya and Zika epidemics, as well as tick-borne Lyme disease, canine babesiosis and granulocytic anaplasmosis, increased in incidence and geographical range (Beugnet & Marié, 2009; Rizzoli *et al.*, 2014; Shragai *et al.*, 2017); while the knowledge and importance of tick-borne rickettsioses have evolved radically (Parola *et al.*, 2013). Additionally, a new agent of flea-borne spotted fever in humans has emerged (Beugnet & Marié, 2009), and plague has been classified as re-emerging (Bitam *et al.*, 2010). Ixodid ticks are second only to mosquitoes as vectors of human diseases and the main vectors of animal diseases (De la Fuente *et al.*, 2016). All tick-borne diseases are maintained in enzootic cycles between their animal reservoirs and vectors. Therefore, to assess the risks vector-borne infections pose to animal or human health and develop strategies for disease control and prevention, it is not only important to identify their competent vector species, their distribution and infection rates, but also the reservoirs of these pathogens in nature.

### 1.2 Tick-borne diseases and their ecology

A number of biotic and abiotic factors have been associated with the changing epidemiology of tick-borne diseases - be it the changing patterns of an endemic disease or an establishment of pathogens in new areas. Over the years, changes in conservation policies and land use, and landscape reshaping have had unintentional consequences on the incidence of endemic vector-borne diseases through creation of new ecosystems and thriving wildlife, which are able to support large numbers of ticks (Medlock *et al.*, 2013; Millins

*et al.*, 2017; Rizzoli *et al.*, 2014). Additionally, global warming, among other things, has a positive impact on the survival, development and prolongation of host-seeking behaviour of questing ticks (Beugnet & Chavet-Monfray, 2013; Li *et al.*, 2016; Medlock *et al.*, 2013). Milder winters and warmer springs allow ticks to quest earlier in the year or remain active all year round, and warmer summers favour their interstadial development (Beugnet & Marié, 2009; Medlock *et al.*, 2013). Ticks have now been reported in new locations and their abundance appears to have increased in known endemic areas (Cull *et al.*, 2018; Jameson & Medlock, 2011; Medlock *et al.*, 2013; Scharlemann *et al.*, 2008); and predictive models show that climate warming is likely to lead to further increases in their distribution and abundance (Li *et al.*, 2016). Additionally, ticks typically engorge slowly, hence they can be dispersed by their hosts (Medlock *et al.*, 2009). For instance, the carriage of feeding *I. ricinus* by birds and large mammals is crucial for their short- and long-range dispersal (Medlock *et al.*, 2013). Further still, carriage of infected ticks on pets may result in the introduction of tick-borne pathogens and their establishment in previously non-endemic areas (Fernández de Marco *et al.*, 2017; Sánchez-Vizcaíno *et al.*, 2016; Wright, 2018). Certain outdoor leisure activities of people can increase their exposure, or their canine companions, to ticks and thus can increase the likelihood of a tick-bite (Mannelli *et al.*, 2012; Rizzoli *et al.*, 2014). However, it has to be emphasised that an increasing awareness of ticks and the diseases they transmit among clinicians and the general public, recognition of disease manifestations, and better reporting, have likely partially driven the rising incidence of some tick-borne diseases (Alvarado-Rybak *et al.*, 2016; Ostfeld & Brunner, 2015).

For a host to fulfil the role of a competent reservoir for a given pathogen, the host must be fed on by an infected tick, get infected, and allow the organism to multiply and survive, to be transmitted to subsequently feeding ticks (Hillyard, 1996; Stuen *et al.*, 2013). Consequently, the local composition and abundance of competent reservoir hosts and competent vectors for these pathogens are key factors in the maintenance and transmission of tick-borne infections in a given area (Mannelli *et al.*, 2012; Rizzoli *et al.*, 2014). The three most commonly encountered species of Ixodid ticks parasitizing domestic animals and



humans in the UK are the exophilic *Ixodes ricinus* (Figure 1), and the nidicolous *Ixodes hexagonus* and *Ixodes canisuga* (Figure 2) (Abdullah *et al.*, 2016; Cull *et al.*, 2018; Ogden *et al.*, 2000; Smith *et al.*, 2011). Particular attention has been given to *I. ricinus* but not much to the latter two species. These ticks are known to parasitize a number of wildlife animals (Table 1), including Eurasian badgers (*Meles meles*) (Bartley *et al.*, 2017; Hillyard, 1996), and whilst badgers are known to host a wide range of pathogens, information on haemoparasites of British badgers and their role as reservoirs of tick-borne infections is limited.

### 1.3 Ixodid ticks

Ixodid ticks have three active life stages, also known as instars - larval, nymphal, and adult (female and male). Each instar takes a single large bloodmeal from a single host in order to moult from one life stage to another and to eventually complete their life cycle and reproduce (Hillyard, 1996; Kazimírová & Štibrániová, 2013), except for the non-feeding males of some species (Capinera, 2008). *I. ricinus* leave their ground microhabitat of foliage, detritus and moss and climb up the vegetation to find a host (Tomkins *et al.*, 2014). On the other hand, nidicolous or nest-dwelling ticks are more likely to be encountered by dogs and cats, and only occasionally by humans (Table 1), due to their close association with their primary hosts (Bowman & Nuttall, 2008; Hillyard, 1996; Jameson & Medlock, 2011). Infection rates among ticks typically vary depending on the region as well as the site from which they have been collected (Abdullah *et al.*, 2017; Bettridge *et al.*, 2013; Hall *et al.*, 2017; Smith *et al.*, 2012, 2013; Tjisse-Klasen *et al.*, 2011).

**Table 1:** Commonly encountered species of Ixodid ticks in the UK

Tick species (common name)	Known hosts	Pathogens found in the UK *
<i>Ixodes ricinus</i> (sheep/deer tick)	Commonly attaches to a wide range of small-large wild and domestic mammals, birds, companion animals, and humans <sup>9,10</sup>	<i>B. burgdorferi</i> s. l. <sup>1,2,4,6</sup> <i>Borrelia miyamotoi</i> <sup>4,5</sup> <i>A. phagocytophilum</i> <sup>5,7</sup> <i>Babesia divergens</i> <sup>1,7</sup> <i>Babesia venatorum</i> <sup>1,3,7</sup> <i>Babesia gibsoni</i> <sup>7</sup> <i>Babesia annae</i> <sup>1</sup> (formerly <i>Theileria annae</i> ) <i>Rickettsia helvetica</i> <sup>8</sup>
<i>Ixodes hexagonus</i> (hedgehog tick)	Badgers, foxes, hedgehogs, dogs, cats, occasionally humans, rarely sheep or cattle <sup>9,10</sup>	<i>Borrelia afzelii</i> <sup>1</sup> <i>B. venatorum</i> ( <i>Babesia</i> sp. EU1) <sup>1</sup> <i>B. annae</i> <sup>1,3</sup> <i>A. phagocytophilum</i> <sup>7</sup>
<i>Ixodes canisuga</i> (fox tick)	Badgers, foxes, dogs, and cats <sup>9,10</sup>	<i>B. venatorum</i> <sup>1,7</sup>

\* Based on DNA-based studies

1 Abdullah *et al.*, 2017

2 Bettridge *et al.*, 2013

3 Davies *et al.*, 2017

4 Hall *et al.*, 2017

5 Hansford *et al.*, 2015

6 Smith *et al.*, 2012

7 Smith *et al.*, 2013

8 Tijssen-Klasen *et al.*, 2011

9 Arthur, 1963

10 Hillyard, 1996

### 1.3.1 Pathogen transmission

In order to fully engorge and complete their feed, ticks inject saliva with a large number of biologically active molecules including anticoagulants, cytolytic, vasoactive mediators and histamine blocking agents (Zintl *et al.*, 2017) into the host's bloodstream during the process of penetration and feeding. Tick-borne pathogens exploit this process for transmission to susceptible hosts (Kazimírová & Štibrániová, 2013). Once a tick acquires

the infectious agent from an infected animal through a bloodmeal, it disseminates throughout the tick's organs, usually without affecting the fitness of the tick (Zintl *et al.*, 2017), where its presence is maintained through successive moults from one life stage to another. It is then transmitted to other hosts during subsequent blood meals. This is known as transstadial transmission (Hillyard, 1996; Rikihisa, 2011). In reservoir competent ticks, the organism is further passed transovarially from one generation to another (Hillyard, 1996).

Through the years, the DNA of *Borrelia burgdorferi* s.l., *Babesia* spp., *Rickettsia helvetica* and *A. phagocytophilum* have been detected in tick larvae (Hall *et al.*, 2017; Jahfari *et al.*, 2014a; Krücken *et al.*, 2013; Schorn *et al.*, 2011; Sprong *et al.*, 2009; Tappe *et al.*, 2014; Welc-Falęciak *et al.*, 2012), indicating that ticks could serve both as vectors and reservoirs for these pathogens. However, a pathogen's capacity for transovarial transmission cannot be based solely on detection of its DNA in larval ticks, and an acquisition of the organism by larvae from the previous generation and a possibility of subsequent transmission to susceptible hosts must be demonstrated. Transovarial transmission has now been confirmed for numerous species of *Babesia* (Bonnet *et al.*, 2007a, 2007b, 2009; Higuchi *et al.*, 1995; Howell *et al.*, 2007), and *R. helvetica* (Burgdorfer *et al.*, 1979). As for *Borrelia*, while the potential for transovarial transmission has been demonstrated for *B. afzelii*, and possibly *B. burgdorferi* (Bellet-Edimo *et al.*, 2005; Van Duijvendijk *et al.*, 2016; Toutoungi & Gern, 1993), the topic of transovarial transmission of *Borrelia burgdorferi* s. l. in ticks remains controversial. Further studies are necessary to investigate the possibility of transovarial transmission for *A. phagocytophilum* (Jahfari *et al.*, 2014a).

### 1.3.2 *Ixodes ricinus*

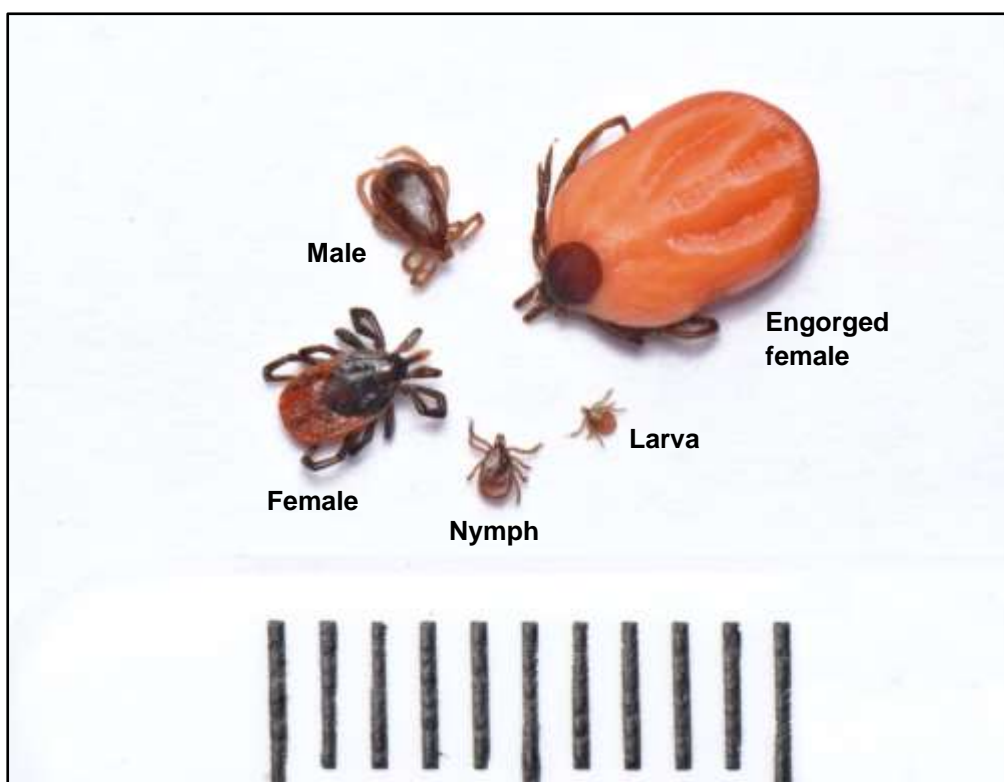
*I. ricinus*, commonly known as the castor bean, sheep or deer tick (Figure 1), is the most abundant and widespread species of ticks in Europe (Cull *et al.*, 2018; Rizzoli *et al.*, 2014). Its success is driven by its ability to withstand a wide range of temperatures and climatic conditions, and feed on virtually all vertebrates sharing its habitat (Medlock *et al.*,

2013). *I. ricinus* is opportunistic and will feed on humans when the chance arises (Medlock *et al.*, 2013). Typical habitats of *I. ricinus* range from deciduous and mixed woodlands to shrubs, pasture grassland and urban parks, in which foliage provides the critical humid microclimatic conditions for their survival and development (Hillyard, 1996; Medlock *et al.*, 2008, 2013). This exceptional adaptability makes it the most ubiquitous tick species in Europe (Jameson & Medlock, 2011). Its life cycle typically takes about 2 to 3 years to complete, depending on the climate, host availability and the effects of development-delaying diapause mechanisms (Hillyard, 1996; Stanek *et al.*, 2012).

As a result of feeding on a broad range of animals, this tick can transmit a great variety of pathogens of veterinary and medical importance (Hillyard, 1996; Raileanu *et al.*, 2017). Tick bites are generally painless and the risk of infection increases with the longer the tick remains attached (Public Health England, 2015). To date, British *I. ricinus* have been found to carry *Borrelia burgdorferi* sensu lato (s. l.), *Borrelia miyamotoi*, *Anaplasma phagocytophilum*, *Babesia divergens*, *Babesia venatorum*, *Babesia gibsoni*, *Babesia annae/Babesia vulpes* (formerly *Theileria annae*), and *Rickettsia helvetica* (Table 1). Questing larvae (Figure 1) can only be infected with pathogens capable of transovarial transmission in ticks (Moutailler *et al.*, 2016), and while the rates of infection with these pathogens among larvae may be relatively low or may not have been fully proven, this life stage should not be disregarded as a vector (Hall *et al.*, 2017). By contrast, questing nymphs (Figure 1) could have already been exposed to more pathogens during their bloodmeal as larvae. Nymphal ticks are typically the main sources of infections due to their relative abundance (Zintl *et al.*, 2017) and because they can be easily overlooked due to their relatively small size (Jameson and Medlock, 2011; Moutailler *et al.*, 2016). Subsequently, some tick bites go unnoticed and a history of a tick bite is not always available when diagnosing a patient (O'Connell, 2009).

*I. ricinus* may also harbour more than one pathogen simultaneously. Co-infections can cause atypical presentations of disease (O'Connell, 2009), may exacerbate symptoms of the disease, or may have consequences in terms of diagnosis and treatment (Diuk-Wasser *et al.*, 2016). Co-infections in questing adults are even more likely than in questing

nymphs since they would have ingested two bloodmeals already (during their larval and nymphal stages) (Moutailler *et al.*, 2016). However, they are also more likely to be brushed off before attaching to humans (Jameson & Medlock, 2011), or removed earlier than nymphal ticks (Moutailler *et al.*, 2016), thus minimising the chance of pathogen transmission. Transmission by co-feeding of infected and uninfected ticks, in the absence of a systemic infection in the host, has also been demonstrated *in vitro* (Gern & Rais, 1996; Randolph *et al.*, 1996).



**Figure 1:** Life stages of *I. ricinus*. Measuring scale is 1 cm long.

(photo by Andrea Gbobaniyi)

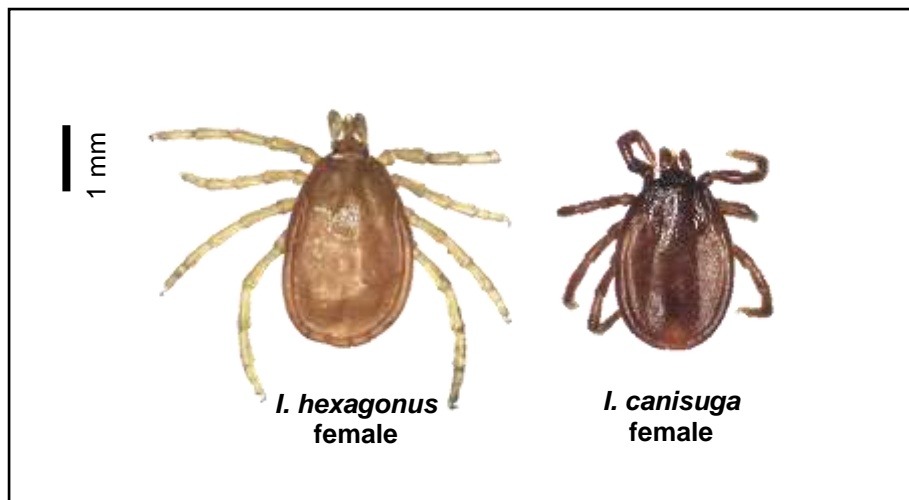
### 1.3.3 *Ixodes hexagonus*

*I. hexagonus*, also known as the hedgehog tick (Hillyard, 1996), is the second most commonly encountered species of ticks by pets in the UK (Abdullah *et al.*, 2016; Davies *et al.*, 2017; Ogden *et al.*, 2000; Smith *et al.*, 2011). The species is found all over the UK, although less so in the North (Davies *et al.*, 2017; Smith *et al.*, 2011). The incidence of *I.*

*hexagonus* in urban environments is attributed to presence of suitable hosts in these areas (Gray *et al.*, 2002). The species has a wide range of hosts, although more restricted than that of *I. ricinus* (Table 1), and can be found in and around nests and burrows of their hosts (Hillyard, 1996).

Whereas the tick once commonly used to infest humans under crowded war-time conditions in the London area and in underground air raid shelters where hedgehogs also sought refuge (Arthur, 1963; Medlock *et al.*, 2009), humans do not tend to encounter these ticks as frequently no more due to the ticks' close association with their primary hosts (Bowman & Nuttall, 2008; Cull *et al.*, 2018, Jameson & Medlock, 2011). While *I. ricinus* is the main vector for *B. burgorferi* s. l., *in vitro* studies demonstrated the competence of *I. hexagonus* towards the transmission of Borreliae as well (Gern *et al.*, 1991; Toutoungi & Gern, 1993), and Borreliae have been found in British *I. hexagonus*, albeit at a low prevalence (Abdullah *et al.*, 2017; Smith *et al.*, 2012). As the tick rarely feeds on humans, their main significance is their contribution to the natural circulation of the spirochaetes (Mannelli *et al.*, 2012). Additionally, *I. hexagonus* and *I. ricinus* often feed on their common hosts at the same time, thus some pathogens may be shared via co-feeding or host infection (Pfäffle *et al.*, 2011). Furthermore, the DNA of *B. venatorum* & *B. annae*, organisms of medical and veterinary importance, respectively, as well as of *A. phagocytophilum*, have also been detected in British *I. hexagonus* (Table 1). However, no further analysis to identify the genetic variants of *A. phagocytophilum* detected in *I. hexagonus* was carried out (Smith *et al.*, 2013), meaning that limited information on *A. phagocytophilum* infecting the hedgehog tick in Britain is available. Although, vector competence of *I. hexagonus* for *A. phagocytophilum* has not been established and thus requires further investigation (Dumitrache *et al.*, 2015). As for Rickettsiae, while no DNA of *Rickettsia* spp. has been detected in *I. hexagonus* in the UK, *R. helvetica* has been previously detected in *I. hexagonus* removed from hedgehogs in Belgium (Jahfari *et al.*, 2017). Additionally, the tick has been proposed as a likely vector of *Babesia annae*, because endemic areas of *B. annae*

infections in Spain closely match the distribution range of *I. hexagonus* (Camacho *et al.*, 2003).



**Figure 2:** *I. hexagonus* and *I. canisuga*

(Figure adapted from Bristol University Tick ID, University of Bristol, 2017)

#### 1.3.4 *Ixodes canisuga*

*I. canisuga*, the least documented and studied species of the three, is also found throughout the UK, albeit at lower numbers than *I. hexagonus* (Abdullah *et al.*, 2017; Ogden *et al.*, 2000; Smith *et al.*, 2011). The tick tolerates much drier conditions than *I. ricinus* (Hillyard, 1996), and typically feeds on medium to large-sized mammals living in burrows and kennels - such as badgers, red foxes, and dogs (Arthur, 1963; Hillyard, 1996). To date, only the DNA of zoonotic *B. venatorum* has been detected in these ticks in the UK (Abdullah *et al.*, 2017; Smith *et al.*, 2013). It has to be emphasised however, that the detection of a pathogen's DNA in a tick does not demonstrate its vector competence and further studies are required to ascertain the tick's competence for *B. venatorum*. Furthermore, *B. annae* was found in *I. canisuga* parasitizing red foxes in Germany, suggesting that the tick could possibly act as a vector of this pathogen, additionally to *I. hexagonus* (Najm *et al.*, 2014). Afterall, one fairly understudied aspect of *Babesia* transmission is the use of "bridge vectors"

(Yabsley & Shock, 2013), which may contribute to the maintenance cycles of this pathogen in nature.

## 1.4 Tick-borne pathogens and their known reservoirs

### 1.4.1 *Borrelia burgdorferi* s. l.

Bacteria of the *B. burgdorferi* s. l. complex that cause Lyme borreliosis, also referred to as Lyme disease, are highly motile spirochaetes able of swimming through highly viscous media, penetrating host tissues, and disseminating throughout their host (Tilly *et al.*, 2008). Lyme disease is the most prevalent and widespread vector-borne zoonosis of the temperate northern hemisphere, despite substantial surveillance and control efforts (O'Connell, 2009; Rizzoli *et al.*, 2011), and the UK is not an exception. Approximately 1,000 to 1,300 laboratory-confirmed cases of borreliosis are reported in the UK annually, the majority of which are indigenously acquired (Public Health England, 2017). Since not all cases are confirmed by laboratory testing (Public Health England, 2015) and many more infections go unnoticed, it has been estimated that as many as 2,000 to 3,000 people develop Lyme disease each year in England and Wales alone (Public Health England, 2015).

While the earliest clinical reference to Lyme borreliosis dates back to as early as 1883 (Burgdorfer, 1993), it was not until the 1970s that the disease became widely known as Lyme disease - after the town of Lyme in Connecticut, where a number of children were thought to have developed juvenile rheumatoid arthritis, which later became clear was a late manifestation of a tick-transmitted disease (Steere *et al.*, 2004). This was followed by the identification of *B. burgdorferi* by Burgdorfer and colleagues (Burgdorfer *et al.*, 1982, 1983). Over the years, different genospecies of what is now known as the *B. burgdorferi* s. l. complex have been identified. These genospecies are associated with different niches, and vary in terms of pathogenicity and clinical manifestations (James *et al.*, 2014). Four genospecies associated with disease in humans are endemic in the UK – *Borrelia garinii*, *Borrelia afzelii*, *Borrelia burgdorferi* sensu stricto (s. s.), and *Borrelia valaisiana* (Bettridge *et al.*, 2013; Hall *et al.*, 2017; Hansford *et al.*, 2017; Millins *et al.*, 2016). Several more



genospecies of *Borrelia* – *Borrelia lusitaniae*, *Borrelia bissettii*, *Borrelia spielmanii* and *Borrelia bavariensis* – are encountered in continental Europe (Mannelli *et al.*, 2012). While *B. garinii* is associated only with neuroborreliosis (NB) (O’Connell, 2014), *B. afzelii* is associated with a skin condition referred to as acrodermatitis chronica atrophicans as well as neurological manifestations (O’Connell, 2014; Stanek *et al.*, 2011). *B. garinii* was previously thought to circulate in birds (Hanincová *et al.*, 2003b; Humair *et al.*, 1998; Kurtenbach *et al.*, 1998) but studies suggest that small mammals, such as grey squirrels (*Sciurus carolinensis*) and hedgehogs (*Erinaceus europaeus*), may play a role as well (Gern *et al.*, 1997; Millins *et al.*, 2015; Skuballa *et al.*, 2007). By contrast, *B. afzelii* appear to circulate in small and medium-sized mammals, including small rodents (Gassner *et al.*, 2013; Hanincová *et al.*, 2003a; Humair *et al.*, 1999), grey squirrels (Millins *et al.*, 2015), foxes (Dumitrache *et al.*, 2015), hedgehogs (Gern *et al.*, 1997), and badgers (Gern & Sell, 2009; Wodecka *et al.*, 2016). *B. burgdorferi* (s. s.) is mostly linked to arthritis (Stanek *et al.*, 2012), which is uncommon in the UK (O’Connell, 2014), and appears to be maintained at low levels by certain species of rodents (Kurtenbach *et al.*, 1998; Michalik *et al.*, 2005). As for *B. valaisiana*, the DNA of which was previously detected in a cerebrospinal fluid of a patient with a progressive spastic paraparesis (Diza *et al.*, 2004), the pathogenicity of this genospecies remains to be clarified (Mannelli *et al.*, 2012). This genospecies appears to circulate in birds (Humair *et al.*, 1998; Heylen *et al.*, 2014). However, earlier this year emerged the first report of *Borrelia spielmanii* in a tick infesting a domestic dog with no recent travel outside the UK (Abdullah *et al.*, 2017). The genospecies was previously thought to be found in mainland Europe only and appears to be associated with skin lesions (Mannelli *et al.*, 2012, Wang *et al.*, 1999). These *Borrelia* differ from *Borrelia miyamotoi*, a relapsing fever (RLF) spirochete (Fukunaga *et al.*, 1995; Jahfari *et al.*, 2014b; Platonov *et al.*, 2011), which is generally associated with non-specific relapsing febrile illness episodes accompanied by flu-like symptoms (Krause *et al.*, 2015; Platonov *et al.*, 2011), and rarely meningoencephalitis (Gugliotta *et al.*, 2013; Hovius *et al.*, 2013).

Clinical manifestations of borreliosis are customarily divided into three stages. An “early localized infection (stage I)” is commonly manifested by erythema migrans (Schánilec *et al.*, 2010), a localized red or faint pink lesion (or a red patch) at the site of a bite, with or without central clearing where previously affected skin returned to normal (O’Connell, 2014; Stanek *et al.*, 2011). Yet, around one in every three people presenting with Lyme disease do not recall preceding erythema migrans (Public Health England, 2015). Patients can also experience non-specific flu-like symptoms; although some early infections can go asymptomatic (O’Connell, 2014). This is followed by an “early disseminated infection (stage II)” involving more than one organ system and manifesting itself by musculoskeletal pains, variable neurological symptoms and occasionally cardiac manifestations (O’Connell, 2014; Schánilec *et al.*, 2010); Lyme carditis is among the rarer manifestations and cardiac conduction abnormalities are typically reversible (Scheffold *et al.*, 2015). “Late persistent infections (stage III)” are manifested by chronic symptoms such as recurrent arthritis, chronic neurological disorders and acrodermatitis chronica atrophicans, depending on the genospecies of *Borrelia* involved (O’Connell, 2009; Schánilec *et al.*, 2010).

A full recovery of patients with neuroborreliosis can take several months following an appropriate treatment, but in rare cases, when the diagnosis is made late in the course of the disease, patients may experience incomplete recovery from severe neurological symptoms. Some patients also tend to report non-specific, recurrent or persistent symptoms such as musculoskeletal pains, disturbances in concentration and memory, reduced performance, or increased fatigue following an appropriate treatment. These are collectively known as the post-Lyme syndrome (Stanek *et al.*, 2011, 2012). Dogs also show different manifestations of Lyme disease, however erythema migrans is absent in canines (Skotarczak, 2014). *B. burgdorferi* s. s. appears to be the most dominant genospecies detected in dogs (Skotarczak, 2014), with disease commonly manifested by malaise, fever, lameness, swelling of joints, loss of body weight and migratory arthritis (Skotarczak *et al.*, 2005; Skotarczak, 2014). Neurological manifestations caused by *B. garinii* (Kybicová *et al.*, 2009; Schánilec *et al.*, 2010) and myocarditis (Janus *et al.*, 2014) have also been described.

### 1.4.2 *Babesia* spp.

*Babesia* spp. are intraerythrocytic piroplasmids of the phylum Apicomplexa. They have been known pathogens in veterinary medicine since the end of the 19th century (Solano-Gallego & Baneth, 2011; Solano-Gallego *et al.*, 2016), and are of increasing threat to public health (Ord, 2015; Rizzoli *et al.*, 2014). Infections can have a broad spectrum of manifestations, ranging from an apparently silent infection to a malaria-like disease known as babesiosis (Homer *et al.*, 2000); clinical features of babesiosis can vary from asymptomatic to life threatening, depending on the condition of the patient and the species of parasite involved (Hunfeld *et al.*, 2008; Solano-Gallego *et al.*, 2016).

The common cause of human babesiosis, and redwater fever in cattle, is *Babesia divergens*, species capable of causing severe disease in asplenic individuals and those with weakened or compromised immune system (Hunfeld *et al.*, 2008; Schnittger *et al.*, 2012; Vannier *et al.*, 2008); although cases remain rare (Zintl *et al.*, 2017). Other zoonotic species of Babesiae in Europe include *Babesia venatorum* (also known as *Babesia* sp. EU1) and *Babesia microti*. *B. venatorum* causes mild to moderate disease and *Babesia microti* is only of limited concern to humans in mainland Europe (Homer *et al.*, 2000; Meer-Scherrer *et al.*, 2004; Yabsley & Shock, 2013), and is transmitted by *Ixodes trianguliceps* in the UK – a tick that does not readily bite humans (Bown *et al.*, 2011; Turner *et al.*, 2014). While zoonotic *B. divergens* and *B. venatorum* are maintained in wild and domestic ruminants (Andersson *et al.*, 2016; Bonnet *et al.*, 2007a; Cézanne *et al.*, 2017; Kauffmann *et al.*, 2017; Michel *et al.*, 2014; Zintl *et al.*, 2003), and *B. microti* in rodents (Bown *et al.*, 2008; 2011), wild carnivores appear to be the reservoirs of species of canine health importance.

Several species of Babesiae of canine health importance are found in Europe (Solano-Gallego & Baneth, 2011), including *Babesia canis* (which is now endemic in the UK), *Babesia gibsoni*, and *Babesia annae*. While *B. canis* is transmitted by *Dermacentor reticulatus* (De Marco *et al.*, 2017), *B. gibsoni* and *B. annae* (two closely related emerging pathogens of dogs in mainland Europe) have been detected in both *I. ricinus* and *I. hexagonus* (Abdullah *et al.*, 2017; Smith *et al.*, 2013). *B. annae*, was first observed in

Northwest Spain in 1996 (Camacho *et al.*, 2001) and a German dog with a history of travel to northern Spain (Zahler *et al.*, 2000). Subsequently, a species name of *T. annae* was proposed (Zahler *et al.*, 2000), however, the pathogen is also known as *Babesia* 'Spanish dog' isolate (Yeagley *et al.*, 2009), *Babesia microti*-like (Camacho *et al.*, 2004; Simões *et al.*, 2011) and *Babesia vulpes* (Baneth *et al.*, 2015). The species is known to cause severe disease in dogs in Spain (Camacho *et al.*, 2001, 2004; García, 2006; Miró *et al.*, 2015), but has also been reported in Portugal (Simões *et al.*, 2011), France (René-Martellet *et al.*, 2015), Sweden (Falkenö *et al.*, 2013) and Croatia (Beck *et al.*, 2009). Infections are characterized by fever, lethargy, pale mucous membranes, tachycardia, tachypnoea, severe haemolysis, haemoglobinuria, moderate to severe regenerative anaemia, thrombocytopenia, anorexia, acute renal failure, and azotemia – the main cause of death (Camacho *et al.*, 2004; Falkenö *et al.*, 2013; Miró *et al.*, 2015; Simões *et al.*, 2011). Alternative routes of transplacental transmission (Falkenö *et al.*, 2013; Fukumoto *et al.*, 2005; Simões *et al.*, 2011) and via biting wounds (Jefferies *et al.*, 2007; Yeagley *et al.*, 2009) have also been described for both *B. annae* and *B. gibsoni*.

Red foxes (*Vulpes vulpes*) are the only known natural reservoir of *B. annae* to date (Baneth *et al.*, 2015). The pathogen has been detected in European (Barandika *et al.*, 2016; Cardoso *et al.*, 2013; Criado-Fornelio *et al.*, 2003; Deždek *et al.*, 2011; Duscher *et al.*, 2014; Farkas *et al.*, 2015; Najm *et al.*, 2014) and British foxes (Alharbi, 2018; Bartley *et al.*, 2016) alike. As for *B. gibsoni*, limited information is available on the natural reservoir of this species. Foxes may harbour this species as well, as the pathogen was detected in foxes from Egypt in 1970, although caution should be exercised when making inferences about species of *Babesia* from historical literature (Penzhorn, 2006). While both species of *Babesia* have been detected in ticks collected from dogs from across Great Britain (Abdullah *et al.*, 2017, Smith *et al.*, 2013), there are no records of *B. annae* having been previously detected in British dogs (Bartley *et al.*, 2016), and little is known about the extent to which *B. gibsoni* contributes to canine babesiosis in the UK. Currently, most cases appear

to be most likely acquired through overseas travels (Sánchez-Vizcaíno *et al.*, 2016) and caused by the now endemic *B. canis* (De Marco *et al.*, 2017).

### 1.4.3 *Anaplasma phagocytophilum*

*A. phagocytophilum*, previously known as *Ehrlichia phagocytophila* and *Ehrlichia equi* (Kohn *et al.*, 2008), are intracellular bacteria infecting granulocytic cells (particularly neutrophils but also eosinophils) of their hosts (Carrade *et al.*, 2009; Woldehiwet, 2006). *A. phagocytophilum* has been known since the 1930s as the agent of tick-borne fever in sheep and since the 1950s as the agent of pasture fever in cattle (Beugnet & Marié, 2009). Since then, multiple genetic variants of the pathogen have been described, each displaying a different vector tropism, host range, degree of host pathogenicity, and a potential to be of public health importance (Bown *et al.*, 2009; Kallio *et al.*, 2014; Stuen *et al.*, 2013; Welch-Faleciak *et al.*, 2014). *A. phagocytophilum* is the most common tick-borne pathogen of ruminants, causes considerable morbidity among the livestock, renders them susceptible to secondary infections with opportunistic pathogens, and contributes to milk yield losses in dairy cattle (Woldehiwet, 2006). However, in recent years, *A. phagocytophilum* also emerged as a pathogen of human and canine health importance (Nováková & Víchová, 2010; Vorou *et al.*, 2007). Strains pathogenic to livestock appear to be harboured by wild ruminants (Dugat *et al.*, 2014; Huhn *et al.*, 2014) and possibly bacteraemic sheep (Thomas *et al.*, 2012; Woldehiwet, 2006); while those pathogenic to human and dogs, appear to be maintained in wild boars (*Sus scrofa*) (Huhn *et al.*, 2014; Michalik *et al.*, 2012; Petrovec *et al.*, 2003), hedgehogs (*Erinaceus europaeus*) (Huhn *et al.*, 2014; Silaghi *et al.*, 2012), or red foxes (*Vulpes vulpes*) (Canelas Domingos *et al.*, 2011; Härtwig *et al.*, 2014). The precise roles of some wildlife species are yet to be determined (Dugat *et al.*, 2015). Transmission by blood transfusion in dogs (Egenvall *et al.*, 1998) and humans (Jereb *et al.*, 2012) have also been described.

Canine granulocytic anaplasmosis is often manifested by fever, pale mucous membranes, lethargy, anorexia, vomiting and diarrhoea, polyuria, thirst, lameness (as a

result of secondary neutrophilic polyarthritis), neurological symptoms and tachypnoea (Beugnet & Marié, 2009; Kohn *et al.*, 2008; Sainz *et al.*, 2015); malfunction of infected phagocytes hinders immune defense mechanisms, facilitating secondary infections (Kohn *et al.*, 2008). A case of immune-mediated haemolytic anaemia and thrombocytopenia due to destruction of erythrocytes and platelets has also been described (Bexfield *et al.*, 2005). Cases of canine granulocytic anaplasmosis have been reported in British dogs (Bexfield *et al.*, 2005; Clark *et al.*, 1996), however, the disease is generally thought to be underdiagnosed since some of the clinical signs may be misinterpreted for Lyme disease or babesiosis (Beugnet & Marié, 2009).

Infections have also been reported in domestic cats, although less commonly (Carrade *et al.*, 2009). Feline granulocytic anaplasmosis presents with fever, lethargy, anorexia, dehydration, tachypnoea, and hyperglycaemia (Bjöersdorff *et al.*, 1999; Heikkilä *et al.*, 2010). The pathogen can also affect horses. Initial symptoms include weakening, apathy and increased body temperature; followed by aversion to movement, stiff gait, anorexia, ataxia, depression, painful oedemas of lower limbs, and occasionally lameness (Dzięgiel *et al.*, 2013; Silaghi *et al.*, 2011). Other manifestations of acute anaplasmosis include bleeding from mucosa, weight loss, enlarged spleen and lymph nodes, diarrhoea, vomiting, inflammation of joints, and convulsions (Dzięgiel *et al.*, 2013).

While *A. phagocytophilum* infections in dogs are not uncommon and infections in livestock are of a considerable economic importance to the industry, limited data is available on human granulocytic anaplasmosis (HGA) in the UK, and reports of infections remain rare (Hagedorn *et al.*, 2014; Sumption *et al.*, 1995). It is believed however, that such as in veterinary medicine, cases are considerably underreported (Stuen *et al.*, 2013). Infections typically result in self-limiting flu-like illness accompanied by myalgias, headaches and malaise in healthy individuals (Carrade *et al.*, 2009), although there is a risk of severe illness in immunocompromised and elderly patients (Bexfield *et al.*, 2005; Jahfari *et al.*, 2017; Stuen *et al.*, 2013). Secondary infections with opportunistic pathogens can also cause complications (Nováková & Víchová, 2010; Rikihisa, 2011; Smith *et al.*, 2013), and an *in*

*vitro* study of an *A. phagocytophilum*-*B. burgdorferi* coinfection demonstrated that *A. phagocytophilum* might enhance *B. burgdorferi* transmigration and dissemination across the blood-brain and other vascular barriers (Grab *et al.*, 2007).

#### 1.4.4 *Rickettsia* spp.

*Rickettsia* spp. are small Gram-negative intracellular bacteria (Nilsson, *et al.*, 2011; Rizzoli *et al.*, 2014). Nine species of Spotted fever rickettsiae (SFR) have been described as emerging pathogens in Europe – including *R. helvetica*, the agent of tick-borne spotted fever syndrome in humans (Nilsson, *et al.*, 2011). *R. hevetica* infections are typically manifested by mild, self-limiting disease with fever, headaches, myalgias and arthralgias, occasionally with a rash and/or an eschar (Nilsson, *et al.*, 2011; Rizzoli *et al.*, 2014). Although, the species has also been associated with fatal perimyocarditis in two young men (Nilsson *et al.*, 1999), as well as a subacute meningitis (Nilsson *et al.*, 2010).

*R. helvetica* has been detected in *I. ricinus* both in mainland Europe (Bonnet *et al.*, 2017; Cotté *et al.*, 2010; Lindblom *et al.*, 2016; Nijhof *et al.*, 2007; Sekejová *et al.*, 2012; Stańczak *et al.*, 2008, 2009; Venclikova *et al.*, 2014), and the UK (Tijssse-Klasen *et al.*, 2011); as well as *I. hexagonus* from hedgehogs (Speck *et al.*, 2013). The pathogen appears to have a widespread distribution across the SW of England and some parts of Scotland (Speck *et al.*, 2013; Tijssse-Klasen *et al.*, 2011), although the burden this pathogen has on human health in the UK is unknown. Other species of Rickettsiae detected in British *I. ricinus* by Tijssse-Klasen *et al.* (2011) include *R. massiliae*-like, *R. canadensis*-like, *R. limoniae*-like and *R. bellii*-like species. Two additional species of Rickettsiae pathogenic to humans have been detected in *I. ricinus* from mainland Europe – *Rickettsia monacensis* (Scarpulla *et al.*, 2016; Simser *et al.*, 2002) and *Rickettsia conorii* (Ciervo *et al.*, 2006). Many questions regarding the reservoir competence of birds and other vertebrates for Spotted fever group Rickettsiae remain unresolved or not fully understood (Elfving *et al.*, 2010; Parola *et al.*, 2013), however, evidence suggests that both avian (Berthová *et al.*, 2016; Hornok *et al.*, 2014) and mammalian hosts (Jahfari *et al.*, 2017; Schex *et al.*, 2011; Sprong *et al.*, 2009)

may play important roles in the maintenance and geographical dispersion of Rickettsiae. Additionally, Rickettsiae are transmitted in ticks transovarially with a high efficiency, meaning that ticks also serve as efficient reservoirs of Rickettsiae in nature (Burgdorfer *et al.*, 1979), and ticks from migratory birds appear to contribute to their wide geographic distribution and reports in new locations (Elfving *et al.*, 2010).

### 1.5 Eurasian badgers as reservoirs of vector-borne pathogens

Eurasian badgers (*Meles meles*), members of the family Mustelidae, are territorial and group-living carnivores that typically live within a shared communal home range (Balestrieri *et al.*, 2009; Macdonald *et al.*, 1999), where they inhabit complex underground burrows consisting of a number of tunnels and nesting chambers known as setts (Corbet & Harris, 1991). These are typically handed on from one generation to another within a social group (Butler & Roper, 1996). While their social groups in Britain are significantly larger than social groups from continental Europe (Byrne *et al.*, 2012), smaller non-territorial groups of individuals have also been reported (Do Linh San *et al.*, 2007). Dispersal of badgers from natal social groups in rural habitats is uncommon, although disturbance to a population increases the likelihood of movements occurring (Corbet & Harris, 1991; Donnelly *et al.*, 2007). While aggressive behaviour among individuals may occur, this is mainly associated with territorial defence and mating activity. Sows typically give birth in February and cubs emerge from their nests in April (Corbet & Harris, 1991).

In the UK, opinions on badgers are often divided. While badgers are one of the iconic wildlife species of the British Isles, they are also known reservoirs of a number of pathogens, one of the most important of which is *Mycobacterium bovis* – a non-vector-borne pathogen causing bovine tuberculosis in cattle (Gortázar *et al.*, 2012; Hancox, 1980). Several blood-feeding ectoparasites can be found on badgers at any time – including the host-specific badger flea (*Paraceras melis*), the biting louse (*Trichodectes melis*) (Butler & Roper, 1996; Do Linh San, 2007; Hancox, 1980), and ticks belonging to species *I. hexagonus*, *I. canisuga*, and occasionally *I. ricinus* (Bartley *et al.*, 2017; Corbet & Harris, 1991; Hillyard, 1996).



Limited data is available on ectoparasites burdens of badgers (Butler & Roper, 1996; Do Linh San, 2007), however, they are regularly known to engage in behaviours that appear to have an anti-parasite function; including grooming after leaving their setts, 'airing' and renewing their bedding, and switching between nesting chambers of their setts (Butler & Roper, 1996).

The existence of haemoparasites in badgers was first confirmed in 1905 when Bettencourt & Franca reported *Trypanosoma pestanai* from a badger in Portugal. This finding was followed by discoveries of three apparently similar intraerythrocytic parasites (piroplasmids) of badgers in Europe and Asia. The first discovery was made in Russia and named *Theileria (Piroplasma) meles* by Ruchjadew in 1948. Then in 1952, Biocca and Corradetti found a similar parasite in badgers near Rome and named it *Babesia missirolii*. The third discovery was made in badgers in Kyrgyzstan and the organism was named as *Piroplasma meles* by Krivkova in 1960; later corrected to *Babesia meles* by Krylov in 1981 (Macdonald *et al.*, 1999). These discoveries were followed by records of piroplasmids in badgers from Cornwall (Peirce & Neal, 1974a) and from Wytham Woods, Oxfordshire (Macdonald *et al.*, 1999). Macdonald *et al.* (1999), in their studies on haemoparasites of badgers from Oxfordshire, referred to these piroplasmids as *Babesia missirolii* and reported that based on microscopic examinations of blood smears, 77.2% of individuals tested positive at least once over 3 years. These piroplasmids are thought to be transmitted by *I. canisuga* or *I. hexagonus* (Barandika *et al.*, 2016; Peirce & Neal, 1974a), however, no definite vector has been confirmed.

Badgers often live in sympatry with red foxes (Young *et al.*, 2015), and there have even been reports, from mainland Europe at least, of badgers cohabiting with foxes in their setts (Do Linh San, 2007; Kowalczyk *et al.*, 2008; Mori *et al.*, 2015). While no evidence of such behaviour was found in British literature, the two carnivores are parasitized by the same species of ticks (Table 1), and thus one may argue that the animals may share certain tick-borne pathogens. Due to their close vicinity to domestic habitats, foxes and badgers may act as a transmission interface for Babesiae to dogs (Duscher *et al.*, 2014). Over the years,

molecular studies detected piroplasmids both in badgers (Gimenez *et al.*, 2009) and foxes (Bartley *et al.*, 2016, Duscher *et al.*, 2014, Najm *et al.*, 2014), and *Babesia annae* was detected in *I. canisuga* collected from badgers in Spain (Millán *et al.*, 2016). However, this could not be considered as a conclusive proof that the ticks were capable of transmitting *B. annae* to badgers, nor that they acquired the parasites from them (Bartley *et al.*, 2017). Subsequently, two studies targeting the 18S rRNA gene of *Babesia* spp. (Barandika *et al.*, 2016; Bartley *et al.*, 2017) shed more light on piroplasmids in badgers. Barandika *et al.* (2016) reported that 33.6% of badgers from northern Spain tested positive for *Babesia* spp. and that two distinct sequence types of Babesiae were found, both of which were related to but clearly different from *B. annae* associated with foxes. Bartley *et al.* (2017), in their studies of Scottish badgers, found that 59.6% of all badger blood samples tested positive for *Babesia* spp. and three distinct sequence types closely related to *B. annae* were discovered. These badgers were routinely infected with more than one parasite isolate at the time, and a further sequence analysis revealed that there also was an evidence of genetic recombination between these isolates (Bartley *et al.*, 2017). To date, there has been no publication on *Babesia* spp. in badgers from England, however previous testing of badger blood samples from Woodchester park in Gloucestershire, using *Babesia*-specific  $\beta$ -*tubulin* primers, revealed that 98.9% of all blood samples were positive for *Babesia* spp. and that each individual tested positive at least once (Gbobaniyi, 2016)

Historically, classification of piroplasmids has relied mainly on morphological and biological observations, and identification based on host origin was typically founded on the believe that these parasites are host-specific. It is now known however, that this is not the case for many species (Yabsley & Shock, 2013). By contrast, molecular studies of the organisms' DNA provide means of species identification, differentiation and establishment of evolutionary relationships that visual observations do not. Furthermore, observations of *Babesia* in blood smears is difficult, particularly when parasitaemia is low (Barandika *et al.*, 2016), hence the true prevalence of the parasite among badgers, particularly among close-knit social groups, may be much higher. On the other hand, while DNA-based techniques are

able to detect even very low host parasitaemias, it has to be emphasised that the level of parasitaemia can affect the success of parasite transmission from one host to another.

Furthermore, two recent studies from mainland Europe (Gern and Sell, 2009; Wodecka *et al.*, 2016) recognised badgers as reservoir hosts for *B. afzelii* and *B. valaisiana*. Additionally, *Borrelia* spp. were detected in *I. hexagonus* (Couper *et al.*, 2010) and *I. canisuga* (Couper *et al.*, 2010; Wodecka *et al.*, 2016) ticks removed from badgers in the UK and mainland Europe. Wodecka *et al.* (2016) detected the DNA of *B. garinii* in nymphal *I. canisuga* and a large proportion of *T. melis* collected from badgers. Considering that *Trichodectes* lice spend their entire life cycle on one host while feeding on skin debris, skin-fluid exudates, dried blood and occasionally whole blood (Bádr *et al.*, 2005), the role of badgers as potential reservoirs of *B. garinii* cannot be ruled out (Wodecka *et al.*, 2016). With regards to *A. phagocytophilum* and *Rickettsia* spp., no evidence of studies involving a large number of badgers to examine whether they play any roles in the maintenance of these pathogens in nature was found.

As for *T. pestanai*, the parasite has been reported in badgers from France (Rioux *et al.*, 1966), Ireland (McCarthy *et al.*, 2009); and in the UK from Cornwall (Peirce & Neal, 1974b), Oxfordshire (Lizundia *et al.*, 2011; Macdonald *et al.*, 1999), and Gloucestershire (Ideozu *et al.*, 2015). Molecular studies by Lizundia *et al.* (2011) and Ideozu *et al.* (2015) detected the parasite in 31% and 35.4% of individuals tested, respectively. *P. melis* was shown to be the primary vector of *T. pestanai* (Lizundia *et al.*, 2011), yet earlier this year a report of *T. pestanai* and *A. phagocytophilum* co-infection in a dog emerged from Germany, meaning that infections with unusual opportunistic vector-borne pathogens in dogs should not be ruled out (Dyachenko *et al.*, 2017).

## 1.6 Aims and objectives

Whilst badgers are known to host a wide range of pathogens, information on haemoparasites of British badgers is limited. Historically, classification and identification of these haemoparasites used to rely on morphological and biological observations and

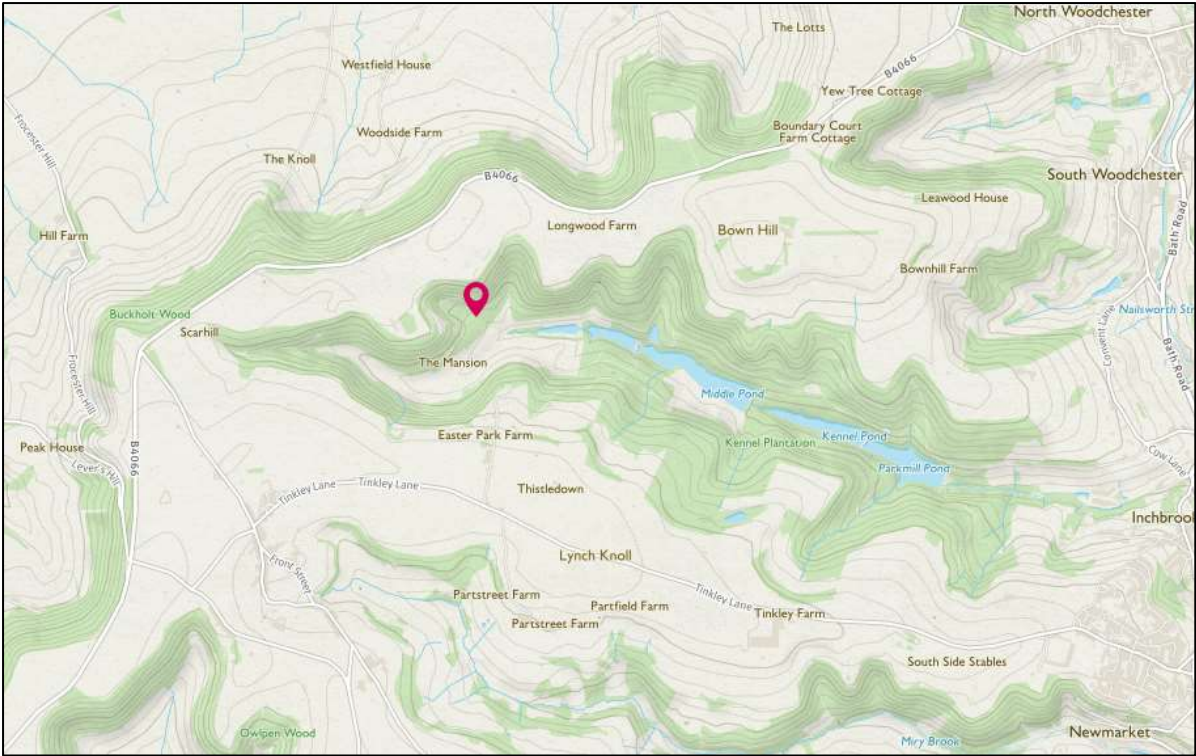
assumptions of host-specificity, however, molecular techniques provide means of species identification and differentiation that visual observations cannot. There have been two comprehensive studies on Babesiae in badgers, one in Spain and another in Scotland, yet no such or similar research has been done in England. As for other vector-borne organisms, the importance of badgers as reservoirs of *A. phagocytophilum*, *Rickettsia* spp., *Borrelia* spp. and *T. pestanai* is slowly emerging.

This study, as a continuation of a BSc project, was aimed at investigating whether badgers in England are reservoirs of vector-borne infections of medical or veterinary health importance. To achieve this, the study assessed the extent to which piroplasmids infecting badgers differ from each other and from those infecting foxes, and whether badgers contribute to the maintenance of *A. phagocytophilum* or *Rickettsia* spp. in nature. Additionally, badgers from Northeast England were screened for *T. pestanai*, to further deepen our knowledge on Trypanosome infections of badgers in England. Lastly, an attempt was made to isolate some of the pathogens carried by questing *I. ricinus* from Gloucestershire, in particular *Babesia* spp., for further studies.

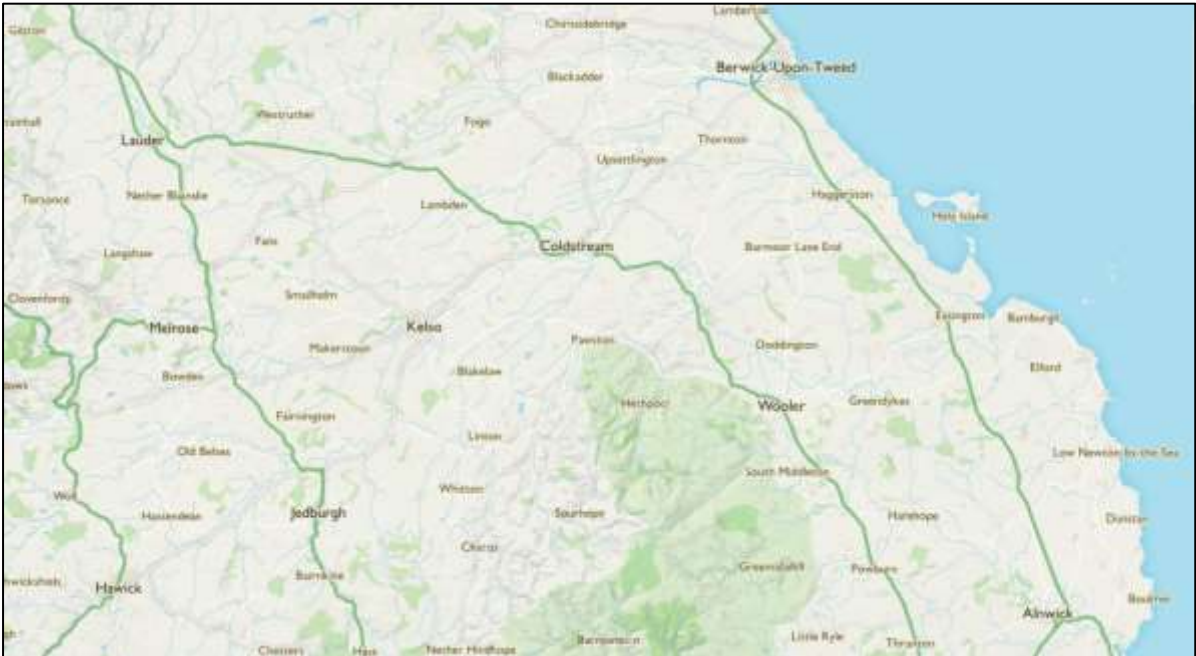
## **Chapter 2: Materials and methods**

### **2.1. Study areas and sample specifications**

Woodchester Park in Gloucestershire, Southwest England (Figure 3), is an area comprising steep-sided wooded valley consisting of deciduous and mixed woodlands, surrounded by agricultural grasslands used for sheep and cattle grazing (Robertson *et al.*, 2015; Rogers *et al.*, 1998). The park is very popular with walkers and families alike, with a range of walking routes and activities suitable for all age categories all year round (National Trust, 2017). Samples from Northeast England (Figure 4) came from areas consisting of mixed farmlands and woodlands (Dr M. Silk, personal communication, 28 June 2018).



**Figure 3:** Ordnance survey map of Woodchester Park, Gloucestershire (Ordnance Survey, 2018)



**Figure 4:** Ordnance survey map of Northeast England (Ordnance Survey, 2018)

In total, 181 blood samples were obtained from 99 badgers at Woodchester Park at two to three-month intervals between 2007 and 2012 as part of ongoing long-term capture-mark-recapture studies (Delahay & Frölich, 2000; Robertson *et al.*, 2015; Rogers *et al.*, 1998). Heparinated blood samples taken from captured animals were stored at -20°C. These samples were previously tested for *Babesia* spp. and *A. phagocytophilum* (Gbobaniyi, 2016), and to further the study, these samples were also tested for *Rickettsia* spp. Additionally, an attempt was made to isolate some of the pathogens carried by *I. ricinus* at Woodchester park. Therefore, in April 2017, 107 host-seeking ticks were collected off the vegetation by blanket dragging at various locations around Woodchester Park. The ticks were kept alive in a humidified chamber, identified to life-cycle stage, and split into 2 groups for further processing. Only the nymphs and adult females were used in this study, giving a total of 104 ticks, as the males of *I. ricinus* do not engorge on blood. As for badgers from Northeast England, 131 blood samples from 94 badgers were obtained between 2015 and 2016 in a survey including badgers from a capture-mark-recapture group, and a second group from which captured badgers have been permanently removed. Each sample was collected into a tube containing EDTA and stored at -80°C until further processing. Ectoparasites removed from these badgers at the point of capture were kept in 70% ethanol at a room temperature, and their identification was carried out using a range of keys. Each tick was identified to species and a life-cycle stage (Arthur, 1963; Hillyard, 1996). Fox blood samples were collected from road-kill carcasses in Bristol, Southwest England, and tested for *Babesia* spp. in a different study (Alharbi, 2018). One known *B. annae* positive sample from Bristol was sent off for sequencing for further comparison against sequences obtained from badgers.

## **2.2 Aseptic dissection of ticks and the maintenance of tick cell cultures**

Of the 104 collected ticks, a total of 38 nymphal and 11 female ticks were washed for 5 min in 0.1% benzalkonium chloride, followed by 1 min wash in 70% ethanol, rinsed twice in a sterile deionised water to remove residual alcohol, and left to dry on a sterile filter paper. All ticks were dissected under aseptic conditions in a vertical laminar flow cabinet. Each tick was

then covered with sterile PBS and halved (Dr L. Bell-Sakyi, personal communication, 15 March 2017). One half of each tick was placed in a tube with *I. ricinus*-derived IRE/CTVM19 tick cells (Tick Cell Biobank, The Pirbright Institute), while the second half was stored in 70% ethanol at -20°C until DNA extraction.

*I. ricinus* embryo-derived tick cell line IRE/CTVM19 cultures at a passage level 224 were maintained at 28°C in a L-15 (Leibovitz) medium supplemented with 20% heat inactivated foetal bovine serum, 10% tryptose phosphate broth, 2mM L-glutamine, and Penicillin-Streptomycin (PEN/STREP; 100U/ml penicillin and 100µg/ml Streptomycin). All culture media and supplements were produced by Gibco/Life Technologies. Cell cultures were grown in approximately 2ml volumes in sealed, flat-sided cell culture tubes (Nunc) with weekly medium changes. Culture media were prepared freshly on the day of use and a medium change was performed by removing three quarters of the old medium from each tube and replacing it with a newly prepared medium. Subcultures were prepared by adding 2.2ml of a fresh medium, resuspending the cells by pipetting, and transferring 2.2ml of the resultant cell suspension into a new tube. All media were prepared and changed in a Class II cell culture laminar flow cabinet up until the point of inoculation with dissected ticks, after which a Class II pathogen biosafety cabinet had to be used instead. All manipulations, including media preparations, were carried out under strict aseptic conditions, and all work surfaces of the hoods, gloves and bottles of media were wiped down with 70% ethanol.

Tick cells do not exhibit contact inhibition and continue to grow in three dimensions, and can be therefore maintained at high cell densities without the need for regular subculturing. These properties make them suitable for isolation of even slow-growing microorganisms (Bell-Sakyi *et al.*, 2007). The PEN/STREP supplement was withdrawn from the medium of cell cultures that were to be infected prior to inoculation, and the cultures were maintained with weekly medium changes. Cultures were monitored for general health by visual inspection and the cells were observed for any growth 8 weeks after inoculation in Giemsa-stained cytocentrifuge smears, which were prepared as described by Dyachenko *et al.* (2013).

### 2.3 DNA extraction

The DNA from Woodchester Park blood samples were extracted in early 2016 by means of alkaline digestion, as described by Bown *et al.* (2003). An extraction control containing ammonium hydroxide solution only was processed with every four blood samples as a contamination control. Briefly, 500 µl of 1.25% ammonium hydroxide solution was added to each 50 µl of a blood sample in a 1.5 ml Safelock microcentrifuge tube (Fisher Scientific), and the samples were heated at 100°C for 20 minutes with lids locked. Afterwards, following a brief centrifugation, the tubes were opened and heated again until half the initial volume had evaporated. The extracted DNA samples and negative controls were then diluted to 1 in 5 in sterile deionized water. A QIAamp® DNA Blood Mini Kit (QIAGEN) was used to extract the DNA from NE England blood samples as specified by the manufacturer. One extraction control containing sterile deionized water was processed with every five blood samples. Lastly, DNA extractions from all ticks (both halved and whole ticks) were carried out using the ZR Tissue & Insect DNA MiniPrep™ Kit (Zymo Research) according to manufacturer's instructions. Randomly picked DNA extracts were assessed for purity and concentration using the NanoDrop (Thermo Scientific) to confirm the success of DNA extractions. All DNA extracts were stored at -20°C until further analysis.

Extracted samples were analysed with a battery of assays targeting the DNA of *Babesia* spp., *A. phagocytophilum*, *Rickettsia*, *Borrelia* and *Trypanosoma* spp. as described below. DNA extractions, Polymerase chain reaction (PCR) reagent setups and addition of extracted DNA were carried out in separate rooms, and gel electrophoresis along with Reverse line blotting (RLB) were carried out in a separate laboratory in order to minimise the risk of cross-contamination.

### 2.4 RLB for *Babesia* spp., *A. phagocytophilum*, and *Rickettsia* spp.

DNA extracts from all questing ticks were tested using a genus-specific RLB assay for *Babesia* spp., *A. phagocytophilum* and *Rickettsia* spp. Firstly, the extracts were subjected to PCR amplifications using the primers specified in Table 2, one of the set of which was



biotin-conjugated at the 5' end to enable the detection of hybridized PCR products during reverse line blotting. The RLB was carried out on a re-usable Biodyne C blotting membrane (Lorusso *et al.*, 2016) containing a range of covalently linked probes (including those specified in Table 3), which could be stripped and reused up to 25 times and enabled simultaneous screening of each sample for different pathogens. Additionally, historic badger blood samples from Gloucestershire were tested using this technique for *Rickettsia* spp. All solutions were prepared as detailed in Appendix 1.

#### 2.4.1 PCR amplification

Samples were subjected to PCR amplifications using *Babesia/Theileria* genus-specific RLB-F2, RLB-R2 primers (Matjila *et al.*, 2004); *Ehrlichia/Anaplasma* Ehr-F (16S8FE) (Lorusso *et al.*, 2016; Schouls *et al.*, 1999) and Ehr-R (BGA1B) (Bekker *et al.*, 2002; Lorusso *et al.*, 2016) primers; and *Rickettsia* genus-specific Rick-F, Rick-R primers (Jado *et al.*, 2006). DNA amplifications for each of the four groups of pathogens was performed in a reaction volume of 25 µl per sample, containing 10 µl of 2x MyTaq Red Mix (Bioline), 1 µl of each primer at a concentration of 20 pmol/µl, 10.5 µl of sterile deionized water and 2.5 µl of extracted DNA. PCR reagent setups and addition of DNA were carried out in separate rooms to minimise the risk of cross-contamination, and each PCR incorporated cross-contamination/negative controls and known positives for *Babesia*, *Ehrlichia/Anaplasma* and *Rickettsia* spp. as positive controls.

To minimize non-specific annealing a touchdown PCR protocol, as described by Lorusso *et al.* (2016), was used. Each amplification consisted of an initial DNA denaturation and polymerase activation step of 30 sec at 98°C; followed by 10 cycles of 5 sec denaturation at 98°C, 5 sec annealing (decreasing from 67 to 57°C at 1°C per cycle), and 7 sec extension at 72°C; then further 40 cycles of 5 sec denaturation at 98°C, 5 sec annealing at 57°C and 7 sec extension at 72°C; followed by a final 1 min extension step at 72°C. Following amplification, the positive and negative controls were run on a 1.5% agarose gel stained with

GelRed (Cambridge Bioscience) to confirm the success of reaction, and the obtained PCR products were stored at 4°C until RLB analysis.

#### 2.4.2 The RLB protocol

Briefly, 10 µl of each PCR product obtained from each DNA sample were mixed, giving a volume of 40 µl, and added to 120 µl of 2x SSPE/0.1% SDS solution in a sterile 1.5 ml microcentrifuge tube. For each positive and negative controls, 10 µl of each respective PCR product were diluted in 150 µl of 2x SSPE/0.1% SDS, giving a total volume of 160 µl. All samples were then heated for 10 min at 100°C for denaturation to take place, cooled rapidly on ice to prevent re-annealing, and centrifuged for 30s at 11,000 × g. Reverse line blotting was performed as detailed by Gubbels *et al.* (1999) and Lorusso *et al.* (2016). At first, the membrane was activated by a 5-minute incubation in 100 ml of 2x SSPE/0.1% SDS at a room temperature. Thereafter, 160 µl of each denatured sample and control preparations were loaded onto the membrane in a miniblotted, perpendicular to the previously applied oligonucleotides, and incubated for 60 min at 42°C. Following hybridization, the PCR products were aspirated, and the membrane was removed from the miniblotted and washed twice in 125 ml of 2x SSPE/0.5% SDS for 10 min at 50°C under gentle rocking. The membrane was then incubated in 50 ml of 2xSSPE/0.5% pre-heated to 42°C and 5 µl of Streptavidin-POD conjugate (Roche) for 30 min at 42°C. Afterwards, the membrane was washed twice in 125 ml of 2x SSPE/0.5% SDS for 10 min at 42°C under gentle rocking, then further two times in 125 ml of 2x SSPE for 5 min at room temperature to rinse off the SDS. To detect the hybridized products the blot was incubated for 1 min in 10 ml of ECL detection fluid (Amersham) at a room temperature and exposed to an ECL hyperfilm (Amersham) for 6 min, which was subsequently manually developed.

After use, all PCR products were stripped from the membrane by two washes in 1% SDS for 30 min, each time at 70°C, followed by a single wash in 20 mM EDTA for 15 min at a room temperature. The washed membrane was then stored in a fresh 20mM EDTA solution at 4°C until further use. Randomly picked positive samples were amplified by end-

point PCR (Table 2) to further ascertain species identity. Following detection of apparent crossreactivities, it was decided that only standard end-point and RT-PCR protocols should be used thereafter.

## 2.5 Additional polymerase chain reaction assays

Extracts from badger bloods and ticks off badgers from NE England were subjected to end-point and real-time PCR assays using the following protocols and primers specified in Table 2. Additionally, a selection of RLB positive samples was subjected to end-point PCRs for *Babesia* and *Rickettsia* spp. in preparation for sequencing. Each PCR contained one extraction/cross-contamination control for every five samples and a known positive control. All products of end-point/nested PCRs were visualised on 1.5% agarose gels stained with GelRed (Cambridge Bioscience) and their relative size was determined by means of HyperLadder 1kb molecular weight marker (Bioline). As for RT-PCR, all samples with a cycle threshold (Ct) lower than 35 were considered positive. The same *Babesia* spp. and *A. phagocytophilum* PCR assays, as described below, were previously used on badger blood samples from Gloucestershire (Gbobaniyi, 2016).

**Table 2: List of PCR primers, probes and their targets**

Reverse line blot PCR (RLB-PCR)			
Pathogen	Name	Primer sequence (5' → 3')	Target/fragment length (bp)
<i>Babesia/ Theileria</i> spp.	RLB-F2	GACACAGGGAGGTAGTGACAAG	18S rRNA / ~400 genus-specific
	RLB-R2	BIOTIN-CTAAGAATTTACCTCTGACAGT	
<i>Anaplasma</i> spp.	Ehr-F (16S8FE)	GGAATTCAGAGTTGGATC(A/C)TGG(C/T)TCAG	16S rRNA / ~400 <i>Ehrlichia/Anaplasma</i> -specific
	Ehr-R (BGA1B)	BIOTIN-CGGGATCCCGAGTTTGCCGGGACTT (C/T)TTCT	

<i>Rickettsia</i> spp.	Rick-F Rick/23-5-F Rick-R Rick/23-5-R	GATAGGTCRGRGTGTGGAAGCAC BIOTIN-TCGGGAYGGGATCGTGTGTTTC	23–5S ISR / 345 genus-specific
<i>Borrelia</i> spp.	B-5SBor 23SBor	BIOTIN-GAGTTCGCGGGAGAGTAGGTTATT TCAGGGTACTTAGATGGTTCACTT	5S–23S ISR / ~170 genus-specific
<b>End-point and Real-time PCR</b>			
Pathogen	Name	Primer/probe sequence (5' → 3')	Target/fragment length (bp)
<i>Babesia/ Theileria</i> spp.	F34	TGTGGTAACCAGAT(t/c)GG(a/t)GCCAA	β-tubulin / 150-280 genus-specific
	R323	CnGT(a/g)TA(a/g)TGnCC(t/c)TT(a/g)GCCCA	
	F79	GA(a/g)CA(t/c)GGnATnGA(t/c)CCnGTAA	
	R206	AC(a/t/g)GA(a/g)TCCATGGT(a/t/g)CCnGG(t/c)T	
	BmF1	GCGATGTATCATTCAAGTTTCTG	18S rRNA / ~600 Apicomplexa- specific
	BmR1	TGTTATTGCCTTACACTTCCTTGC	
	BmF2	ACGGCTACCACATCTAAGGAAGGC	
	BmR2	TCTCTCAAGGTGCTGAAGGA	
<i>Anaplasma phagocytophilum</i>	ApMSP2f	ATGGAAGGTAGTGTGGTTATGGTATT	msp2 / 77 species-specific
	ApMSP2r	TTGGTCTTGAAGCGCTCGTA	
	ApMSP2p	FAM-TGGTGCCAGGGTTGAGCTTGAGATTG-TAMRA	
<i>Rickettsia</i> spp.	RKND03F	GTGAATGAAAGATTACACTATTTA	gltA gene genus-specific
	RKND03R	GTATCTTAGCAATCATTCTAATAGC	
	RKND03P	FAM-CTATTATGCTTGCGGCTGTCGGTTC-TAMRA	
<i>Borrelia</i> spp.	CS409d	CCTATGGCTATTATGCTTGC	gltA / 750 genus-specific
	RP1258n	ATTGCAAAAAGTACAGTGAACA	
<i>Borrelia</i> spp.	Bb23Sf	CGAGTCTTAAAAGGGCGATTTAGT	23S rRNA / 75 genus-specific
	Bb23Sr	GCTTCAGCCTGGCCATAAATAG	
	Bb23SP	FAM-AGATGTGGTAGACCCGAAGCCGAGTG-TAMRA	
<i>Trypanosoma</i> spp.	TRY927F	GAAACAAGAAACACGGGAG	18S rRNA / 420-500 genus-specific
	TRY927R	CTACTGGGCAGCTTGGA	
	SSU561F	TGGGATAACAAAGGAGCA	
	SSU561R	CTGAGACTGTAACCTCAAAGC	

ISR - intergenic spacer region

All primers were synthesized by Eurofins (UK)

**Table 3: List of RLB probes and their targets**

<b><i>A. phagocytophilum, Babesia and Rickettsia spp.</i></b>		
<b>Pathogen</b>	<b>Probe sequence (5' → 3')</b>	<b>Reference</b>
<i>Babesia/Theileria</i> catch-all	TAATGGTTAATAGGA(A/G)C(A/G)GTTG	Matijla <i>et al.</i> , 2008
<i>Theileria</i> catch-all	ATTAGAGTGCTCAAAGCAGGC	Matijla <i>et al.</i> , 2008
<i>Babesia divergens</i>	ACT(A/G)ATGTCGAGATTGCAC	Oosthuizen <i>et al.</i> , 2009
<i>Anaplasma</i> catch-all	GGGGAAAGATTTATCGCTA	Bekker <i>et al.</i> , 2002
<i>Anaplasma phagocytophilum</i>	TTGCTATAAAGAATAATTAGTGG TTGCTATGAAGAATAATTAGTGG TTGCTATAAAGAATAGTTAGTGG TTGCTATAGAGAATAGTTAGTGG	Schouls <i>et al.</i> , 1999
SFG <i>Rickettsia</i> spp.	ACTCACAARGTTATCAGGT	Jado <i>et al.</i> , 2006
<b><i>Borrelia spp.</i></b>		
<b>Pathogen</b>	<b>Probe sequence (5' → 3')</b>	<b>Reference</b>
<i>Borrelia burgdorferi</i> s. l.	CTTTGACCATATTTTTATCTTCCA	Rijpkema <i>et al.</i> , 1995
<i>Borrelia burgdorferi</i> s. s.	AACACCAATATTTAAAAACATAA	Rijpkema <i>et al.</i> , 1995
<i>Borrelia afzelii</i>	AACATTTAAAAATAAATTCAAGG	Rijpkema <i>et al.</i> , 1995
<i>Borrelia valaisiana</i>	TATATCTTTTGTTCAATCCATGT	Poupon <i>et al.</i> , 2006
<i>Borrelia garinii</i>	CAAAAACATAAATATCTAAAAACATAA AACATGAACATCTAAAAACATAAA	Poupon <i>et al.</i> , 2006 Rijpkema <i>et al.</i> , 1995
<i>Borrelia spielmanii</i>	GAATGGTTTATTCAAATAACATA GAATAAGCCATTTAAATAACATA	Gern <i>et al.</i> , 2010
<i>Borrelia lusitaniae</i>	TCAAGATTTGAAGTATAAAATAAAA AAATCAAACATTCAAAAAATAAAC	Poupon <i>et al.</i> , 2006 Gern <i>et al.</i> , 2010
<i>Borrelia bissettii</i>	AAACACTAACATTTAAAAACAT AACTAACAAACATTTAAAAACAT	Gern <i>et al.</i> , 2010
RLF <i>Borrelia</i> spp.	CTATCCATTGATCAATGC	Gern <i>et al.</i> , 2010

### 2.5.1 RT-PCR for *A. phagocytophilum*, *Rickettsia* and *Borrelia* spp.

Blood samples and ticks collected from badgers were screened for *A. phagocytophilum* and *Rickettsia* spp. with the ApMSP2f, ApMSP2r primers and ApMSP2p probe targeting a portion of the *msp2* gene of *A. phagocytophilum* (Courtney *et al.*, 2004), and RKND03F, RKND03R primers and RKND03P probe targeting the *gltA* of *Rickettsia* spp. (Socolovschi *et al.*, 2010), respectively. Additionally, all ticks collected from badgers in NE England and questing ticks from Gloucestershire were screened with *Borrelia* genus-specific BB23Sf, Bb23Sr primers and Bb23SP probe targeting the 23S rRNA gene of *Borrelia* spp. (Courtney *et al.*, 2004). All probes were dual-labelled – labelled at the 5' and 3' ends with 6-carboxy-fluorescein (6-FAM) and 6-carboxyl-tetramethyl-rhodamine (TAMRA) respectively (Courtney *et al.*, 2004; Socolovschi *et al.*, 2010).

Amplifications for *A. phagocytophilum* and *Borrelia* spp. were performed in a volume of 20 µl per sample, containing 10 µl of 2x MyTaq Mix (Bioline), 1 µl of each primer (at a concentration of 22 pmol/µl for ApMSP2f/ApMSP2r primers and 10 pmol/µl for BB23Sf/Bb23Sr primers), 1 µl of probe (at 3.3 pmol/µl for ApMSP2p and 3 pmol/µl for Bb23SP), 5 µl of sterile deionized water, and 2µl of extracted DNA. Cycling conditions consisted of an initial denaturation step of 5 min at 95°C, followed by 40 cycles of 15 sec at 95°C, and an annealing-extension step of 1 minute at 57°C (Courtney *et al.*, 2004). PCRs for *Rickettsia* spp. were performed in a final volume of 20 µl, containing 10 µl of 2x MyTaq Mix, 0.5 µl of each primer (at 20 pmol/µl), 2 µl of probe (at 2 pmol/µl), 2 µl of sterile deionized water and 5µl of extracted DNA. Amplifications consisted of an initial denaturation step of 3 min at 95°C, followed by 40 cycles of 1 sec at 92°C and 35 sec at 60°C. All *Borrelia* positive samples were further analysed by RLB for genotyping of *Borrelia* spp., as described in section 2.6.

### 2.5.2 End-point PCR for *Babesia*, *Rickettsia* and *Trypanosoma* spp.

Blood samples and ticks collected from badgers were screen with an end-point PCR targeting the  $\beta$ -tubulin gene of *Babesia* spp. A selection of *Babesia* positive samples,

including badger bloods and ticks from NE England and Gloucestershire, and foxes from Bristol, were subjected to further PCRs for two independent markers (namely the 18S rRNA and the  $\beta$ -tubulin) to ascertain species identity and examine the species of *Babesia* infecting badgers, foxes, and ticks parasitizing these animals. Furthermore, end-point PCRs were used to screen badger bloods from NE England for *T. pestanai*, and to further ascertain species identity of randomly picked *Rickettsia* positive samples.

For *Babesia* spp., a nested PCR with the external F34/R323 and internal F79/R206 primers targeting the  $\beta$ -tubulin gene of *Babesia/Theileria* spp. (Cacciò *et al.*, 2000), and the BmF1/BmR1 and BmF2/BmR2 primers targeting the Apicomplexan 18S rRNA gene (Simpson *et al.*, 2005), as detailed in Table 2, were chosen. Amplifications were performed in a volume of 20  $\mu$ l per sample. The first round of amplification contained 10  $\mu$ l of 2x MyTaq Mix (Bioline), 1  $\mu$ l of forward and reverse primers (at 20 pmol/ $\mu$ l each), 6  $\mu$ l of sterile deionized water and 2 $\mu$ l of extracted DNA. The second round consisted of 10  $\mu$ l of 2x MyTaq Red Mix, 1  $\mu$ l of forward and reverse primers (at 20 pmol/ $\mu$ l), 7  $\mu$ l of sterile deionized water and 1 $\mu$ l of the first round product. The same PCR protocol was used for both genetic markers, and for both first and second round amplifications, following cycling conditions of an initial denaturation for 5 min at 94°C; 40 cycles of 30 sec at 95°C, 30 sec at 55°C, and 50 sec at 72°C; then a final extension step for 5 min at 72°C.

As for *Rickettsia* spp., the genus-specific CS409d and RP1258n primers targeting the *gltA* gene of *Rickettsia* spp. (Roux *et al.*, 1997) were chosen. Amplifications were performed in a volume of 20  $\mu$ l, containing 10  $\mu$ l of 2x MyTaq Red Mix (Bioline), 1  $\mu$ l of each primer (at 10pmol/ $\mu$ l), 6  $\mu$ l of sterile deionized water and 2 $\mu$ l of extracted DNA. Cycling conditions consisted of an initial denaturation step of 15 min at 95°C, followed by 40 cycles of 30 sec at 94°C, 30 sec at 54 °C and 55 sec at 72 °C, then a final elongation step for 7 min at 72°C (Tijssen-Klasen *et al.*, 2011). Lastly, the blood samples were screened with the external TRY927F, TRY927R primers (Noyes *et al.*, 1999, 2000) and internal SSU561F, SSU561R primers (Noyes *et al.*, 1996) for *Trypanosoma* spp. The first round of amplification

contained 12.5 µl of 2x MyTaq Mix, 1 µl of TRY927F and TRY927R primers (at 20 pmol/µl each), 8.5 µl of sterile deionized water and 2µl of extracted DNA; while the second round consisted of 12.5 µl of 2x MyTaq Red Mix, 1 µl of SSU561F and SSU561R primers (at 20 pmol/µl), 9.5 µl of sterile deionized water and 1µl of the first round product as a template. Both amplifications followed cycling conditions of an initial denaturation for 2 min at 95°C, followed by 35 cycles of 20 sec at 95°C, 20 sec at 58°C, and 50 sec at 72°C; then a final extension step for 5 min at 72°C.

## **2.6 RLB for genotyping of *B. burgdorferi* s. l.**

Positive tick samples were subjected to genotyping of *Borrelia* spp. B-5SBor primer was biotin-conjugated at the 5' end (Table 2) to enable detection of hybridized PCR products, and all oligonucleotide probes presented in Table 3 were 5' amine modified to allow fixation to the membrane.

### **2.6.1 PCR amplification**

Samples were subjected to PCR amplifications using the B-5SBor and 23SBor primers targeting the variable intergenic spacer region between the 5S and 23S rRNA genes (Alekseev *et al.*, 2001). DNA amplification was performed in a reaction volume of 20 µl per sample, containing 10 µl of 2x MyTaq Red Mix (Bioline), 1 µl of each primer (at 10pmol/µl), 6 µl of sterile deionized water and 2µl of extracted DNA. Negative controls containing reagents only and known positives for *B. burgdorferi* s. s., *B. afzelii*, *B. garinii*, and *B. valaisiana* as positive controls were included in the PCR. To minimize nonspecific amplifications a touchdown PCR program, as detailed by (Burri *et al.*, 2007), was used. Amplification consisted of an initial denaturation step of 3 min at 94°C, followed by a 20 sec denaturation at 94°C, 30 sec annealing stage at 60°C, and a 30 sec extension at 72°C. During subsequent cycles, the annealing temperature was lowered by 1°C until it reached 52°C. After the final annealing temperature was reached, further 40 cycles using 52°C were



carried out, followed by a final extension for 10 minutes at 72°C. Obtained PCR products were stored at 4°C until RLB analysis.

### **2.6.2 Membrane probing and RLB analysis**

Membrane probing was carried out as previously described by Rijpkema *et al.* (1995). A Biotodyne C blotting membrane (Pall Life Sciences) was activated by incubation in 16% 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) solution for 15 min at a room temperature, and rinsed with distilled water for 2 min under gentle rocking. The membrane was then placed in a miniblottedter and the remaining solution was removed by aspiration. Amino-linked oligonucleotide probes (Table 3) were then covalently coupled to the activated Biotodyne C membrane as followed. Each oligonucleotide probe was diluted in 150 µl of sterile 500mM NaHCO<sub>3</sub> (pH 8.4) to a concentration of 10pmol/150 µl. 140 µl of diluted ink (1 µl of ink in 100 µl of 2xS SPE) was added to the first and the last lane, and the remaining lanes were loaded with 150 µl of each oligonucleotide suspension. Following a 1-minute incubation at room temperature, the excess solution as well as the ink were removed by aspiration. Afterwards, the membrane was removed from the miniblottedter and inactivated by incubation with 100 ml of 100mM NaOH for 10 min at a room temperature, under gentle rocking. The membrane was then washed with distilled water to remove excess NaOH, incubated for 10 min with 100 ml of 2x SSPE/0.1% SDS at 50°C, washed for 15 min with 100 ml of 20mM EDTA at room temperature, and stored at 4°C until used. PCR products were hybridized to 13 different oligonucleotide probes (Table 3) using the same protocol described in section 2.4.2, with the exception of 10 µl of each PCR product being suspended in 140 µl of 2x SSPE/0.1% SDS to give a final volume of 150 µl, and the membrane being disposed off after use.

### **2.7 DNA sequencing and phylogenetic analysis**

PCR amplicons chosen for sequence analysis were run through 1.5% agarose gels stained with GelRed (Cambridge Bioscience), bands containing amplified DNA were

excised, and the DNA was purified from the gel using the Isolate II PCR and Gel Kit (Bioline) as specified by the manufacturer. All samples were then assessed for purity and concentration using the NanoDrop (Thermo Scientific), diluted down to specified concentrations, and sequenced in both directions by Source BioScience (Cambridge and Nottingham). Sequence inspections, cleaning and assembly were carried out using the ChromasPro software, and species identification was performed by comparing derived sequences with sequences published in the GenBank database with the use of the Basic Local Alignment Search Tool (BLAST). To determine the relatedness of *Babesia* spp. detected in badgers and ticks to other known species of *Babesia*, two phylogenetic trees were constructed based on partial sequences of the 18S rRNA and  $\beta$ -tubulin genes of *Babesia* spp. from this study and from already published sequences. Sequences were aligned using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) tool, and trees were generated using the Neighbour-Joining method and the Jukes-Cantor model in the MEGA6 software.

## 2.8 Statistical analysis

Binomial proportion confidence intervals were calculated for all prevalence results in Microsoft Excel. The binomial proportion confidence interval is a confidence interval for the probability of success if an experiment was repeat multiple times (Wallis, 2013).

## Chapter 3: Results

### 3.1 Badgers from Gloucestershire

A total of 181 blood samples collected from 58 female and 41 male badgers were tested for presence of *Babesia* spp. and *A. phagocytophilum* as part of a previous project (Gbobaniyi, 2016). This was further extended through this study with an additional screening of these samples for *Rickettsia* spp., and an attempt to isolate some of the pathogens, particularly *Babesia* spp., carried by questing ticks at Woodchester Park in Gloucestershire. Badgers from Gloucestershire were found to be infected with *Babesia* spp., but not *A.*

*phagocytophilum* (Gbobaniyi, 2016) or *Rickettsia* spp. The findings are summarised in Table 4, in which historic data was included for comparison of infection rates among badgers from Gloucestershire and NE England. No testing of bloods for *T. pestanai* was carried out as the prevalence of *T. pestanai* in badgers from Gloucestershire has already been determined. As for *Borrelia* spp., the spirochaetes are commonly found in skin and internal organ biopsies but not in the peripheral blood, and thus badger bloods were not screened for *Borrelia* spp. neither.

During previous studies, the DNA of *Babesia* spp. was detected in 179 of the 181 blood samples tested, giving a prevalence of 98.9% (95% CI 96.1 – 99.9). Each individual tested positive at least once, and an occurrence of chronic infections was apparent. Of the two blood samples that were found to be negative, one was of a male cub and the other one of an adult male. They both tested positive for *Babesia* spp. three months later (Gbobaniyi, 2016). A closer look at the body temperatures of these two individuals taken at times of sampling did not reveal any marked changes. As for the rest of the badgers, which tested positive for *Babesia* spp., some fluctuations in body temperatures were observed; however, it is difficult to say whether this was a result of *Babesia* infections or an infection with a different pathogen. Overall infection rates ranged from 39/41 (95.1%, 95% CI 83.4 – 99.4) among males to 58/58 (100%, 95% CI 93.8 – 100) among the females. An observation that cubs as young as one month old were already positive suggested the possibility of other means of transmission than by a tick, such a vertical transmission from a mother to her offspring. While the infection rate of male badgers were slightly lower than that of the females, there were no apparent associations between the age or social groups and the prevalence of infections observed, thus no further statistical analysis was carried out.

**Table 4:** Infections detected in badgers and questing ticks from Gloucestershire

Pathogen	Badger Infection rates (%)	<i>I. ricinus</i> Infection rates (%)		
		Nymphal	Female	Overall
<i>Babesia</i> spp.*	179/181 (98.9%)*	10/93 (10.8%)	0/11 (0%)	9.6%
<i>Anaplasma phagocytophilum</i> *	0/201 (0%)*	1/93 (1.1%)	1/11 (9.1%)	1.9%
<i>Rickettsia</i> spp.	0/181 (0%)	3/93 (3.2%)	0/11 (0%)	2.9%
<i>Borrelia</i> spp.	N/A	8/93 (8.6%)	3/11 (27.3%)	10.6%

\* Historic data (Gbobaniyi, 2016)

### 3.2 Ticks from Gloucestershire

In total, 107 host-seeking ticks, of which 93 were nymphs, 11 females and 3 males were collected off the vegetation, all of which belonged to species *I. ricinus* as expected. An attempt to isolate pathogens infecting *I. ricinus* at Woodchester Park was made, using IRE/CTVM19 tick cell line cultures. Upon inoculation with halved ticks, cell cultures were maintained for 3 weeks, after which changes in turbidity were observed. What was initially thought to be an overgrowth of tick cells was confirmed to be a yeast contamination on week 6 post-innoculation. While the attempt to isolate any pathogens was unsuccessful, the screening of 104 of the collected ticks (Table 4) revealed that 9.6% (95% CI 4.7 - 17) of ticks, 10 nymphal (10.8%, 95% CI 5.3 - 18.9) and no females were positive for *Babesia* spp. A relatively high number of nymphal ticks appeared to be infected with *Babesia* spp., while no Babesiae were found in females. Of these, one harboured a co-infection with *Rickettsia* spp. and another one with *A. phagocytophilum*. It has to be noted however, that there were considerable cross-reactivity issues encountered on RLB and due to insufficient amount of DNA left in some of the samples, they could not be retested to confirm this high prevalence by a standard PCR. As for *A. phagocytophilum* and *Rickettsia* spp., 1.9% (95% CI 0.2 - 6.8)

of ticks (1 nymph and 1 female) were infected with *A. phagocytophilum* and 2.9% (95% CI 0.6 - 8.2) (3 nymphs and no females) with *Rickettsia* spp. The results of DNA sequencing of randomly picked *Babesia* and *Rickettsia* spp. positive samples detected in ticks can be found in section 3.5.

Additionally 10.6% (95% CI 5.4 - 18.1) of ticks, 8.6% on nymphal (95% CI 3.8 - 16.3) and 27.3% of female ticks (95% CI 6 - 61) were infected with *Borrelia* spp. A considerably higher proportion of female (3/11) compared to nymphal ticks (8/93) were found to be infected with *Borrelia* spp. Genotyping of *Borrelia* spp. positive samples revealed that these ticks were infected with *B. garinii* (5.8%, 95% CI 2.2 - 12.1), *B. valaisiana* (5.8%, 95% CI 2.2 - 12.1) and *B. afzelii* (0.96%, 95% CI 0.02 - 5.2). Four of the nymphal ticks were infected with *B. garinii*, two with *B. valaisiana*, and one harboured a co-infection with *B. garinii* and *B. valaisiana*. The DNA from one additional nymph failed to react with the probes. Of the three positive females, one was infected with *B. valaisiana* only, while the second and third females harboured a *B. afzelii*-*B. valaisiana* and *B. garinii*-*B. valaisiana* co-infections, respectively. No *B. miyamotoi* was found. A co-infection with *B. valaisiana* and *Rickettsia* spp. was found in one nymphal tick.

### 3.3 Badgers from Northeast England

A total of 131 blood samples collected from 44 female and 50 male badgers were screened for *Babesia* spp., *Rickettsia* spp., *A. phagocytophilum* and *T. pestanai* (Table 5). All 131 blood samples were positive for *Babesia* spp., giving an overall infection rate of 100% (95% CI 97.2 - 100), and further supporting a previous observation of chronic infections and a possibility of vertical transmission among badgers. Only the data on body condition and no body temperature status were collected at each capture, however, no marked differences in the collected data were observed. Furthermore, no *A. phagocytophilum*, *Rickettsia* spp. or *T. pestanai* were detected, and no screening was done for *Borrelia* spp. as the spirochaetes can be commonly found in skin and internal organ biopsies but not the peripheral blood.

**Table 5:** Infections detected in badgers and ticks from Northeast England

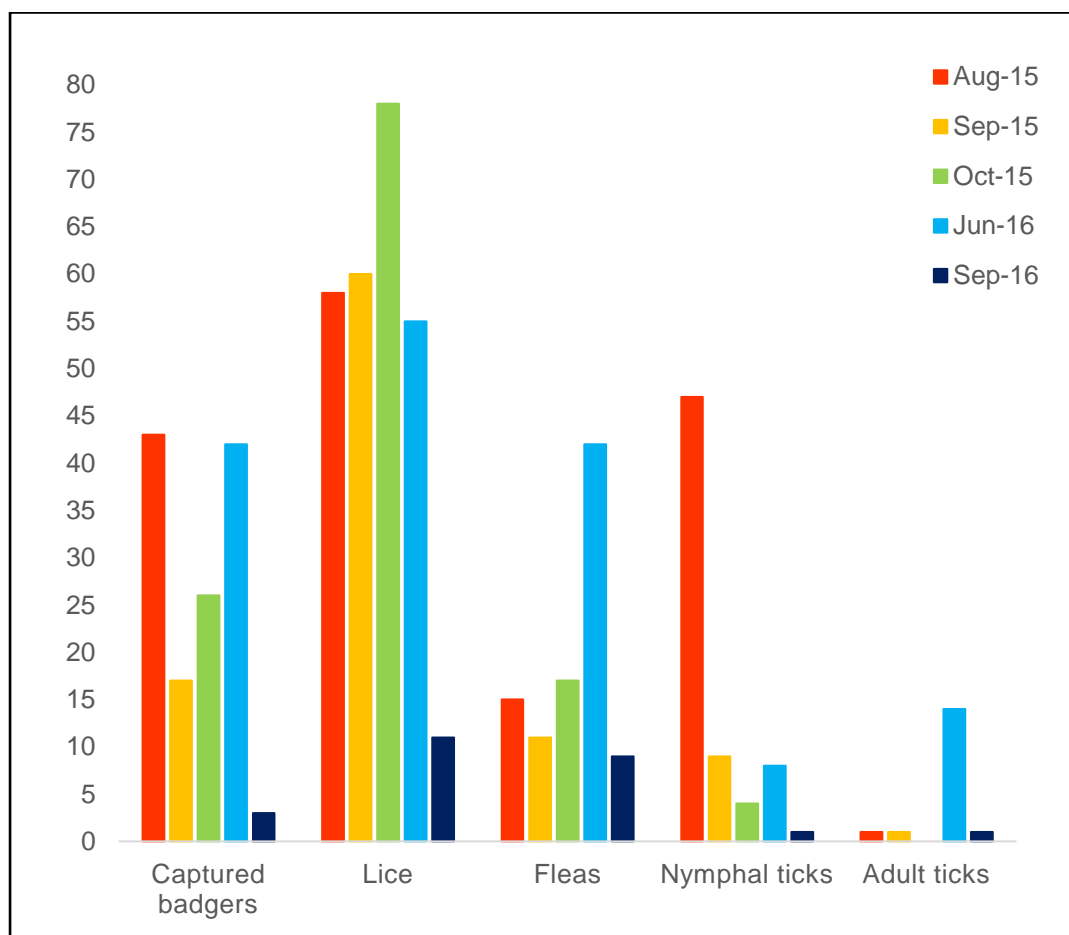
Pathogen	Infection rates (%)			
	Badgers	<i>I. ricinus</i>	<i>I. hexagonus</i>	<i>I. canisuga</i>
<i>Babesia</i> spp.	131/131 (100%)	3/3 (100%)	0/3 (0%)	27/91 (29.7%)
<i>Anaplasma phagocytophilum</i>	0/131 (0%)	0/3 (0%)	0/3 (0%)	0/91 (0%)
<i>Rickettsia</i> spp.	0/131 (0%)	0/3 (0%)	0/3 (0%)	0/91 (0%)
<i>Borrelia</i> spp.	N/A	0/3 (0%)	0/3 (0%)	0/91 (0%)
<i>Trypanosoma pestanai</i>	0/131(0%)	N/A	N/A	N/A

### 3.4 Ectoparasites from Northeast England

Of the 459 ectoparasites collected from badgers in NE England, 267 were *T. melis* lice (58.2%, 95% CI 53.5 - 62.7), 93 *P. melis* fleas (20.3%, 95% CI 16.7 - 24.2), and 99 ticks (21.6%, 95% CI 17.9 - 25.6). Of the 99 ticks, 3 (3%, CI 0.6 - 8.6) were *I. ricinus* (all female), 3 (3%, CI 0.6 - 8.6) *I. hexagonus* (2 nymphs, 1 female), and 93 (94%, CI 87.3 - 97.4) *I. canisuga* ticks (1 larva, 78 nymphs and 14 female). However, only 91 of *I. canisuga* ticks were screened as two of the nymphs were lost during processing. No male ticks were found. All ticks were at least partially engorged except for one *I. canisuga* nymph, which appeared unfed and had its mouthparts intact. Only the ticks belonging to *I. canisuga* (29.7%, 95% CI 20.6 - 40.2) and *I. ricinus* species (100%, 95% CI 29.2 - 100) were found to be positive for *Babesia* spp. (Table 5). Even partially fed *I. canisuga* nymphs gave a faint band on a gel, whereas no DNA of the piroplasmid was detected in the three *I. hexagonus* ticks retrieved from badgers. These three ticks were collected from a single badger each, and no other species of ticks were collected from these badgers. Therefore, it could not be established whether the lack of *Babesia* in these ticks was a result of very low blood parasitaemias in

badgers, or whether the tick is not a competent vector for *Babesia* spp. infecting badgers. Further analysis of *Babesia* spp. detected in badgers is detailed in section 3.5.

When compared to the number of badgers captured, as shown in Figure 5, infestations with ticks appeared to be more prevalent in the months of June, August and September. Nymphal tick infestations were at their highest in August 2015 where 47 of the 48 ticks collected from 43 badgers were nymphal, and in September 2015 where 9 of the 10 ticks collected from 17 individuals were nymphs. On the other hand, infestations with lice were at the highest in the months of September and October, with 60 lice collected from 17 badgers in September 2015, 78 lice collected from 26 badgers in October 2015, and 11 lice from 3 badgers in September 2016. Infestation rates with fleas showed marked fluctuations.



**Figure 5:** Infestation rates of captured badgers

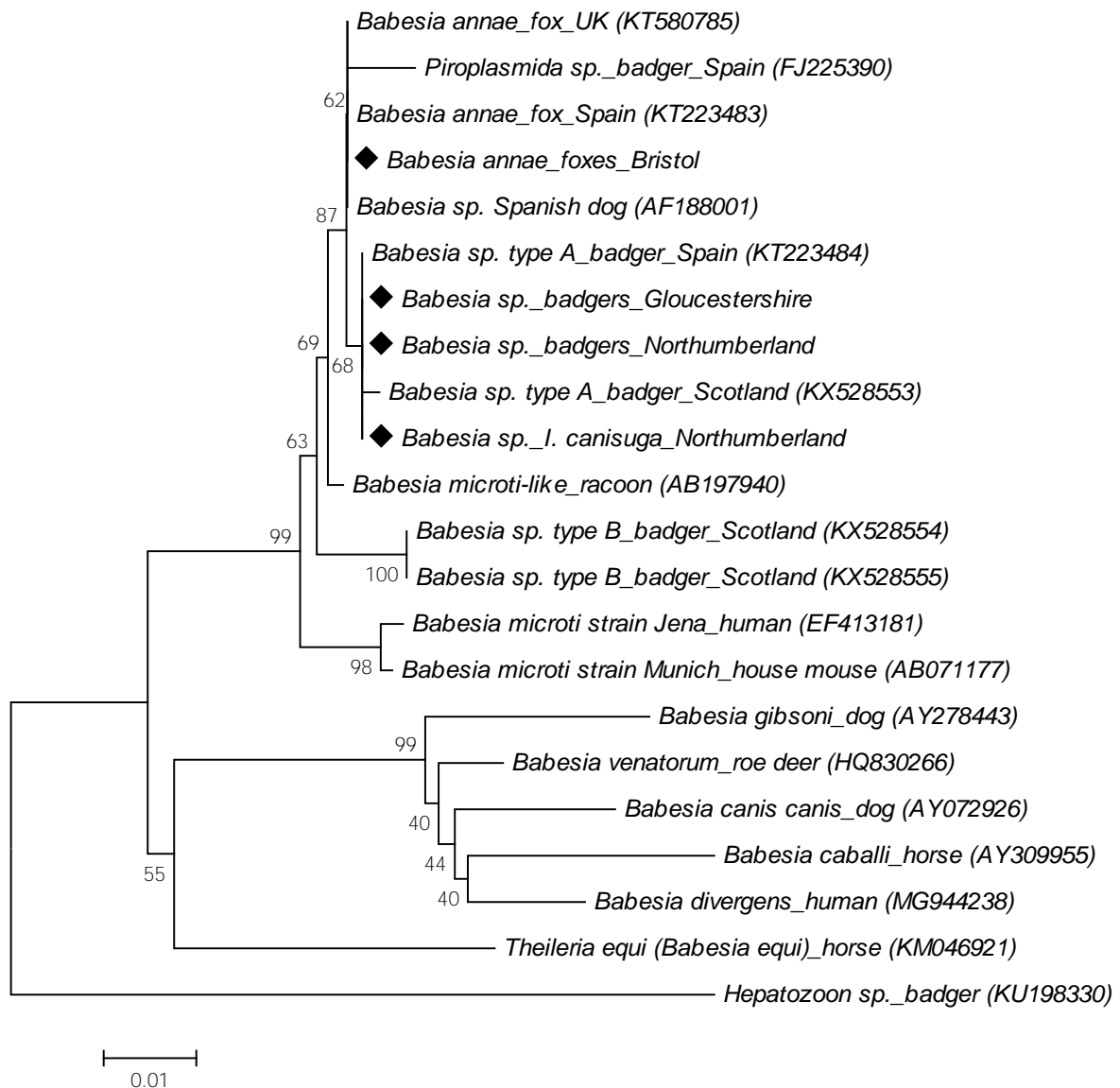
### 3.5 DNA sequencing and phylogenetic analysis

Two genetic markers were used to identify the species of *Babesia* detected in badgers and ticks from NE England, namely the 18S rRNA and the  $\beta$ -tubulin genes. DNA sequencing of 622 bp long fragments of the 18S rRNA gene from four positive badgers from Gloucestershire, Southwest England and four from Northeast England revealed that the sequences were identical to each other and to *Babesia* sp. type A infecting badgers in Northern Spain (KT223484). The sequences were additionally found to be 99% identical to *Babesia* sp. type A isolates from British badgers (KX528553), and only 96% identical to *Babesia* sp. type B from British badgers (KX528554, KX528555). Furthermore, the species of Babesiae infecting badgers were 99% similar to *B. annae*/*B. vulpes* infecting foxes in Spain and the UK (KT223483, KT580785), including those from Bristol. The sequencing of about 150 bp long fragments of the  $\beta$ -tubulin gene showed an 89% homology to *B. microti* Munich strain (AB124587) and an 87% homology to a *Babesia microti*-like species (*B. annae*) from a fox (AY144707).

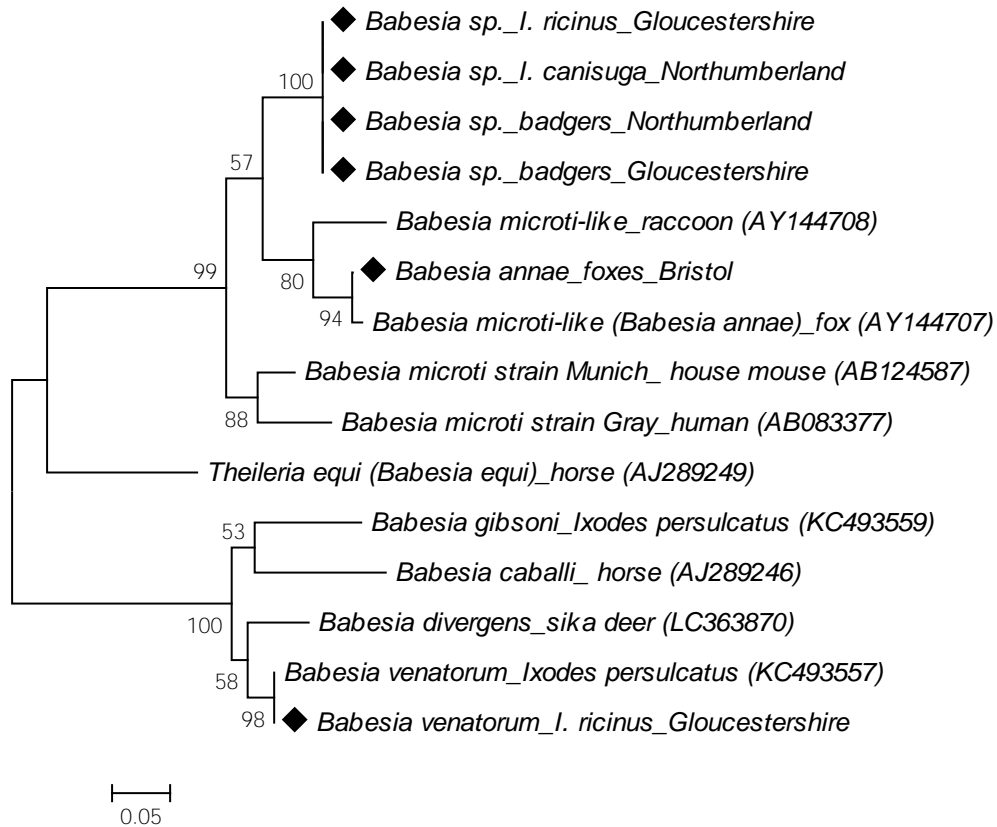
The sequencing of a  $\beta$ -tubulin fragment from 3 host-seeking ticks collected off the vegetation at Woodchester Park revealed that one of the ticks was infected with *B. venatorum* (99% identical to *B. venatorum*, accession number KC493557) and the other two ticks with *Babesia* spp. infecting badgers. The DNA sequences found in these two ticks were identical to the DNA sequences of *Babesia* spp. infecting badgers in both the Southwest and Northeast England, meaning that *I. ricinus* could serve as its vector. Furthermore, one of the ticks was co-infected with *A. phagocytophilum*, which co-incidentally was not detected in badgers. Considering this tick was at its nymphal stage, *Babesia* spp. infecting badgers appear to be transmitted in *I. ricinus* transovarially, and the tick appeared to have picked up *A. phagocytophilum* during its bloodmeal at the larval stage. As for ticks from NE England, a sequencing of both  $\beta$ -tubulin and 18S rRNA genes of *Babesia* spp. from two *I. ricinus* and one *I. canisuga* ticks was attempted. While the DNA sequencing of a  $\beta$ -tubulin fragment detected in one of the *I. ricinus* removed from badgers produced only a partial readable



sequence, this sequence was found to be highly similar to the one detected in badgers and clearly different from the one detected in foxes. The  $\beta$ -tubulin sequence from the second tick was unclear, suggesting a co-infection with another species of *Babesia*. The sequencing of 18S rRNA gene was unsuccessful. The  $\beta$ -tubulin and 18S rRNA sequences detected in *I. canisuga* were identical to those found in badgers.



**Figure 6:** Phylogenetic tree based on alignment of partial sequences of the 18S rRNA gene of *Babesia* spp. with *Hepatozoon* sp. from a badger included as an outgroup, demonstrating the relatedness of *Babesia* spp. detected in badgers and ticks in this study to other known species of *Babesia*. The phylogenetic tree was generated using the Neighbor-Joining method and the Jukes-Cantor model in MEGA6.



**Figure 7:** Phylogenetic tree based on alignment of partial sequences of the  $\beta$ -tubulin gene of *Babesia* spp., demonstrating the relatedness of *Babesia* spp. detected in badgers and ticks in this study to other known species of *Babesia*. The phylogenetic tree was generated using the Neighbor-Joining method and the Jukes-Cantor model in MEGA6.

The phylogenetic trees in Figures 6 and 7 are based on about 600 bp long fragments of the 18S rRNA gene and 150-270 bp long fragments of the  $\beta$ -tubulin gene of different species of *Babesia*. The trees branch into groups of *Babesia* spp. infecting ruminants, humans, horses and dogs, and groups of *Babesia* spp. infecting rodents and humans (*B. microti*) and those infecting dogs and wild carnivores. *Hepatozoon* spp. from a badger was used as an outgroup. The results of phylogenetic analysis demonstrate that the species of *Babesia* infecting badgers in the Southwest and the Northeast of England are Type A species, and closely related to *B. annae* associated with foxes (which causes disease dogs)

and *Babesia microti*-like species infecting racoons; and only distantly related to *B. microti* genotypes harboured by rodents, some of which are pathogenic to humans.

As for *Rickettsia* spp., the sequencing of 771 bp long fragments of the *gltA* gene from two host-seeking *I. ricinus* showed a 99% homology to *R. helvetica* detected in *I. ricinus* from Switzerland (U59723) and *I. ricinus* removed from dogs in Gloucestershire. No other species of *Rickettsia* were found.

#### Chapter 4: Discussion

The aim of this study was to clarify what vector-borne infections are harboured in Eurasian badgers in the UK, and whether ticks parasitizing these badgers pose any risk to animal or human health. Two distinct locations were chosen, one with a large population of badgers involved in capture-recapture studies, and another one where one group of badgers was exposed to capture-recapture and another from which captured badgers have been permanently removed. This study has reiterated the findings of another, smaller study, that badgers do not appear to be competent reservoir hosts for *A. phagocytophilum* (Jahfari *et al.*, 2014a), and that they do not appear to harbour *Rickettsia* spp. However, they can be heavily infected with piroplasmids. In this study, all badgers, both in Gloucestershire, Southwest England and in Northeast England, tested positive for *Babesia* spp. at least once, and an evidence of chronic infections was apparent. No contamination was detected, all negative and contamination controls remained negative. There was no marked difference in the infection rates among badgers from Gloucestershire and NE England, or the two groups of badgers from which samples were taken in the Northeast, and there was no apparent association between the badgers' age, sex or social groups and the observed rates of infection.

Overall, the infection rates among badgers in Gloucestershire and NE England were much higher than among badgers in Scotland, where only 70.2% of badgers were found to be positive for *Babesia* spp. (Bartley *et al.*, 2017). Only one type of *Babesia* was found in badgers in this study, however, it has to be emphasised that the proportion of sequenced

samples was much smaller compared to those sequenced by Bartley *et al.* (2017). Studies from elsewhere in Britain found badgers infected with *Babesia* spp. in Cornwall, South England (Peirce & Neal, 1974a) and Oxfordshire, Southwest England (Macdonald *et al.*, 1999). Macdonald *et al.* (1999) referred to these piroplasmids as *Babesia missirolii* and reported that based on their microscopic examinations of badger blood smears, 77.2% of individuals tested positive at least once over 3 years. Furthermore, there was an evidence of some age- and sex-related variations in the infection rates of badgers, but not in the year of the highest prevalence, and no significant difference in the infection rates among different social groups was observed (Macdonald *et al.*, 1999). The findings from this study, to some degree, correlate with their findings. The marked differences in infection rates among badgers from this study and in the findings of Macdonald *et al.* (1999) appear to be a result of the two different approaches employed to screen the bloods of badgers; that is the use of DNA-based and microscopical techniques. A microscopical detection of piroplasmids in chronically infected and carrier animals can be challenging due to lower and intermittent parasitaemias, as previously seen in dogs infected with *B. gibsoni* (Jefferies *et al.*, 2007). On the other hand, while DNA-based studies provide means of species identification and differentiation that visual observations cannot, they are also able to detect very low parasitaemias in chronically infected animals. It has to be emphasised, however, that the level of parasitaemia can affect the success of parasite transmission from one host to another. This has been demonstrated in an *in vitro* study on the transmission of *B. microti* by *Ixodes trianguliceps* among voles (Randolph, 1995). Randolph (1995) showed that there is a very brief duration of infectivity in natural hosts, and that the level of host parasitaemia affects the probability of parasite transmission from a host to tick, as well as the transstadial transmission within the tick, therefore the probability of transmission from one host to another. It has to be borne in mind, however, that animal *in vitro* studies in the laboratory may not be truly representative of infections in the wildlife. Animals out in the wild face different conditions and environmental pressures, which may affect their immune responses or the levels of parasitaemia in their bloodstream in different ways.

Detection of *Babesia* spp. in cubs as young as one month old either indicates that cubs become infected while still inhabiting their setts, soon after their emergence, or that the piroplasmids may be transmitted among badgers by means other than by a tick – such as a vertical transmission from a mother to her offspring. Additional means of transmission among adult badgers may include transmission of Babesiae via bite wounds. Territorial defence by badgers may involve aggressive encounters between members of different social groups, which may lead to bite wounds that may be a potential route for pathogen transmission (Delahay *et al.*, 2006). After all, the possibility of alternative routes of transplacental transmission (Falkenö *et al.*, 2013; Fukumuto *et al.*, 2005; Simões *et al.*, 2011) and via biting wounds (Jefferies *et al.*, 2007; Yeagley *et al.*, 2009) have already been described for other species of *Babesia* infecting carnivores. The presence of chronic infections with *Babesia* spp. has been also been reported in a number of other animals, including rodents (Bown *et al.*, 2008) and dogs (Jefferies *et al.*, 2007). It is not known what precise effect these piroplasmids have on the overall health of badgers; further studies are needed to determine that.

The results of phylogenetic analysis based on two genetic markers demonstrated that the species of *Babesia* harboured in badgers clustered together with the species of *Babesia* harboured in foxes and racoons, as expected. The piroplasmids from badgers are closely related to, but clearly different from, *B. annae* associated with foxes, which is pathogenic to dogs. Another difference between *Babesia* spp. from badgers and *B. annae* is in their respective levels of infectivity in their natural hosts. While the infection rates with *Babesia* spp. among badgers can surpass 70%, the infection rates with *B. annae* among foxes have found to be much lower – an average of 14.1 – 34.2% in England (Alharbi *et al.*, 2018; Bartley *et al.*, 2016), 7.5% in Scotland (Bartley *et al.*, 2016), and 16.7 - 45.8% in Northern Spain (Barandika *et al.*, 2016; Millán *et al.*, 2016). Furthermore, while the DNA of *B. annae* was previously detected in *I. canisuga* collected from badgers in Spain (Millán *et al.*, 2016), no *B. annae* has been found in badgers in this study or other studies (Barandika *et al.*, 2016; Bartley *et al.*, 2017; Gimenez *et al.*, 2009). It is therefore safe to say that the

piroplasmids detected in badgers in Gloucestershire and NE England are identical to *B. missirolii* and that badgers are not competent reservoir hosts for *B. annae*.

*I. canisuga* and *I. hexagonus* have been suspected of transmitting *B. missirolii* among badgers (Barandika *et al.*, 2016; Peirce & Neal, 1974a); however, no definite vector has been confirmed. Ticks removed from badgers in NE England belonged to the species of *I. canisuga* (93/99), *I. ricinus* (3/99) and *I. hexagonus* (3/99); all of which were at least partially engorged, except for one *I. canisuga* nymph. A considerably higher proportion of *I. hexagonus* and *I. ricinus* ticks were found to parasitize badgers from Gloucestershire, compared to NE England (Perrin, 2011; Prof R. Birtles, personal communication, 27 June 2018). While *I. hexagonus*, being a nidicolous tick which primarily resides in the nests and burrows of their hosts, appears to show less marked seasonal changes than *I. ricinus* (Cull *et al.*, 2018), studies show that the tick is encountered less frequently in the North of the country (Davies *et al.*, 2017; Smith *et al.*, 2011). The findings of this study support that. Furthermore, while the three *I. hexagonus* ticks from NE England were negative for *Babesia* spp., 27/91 (29.7%) *I. canisuga* and 3/3 (100%) *I. ricinus* ticks were found to be positive. This observation could be explained in two ways. Either *I. hexagonus* are not competent vectors of *Babesia* spp. infecting badgers; which would explain the relatively low infection rates of 52.5% among badgers from northern Spain that were heavily infected with *I. hexagonus* but not so much with *I. canisuga* or *I. ricinus* (Barandika *et al.*, 2016). Although, no ticks off Scottish badgers were collected or screened by Bartley *et al.* (2017). Alternatively, the blood parasitaemias in those three badgers could have been too low for the ticks to pick up the parasite. Because no other species of ticks were removed from the three badgers in question, it cannot be established whether the lack of *Babesia* in these ticks was a result of very low blood parasitaemias, or whether the species of tick is not a competent reservoir of *B. missirolii*. Therefore, a further study is required to establish the role of *I. hexagonus* as a vector of *Babesia* spp. among badgers in the UK. Considering the very high proportion of *I. canisuga* feeding on badgers, and the detection of exactly the same species of *Babesia* in badgers and *I. canisuga* feeding on badgers, the tick appears to be a

competent vector. No *A. phagocytophilum*, *Borrelia* or *Rickettsia* spp. were detected in ticks from NE England. *Borrelia* spp. have been previously detected in *I. canisuga* retrieved from badgers (Couper *et al.*, 2010). A closer look at a study of *Borrelia* spp. in questing ticks at Kielder Forest, Northeast England, revealed that while the density of ticks there is high, the prevalence of *Borrelia* is low. *B. valaisiana* was the only genospecies found (Bettridge *et al.*, 2013). This either means that *Borrelia* spp. are absent in the sampling locations of NE England, or that their prevalence is extremely low and hence they are hard to detect.

Overall infestation rates of badgers with ectoparasites appear to match the trend of other studies, that infestations with lice (*T. melis*) are at their highest, followed by ticks and fleas (*P. melis*) (Butler & Roper, 1996; Do Linh San, 2007). Infestations with ticks appeared to be more prevalent in the months of June, August and September, with the number of nymphal ticks being at their highest in the months of August and September; corresponding with findings from Switzerland (Do Linh San, 2007). On the other hand, infestations with lice were at their highest in the months of September and October, contrary to the findings of Do Linh San (2007) that infestation rates with lice among badgers were at their highest from spring to autumn. The occurrence of fleas on badgers showed marked fluctuations. It has to be emphasised though that a human error factor resulting in an unconscious omission of some ectoparasites at the time of collection must be taken into consideration.

Only *I. ricinus* ticks were collected off the vegetation at Woodchester Park, as expected. Because of their close associations with their hosts' nests and burrows, *I. hexagonus* and *I. canisuga* are not readily picked up by blanket dragging. No larval *I. ricinus* were found on the vegetation in April, which correlates with the findings of other studies, that larvae tend to start questing around the time of May (Cull *et al.*, 2018; Dobson *et al.*, 2011; Jameson & Meldock, 2011; Randolph *et al.*, 2002). An attempt to isolate pathogens infecting *I. ricinus* at Woodchester Park, in particular *Babesia* spp., was unsuccessful due to yeast contamination of cell cultures. Bacteria, molds and yeasts are found virtually everywhere. They can enter cell cultures through a number of ways, including airborne particles and aerosol fallout during culture manipulation, contact with nonsterile surfaces when performing cell

culture manipulations, and human error. Cell culture contaminants, such as yeast, are able to quickly colonise and thrive in the rich environment within cell cultures, and achieve high densities due to their relatively fast growth rates. Eventually, these contaminants can alter the growth and characteristics of the infected cultures (Corning Life Sciences, 2008). In this case, the source of contamination is thought to have been either an airborne particle fallout during culture manipulations or the cabinet used to perform culture manipulations. A number of other people using the same cabinet also detected yeast contamination in their work. Considering the timeline of this project and logistical problems associated with the collection of a new batch of ticks from Woodchester Park, it was decided that no further attempt at the isolation of tick-borne pathogens in tick cell cultures would be made.

While an attempt to isolate any pathogens was unsuccessful, the screening of 104 of the collected ticks revealed that 10.8% of the nymphal ticks and no females were infected with *Babesia* spp. A relatively high number of nymphal ticks appeared to be infected, which may be partially attributed to the crossreactivity issues detected on RLB. Of the three *Babesia* positive samples sequenced, one was found to be *B. venatorum*, a zoonotic species of *Babesia* harboured in roe deer (*Capreolus capreolus*) (Andersson *et al.*, 2016; Bonnet *et al.*, 2007a) and endemic to UK (Abdullah *et al.*, 2017; Davies *et al.*, 2017; Smith *et al.*, 2013); the other two were identical to the species infecting badgers. Furthermore, one of the ticks infected with *B. missirolii* was found to be co-infected with *A. phagocytophilum*. Considering that badgers do not appear to be competent reservoirs of *A. phagocytophilum*, and while the DNA of *A. phagocytophilum* has been detected in questing *I. ricinus* larvae in Europe (Jahfari *et al.*, 2014a; Krücken *et al.*, 2013) but no transovarial transmission followed by a successful transstadial transmission of *A. phagocytophilum* in ticks has been proven, it is likely that the tick would have picked up this pathogen during its bloodmeal at the larval stage. In this case, the tick would have acquired the piroplasmid transovarially and allowed for their further transstadial transmission. It can be therefore argued that this finding substantiates vector competence of *I. ricinus* for *Babesia* spp. infecting badgers, additionally to *I. canisuga*. Furthermore, another *Babesia* spp. infected nymph, species of which was not determined,



harboured a *Rickettsia* spp. co-infection. Overall, 1.9% of ticks were found to be infected with *A. phagocytophilum* and 2.9% with *R. helvetica*, further reiterating the findings of a previous study (Tijssen-Klasen *et al.*, 2011) showing that *R. helvetica* is present in the UK, especially in the Gloucestershire area. One of the *R. helvetica* infected ticks harboured a co-infection with *B. valaisiana*.

As for *Borrelia* spp., 10.6% of questing ticks from Gloucestershire were infected with *Borrelia* spp. A considerably higher proportion of female (3/11) compared to nymphal ticks (8/93) were found to be infected. However, it has to be noted that the sample size of female to nymphal ticks was considerably small, therefore the true rate of infection among female *I. ricinus* could not be properly quantified and is likely overestimated. Whereas no *Borrelia* were detected in NE England, three genospecies of *Borrelia* spp. appear to circulate in Gloucestershire - *B. garinii*, *B. valaisiana* and *B. afzelii*. While these pathogens were detected in questing ticks, rather than ticks removed from badgers, badgers have been previously shown to be reservoir competent hosts for *B. afzelii* and *B. valaisiana* (Gern and Sell, 2009; Wodecka *et al.*, 2016); and possibly even for *B. garinii* (Wodecka *et al.*, 2016). It may be therefore assumed that badgers from Gloucestershire contribute to the circulation of at least *B. afzelii* and *B. valaisiana*. A closer look at co-infections revealed that one nymphal tick was co-infected with *B. garinii* and *B. valaisiana*, and two female ticks with *B. afzelii* and *B. valaisiana*, and *B. garinii* and *B. valaisiana*, respectively.

Last of all, badgers from the Northeast were screened for the *P. melis*-transmitted *T. pestanai*, since the prevalence of trypanosomes among badgers from Gloucestershire has already been determined (Ideozu *et al.*, 2015). While 35.4% of badgers from Gloucestershire were infected with *T. pestanai* (Ideozu *et al.*, 2015), closely matching infection rates (31%) of badgers in neighbouring Oxfordshire (Lizundia *et al.*, 2011), no *T. pestanai* was detected in badgers from NE England. This shows that while *P. melis* is common among badgers in the Northeast, trypanosomes are not. Woodchester Park in Gloucestershire has a high-density population of badgers, which is not the case in NE England. It may be therefore argued that the low-density population of badgers in the Northeast compared to

Gloucestershire could have been the reason why *T. pestanai* was not detected in badgers. Future studies may investigate whether *T. pestanai* may be of critical health importance either to badgers or other animals, and whether it may be transmitted by ticks, in addition to *P. melis*.

In conclusion, it was demonstrated that badgers are not competent reservoir hosts for *A. phagocytophilum*, or *Rickettsia* spp., and that the species of *Babesia* infecting badgers (*B. missirolii*) are clearly distinct from *B. annae* or other species of *Babesia* pathogenic to dogs. To date, there have been no reports of small *Babesia* other than *B. gibsoni* or *B. annae* (*T. annae*) causing disease in domestic canines. It is unknown, however, to what extent this species of *Babesia* have on the overall health of badgers; further studies are needed to determine that. *Ixodes ricinus* and *I. canisuga* appear to be transmitting *Babesia* spp. among badgers, however, other routes of transmission (such as transplacentally or via biting wounds) should also be considered. Future studies are required to confirm the role of *I. hexagonus* in the transmission of Babesiae among badgers. Badgers may however, contribute to the circulation of *Borrelia* spp. in Gloucestershire.

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## Appendix 1: Reverse line blot solutions

<b>Final solution</b>	<b>Formula</b>
<b>2x SSPE</b>	50 ml of 20x SSPE + 450 ml of dH <sub>2</sub> O
<b>2x SSPE/0.1% SDS</b>	50 ml of 20x SSPE + 5 ml of 10% SDS + 455 ml of dH <sub>2</sub> O
<b>2x SSPE/0.5% SDS</b>	50 ml of 20x SSPE + 25 ml of 10% SDS + 425 ml of dH <sub>2</sub> O
<b>1% SDS</b>	50 ml of 10% SDS + 450 ml of dH <sub>2</sub> O
<b>20 mM EDTA</b>	20 ml of 0.5M EDTA + 480 ml of dH <sub>2</sub> O