

## Original article

# Voles, shrews and red squirrels as sources of tick blood meals and tick-borne pathogens on an island in southwestern Finland

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## ABSTRACT

Molecular identification of the previous blood meal source of a questing tick (Acari: Ixodidae) from blood meal fragments was proposed a few decades ago. Following this, several blood meal assays have been developed and published, but none of them have been taken into widespread use. Recently, novel retrotransposon-based qPCR assays designed for detecting blood meal fragments of North American host species were published. We wanted to assess their function with host species present in Finland.

Questing ticks were collected by cloth dragging in August–September 2021 from an island in southwestern Finland. DNA was extracted from *Ixodes ricinus* nymphs (n=438) and qPCR assays applied to identify larval blood meal sources (voles, shrews and red squirrels) and screen for several tick-borne human pathogens and other microbes with pathogenic potential [*Borrelia* spp. (including specific assays for *Borrelia afzelii*, *Borrelia garinii*, *Borrelia valaisiana*), *Anaplasma phagocytophilum*, *Babesia* spp., *Rickettsia* spp., and *Neoehrlichia mikurensis*].

The probability of a nymph having fed as larva on either a vole, shrew or red squirrel was 0.34 (0.30 – 0.38; 95% confidence interval). Bacteria of the genus *Borrelia* were the most common pathogens detected, with host-specific probabilities of carrying *Borrelia* of 0.30 (0.18 – 0.44) for nymphs that had fed on voles, 0.23 (0.14 – 0.35) for nymphs that had fed on shrews, and 0.42 (0.28 – 0.58) for nymphs that had fed on red squirrels. Other microbes were rarely acquired from these hosts, apart from *N. mikurensis* from voles.

This study highlights that shrews and red squirrels may equal voles as blood meal sources for *I. ricinus* larvae. Overall, variation in proportions of blood meals provided by these animals may be high across even proximate study areas. All studied host species appeared to be important sources for particularly *Borrelia afzelii*, and voles also for *N. mikurensis*.

## 1. Introduction

Ticks (Acari: Ixodidae) and tick-borne diseases (TBDs) form a significant and growing threat to human health and well-being in Europe, with some hundreds of thousands of cases of TBDs diagnosed annually (Marques et al., 2021). In Europe, Lyme borreliosis caused by *Borrelia burgdorferi* sensu lato (henceforth Bbsl) spirochetes and tick-borne encephalitis caused by the tick-borne encephalitis virus (TBEV) are the most common and serious tick-borne diseases reported (Süss, 2011; Marques et al., 2021). The ongoing global warming is expected to be beneficial to the most common vectors of agents causing TBDs in the northern parts of Europe, the sheep tick (*Ixodes ricinus*) and the taiga tick

(*Ixodes persulcatus*), leading to expansion of distribution ranges and increases in densities (Tokarevich et al., 2011; Jaenson et al., 2012). As they move to new areas and increase in number, the probability of humans encountering infected ticks concurrently rises, leading to higher disease risk (Mather et al., 1996) and, consequently, an increasing need to control this risk. Several methods proposed for controlling ticks and/or tick-borne pathogens target specific host animals or host animal groups, which are thought to be particularly important as tick blood meal hosts or pathogen reservoirs (Daniels et al., 1991; Solberg et al., 2003; Pelletier et al., 2020). However, for such control schemes to be effective, the (locally) most important hosts need to be correctly identified.

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For the most common tick-borne pathogens (TBPs) causing disease in humans in Europe, *Borrelia burgdorferi* sensu lato, transovarial transmission does not occur or is considered to be an exceedingly rare occurrence (Richter et al., 2012; Helble et al., 2021). Consequently, ticks most commonly acquire these pathogens during blood meals from infected (reservoir) hosts (Helble et al., 2021). The contributions of different European host species to *I. ricinus* blood meals and circulation of BbS1 and other TBPs have most commonly been studied by capturing host individuals and removing ticks from them, often followed by analysis of TBPs from ticks and hosts (Matuschka et al., 1991; Tälleklint and Jaenson, 1993; Tälleklint and Jaenson, 1994; L'Hostis et al., 1996; Humair and Gern, 1998; Pichon et al., 2003; Bown et al., 2011; Pérez et al., 2012; van Duijvendijk et al., 2016). While the roles and importance of several hosts have been determined this way, some uncertainties remain in extrapolating results to nature. Removing ticks from hosts or allowing collected ticks to finish blood meals and subsequent development in a laboratory/captivity means that we cannot conclusively assess the quality of the hosts in providing blood meals leading to successful production of questing ticks of the next life stage and, consequently, further circulation of acquired pathogens. For example, even if equal numbers of ticks are counted from two different animal species, they may in the wild move in different habitats, leading to differences in the mortality of ticks dropping off. Consequently, conclusively assessing the relative contributions of each host species to tick or TPB maintenance this way is difficult, particularly when only one or a few species are sampled. However, the main issue with these methods is simply that they are labor-intensive and often require ethical permits that have to be applied for, further adding to the workload of researchers (Pichon et al., 2003).

After molting to the next life stage following a blood meal, fragments of the previous blood meal remain in the midgut of the ticks (Kirstein and Gray, 1996). The possibility of directly identifying the previous host and source of pathogens from these fragments was recognized a few decades ago, with several assays having been developed to identify blood meal sources (Kirstein and Gray, 1996; Pichon et al., 2003; Cadenas et al., 2007; Humair et al., 2007; Collini et al., 2016; Wodecka and Skotarczak, 2016). However, none of these assays appear to have been taken into regular or widespread use, possibly signifying replicability issues in other laboratories. Regarding such methods, the simplest and least labor-intensive protocols would likely be real-time quantitative PCR (qPCR) assays. Such assays are fast, highly precise and already commonly used to screen TBPs from tick samples, so adding primers (and probes) to study blood meal sources would be feasible for many research groups working on ticks and tick-borne pathogens. While few qPCR assays to study blood meal sources have been published (Collini et al., 2016), recent publications from the USA have reported successful and repeated use of a novel qPCR assay based on retrotransposons (Goethert et al., 2021a, 2021b; Goethert and Telford, 2022). While the primers and probes in these studies were designed based on host species present in the USA, at least the primer/probe sets targeting voles, shrews and squirrels appeared to work for the most common species of each group present in Finland equally well, based on simple *in silico* analysis with the primer/probe sequences and sequences of local host species (unpublished own data). Thus, in order to test their function with tick samples, we collected a set of questing *I. ricinus* nymphs from Seili Island in southwestern Finland, which hosts the most well-studied tick population in Finland (Mäkinen et al., 2003; Sormunen et al., 2016b, 2018, 2020a).

In the current study, we assessed the roles of voles (primarily the bank vole, *Clethrionomys glareolus*), shrews (*Sorex* spp.) and the red squirrel (*Sciurus vulgaris*) as sources of previous blood meals for questing *I. ricinus* nymphs on a tick-infested island in southwestern Finland using Taqman-based qPCR assays. Likewise, we screened nymphs for the presence of several tick-borne pathogens and assessed to what extent they have been acquired from the hosts/host groups under study.

## 2. Materials and methods

### 2.1. Tick collection

Ticks were collected by cloth dragging with a 1 × 1 m cloth from six fixed study transects on Seili Island in August and September 2021 (Fig. 1). Seili is a small (1.6 km<sup>2</sup>), rural island located in the Archipelago Sea in southwestern Finland, which houses the Archipelago Research Institute of the University of Turku. The study transects consist of 50 m lines, which were dragged in three roughly equal parts. Each transect was visited once or twice per month. Ticks were collected from the cloth using tweezers and placed in 1.5 ml Eppendorf tubes containing 70% ethanol. Brief textual descriptions of the study transects have previously been published (Sormunen et al., 2019). In general, “C” transects represent habitats dominated by coniferous trees, “D” transects habitats dominated by deciduous trees/shrubs and “A” transects habitats dominated by common alders (*Alnus glutinosa*). Representative camera trap pictures from each study transect are provided in Fig. 1. During 2012–2020, some thousands of *Ixodes* ticks collected by cloth dragging from the island have undergone morphological or molecular species identification, and *I. ricinus* is the sole species that has been detected (Sormunen et al., 2016a, 2018, 2020a).

### 2.2. Blood meal and tick-borne pathogen analyses

We focused on *I. ricinus* nymphs for blood meal and TBP analyses. Questing larvae have naturally had no time to feed yet, whereas questing adults have fed on two animals, making determination of TBP sources more difficult. Likewise, the chosen assays target small host animal species/groups that are typically associated with providing larval blood meals. We used *I. ricinus* nymphs collected in August or September, as success rates have seemed higher for late season samples in previous trials with various blood meal assays. The reason for this could be that nymphs collected in the spring or early summer have overwintered (either as nymphs or engorged larvae), meaning that the blood meal fragments have likely been degrading inside the ticks for over six months, since the previous autumn. In contrast, at least a portion of nymphs active in August or September may have acquired the blood meal earlier in spring or early summer, leading to less time for DNA degradation. Total DNA was extracted from nymphs using Macherey-Nagel (Duren, Germany) NucleoSpin® RNA kits and RNA/DNA buffer sets, following kit protocols. DNA extracts were stored at -20 °C to await analysis.

We used Taqman-based qPCR assays to screen nymph samples for blood meal sources (voles, shrews and red squirrels) and several tick-borne microbes: *Borrelia* spp. (including specific assays for *Borrelia afzelii*, *Borrelia garinii* and *Borrelia valaisiana*), *Anaplasma phagocytophilum*, *Babesia* spp., *Rickettsia* spp., and *Neorhlichia mikurensis*. The primers and probes used are reported in Table 1. The primers “Bb23S” (from Courtney et al. 2004), originally designed for screening *B. burgdorferi* s.l., have been observed to also amplify *Borrelia miyamotoi*, a relapsing fever spirochete not part of the *B. burgdorferi* sensu lato species complex (unpublished own data). As such, positive samples that could not be identified as any of the specific *Borrelia* species analyzed have to be considered as *Borrelia* spp. rather than *B. burgdorferi* s.l. Consequently, we refer to *Borrelia* spp. when collectively discussing all detections of *Borrelia* made in the current study.

The assay protocols and master-mix contents are reported in the Technical Appendix (Table A1). Prior to blood meal analyses, we tested the sensitivity of the assays using host DNA acquired from tissue samples with serial dilutions up to 1 × 10<sup>-7</sup> ng/μl, using the reported protocols. We used DNA extracts from a bank vole (*C. glareolus*), a common shrew (*Sorex araneus*) and a red squirrel (*S. vulgaris*) for the sensitivity tests and, subsequently, as positive controls in respective qPCR runs.

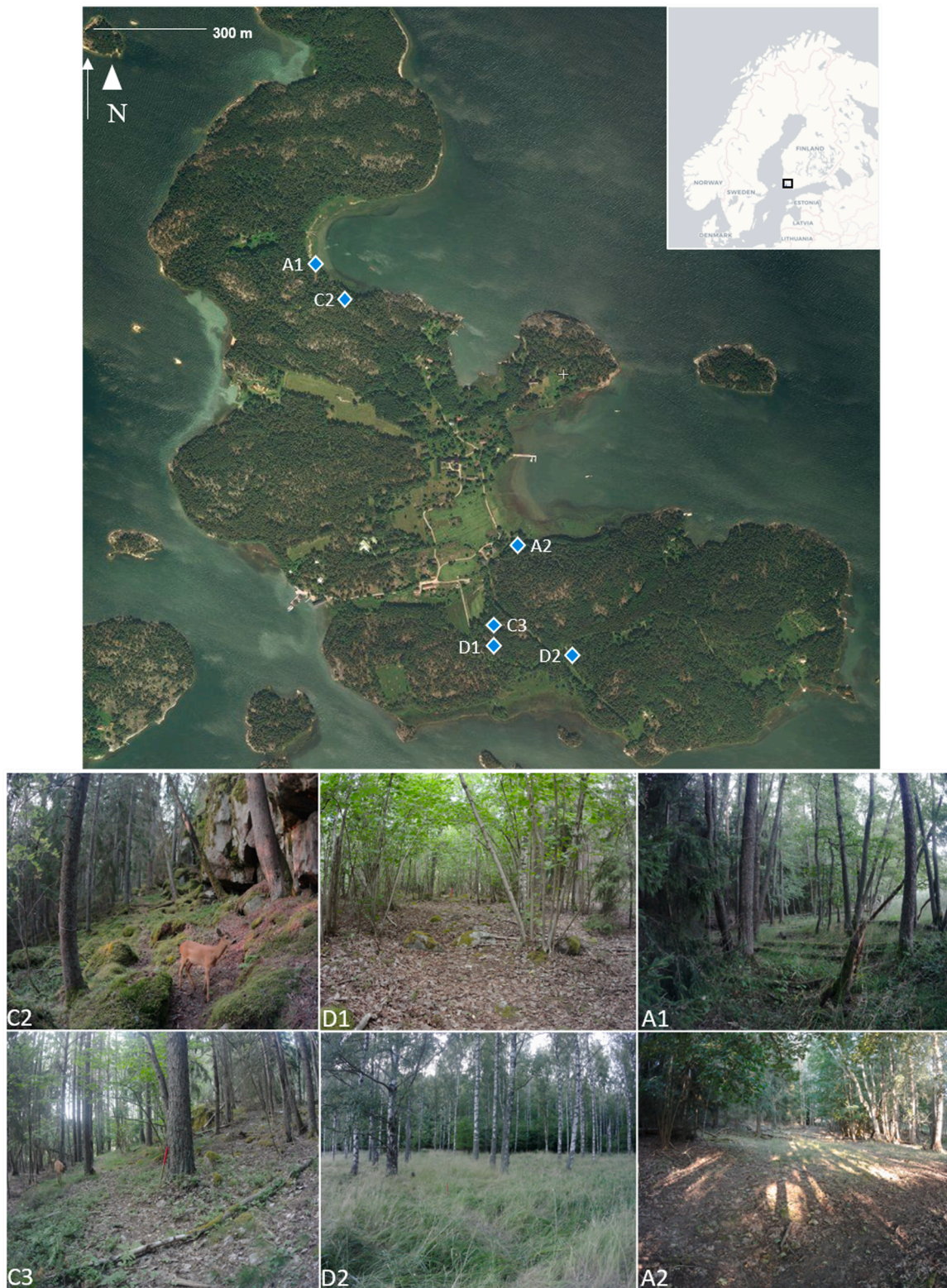


Fig. 1. Locations and representative camera trap pictures of study transects on Seili Island in southwestern Finland.

### 2.3. Statistical analysis

We used generalized linear models (GLM) with binomial distribution and logit link function to analyze differences in the probabilities of *I. ricinus* nymphs having fed on specific hosts (voles, shrews, red squirrels, or unidentified hosts; separate model for each) among study transects. Likewise, we used similar models to analyze differences in the

probabilities of nymphs having fed on specific hosts carrying the analyzed tick-borne pathogens. Due to low numbers of positive detections for other pathogens, these analyses were only made for *Borrelia* spp. and *N. mikurensis*. In cases of 0 detections (e.g. transect A2 for blood meal sources), the transects were not included in analysis, as the binomial GLMs cannot properly handle such data (a problem known as complete separation) (Albert and Anderson, 1984).

**Table 1**  
List of primers and probes used for detection of tick host blood meals and pathogens.

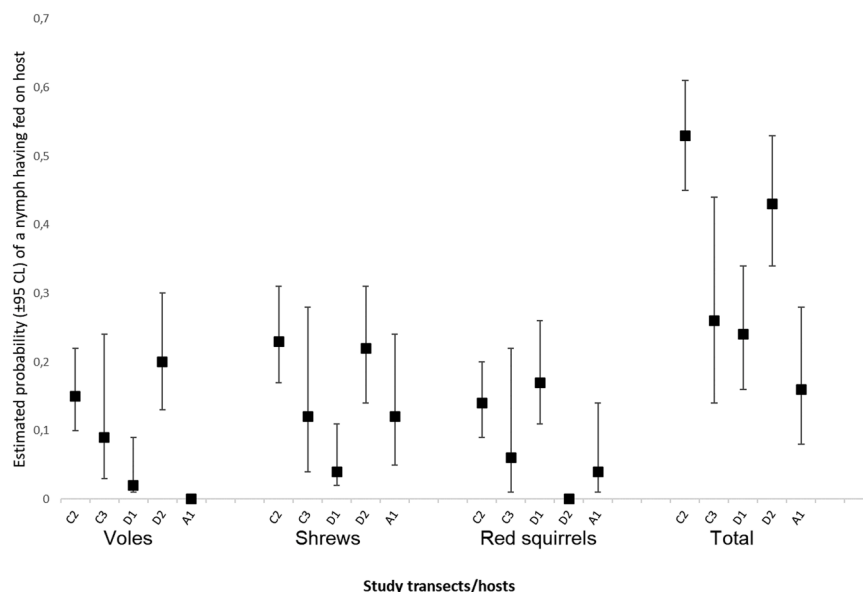
Primer/probe name	Primer/probe target	5' → 3'	References
qPCR:			
SorexSINE-164f	Shrew SINE SA_Ba-325L16	GATTCCCAGCATCCCATATG	Goethert 2021b
SorexSINE-235rmod	Shrew SINE SA_Ba-325L16	RTTACTCCTGGCTCTGCA	
SorexSINE185PCA	Shrew SINE SA_Ba-325L16	[HEX]-GTCCCCAAGCACC GCCAGG-[BHQ1]	
SqrlSINE-103F	Squirrel SINE family CM55	CCCTGTCTCTAAATAAAAATACA	Goethert 2021b
SqrlSINE-183R	Squirrel SINE family CM55	TACCAGGGATTGAACCTCAG	
SqrlSINE-132P	Squirrel SINE family CM55	[Cy5]-GGGCTAGGGATGTGGCTCAGTGG-[BHQ3]	
MicrB2SINE-55F	Vole SINE family B2	TGAGITCAATTCCCAGCAAC	Goethert 2021b
MicrB2SINE-160R	Vole SINE family B2	TGTATACAATATTCTGTCTGTGTG	
MicroB2SINE-113P	Vole SINE family B2	[HEX]-GCCTCTCTGGCCTGCAGA-[BHQ1]	
Bb23Sf	<i>Borrelia</i> 23S RNA	CGAGTCTTAAAAGGGCGATTTAGT	Courtney et al. 2004
Bb23Sr	<i>Borrelia</i> 23S RNA	GCTTCAGCCTGGCCATAAATAG	
Bb23Sp	<i>Borrelia</i> 23S RNA	[FAM]-AGATGTGGTAGACCCGAAGCCGAGTG-[BHQ1]	
Baf_recA-F	<i>B. afzelii</i> recA	AGTCAGCCTGATACC GGAGA	Own
Baf_recA-R	<i>B. afzelii</i> recA	ATTTTGGGGTCAAAGCTGCC	Own
Baf_P	<i>B. afzelii</i> recA	[FAM]-TGCCGAACATTTAATTAGAAG-[BHQ1]	Tveten 2013
Bga_recA-F	<i>B. garinii</i> recA	ATGCCAAAAGCTTTGGGGGTT	Own
Bga_recA-R	<i>B. garinii</i> recA	AGGGGTAAAGCTGCTACAGA	Own
Bga_recA-P	<i>B. garinii</i> recA	[HEX]-TTGCCGAACATTTAATCAGAA-[BHQ1]	Tveten 2013
Bva_recA-F	<i>B. valaisiana</i> recA	TGGTCTGAGTCGTCTGGTA	Own
Bva_recA-R	<i>B. valaisiana</i> recA	CTTGCTCTCCGGTGTCCAGG	Own
Bva_recA-P	<i>B. valaisiana</i> recA	[Cy5]-AGGTTCAAAAAGAAGGTGGTAT-[BHQ2]	Tveten 2013
Rssp-F	<i>Rickettsia</i> gltA	GAGAGAAAATTATATCCAAATGTTGAT	Labruna et al. 2004
Rssp-R	<i>Rickettsia</i> gltA	AGGGTCTTCGTGCATTTCTT	
Rssp-P	<i>Rickettsia</i> gltA	[CY5]-CATTGTGCCATCCAGCCTACGGT-[BHQ3]	
CNeGroEL-F	<i>N. mikurensis</i> groEL	CCTTGAAAATATAGCAAGATCAGGTAG	Jahfari et al. 2012
CNeGroEL-R	<i>N. mikurensis</i> groEL	CCACCACGTAACCTTATTAGCACTAAAG	
CNeGroEL-P	<i>N. mikurensis</i> groEL	[FAM]-CCTCTACTAATTATTGCWGAAGATGTAGAAGGTGAAGC-[BHQ1]	
ApMSP2F	<i>A. phagocytophilum</i> Msp2	ATGGAAGGTAGTGTGGTTATGGTATT	Courtney et al. 2004
ApMSP2R	<i>A. phagocytophilum</i> Msp2	TTGGTCTTGAAGCGCTCGTA	
ApMSP2P	<i>A. phagocytophilum</i> Msp2	[CY5]-TGGTGCCAGGGTTGAGCTTGAGATTG-[BBQ650]	
Bab18S-F	<i>Babesia</i> 18S rRNA	CAGCTTGACGGTAGGGTATTGG	Radzijeuskaja et al. 2008
Bab18S-R	<i>Babesia</i> 18S rRNA	TCGAACCCTAATTCCCCGTTA	
Bab18S-P	<i>Babesia</i> 18S rRNA	[HEX]-CGAGGCAGCAACGG-[BHQ1]	

All the GLMs were run with the GLIMMIX procedure of SAS v. 9.4. (SAS Institute, Cary, North Carolina, USA) using maximum likelihood estimation. Multiple, a posteriori, pairwise comparisons (among transects) for differences of the estimated marginal means (i.e. ls-means in SAS) were adjusted by the Tukey–Kramer method. The binary GLMs provide estimates on the probability of a nymph carrying the studied pathogen, not prevalence rates. All the probabilities (and 95% confidence intervals) of analyzed nymphs being positive for hosts or microbes

given in the text, tables, or figures are estimated marginal means from the GLMs.

### 3. Results

In total, 438 *I. ricinus* nymphs collected from six study transects on the island were analyzed for blood meal sources and several tick-borne microbes. The sensitivities of the host assays, using DNA extracts from



**Fig. 2.** Probabilities (with 95% confidence limits) of questing *Ixodes ricinus* nymphs having fed on specific host animals on different transects. Transect A2 was omitted due to no findings of ticks having fed on the studied animals.

host animal tissue samples, were determined to be  $1 \times 10^{-6}$  ng/ $\mu$ l for shrews,  $1 \times 10^{-6}$  ng/ $\mu$ l for squirrels and  $1 \times 10^{-4}$  ng/ $\mu$ l for voles (cutoff  $C_T < 40$ ). The overall probability of a questing nymph having fed on a vole, shrew, or red squirrel was 0.34 (0.30 – 0.38; 95% confidence interval). An additional three nymphs that were positive for both vole and shrew DNA were not included in these numbers and are not included in analyses regarding blood meal sources.

There was noticeable variation in the probabilities of blood meals being provided by the examined host species/groups across study transects (GLM for voles, shrews and squirrels pooled,  $n=399$ ,  $F_{4, 394}=7.89$ ,  $p<.0001$ ) (Fig. 2). Shrews appeared to be the most common blood meal sources, with the probability of a nymph having previously fed on a shrew at 0.15 (0.12 – 0.19), followed by voles at 0.10 (0.08 – 0.13) and squirrels at 0.09 (0.06 – 0.12). Variation in the probabilities of blood meals being provided by each host species/group among study transects was also observed (Fig. 2). Transect-specific differences, based on host species/group-specific GLMs, in probabilities of hosts providing blood meals are reported in the Technical Appendix (Table A2).

*Borrelia* spp. were the most common pathogens detected from nymphs, with the mean probability of a nymph carrying *Borrelia* estimated as 0.24 (0.20 – 0.28) (Table 2). Voles, shrews or squirrels could be identified as sources in 42% ( $n=45$ ) of *Borrelia* detections. Out of 54 detections of *B. afzelii*, 56% ( $n=30$ ) were from these sources, whereas no detections of *B. garinii* or *B. valaisiana* were made from nymphs that had fed on the animals. The probability of being positive for *Borrelia* was 0.30 (0.18 – 0.44) for nymphs that had fed on voles, 0.23 (0.14 – 0.35) for nymphs that had fed on shrews, and 0.42 (0.28 – 0.58) for nymphs that had fed on red squirrels (Table 2). The probability of carrying *Borrelia* was higher for nymphs that had fed on red squirrels than for nymphs that had fed on unidentified sources (GLM,  $n = 438$ ,  $F_{3, 434}=2.96$ ,  $p=0.03$ ; Table A2). Nymphs that had fed on squirrels carried *Borrelia* more commonly than expected by random co-occurrence [ $X^2$  (1,  $N=438$ ) = 37.3,  $p < 0.0001$ ].

*Anaplasma phagocytophilum*, *Rickettsia* spp., *Babesia* spp. and *N. mikurensis* were rare (Table 2). Nymphs that had fed on voles carried no *A. phagocytophilum*, *Babesia* spp. or *Rickettsia* spp., whereas a single nymph that fed on a shrew carried *Babesia* spp. and another nymph that fed on a red squirrel carried *A. phagocytophilum* (Table 2). Altogether 11 nymphs that had fed on voles and one nymph that had fed on a shrew

carried *N. mikurensis*, whereas the source remained unknown in 10 detections. The probability of carrying *N. mikurensis* was higher for nymphs that had fed on voles than for nymphs that had fed on shrews, squirrels or unidentified sources (GLM,  $n = 400$ ,  $F_{2, 397}=11.86$ ,  $p<.0001$ ; Table 2). Nymphs that had fed on voles carried *N. mikurensis* more commonly than expected based on random co-occurrence [ $X^2$  (1,  $N=438$ ) = 7.5,  $p = 0.01$ ]. Of 11 detections of *N. mikurensis* from nymphs that had fed on voles, three were co-infections with *B. afzelii* and a further one was a co-infection with an unidentified *Borrelia* species.

#### 4. Discussion

This study highlights that shrews and red squirrels may equal voles as blood meal and TBP sources for *I. ricinus* larvae. Furthermore, the overall contribution of these animals to larval blood meals was high. While the proportion of successful blood meal identifications remained at less than half in the current study, voles (2 species present on the island), shrews (1-2 species) and squirrels (1 species, *S. vulgaris*) only form a small part of the tens of potential host animals on the island, including deer, hares, canids, mustelids, mice, reptiles, hedgehogs and birds. In this context, the proportion of larvae having fed on these few species is high, reaching up to half of all previous blood meals on one transect.

Regarding the reliability of the obtained results, it is possible that older nymphs that had been active since spring were also caught with cloth dragging and analyzed, which may have influenced the success of blood meal identification due to degradation of host DNA, as suggested previously (Goethert et al., 2021a). However, this is likely to only lead to potential underestimation of the relative importance of the host species in focus. Furthermore, the sensitivity of the vole primers was observed to be noticeably lower for bank voles than previously reported for North American vole species (Goethert et al. 2021b;  $10^{-4}$  vs  $10^{-7}$ ) and also lower than those of the shrew and red squirrel primers. In addition to potentially undermining the overall contributions of these hosts to larvae blood meals, this may also have led to underestimation of the role of voles relative to shrews and red squirrels. While higher sensitivity may in general be expected to lead to less false negatives, the extent of potential underestimation here remains completely unknown.

The roles of the three animal groups as hosts for immature stages of

**Table 2**

Numbers of ticks infected with the screened microbes (bolded) and corresponding infection prevalence in *Ixodes ricinus* nymphs collected from Seili Island, Finland. Presented with 95% confidence intervals. For *Borrelia* spp. and *Neoehrlichia mikurensis*, probabilities of carrying the pathogen, derived from the used generalized linear models, are provided instead of prevalence estimates.

Blood source	n	<i>Borrelia</i> spp.	<i>Borrelia afzelii</i>	<i>Borrelia garinii</i>	<i>Borrelia valaisiana</i>	Unidentified <i>Borrelia</i>	<i>Anaplasma phagocytophilum</i>	<i>Babesia</i> spp.	<i>Rickettsia</i> spp.	<i>Neoehrlichia mikurensis</i>
Vole	47	<b>14</b> 0.30 <sup>a,b</sup> (0.18 – 0.44)	<b>10</b> 0.21 (0.12 – 0.35)	-	-	<b>4</b> 0.09 (0.03 – 0.21)	-	-	-	<b>11</b> 0.23 <sup>c</sup> (0.13 – 0.38)
Shrew	68	<b>15</b> 0.23 <sup>a,b</sup> (0.14 – 0.35)	<b>12</b> 0.18 (0.11 – 0.30)	-	-	<b>3</b> 0.05 (0.01 – 0.13)	-	<b>1</b> 0.02 (0.002 – 0.10)	-	<b>1</b> 0.02 <sup>d</sup> (0.002 – 0.10)
Squirrel	38	<b>16</b> 0.42 <sup>a</sup> (0.28 – 0.58)	<b>8</b> 0.21 (0.11 – 0.37)	-	-	<b>8</b> 0.21 (0.11 – 0.37)	<b>1</b> 0.03 (0.003 – 0.17)	-	-	-
Unidentified host	285	<b>61</b> 0.21 <sup>b</sup> (0.17 – 0.26)	<b>24</b> 0.08 (0.06 – 0.35)	<b>12</b> 0.03 (0.01 – 0.04)	<b>11</b> 0.03 (0.01 – 0.04)	<b>14</b> 0.05 (0.03 – 0.08)	<b>8</b> 0.03 (0.01 – 0.05)	<b>5</b> 0.02 (0.01 – 0.04)	<b>6</b> 0.02 (0.01 – 0.05)	<b>10</b> 0.03 <sup>d</sup> (0.02 – 0.06)
Total	438	<b>106</b> 0.24 (0.20 – 0.28)	<b>54</b> 0.12 (0.10 – 0.16)	<b>12</b> 0.03 (0.01 – 0.04)	<b>11</b> 0.03 (0.01 – 0.04)	<b>29</b> 0.07 (0.05 – 0.09)	<b>9</b> 0.02 (0.01 – 0.04)	<b>6</b> 0.01 (0.01 – 0.03)	<b>6</b> 0.01 (0.01 – 0.03)	<b>22</b> 0.05 (0.03 – 0.08)

<sup>a-b</sup>Estimates for hosts with the same letter are not significantly ( $p<0.05$ ) different (*Borrelia*; GLM,  $n=435$ ,  $F_{3, 431}=3.15$ ,  $p=0.02$ ).

<sup>c-d</sup>Estimates for hosts with the same letter are not significantly ( $p<0.05$ ) different (*N. mikurensis*; GLM,  $n=397$ ,  $F_{2, 394}=12.64$ ,  $p<0.0001$ ).

*I. ricinus* have been examined previously, but mainly voles are commonly studied and quoted as particularly important hosts (Matuschka et al., 1991; L'Hostis et al., 1996; Gray et al., 1999; Humair et al., 2007; Pérez et al., 2012; Collini et al., 2016; Wodecka and Skotarczak, 2016; Cayol et al., 2017; van Duijvendijk et al., 2016; Honig et al., 2017). In contrast, fewer studies have highlighted the importance of shrews or red squirrels in *I. ricinus* population upkeep (Tälleklint and Jaenson, 1994; Humair and Gern, 1998; Bown et al., 2011; Pisanu et al., 2014). However, by analyzing blood meal sources from wild, questing *I. ricinus* nymphs, we were able to determine that the relative contributions of each of these species/groups as larval blood meal sources were locally approximately equal. In fact, shrews appeared to have provided blood meals to *I. ricinus* larvae more commonly than voles, with red squirrels also nearly equal to voles in this regard. However, due to the lower sensitivity of the vole assay, voles may be underrepresented in the data. The relative role of voles as blood meal sources may thus be greater than observed here.

Red squirrels thrive in urban environments, leading to higher population densities therein compared to forests (Jokimäki et al., 2017). As such, their role may be even more significant in urban green spaces, which are increasingly being recognized as potential threat areas regarding tick-borne diseases (Rizzoli et al., 2014; Hansford et al., 2022). In such areas, the risk of tick-borne diseases may be particularly high, as human activity is also at its highest (Sormunen et al., 2020b). If squirrels form an important part of urban life cycles of ticks, control measures targeted at them may be effective in reducing the risk of tick-borne diseases therein. The role of squirrels in the upkeep of specifically urban tick populations should be further evaluated.

The overarching pattern regarding the role of these animals as blood meal sources appears to be that of high spatial variation. While collectively they formed an important source of previous blood meals for *I. ricinus* nymphs on the island, there appears to be noticeable variation in their contributions even between proximal study transects. The contribution of voles and shrews was particularly high on transects C2 and D2. Transect C2 is characterized by relatively extensive canopy cover provided by conifers (mainly Norway spruce, *Picea abies*) and lots of moss and moss-covered stones on the ground floor, forming tunnels and holes that can be used as shelters by small mammals. As for transect D2, while canopy cover is not as extensive, dense growths of tall grasses provide shelter for both voles and shrews. In addition, on transect C2, there are lots of spruce cones on the ground for voles to consume. Consequently, these transects seem to offer good cover from predators for both species and also an abundant food source for voles on C2, likely contributing to the apparently high occurrence of the animals on these transects.

The relative contribution of red squirrels was highest on study transects that had lots of trees and clearly identifiable food sources for squirrels (spruce cones on C2 or hazelnuts on D1). In contrast, no nymphs that had fed on squirrels were identified on transect D2, where nearly half of larval blood meals were provided by voles and shrews. This transect is characterized by a monoculture of birch and dense growths of tall grasses covering the ground floor, providing poor feeding opportunities for squirrels. Transects A1 and C3 with lower numbers of blood meals provided by squirrels had plenty of trees, but food sources were more scarce (mostly Scots pine, *Pinus sylvestris*, on C3 and common alder on A1).

Surprisingly, no nymphs that had fed on voles, shrews or red squirrels were detected from transect A2. This transect is quite open, has little ground floor cover and provides few hiding places for voles and shrews. Likewise, canids and mustelids [red foxes (*Vulpes vulpes*), raccoon dogs (*Nyctereutes procyonoides*), and badgers (*Meles meles*)] move in the area frequently (2.1 detections/camera trap day in May–September 2021). The high activity of predators and lack of shelter likely makes the area suboptimal particularly for small mammals. All in all, the results of the blood meal assays suggest that knowledge of habitat preferences, food sources and predator activity may be used, at least to some degree, to estimate the role of different animals in local tick population upkeep.

However, variation may be expected to be high and results highly localized. Likewise, estimation is sure to be more difficult for animals with greater movement ranges, such as birds.

*Borrelia* spp. were the most common pathogens detected, as expected based on both previously made local (Sormunen et al., 2018) and Europe-wide studies (Estrada-Peña et al., 2018). Voles, shrews and red squirrels could be identified as the sources in close to half of all detections, again highlighting the relative importance of these few host species. Most of the findings of *Borrelia* from ticks that had fed on the animals were of *B. afzelii*, a species linked to rodent reservoirs (Kurtenschbach et al., 2002). Conversely, *B. garinii* and *B. valaisiana*, of which particularly the latter has been strongly linked to bird reservoirs (Kurtenschbach et al., 2002; Norte et al., 2020), were not detected from either rodents or shrews. Regarding the remaining 24 detections of *B. afzelii*, it is possible that some of the analyzed nymphs had been active since spring, which may have led to the degradation of host DNA to the level where it is no longer detectable (Goethert et al., 2021a). However, there are also several other potential (reservoir) hosts on the island, including yellow-necked mice (*Apodemus flavicollis*) and European hares (*Lepus europaeus*), from which the pathogen could have been acquired. In this regard, it is also worth noting that we were not able to specifically test the primers on field voles (*Microtus agrestis*) or Eurasian pygmy shrews (*Sorex minutus*), due to lack of suitable samples. At least field voles are known to inhabit the island, although only 1 out of 45 voles trapped from the same study transects in 2020 was a field vole (unpublished own data).

While voles and shrews seemed to mostly carry *B. afzelii*, half of all *Borrelia* detections from red squirrels were of a species that remained unidentified. In addition to the species screened in the current study, *B. burgdorferi* sensu stricto has previously been detected from the island (Sormunen et al., 2016a, 2018) and has been suggested to circulate in sciurids (Humair and Gern, 1998; Pisanu et al., 2014). Furthermore, the primers and probe used to screen *Borrelia* also detect *Borrelia miyamotoi* (not a member of the Bbsl group), which has also been previously detected on the island (Sormunen et al., 2016a, 2018) and from red squirrels (Ruyts et al., 2017). For *B. miyamotoi*, transovarial transmission has been recorded, and infections may thus have not been acquired from the observed blood meal source. However, the prevalence of *B. miyamotoi* has consistently been low on the island (0.003 – 0.013; Sormunen et al. 2018), so it may be expected to not significantly influence the results. In any case, the high prevalence of *Borrelia* in nymphs that had fed on red squirrels as larvae, as well as their role as blood meal sources also for nymphs, suggests that they are likely important in the upkeep of *Borrelia* where present, as has been previously suggested in studies involving these animals (Humair and Gern, 1998; Pisanu et al., 2014).

Regarding *A. phagocytophilum*, *Rickettsia* spp. and *Babesia* spp., the role of voles, shrews and squirrels in upkeep appears to be minimal locally. These microbes have mostly been linked to other reservoir hosts or vertical transmission, so this is not particularly surprising (Vichová et al., 2014; Krawczyk et al., 2022). As for *Babesia*, whereas certain species reportedly circulate in rodents (Beck et al., 2011; Karshima et al., 2021), only the species *Babesia venatorum* which is linked to large reservoir hosts has previously been reported from the island (Sormunen et al., 2018). However, the *Babesia* species detected from a shrew in the current study remains unidentified and might signify a novel species to the island. For *N. mikurensis*, observations support previously made conclusions: voles appear to be the main reservoirs for the pathogen (Andersson and Råberg, 2011; Andersson et al., 2014). However, in the current study, a high proportion of detections could not be linked to any of the analyzed host animals, including voles. As previously mentioned, it is possible that older nymphs were collected and analyzed in autumn, preventing successful blood meal determination. There exists also the possibility that the primers do not pick up field voles, another possible reservoir (Andersson and Råberg, 2011). However, it is likewise possible that another, previously unidentified reservoir exists. Further studies

should be conducted to uncover potential novel reservoirs for this emerging tick-borne pathogen.

In conclusion, the novel qPCR assays used to determine tick blood meal sources, developed in the USA (Goethert et al., 2021b) and tested here, appear to be functioning for at least bank voles, common shrews and red squirrels also in Finland. Consequently, the further application of the assays to specific study questions, such as the role of red squirrels in the upkeep of urban tick and TBP populations, may provide important novel data to help in, for example, developing control schemes for ticks or TBPs in urban green spaces. Likewise, wider application may reveal novel connections between hosts and TBPs of medical interest.

#### CRedit authorship contribution statement

**Jani J. Sormunen:** Conceptualization, Formal analysis, Resources, Writing – original draft, Supervision, Funding acquisition. **Satu Mäkelä:** Methodology, Validation, Investigation, Data curation, Writing – review & editing. **Tero Klemola:** Formal analysis, Resources, Writing – review & editing, Project administration. **Theophilus Y. Alale:** Investigation, Data curation, Writing – review & editing. **Eero J. Vesterinen:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition.

#### Declarations of Competing Interest

None.

#### Data availability

Data will be made available on request.

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#### Supplementary materials

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