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Short communication

Short Communication: *Anaplasma phagocytophilum* and *Babesia* spp. in ixodid ticks infesting red foxes (*Vulpes vulpes*) in Great Britain

Karen L Mansfield [a,*], Estela González [a], Stuart McKay [a], Ternenge Apaa [a], Alexander J Kent [b], Paul Cropper [b], Naomi Berry [b], Luis M Hernández-Triana [a], Nicholas Johnson [a,c]

[a] Animal and Plant Health Agency (APHA), Woodham Lane, Addlestone, KT15 3NB, UK
[b] Animal and Plant Health Agency, York Biotech Campus, Sand Hutton, York, YO41 1LZ, UK
[c] Faculty of Health and Medical Sciences, University of Surrey, Guildford, Surrey, GU2 7XH, UK

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ABSTRACT

Red foxes (*Vulpes vulpes*) are found throughout the United Kingdom (UK), and can reach high population densities in urban areas. They are often infested with ticks which may carry tick-borne pathogens, leading to a risk of transmission to domestic animals and humans. This study investigated the prevalence of tick-borne pathogens in ticks sourced from red fox carcasses across Great Britain between 2018 and 2022. Tick species were identified using morphological keys and molecular barcoding, followed by specific pathogen testing using PCR. In total, 227 ticks were collected from 93 foxes. Pooling ($n = 2$) was undertaken for unengorged nymphs from the same tick species and fox host, with 203 homogenates tested in total (24 pools and 179 individual ticks). *Ixodes hexagonus* was the most abundant tick species sampled (73 %), of which 59 % were nymphs and 41 % were females. Less common were *Ixodes ricinus* (12 %) and *Ixodes canisuga* (15 %), the majority of which were females (73 % and 91 %, respectively). One *Ixodes* sp. larva was identified. *Babesia* DNA was identified in seven individual ticks and once in pooled ticks ($n = 2$); seven detections were in *I. hexagonus* and one in *I. canisuga*, with an overall detection rate of 7 % (95 % CI: 6 – 8 %). Sequence analysis confirmed that all *Babesia* detections in *I. hexagonus* were *Babesia vulpes*, with detection of *Babesia* Badger Type A in *I. canisuga*. Screening for *Anaplasma phagocytophilum* DNA through amplification of the *msp2* gene yielded an overall detection rate of 4 % (detected in *I. hexagonus* only). Louping ill virus was not detected by qRT-PCR in any tick RNA tested. The majority of pathogen detections were in ticks from red foxes in rural areas of the UK, although a small number of *Babesia* detections were in ticks collected from semi-rural or urban red foxes. Additionally, *B. vulpes* was detected in GB red fox tissues, suggesting a potential role as a reservoir host. This study confirms the detection of tick-borne pathogens in ticks infesting UK red foxes and highlights the involvement of GB tick species in animal or human disease transmission.

1. Introduction

European red foxes are frequently infested with ticks (Bartley et al., 2016; Lesiczka et al., 2023a), which include exophilic species such as *Ixodes ricinus, Dermacentor reticulatus, Rhipicephalus sanguineus* s.l., or *Haemaphysalis punctata*, and endophilic, nest-dwelling species such as *Ixodes hexagonus* and *Ixodes canisuga* (Sobrino et al., 2012; Lledo et al., 2016; Dwuznik et al., 2020; Lesiczka et al., 2023a). However, along with a role as a mammalian host for ticks, foxes can act as a reservoir species for tick-borne pathogens that may pose a health threat to humans or other animals (Lesiczka et al., 2023a). European red foxes have been associated with a range of tick-borne pathogens, frequently *Hepatozoon canis* and *Babesia vulpes*, but also *Babesia canis, Borrelia miyamotoi, Borrelia burgdorferi* s.l. complex, *Ehrlichia canis, Candidatus* Neoehrlichia sp., *Rickettsia* spp. and *Anaplasma phagocytophilum* (Duscher et al., 2014; Lledo et al., 2016; Ebani et al., 2017; Mierzejewska et al., 2021; Sgroi et al., 2021; Lesiczka et al., 2023a). Several of these pathogens are also detected in ticks infesting foxes, including *E. canis, Borrelia burgdorferi* s.l. complex and *Babesia* spp. (Hornok et al., 2013; Checa et al., 2018; Wodecka et al., 2022).

In UK livestock, *A. phagocytophilum* is an important cause of tick-borne fever, although very few cases of human granulocytic

* Corresponding author.
E-mail address: Karen.Mansfield@apha.gov.uk (K.L. Mansfield).

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anaplasmosis have been reported (Gandy et al., 2022), and *Babesia* species can cause haemolytic anaemia in humans, ruminants and dogs (Rochlin and Toledo, 2020; Johnson et al., 2022). Babesiosis in cattle is widespread throughout England and Wales (McFazdean et al., 2023), although human infection is relatively rare (UKHSA, 2020). Generally, the *Babesia* species that infect cattle and humans are likely to be different to those infecting canids, including foxes (Laha et al., 2015). Similarly, *A. phagocytophilum* can be differentiated into several genetic variants, according to the vertebrate hosts they are associated with (Dugat et al., 2015). Another tick-borne pathogen, louping ill virus (LIV), is endemic in the UK and transmitted by *I. ricinus*, principally causing neurological disease in sheep (Jeffries et al., 2014).

This study assessed ticks collected from red fox carcasses across England, Wales, and Scotland for the detection of tick-borne pathogens. Furthermore, red fox tissues from western England were assessed for the presence of *Babesia* DNA following a regional fox mortality event. These data provide further empirical evidence on the detection of tick-borne pathogens in GB tick populations, enhancing understanding of the relationships between ticks, the foxes from which they blood-feed, and associated tick-borne pathogens.

2. Materials and methods

2.1. Study 1: Analysis of ticks infesting red foxes

2.1.1. Tick collection, processing and identification

Ticks were sourced from red fox carcasses collected in England, Scotland and Wales as part of an ongoing annual *Echinococcus multilocularis* surveillance programme, coordinated by the Animal and Plant Health Agency (APHA). Foxes killed for pest control purposes were necropsied and screened for the presence of this parasite. All available tick specimens (n = 227) were collected from foxes (n = 93) from February 2018 to July 2019 and November 2021 to October 2022, with restricted fox collection during the cubbing season (March to May). The kill date was unknown for one fox. Several counties (other than Gloucestershire, Somerset, Yorkshire) were targeted to increase coverage, and some sampling in eastern England was limited to specific months for operational reasons. Ticks were stored in ethanol at 4 − 24 °C prior to washing once in 70 % ethanol (5 mins) and twice in distilled water (5 mins). Ticks were morphologically identified under a stereo microscope using published pictorial keys (Sándor et al., 2017a, 2017b; Otranto et al., 2017), before homogenisation in Dulbecco's Modified Eagle's medium (D-MEM) with 10 % foetal bovine serum (FBS). Adults and engorged nymphs were homogenised individually. Partially engorged nymphs from the same tick species and fox host at a given time-point were pooled (maximum n = 2). Hence 203 homogenates in total (24 pools, 179 individual ticks) were maintained at −80 °C prior to testing. RNA was extracted using the NucleoSpin® RNA II kit (Macherey-Nagel), and spectrophotometrically quantified to confirm successful extraction. DNA was extracted using the Wizard® Genomic DNA purification kit (Promega). Extraction kits were used according to manufacturer instructions. To support morphological identification, a PCR that amplified a 658-base pair (bp) region of the tick mitochondrial *COI* gene was used with previously published primers (Folmer et al., 1994), detailed in Supplementary File S1.

2.1.2. Geospatial mapping and statistical analysis

Maps were produced using art ArcGIS Pro 3.0.1 and Safe Feature Manipulation Engine (FME) 2022.2, as detailed in Supplementary File S1. The British National Grid (BNG) reference for fox location was used with the UK Grid Reference Finder website (https://gridreferencefinder.com/) to identify urban versus rural foxes, where semi-rural was considered a large village or an area with a network of large roads. Pearson Chi-square test for independence (level of significance, α = 0.05) was used to evaluate associations between (i) number of each tick species and season, (ii) pathogen detection and tick life stage, and (iii) pathogen detection and fox location (urban or rural). GraphPad Prism (v8.4.2) was used to calculate 95 % confidence intervals (CI) for data describing detection of pathogen DNA.

2.2. Study 2: Fox tissue submission

Ten red fox carcasses (unrelated to Study 1) were submitted to the Wildlife Network for Disease Surveillance (Somerset, UK) for post-mortem from October 2021 to December 2022, following a regional fox mortality event in the west of England. Some foxes appeared jaundiced at post-mortem, and initial tests for leptospirosis and canine adenovirus were negative. Liver, kidney and clotted blood samples (if available) were retained for *Babesia* testing, from which DNA was extracted using the DNeasy® Blood and Tissue kit (Qiagen) according to manufacturer instructions.

2.3. Molecular detection of tick-borne pathogens

Babesia DNA detection in tick and fox samples utilised previously published primers to amplify a 423-bp region of the Piroplasm *18S rRNA* coding sequence (Armstrong et al., 1998), as detailed in Supplementary File S1. Sequence generated for *Babesia*-positive samples were subject to NCBI online BLAST® search for *Babesia* identification. Phylogenetic analysis of a 396-bp fragment of the *Babesia 18S* gene (Study 1) was undertaken as detailed in Supplementary File S1, including reference sequences detailed in Supplementary Table S1. Detection of *A. phagocytophilum* DNA in tick samples utilised previously published primers and probe to amplify a 77-bp region of the *A. phagocytophilum msp2* gene (Courtney et al., 2004), as detailed in Supplementary File S1. For viral RNA detection, extracted RNA was screened using a LIV-specific RT-PCR with previously-published primers and probe to amplify a 97-bp region of the envelope gene (Marriott et al., 2006), as detailed in Supplementary File S1.

3. Results

3.1. Study 1: Analysis of ticks infesting UK red foxes

3.1.1. Tick species infesting red foxes

The most common tick species associated with red foxes was *I. hexagonus* (165/227, 73 %), of which 59 % (97/165) were nymphs and 41 % (68/165) were females (Fig. 1A). In comparison, *I. ricinus* (12 %) and *I. canisuga* (15 %) infested foxes to a lesser extent and the majority were females (73 % and 91 %, respectively). The single larva detected was identifiable only as *Ixodes* sp. The mean number of ticks collected per fox was 2.4 (range 1 − 14), and 50 % of foxes (n = 49) were infested with multiple ticks, including twelve cases of concurrent infestation by different tick species (Supplementary Table S2), comprising *I. hexagonus* with either *I. canisuga* (n = 7) or *I. ricinus* (n = 5). A large proportion of ticks were from foxes in western or southwestern regions of England (Supplementary Fig. 1A), although potential sampling bias cannot be ruled out due to geographical targeting. *Ixodes hexagonus* were detected in England and Wales, *I. ricinus* in England and Scotland, and *I. canisuga* predominantly detected in the West Country of England (including Devon and Herefordshire) (Supplementary Fig. 1B). *Ixodes hexagonus* were detected consistently throughout the year, whereas *I. ricinus* appeared more prominent in spring and autumn (Supplementary Fig. 2, Supplementary Table S3). However, seasonal trends were not significant (p = 0.07), likely due to the limited sample size. Ticks were identified morphologically using published pictorial keys, supported by molecular *COI* gene analysis (Supplementary Tables S4 and S5), and results of successful *COI* gene sequencing were consistent with morphological identification. *COI* gene sequence for *I. hexagonus* specimens shared 99.85–100 % identity with available sequence [MG432679] whilst sequence from *I. canisuga* shared 99.22–100 identity with available sequence [KX218106; KY962047]. In 40 % (n = 82) of DNA samples,

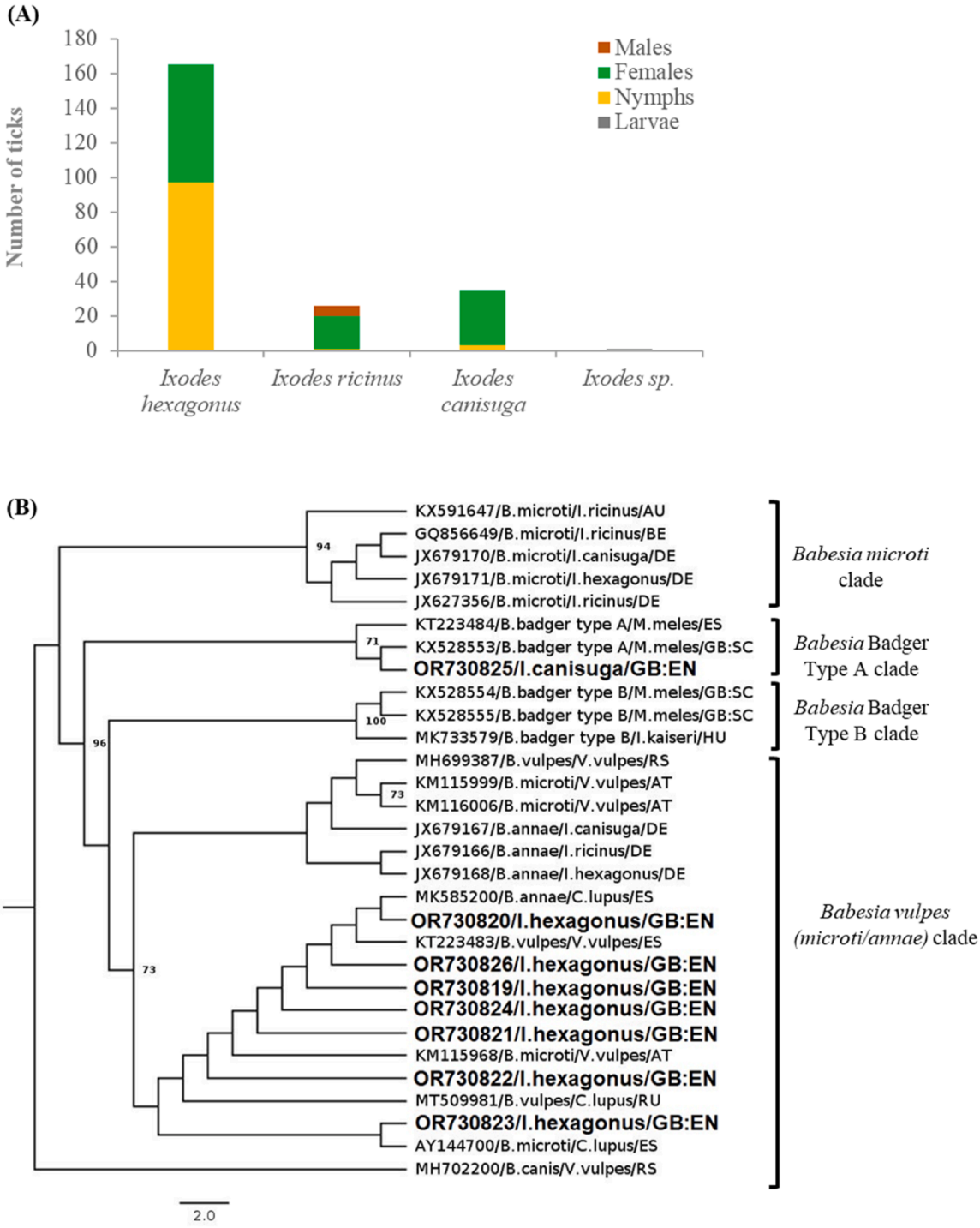

Fig. 1. (A) Total number of ticks collected from red foxes ($n = 93$) in Great Britain between 2018 and 2022, showing species detected and differentiation between life cycle stages of larvae (grey), nymphs (orange), females (green) and males (brown), and (B) phylogenetic analysis of *Babesia 18S* gene sequences (396-bp) detected in ticks collected from red foxes in Great Britain, generated using Maximum Likelihood estimation (10,000 replicates) and rooted with *Babesia canis*. Sequences derived from this study are highlighted in bold. Country abbreviations: AT, Austria; AU, Ukraine; BE, Belgium; DE, Germany; ES, Spain; GB:EN, England; GB:SC, Scotland; HU, Hungary; RS, Serbia; RU, Russia.

COI gene amplification was unsuccessful using conventional PCR, possibly due to specimen quality or assay sensitivity limitations compared to real-time PCR. In total, 121/203 tick DNA extracts were *COI* gene-positive, hence only these were assessed for pathogen detection. For all other analyses, the full dataset was assessed ($n = 227$ ticks), as species identification was reliant primarily on morphological identification.

3.1.2. Detection of tick-borne pathogens

Assessment of *COI* gene-positive DNA yielded 8/121 tick homogenates (seven individual ticks and one pool of two ticks) that were positive for *Babesia* (Table 1, Supplementary Table S4), with a detection rate of 7 % (95 % CI: 6–8 %). Detections were in *I. hexagonus* ($n = 7$) and *I. canisuga* ($n = 1$) from Devon, Somerset, Hampshire, North Yorkshire and Cheshire (Supplementary Fig. 3A). The majority of *Babesia*

Table 1
Detection of tick-borne pathogen nucleic acid in ticks collected from red foxes in Great Britain ($n = 93$) between February 2018 and November 2022. CI, confidence interval.

Tick species	Individual ticks collected (*n*)	Homogenates tested (*n*)*	*COI* gene- positive homogenates (*n*)	*Babesia 18S* gene		*Anaplasma phagocytophilum msp2* gene	
				Positive (*n*)	Detection rate[b] (%)	Positive (*n*)	Detection rate[b] (%)
I. hexagonus	165	143	103	7	7	5	5
I. canisuga	35	34	15	1	7	0	0
I. ricinus	26	26	3	0	0	0	0
Ixodes sp.	1[a]	1	0	0	0	0	0
All ticks	**227**	**203**	**121**	**8**	7	**5**	4
Overall detection rate (all species):				**7 %** (95 % CI: 6–8 %)		**4 %**[c]	

* Either individual ticks or pooled ($n = 2$).
[a] Single larva pooled with one *I. hexagonus* nymph.
[b] Only *COI*-gene positive samples were included in calculations for detection rate.
[c] Confidence interval not determined as all detections were in a single species.

detections were in ticks from rural foxes, however some were in ticks from semi-rural or urban foxes (Supplementary Table S4). *Babesia* detections in *I. hexagonus* were confirmed as *B. vulpes*, based on 99.75–100 % identity with available sequence [MT509981; MK585200]. The *Babesia* in *I. canisuga* was confirmed as '*Babesia* Badger Type A' based on 100 % identity with available sequences [MG799845; KX528553]. Phylogenetic analysis of partial *Babesia 18S* gene sequences (396-bp) is shown in Fig. 1B, where detections of *B. vulpes* DNA (*I. hexagonus*) grouped within a distinct clade with European and Russian *B. vulpes/B. microti/B. annae* sequences from ticks, foxes and domestic dogs (*Canis lupus familiaris*). Two discrete clades of *Babesia* Badger Type A and Type B were also differentiated; the single detection in *I. canisuga* grouped within the 'Badger Type A' clade with similar sequences from badgers (*Meles meles)* (Bartley et al., 2017). Detections of *A. phagocytophilum* DNA ($n = 5$) were in *I. hexagonus* from rural foxes in Somerset, Worcestershire, Gloucestershire and Essex (Supplementary Fig. 3B; Supplementary Table S5), with a detection rate of 4 % (Table 1). In comparison, LIV RNA was not detected ($n = 203$ homogenates). Overall, there was no significant association between pathogen detection and either tick species ($p = 0.999$), tick life stage ($p = 0.999$) or fox kill location (urban versus rural) ($p = 0.060$).

3.2. Study 2: Detection of Babesia in red fox tissues

Assessment of DNA extracted from fox tissues confirmed that 70 % (7/10) of the foxes associated with a mortality event in western England were positive for *Babesia* (Supplementary Table S6). Sequence analysis of a 396-bp *18S* rDNA fragment identified the *Babesia* species as *B. vulpes (B. annae)*, sharing 100 % identity with published GenBank sequence data [KT580785] derived from DNA extracted from lung exudate from UK foxes (Bartley et al., 2016).

4. Discussion

In this study, three tick species were shown to infest UK red foxes, aligning with geographical trends from previous UK tick surveillance (Hansford et al., 2022). *Ixodes hexagonus* and *I. canisuga* were predominantly found on foxes in England, whilst *I. ricinus* were also found on foxes in Scotland. The most common tick infesting foxes was *I. hexagonus* of which the majority were nymphs, with year-round prevalence since it is a nest-based species less affected by external temperature (Cull et al., 2018). In comparison, *I. ricinus* appeared to predominate in spring and autumn. In *I. hexagonus*, we detected *A. phagocytophilum* DNA ($n = 5$) and *Babesia* DNA ($n = 7$) in nymphs and females, where the *Babesia* detections were confirmed as *B. vulpes* (previously *Theileria annae, B. annae, B.* cf. *microti* and *B. microti*-like piroplasm) (Baneth et al., 2015; 2019). Additionally, *Babesia* Badger Type A was detected in one *I. canisuga* female. *Ixodes canisuga* were predominantly collected from foxes in the West Country of England, which has high density badger populations. Since foxes are known to occupy abandoned badger setts or live alongside badgers in setts (Parrott et al., 2012; Mori and Menchetti, 2019), this supports the detection of Badger Type *Babesia* DNA in *I. canisuga* from a fox.

However, there are limitations to the interpretation of pathogen data obtained from analysis of feeding (engorged) ticks. Although removal of a tick from an animal host demonstrates that the tick was either taking or about to take a bloodmeal, it is difficult to differentiate whether the pathogen source is the tick itself or the bloodmeal taken from the host (i.e., the host is the source) (Johnson et al., 2022). Red foxes are a known reservoir for *A. phagocytophilum* and *B. vulpes* (Ebani et al., 2011; Checa et al., 2018; Lesiczka et al., 2023a). Lung exudate from UK foxes has been shown to contain *B. annae* (*B. vulpes*) DNA (Bartley et al., 2016), and we detected *B. vulpes* DNA in blood, liver and kidney tissue from foxes, confirming widespread establishment of *B. vulpes* in the GB red fox population. This highlights the potential role of red foxes in transmission cycles of *Babesia* spp. and *A. phagocytophilum*, where they may have multiple roles as reservoir species, a blood meal source for ticks, and/or facilitating contact between infected ticks and other animals or humans (Lesiczka et al., 2023a). However, the lack of transovarial transmission in ixodid tick species for *A. phagocytophilum* and small *Babesia* such as *B. vulpes* and *Babesia* Badger Type A (Ravindran et al., 2023; Gray et al., 2010) suggests that transstadial transmission between different tick life stages may also be important in maintenance and transmission of these pathogens (Karbowiak et al., 2018).

Only 12 % of ticks collected were *I. ricinus*, with no pathogen detections despite being a key pathogen vector in Europe (Moraga-Fernández et al., 2023). This species is the principal tick vector for LIV, although LIV RNA was not detected. However, this was not unexpected since LIV prevalence in *I. ricinus* is relatively low in parts of the UK, in the order of 3 % (Holding et al., 2020), suggesting that higher numbers of *I. ricinus* would have to be sampled to detect LIV. Although *I. ricinus* is the most abundant UK tick species (Hansford et al., 2022), and they feed on a diverse range of vertebrates (Kahl and Gray, 2023), these data suggest that the red fox is not the primary vertebrate host for *I. ricinus*. This is supported by experimental studies where the feeding performance of *I. ricinus* larvae and nymphs on foxes was poor (Kahl and Geue, 1995), and taken together, suggests that the reservoir potential of the red fox for LIV is limited. Host preference differences influence tick-borne pathogen transmission dynamics and tick-borne disease risk (Cull et al., 2018). Although clinical disease associated with *B. vulpes* or *A. phagocytophilum* has not been reported in red foxes (Ebani et al., 2011; Lesiczka et al., 2023a), both pathogens cause disease in other mammals. Tick surveillance suggests that dogs are the most common mammalian host for UK ticks (39 %), including *I. canisuga* and *I. hexagonus* (Cull et al., 2018). In dogs, *B. vulpes* and badger-associated *Babesia* sp. can cause severe disease including anaemia and thrombocytopenia (Miró

et al., 2015; Hornok et al., 2018; Unterköfler et al., 2023), and *A. phagocytophilum* can cause polyarthritis and thrombocytopenia (Martinescu et al., 2023). Although *B. vulpes* and badger-associated *Babesia* are considered non-zoonotic (Azagi et al., 2021) an *A. phagocytophilum* variant (ecotype 1) with zoonotic potential to cause human granulocytic anaplasmosis has been detected in European foxes (Lesiczka et al., 2023b).

In summary, although interpretations should consider sampling biases stemming from targeted geographic coverage and operational timing constraints, these data provide evidence for tick-borne pathogen detection in ticks infesting both rural and urban foxes in the UK. Environmental enhancement via an increase in urban green areas has increased the number of habitats that support foxes and ticks (Bartley et al., 2016), encouraging fox populations to become increasingly urbanised (Scott et al., 2014) and providing increased potential for tick-borne pathogen transmission.

Data availability

Babesia sequence data from this study have been deposited in NCBI GenBank with primary accession numbers as detailed in Supplementary Table S1 [OR730819–OR730826], and tick *COI* gene sequence data are available under GenBank accession numbers [PP978617–PP978676; PP982737].

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CRediT authorship contribution statement

Karen L Mansfield: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Estela González:** Writing – review & editing, Investigation. **Stuart McKay:** Writing – review & editing, Visualization, Formal analysis. **Ternenge Apaa:** Writing – review & editing, Visualization, Formal analysis. **Alexander J Kent:** Writing – review & editing, Data curation, Conceptualization. **Paul Cropper:** Writing – review & editing, Data curation. **Naomi Berry:** Writing – review & editing, Data curation. **Luis M Hernández-Triana:** Writing – review & editing. **Nicholas Johnson:** Writing – review & editing, Funding acquisition, Conceptualization.

Declaration of competing interests

The authors declare that they have no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2024.102401.

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