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Prevalence of tick-borne pathogens in feeding and questing *Ixodes ricinus* ticks from Southern Sweden

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ABSTRACT

Ixodes ricinus, the most common tick species in Northern Europe, plays a significant role as a vector of several pathogens, with its geographical distribution expanding in recent years. In Southern Sweden, particularly in Region Skåne County (referred to as Skåne), the favorable climate and landscape conditions support extensive proliferation of *I. ricinus*. Despite Lyme borreliosis being common in this region and few annual cases of tick-borne encephalitis (TBE) being reported, data on the circulation of tick-borne pathogens (TBPs) remain limited. This study molecularly investigated the presence of *Anaplasma phagocytophilum, Babesia* spp., *Borrelia* spp., and TBE virus (TBEV) in *I. ricinus* ticks ($n = 1000$). In detail, questing ticks (82 adults and 196 nymphs) were collected from vegetation in forest and meadow areas, while 581, 80 and 8 feeding adults were collected from 39 roe deer, 6 fallow deer and 1 moose, respectively. Additionally, 53 feeding adults were removed from domestic animals (42 from four dogs and 11 from one cat).

The molecular analyses detected *Anaplasma phagocytophilum, Borrelia* spp., and *Babesia* spp. in 54 %, 24 %, 3.2 % of host-feeding ticks and in 0.40 %, 35 %, 3.6 % of questing ticks, respectively. In detail, for *Borrelia* and *Babesia* genera, the following species were detected: *Borrelia miyamotoi, Borrelia afzelii, Borrelia garinii, Borrelia burgdorferi* s.s., *Babesia microti* and *Babesia venatorum*. TBEV was not detected.

Moreover, the relationship between the feeding duration of the roe deer-collected ticks and their PCR-positivity for *Borrelia* spp. and *A. phagocytophilum* was also modeled. The results showed a reduction in the probability of tick infection with *Borrelia* spp. as attachment time increased, supporting evidence that roe deer serum exerts a borreliacidal effect. This study highlights the presence of several zoonotic TBPs in Skåne, emphasizing the need for a structured monitoring plan and preventive strategies within a One Health framework.

1. Introduction

Ticks are globally widespread hematophagous ectoparasites of mammals, birds, and reptiles (Parola and Raoult, 2001). They are considered the second most significant arthropod vectors of human and animal pathogens – including bacteria, viruses and protozoa – following

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mosquitoes (de la Fuente et al., 2017; Parola et al., 2013; Parola and Raoult, 2001). In Europe, *Ixodes ricinus* is regarded as the most important hard tick species in both human and veterinary medicine (Keve et al., 2022; Parola and Raoult, 2001). In Sweden, *I. ricinus* has become increasingly abundant in southern and central regions over the past three decades, gradually spreading northward and westward due to several factors, including climate change. For example, milder winters and longer, more humid vegetation periods enhance the survival, proliferation and distribution of both ticks and their hosts (Jaenson et al., 2012). Skåne County (hereafter referred to as Skåne), the southernmost county of Sweden, has an estimated population of 1.4 million inhabitants as of 2022. Its landscape features a mix of agriculture and woodlands in the northern and north-eastern regions, while the southern and western areas are dominated by agricultural landscapes. The county's temperate climate, characterized by warm, humid summers and mild winters, provides favourable conditions for the survival of ixodid ticks.

Ixodes ricinus is considered one of the primary vectors of multiple tick-borne pathogens (TBPs), including the *Borrelia burgdorferi* sensu lato (s.l.) group, which contains the aetiological agents of Lyme borreliosis in humans. Other pathogens transmitted by *I. ricinus* include *Borrelia miyamotoi, Anaplasma phagocytophilum, Babesia* spp. and Orthoflavivirus encephalitidis (Postler et al., 2023) previously known as tick-borne encephalitis virus (TBEV). Despite its significance, limited information is currently available on the circulation and dynamics of TBPs in ticks in Skåne (Karlsson and Andersson, 2016). Investigating the presence of pathogens and the associated tick-borne diseases (TBDs) in a determined area is a critical first step in developing a structured surveillance plan (Braks et al., 2011; Capelli et al., 2012)

Among TBDs, only tick-borne encephalitis (TBE) is currently notifiable in Sweden. Although TBE is relatively rare in Skåne, with an annual incidence of approximately 1/100,000 population, it is considered an emerging disease in the region and has spread to new areas since the early 2000s (Waldeck et al., 2023). In contrast, Lyme borreliosis, is not a notifiable disease in Sweden, but has long been recognized as common in southern parts of the country (Berglund et al., 1995). Data on the incidence of other TBDs in humans, such as human granulocytic anaplasmosis (HGA), babesiosis and infections caused by *B. miyamotoi*, are scarce in Sweden. Current knowledge on their impact on human health is limited to case reports and sero-epidemiological studies (Bläckberg et al., 2018; Henningsson et al., 2015a; Karlsson et al., 2001; Svensson et al., 2019).

The broad host range of *I. ricinus* (e.g. rodents, ungulate hosts, pets and humans) underscore its significance in public health as vector of several zoonotic agents (Øines et al., 2012). Among wild ungulate hosts, roe deer is the most abundant cervid species in Europe and can act as a reservoir for certain *I. ricinus*-transmitted pathogens, such as *A. phagocytophilum* (Remesar et al., 2020). Conversely, ungulates like deer and cattle are considered incompetent hosts for the transmission of *B. burgdorferi* s.l. spirochetes (Mannelli et al., 2012). Serum of these hosts, including roe deer, has been shown to exert a borreliacidal effect (Jaenson and Tälleklint, 1992; Kjelland et al., 2011).

The aim of this study was to estimate the occurrence of TBPs in both engorged and questing *I. ricinus* collected in Skåne. Furthermore, the relation between the presence of *Borrelia* spp. and *A. phagocytophilum* in ticks feeding on roe deer was explored, and the impact of feeding duration on the likelihood of ticks being infected with these pathogens was modelled.

2. Materials and methods

2.1. Tick collection

Ticks were collected between 2011 and 2016 in Skåne from both mammal hosts (roe deer, fallow deer, moose, dogs, and a cat) and vegetation. Questing ticks were collected by dragging vegetation in forest and meadow areas within a ~1 km radius near the village of Övarp, specifically. The sampling site was chosen according to a previous report from a TBE patient regarding the site where the tick bite resulting in TBE had probably occurred. Sampling of questing ticks occurred between May and September in 2011 and in May 2012.

Data on the specific locations and periods of sampling were collected whenever available (Additional file 1: Table S1). A map showing the collection sites for questing ticks, ticks from dogs and the cat, and of roe deer origins is provided in Fig. 1. Ticks from the cat and dogs were collected by pet owners, recruited from the staff at the Department of Infectious Diseases, Central Hospital Kristianstad (Kristianstad, Sweden), and stored in empty Eppendorf tubes at −80 °C. Engorged adult ticks from ungulates were collected at the Sjunkaröd slaughterhouse (Skåne, Sweden), specialised in wild animals and serving a large area of north-eastern Skåne. All ticks were stored at −80 °C until subsequent analyses.

2.2. Morphological identification and feeding time estimation of the ticks

Morphological identification of ticks was performed under a stereomicroscope (Leica MZ16, Leica Microsystems, Stockholm, Sweden) with magnification up to 200x, according to morphological taxonomic keys (Arthur, 1963; Estrada-Peña et al., 2017; Hillyard, 1996).

The feeding duration of female *I. ricinus* was estimated using scutal and coxal indices (SI and CI, respectively) based on regression equations described by Gray et al. (2005). Measurements were performed with the DinoCapture® software, with an accurate adjustment of specific magnification calibration with a USB-digital microscope (Dino-Lite pro AM413TL, AnMoElectronics Corp., Taiwan) under a magnification of up to 40x.

2.3. Extraction of total NA and cDNA synthesis

Total nucleic acids (NA) extraction was performed for each adult tick and nymph by homogenizing the specimens in 2 ml screw-lock microtubes (Sarstedt AG, Nümbrecht, Germany). Each tube contained a 5 mm sterile stainless-steel bead (Qiagen®, Hilden, Germany) and 450 µl of mixed lysis buffer solution (441 µl of RNeasy Lysis Buffer (Qiagen®, Hilden, Germany) and 9 µl of 2M Dithiothreitol (DTT)). The ticks were disrupted using the TissueLyser instrument (Qiagen®) at a frequency of 30 times/s for 1 min. This process was repeated after rotating the tube position of 180°, followed by centrifugation at 20,000 × g for 3 min.

Subsequently, 90 µl of lysate supernatant were manually transferred to the 96-well extraction plate, each well containing 10 µl of Tritirachium Proteinase K (Sigma® Life Science, Germany). Total NA extraction was automated using the Magnatrix 8000+ extraction robot (NorDiag, Sweden), using Vet Viral NA commercial kit (NorDiag, Sweden).

Complementary DNA (cDNA) synthesis was performed using Illustra Ready-To-Go RT-PCR Beads (GE Healthcare, Amersham Place, UK) following Lindblom et al. (2014), and subsequently stored at −20 °C until further molecular analyses.

2.4. Detection of tick-borne pathogens

Real time PCR assays were conducted to detect *A. phagocytophilum, Babesia* spp., and *Borrelia* spp. in one microliter of the total NA extracted from each individual tick. Samples positive for *Borrelia* spp. were further analysed to determine the presence of selected *Borrelia* species: *B. miyamotoi, B. afzelii, B. garinii*, and *B. burgdorferi* s.s.

The detection of *Borrelia* spp., *B. miyamotoi* and *A. phagocytophilum* was performed using TaqMan real-time PCR assays, as previously described (Gyllemark et al., 2021; Henningsson et al., 2015b; Hovius et al., 2013). Each assay was included a single annealing/elongation step at 60 °C.

For *Babesia* spp., and the three species within the *B. burgdorferi* s.l.

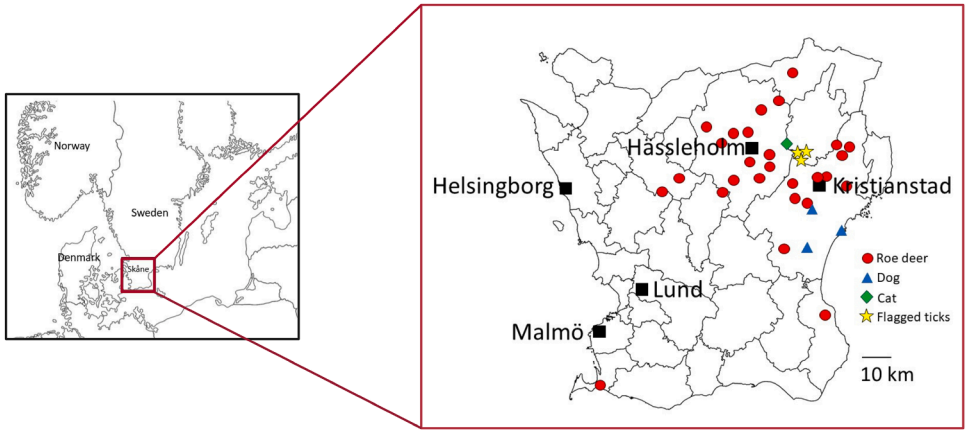

Fig. 1. The collection site for questing ticks, ticks from dogs and cats and the origin of roe deer.

complex, detection was carried out using the SsoAdvanced Universal SYBR Green Supermix (Biorad, Hercules, CA, USA) according to Casati et al. (2006), with an annealing step at 55 °C. The *Borrelia* spp. assay was performed following the protocol of Chiappa et al. (2022), with an annealing step at 52 °C.

TBEV detection was conducted using pooled cDNA samples, with four samples per pool, due to the low expected occurrence of this pathogen (Pettersson et al., 2014). The assay was performed according to Lindblom et al. (2014) using a Multiplex TaqMan™ assay to amplify various TBEV strains, with annealing and elongation combined in a single step at 60 °C.

All analyses were performed on a Bio-Rad CFX96 Real-Time system (Bio-Rad Laboratories, Inc., Hercules, USA). The primers, probes, and their final concentrations are shown in Table 1.

Babesia spp.-positive samples with the lowest threshold cycles (Ct < 30) were selected for sequencing. PCR products were loaded on 2.2 % agarose gel, and the resulting bands were excised and purified using the Wizard SV Gel and PCR Clean-Up System Kit (Promega, Madison, WI, USA) following to the manufacturer's instructions. Purified amplicons were subjected to Sanger sequencing (Eurofins Genomics, Germany). The resulting sequences were analysed and manually curated using BioEdit Software v7.0 (Hall, 1999) and compared against sequences available in GenBank using the Basic Local Alignment Search Tool (BLAST).

2.5. Statistical analyses

To assess the effects of feeding time on the probability of a tick being PCR-positive for *Borrelia* spp. or *Anaplasma* spp., generalized linear mixed models (GLMMs) with a binomial distribution were used.

The response variable was the tick infection status (PCR positivity for *Borrelia* or *Anaplasma*), while the feeding duration was and the explanatory variable. To account for possible non-linear effects of the time, its second-order polynomial effect was considered. Time was therefore standardised. Ticks collected from the same host individual or within the same sampling area might introduce data autocorrelation. Therefore, host ID and sampling area were included as random factors. Furthermore, to examine potential coinfection effects, the infection status of

Table 1
Primers and probes used for the detection of TBPs.

TBP	Primers/Probe	Sequence (5′→ 3′)	Final concentration (nM)	Reference
TBEV	F-TBE	GGGCGGTTCTTGTTCTCC	200	(Lindblom et al., 2014)
(3′ non coding region)	R-TBE	ACACATCACCTCCTTGTCAGACT	200	
	TBE-probe-WT[a]	TGAGCCACCATCACCCAGACACA (BHQ1)	200	
	TBEE-F6	GGCTTGTGAGGCAAAAAAGAA	200	
	TBEE-R2	TCCCGTGTGTGGTTCGACTT	200	
	TBEE-P4[b]	AAGCCACAGGACATGTGTACGACGCC (BHQ1)	200	
A. phagocytophilum	AnF	TTTTGGGCGCTGAATACGAT	600	(Henningsson et al., 2015b)
(*gltA* gene)	AnR	TCTCGAGGGAATGATCTAATAACGT	600	
	ApM[c]-probe	TGCCTGAAC AAGTTATG	150	
Babesia spp.	BJ1	GTCTTGTAATTGGAATGATGG	200	(Casati et al., 2006)
(*18S rRNA* gene)	BN2	TAGTTTATGGTTAGGACTACG	200	
Borrelia spp.	Borrelia_F	GCT GAG TCA CGA AAG CGT AG	200	(Gyllemark et al., 2021)
(*16S rRNA* gene)	Borrelia_R	CAC TTAACACGTTAGCTTCGGTA	200	
	Borrelia_P[a]	CGCTGTAAACGATGCACACTTGGT (MGB)	200	
B. afzelii	B.afzelii_F	ATTCTTGTGGTCCTGGTT	250	(Chiappa et al., 2022)
(*TsaB* gene)	B.afzelii_R	TGAATCAATCTGCCCTAG	250	
B. garinii	B.garinii_F	AAAAAGTGATAGAGAGTTCC	250	(Chiappa et al., 2022)
(*RimP* gene)	B.garinii_R	CCCTCTTCAAATTCATTGTC	250	
B. burgdorferi s.s.	*B.bss*_F	TGTATTCAAGAAACTAAAGCC	250	(Chiappa et al., 2022)
(*ribonuclease III* gene)	*B.bss*_R	GCTCAACTTTTGAATAAATGC	250	
B. miyamotoi	B.miya_F	AGAAGGTGCTCAAGCAG	200	(Hovius et al., 2013)
(*16S rRNA* gene)	B.miya_R	TCGATCTTTGAAAGTGACATAT	200	
	Probe[d]	AGCACAACAGGAGGGAGTTCAAGC(BHQ2)	200	

FAM, 6-carboxy-fluorescine; HEX, 6-carboxy-hexachlorofluorescein; BHQ, Black Hole Quencher;
MGB, minor groove binder.
a, c, d: Fluorescent reporter FAM
b: Fluorescent reporter HEX

Borrelia spp. was added as an additional explanatory variable in the model for *Anaplasma* spp., and vice versa, to test whether the presence of one pathogen influenced the likelihood of infection by the other. A set of models were fitted, including the following combinations of random variables: no random variables, collection area of samples, or animal host ID alone, and both collection area and host ID. The minimal adequate model, was thus obtained by first selecting the random structure and only after, assessing the fixed structure. These two independent evaluations selected the model structure with the lowest Akaike's information criterion (Rhodes et al., 2009). Finally, residual distribution of the selected model was visually inspected to check model fit. Analyses and data visualization were performed using R software (R version 4.3.0), using packages lme4, MuMIn, sjPlot, ggeffects, ggplot2, dplyr, tidyr.

3. Results

3.1. Tick species identification

A total of 1000 ticks collected from mammal hosts ($n = 722$) and vegetation ($n = 278$) were morphologically identified as *I. ricinus*. All feeding ticks collected from hosts were adults, while the questing ticks obtained through flagging included both adults and nymphs. Detailed information on the sampled hosts and the developmental stages of both questing and feeding ticks is provided in Table 2.

3.2. TBPs in feeding ticks

Results from the PCR assays assessing the presence of TBPs in feeding ticks are reported in Table 3. The total number of ticks collected from each host and the positivity for at least one pathogen are detailed in Additional file 2: Table S2. Roe deer were the most frequently sampled hosts, accounting for the majority of collected ticks. DNA of *A. phagocytophilum* was detected in 54 % (390/722) of host-collected ticks, making it the most prevalent TBP identified. Of these positive samples, 339 were collected from roe deer, 50 from fallow deer, and one from cat. DNA of *Borrelia* spp. was detected in 24 % (170/722) of the samples. Species-specific PCR assays on *Borrelia* spp.-positive samples revealed that 8.8 % (15/170) were identified as *B. afzelii*, 10 % (17/170) as *B. garinii*, 4.2 % (7/170) as *B. burgdorferi* s.s., and 7.0 % (12/170) as *B. miyamotoi*.

DNA of *Babesia* spp. was detected in 3.2 % (23/722) of the ticks. Among these, five amplicons with the lowest amplification cycles, were sequenced. Sequencing results identified one sample as *Babesia microti* (acc. no.: PQ041272; 100 % identity with GenBank record OL773537) and four samples as *Babesia venatorum* (acc. no.: PQ041270; 100 % identity with GenBank record GU734773). No PCR-positivity for TBEV was obtained.

Table 2
Number of ticks classified by sex/developmental stage and according to the host species.

Host species/ Questing ticks	Animals ($n = 51$)	Developmental stage of the tick		
		Females ($n = 614$)	Males ($n = 190$)	Nymphs ($n = 196$)
Roe deer (*Capreolus capreolus*)	39	458	123	0
Fallow deer (*Dama dama*)	6	64	16	0
Moose (*Alces alces*)	1	5	3	0
Dog *(Canis lupus familiaris)*	4	42	0	0
Cat (*Felis catus*)	1	7	4	0
Questing ticks	-	38	44	196

Table 3
TBPs positivity in feeding and questing ticks.

TBPs	Feeding ticks	Questing ticks	
	Adults ($n = 722$)	Adults ($n = 82$)	Nymphs ($n = 196$)
A. phagocytophilum	390 (54 %)	1 (1.2 %)	0
Borrelia spp.	170 (23.5 %)	40 (48.8 %)	58 (29.6 %)
B. afzelii	15 (2 %)	23 (28 %)	24 (12.2 %)
B. garinii	17 (2.3 %)	9 (11 %)	10 (5.1 %)
B. burgdorferi s.s	7 (1 %)	0	1 (0.5 %)
B. miyamotoi	12 (1.7 %)	0	3 (1.5 %)
Babesia spp.	23 (3.2 %)	5 (6.1 %)	5 (2.5 %)
TBEV	0	0	0

3.3. TBPs in questing ticks

Results from the PCR assays carried out to assess the presence of TBPs in questing ticks are reported in Table 3. The most frequently detected TBP was *Borrelia* spp., identified in 49 % (40/82) of adults and 30 % (58/196) of nymphs. Among *Borrelia* spp.- positive adult ticks, 58 % (23/40) were identified as *B. afzelii* and 22 % (9/40) as *B. garinii*, while no adults tested positive for *B. burgdorferi* s.s. or *B. miyamotoi*. For *Borrelia* spp.-PCR-positive nymphs, 41 % (24/58), 17 % (10/58), 1.7 % (1/58), and 5.2 % (3/58) were positive for *B. afzelii, B. garinii, B. burgdorferi* s.s., and *B. miyamotoi*, respectively.

The second most common TBP was *Babesia* spp., detected by PCR in 6.1 % (5/82) of adults and 2.6 % (5/196) of nymphs. Sequencing of three selected *Babesia* spp. amplicons with the lowest amplification cycles identified one *B. microti* in a female tick (acc. no.: PQ041271; 100 % identity with GenBank record OL773537) and two *B. venatorum* in nymphs (acc. no: PQ041269; 100 % identity with GenBank record GU734773). In contrast to feeding ticks, DNA of *A. phagocytophilum* was the least detected in questing ticks, with an occurrence of 1.2 % (1/82) in adults (one female). No PCR-positivity for TBEV was detected in questing ticks.

3.4. Co-infections

The total rate of coinfection observed was 11 % (110 out of 1000 samples). Among feeding ticks, *A. phagocytophilum* was detected alongside *B. afzelii* ($n = 4$), *B. garinii* ($n = 7$), *B. burgdorferi* s.s. ($n = 2$), *B. miyamotoi* ($n = 8$), other *Borrelia* spp. ($n = 64$), and *Babesia* spp. ($n = 11$). Coinfections involving *Babesia* spp. included its presence with *B. afzelii* in both questing ticks ($n = 3$) and feeding ticks ($n = 2$) as well as with other *Borrelia* spp. in questing ticks ($n = 1$) and feeding ticks ($n = 5$). In addition, one questing tick tested positive for both *B. afzelii* and *B. miyamotoi*. Triple infections were identified exclusively in feeding ticks (*Borrelia* spp. + A. *phagocytophilum* + *Babesia* spp., $n = 2$; *B. afzelii* + *B. garinii* + *A. phagocytophilum*, $n = 1$).

3.5. Estimated feeding time of the ticks

Feeding time was successfully estimated for 533 out of 614 adult females collected from hosts. In detail, 428 ticks from roe deer, 55 from fallow deer, three from moose, 42 from dog, and five from a cat were analysed. Statistical analyses to evaluate the effect of feeding time on the probability of ticks being infected with *Borrelia* spp. or *A. phagocytophilum* were performed on a subset of 358 ticks collected from roe deer, with both host identification number (ID) and area of sampling available for those samples. Data from fallow deer and moose were excluded due to the low number of individual samples (6 and 1, respectively) and the unavailability of information on sampling area. The mean feeding time from roe deer was estimated at 82.8 h, with a symmetric range spanning from 8 to 204 h around this mean.

The minimal adequate model describing the effects of ticks' feeding time on the probability of testing positive for *Borrelia* spp. included the animal host ID as the sole random variable. A significant positive effect

of time was highlighted, leading to a decreasing relationship between ticks' feeding time and ticks' probability of testing positive for *Borrelia* spp. (Fig. 2, Table 4). The effect of *A. phagocytophilum* coinfection was not retained in the minimal model.

The minimal adequate model describing the effects of ticks' feeding time on the probability of testing positive for *A. phagocytophilum* included both the host ID and the collection area of samples as random variables. A significant positive effect of feeding time, alongside its second-order polynomial term, was observed, resulting in a convex, non-linear relationship between feeding time and the probability of ticks testing positive for *A. phagocytophilum* (Fig. 3, Table 4). The effect of *Borrelia* spp. coinfection was not retained in the minimal model.

Table 4
Factors affecting the probability of a tick collected from roe deer to be infected by *Borrelia* spp. and *A. phagocytophilum*.

Bacterium	Variable	df	Deviance	p-value
Borrelia spp.	Time	1	4.217	0.04
A. phagocytophilum	Time	1	40.589	<0.001
	Time^2	1	10.298	0.001

4. Discussion

The geographical expansion and the increasing abundance of ticks across Europe are driven by several factors, including climate change and the consequent extension of the growing season. These changes enhance the survival and proliferation of ticks, facilitate the spread of the pathogens they may transmit, and impact the distribution of their maintenance hosts over larger geographical areas (Jaenson et al., 2012; Medlock et al., 2013). Although the sampling approach in this study does not allow for generalized prevalence estimates of TBPs or factors influencing them, the obtained results can provide valuable insights for public health management and the planning of preventive strategies.

In this study, all ticks were classified as *I. ricinus* based on morphological identification.

No nucleic acids of TBEV were detected in the analysed ticks. The absence of TBEV-positive ticks might be addressed to the possibility that the viral load could be below the detection limit of the real time PCR (Belova et al., 2012). Additionally, ticks were collected from numerous areas, with a limited number of ticks from each location. TBEV typically occurs in micro-foci, where the Minimum Infection Rate (MIR) among ticks seldom exceeds a few percent (Topp et al., 2022). Therefore, collecting a greater number of ticks from a presumed micro-focus area is necessary to estimate the prevalence of TBEV in ticks. Since TBEV is challenging to detect in questing ticks, testing engorged ticks has been suggested as a more effective method for identifying this pathogen (Stefanoff et al., 2013; Süss et al., 2006).

In the present work, only 1.2 % of questing adult ticks carried DNA of *A. phagocytophilum*, which aligns with the previously reported prevalence of 0.7 % in questing *I. ricinus* adults in Sweden (Wallménius et al., 2012). In contrast, we observed an occurrence of *A. phagocytophilum* DNA in 54 % of feeding adult *I. ricinus* ticks in Skåne.

These results are consistent with previous PCR investigations of blood samples, which indicate that *A. phagocytophilum* is widely prevalent in roe deer across different European countries, with reported rates of 2.9 % in Austria, 37 % in Poland, and 78 % in the Netherlands (Kogler et al., 2021; Welc-Falęciak et al., 2013; Wijburg et al., 2022). Although roe deer are reservoirs for their own strains of *A. phagocytophilum*, previous studies have reported that they can harbour several additional strains, including some pathogenic variants capable of infecting humans and domestic animals (Remesar et al., 2020).

A study from Southern Germany reported that 86 % of engorged ticks collected from roe deer harboured *A. phagocytophilum*, while the prevalence in questing nymphs collected from the same area was as low as 0.8 % (Overzier et al., 2013). This is consistent with our findings, where none of the questing nymphs tested positive. These results support the assumption that roe deer may act as reservoirs for *A. phagocytophilum* and that ticks are primarily infected during the adult stage while feeding on this host. It must be pointed out that zoonotic *A. phagocytophilum* strains represent only a small subset of all known *A. phagocytophilum* genovariants. Zoonotic strains found in Europe belong to a monophyletic group (ecotype I), that encompasses almost all isolates from

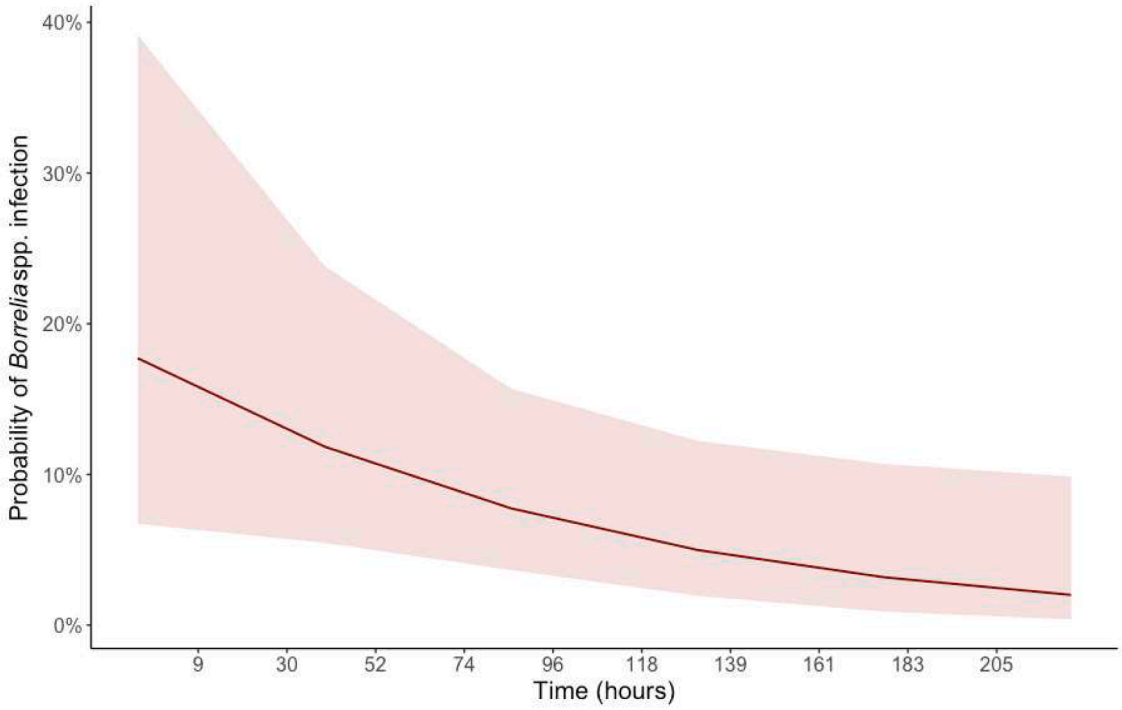

Fig. 2. Predicted marginal effects of *Borrelia* spp. probability to test positive in relation to feeding time (expressed in hours) of ticks collected from roe deer. Shadowed area: 95 % C.I.

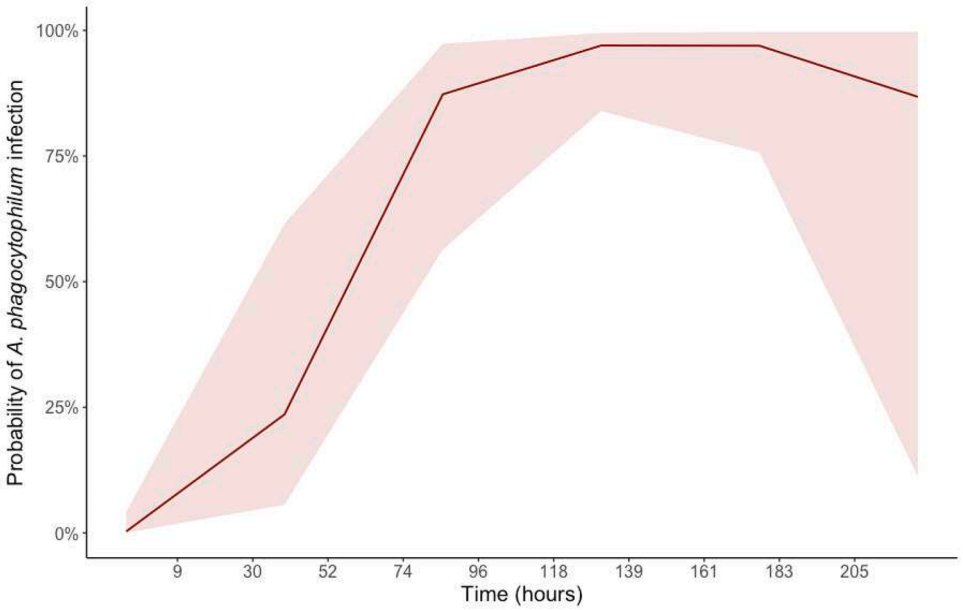

Fig. 3. Predicted marginal effects of *A. phagocytophilum* probability to test positive in relation to feeding time (expressed in hours) of ticks collected from roe deer. Shadowed area: 95 % C.I.

horses, dogs, cats, wild boars, red foxes, hedgehogs, sheep, and goats (Rar et al., 2021). In contrast, strains from roe deer predominantly belong to ecotype II, which is not considered to have zoonotic potential (Rar et al., 2021). Indeed, despite the high occurrence of *A. phagocytophilum* in roe deer and adult ticks, HGA is regarded as a rare disease in Sweden. Moreover, the low number of reported HGA cases might be attributed to humans being more frequently parasitized by *I. ricinus* nymphs than by adult ticks. Further analyses should be focused on evaluating the specific *A. phagocytophilum* strains circulating in the study area to determine the presence of potential zoonotic variants. Finally, the high prevalence of this pathogen in the analysed adult feeding ticks might be attributed to the simultaneous feeding on the same infected host, which is a potential mechanism for tick infection with *A. phagocytophilum*.

The mean occurrence of the investigated *Borrelia* species observed in the present study aligns with findings from a previous work performed in Southern Sweden (Wilhelmsson et al., 2013). With regard to *B. miyamotoi,* the same study reported a prevalence of 0.2 % in ticks removed from humans in the southernmost part of Sweden (Wilhelmsson et al., 2013). In the present work, a PCR-positivity of 1.5 % was observed when considering both questing and feeding ticks. This zoonotic pathogen should, therefore, be included in the list of potential TBPs in this region, as it is associated with neurological symptoms and fever that may be related to prior tick bite (Henningsson et al., 2019).

The differentiation of *Borrelia* species was successful in a proportion of *Borrelia* spp.-positive samples, ranging from 30 % in feeding ticks to 71 % in questing ticks. This variability can be attributed to the focus on detecting selected *B. burgdorferi* s.l. members (*B. afzelii, B. garinii* and *B. burgdorferi* s.s.) and *B. miyamotoi* using species-specific PCR assays rather than Sanger sequencing. For *B. burgdorferi* s.l., we employed a detection approach ('Lydet'; Chiappa et al., 2022) that targets the most frequent genospecies responsible for human Lyme borreliosis in Europe. However, the copy numbers of the genes targeted by the species-specific PCRs are unknown. Consequently, it cannot be ruled out that samples containing low quantities of the targeted *B. burgdorferi* s.l. genospecies might have tested negative in these reactions.

Even though roe deer are considered as one of the most important maintenance hosts for *I. ricinus* populations and the pathogens they transmit, such as *Babesia* spp., *Rickettsia* spp., and *A. phagocytophilum* (Cafiso et al., 2021a; Melis et al., 2024; Mysterud et al., 2014), this is not the case for *B. burgdorferi* s.l. (Jaenson and Tälleklint, 1992; Kurtenbach et al., 1998). Specifically, the complement system of roe deer induces the reduction of the bacterial load in both the host and the vector by lysing spirochaetes in the midgut of feeding ticks. Consequently, the longer the blood meal lasts, the lower is the likelihood of ticks feeding on roe deer testing positive for *Borrelia* spp. (Kurtenbach et al., 2006; Rosef et al., 2009). Another possible explanation for the lower occurrence of *Borrelia* in ticks is that after prolonged feeding, some borreliae might migrate from the feeding tick into the host. For instance, the minimal transmission time of *B. burgdorferi* s.l. from feeding nymphal *I. ricinus* to the host is known to be only 12–24 h (Kahl et al., 1998).

The results of this study showed a progressive reduction in the probability of ticks being infected with *Borrelia* spp. as the time of attachment increased. This trend persisted up to approximately 120 h of feeding, after which a plateau was reached and maintained until around 200 h. Due to the limited sample size of the other host species, the incompetence of hosts for *Borrelia* spp. was assessed for roe deer only. Further analyses are needed to explore whether these findings apply to other cervid species as well.

Both in vertebrate hosts and in ticks, *A. phagocytophilum* evades recognition by the host's innate immune system (Sonenshine and Macaluso, 2017). In contrast to *Borrelia* spp., the probability of feeding ticks testing positive to *A. phagocytophilum* increased with attachment time.

However, it must be pointed out that the GLM models proposed herein rely on regressing equations that estimate feeding time based on a direct proportionality between blood volume in the tick and feeding duration, which have been experimentally validated up to approximately 72 h (Gray et al., 2005). Our results within 72 h feeding period clearly shows an increasing trend for the probability of adult ticks feeding on roe deer testing positive for *A. phagocytophilum*, whereas the opposite trend was observed for *Borrelia* spp. We assumed that tick engorgement increases with rising feeding duration, as previously supposed by (Hofhuis et al., 2017). However, to reinforce these findings for longer feeding periods, experimental confirmation of the equations beyond 72 h should be performed.

In the present study, the prevalence of *Babesia* spp. DNA in questing ticks was 3.6 %. These results are in accordance with those reported in a previous study conducted in Southern Sweden, including Skåne, where the prevalence of *Babesia* spp. in questing ticks was 4.4 % (Karlsson and Andersson, 2016). Additionally, 3.2 % of feeding *I. ricinus* ticks collected

in Skåne tested positive for *Babesia* spp., with 19 out of 23 positive ticks were collected from roe deer, the most represented host species in this study. Limited information is available on the prevalence of *Babesia* spp. in ticks collected from roe deer in this region. Nonetheless, a previous study involving molecular analyses of roe deer blood samples in Southern Sweden reported that 57 % of individuals were infected with *Babesia* spp., including *Babesia capreoli* and *B. venatorum* (Andersson et al., 2016). In the current study, amplicon sequencing of selected *Babesia* spp.-positive samples (five from roe deer and three from questing ticks) confirmed the circulation of *B. venatorum* and *B. microti* in the study area, as previously reported by Karlsson and Andersson (2016). However, since only samples with the lowest amplification cycles were sequenced, the occurrence of *B. capreoli* and other *Babesia* spp. in the analysed samples should not be ruled out.

Babesia venatorum and *B. microti* are known zoonotic agents that can cause human disease, especially in immunocompromised individuals, such as splenectomised patients (Bläckberg et al., 2018; Young et al., 2020).

Conversely, in immunocompetent individuals, diseases caused by *B. venatorum* are less frequent (Sun et al., 2014). However, it cannot be excluded that infected, asymptomatic blood donors may act as an infection source of *Babesia* species through blood transfusions, particularly in the absence of routine pathogen testing in blood samples (Hildebrandt et al., 2007; Moritz et al., 2016).

Furthermore, co-infections warrant careful consideration, since 11 % of the ticks in this study were found to be infected with at least two different TBPs. This increases the risk of simultaneous exposure of susceptible vertebrate hosts to multiple TBPs. Co-infections in humans and other vertebrates are widely recognized to have critical clinical, diagnostic, and therapeutic implications (Cutler et al., 2021). Although the interplay between microbial agents within ticks require further investigation, it is already well-established that disease severity in vertebrate hosts can be modulated by synergistic, neutral, or antagonistic interactions among pathogens (Cafiso et al., 2021b; Cutler et al., 2021; Pawełczyk et al., 2021). Multiple infections in hosts may complicate the diagnostic process, leading to misdiagnoses, or may exacerbate disease progression (Wójcik-Fatla et al., 2009). For instance, the modulation of the immune response by one pathogen can increase susceptibility to various secondary infections (Boyer et al., 2022).

Overall, co-infections were more prevalent in feeding ticks compared to the questing ones. This finding could be attributed to the increased opportunities engorged adult ticks have had to acquire TBPs during multiple blood meals, compared to questing ticks. The most common co-infection observed in this study was the combination of *Borrelia* spp. and *A. phagocytophilum*, a result consistent with findings reported for *I. ricinus* ticks by Civitello et al. (2010). Notably, only a small proportion of co-infected ticks harboured *Borrelia* species associated with Lyme disease or *B. miyamotoi*. This might be preliminary interpreted as a low risk of exposure to multiple pathogenic TBPs from a single tick bite.

Considering the circulation of potentially zoonotic agents in Skåne, the current study provides critical baseline data useful to support the development of future surveillance plans targeting both tick vectors and their hosts, aligned with the One Health approach.

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CRediT authorship contribution statement

Chiara Cialini: Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Alessandra Cafiso:** Writing – review & editing, Writing – original draft. **Mattias Waldeck:** Writing – review & editing, Visualization, Supervision, Resources, Funding acquisition, Conceptualization. **Åsa Lundgren:** Resources, Conceptualization. **Johan Fält:** Resources, Conceptualization. **Bo Settergren:** Writing – review & editing, Supervision, Resources, Conceptualization. **Phimphanit Choklikitumnuey:** Investigation, Formal analysis. **Giulia Chiappa:** Investigation, Formal analysis. **Eleonora Rosso:** Investigation, Formal analysis. **Laura Roveri:** Investigation, Formal analysis. **Elisa Fesce:** Visualization, Methodology. **Nicola Ferrari:** Visualization, Methodology. **Per-Eric Lindgren:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Chiara Bazzocchi:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Giulio Grandi:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

PEL was a senior, scientific advisor to Pfizer Inc., and Bavarian-Nordic A/S. The other authors declare no conflicts of interest.

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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.2025.102453.

Data availability

Data will be made available on request.

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