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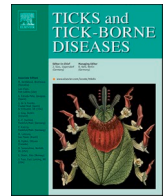
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Original article

Identifying breeding hosts of *Ixodes ricinus* ticks using stable isotope analysis of their larvae – Proof of concept

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ABSTRACT

Ticks are important vectors of zoonotic pathogens. Ticks are parasites that are dependent on their hosts for blood meal to develop and reproduce. The abundance of ticks is dependent on the availability of suitable breeding hosts, often medium- and large-sized mammals. So far there has been a shortage of direct methods identifying the breeding hosts for the female ticks. In this study, we introduce a stable isotope analysis (SIA) method that enables us to identify the trophic group of the breeding host, i.e. the host on which the tick mother fed, by sampling larval ticks from the field. We established a reference database on the stable isotope (SI) values ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of the blood of potential tick host species, and of larvae from *Ixodes ricinus* females, which have fed on known hosts. By comparing the SI values from field collected larval ticks to our reference data, we can determine their most likely host species group. Our results show that the isotopic signatures of *I. ricinus* tick larvae reflect the diet of the breeding host of the mother tick. SIA proved reliable in categorizing the breeding hosts of *I. ricinus* into two distinguishable trophic groups; herbivores and carni-omnivores. To our knowledge, this is the first time that stable isotope analyses have been applied to detect transovarial (i.e. over-generational) traces of a blood meal in ticks. The method provides an efficient, novel tool for directly identifying tick breeding hosts by sampling field collected larvae. *Ixodes ricinus* is the most important vector of TBPs (tick-borne pathogens) in Europe, and to predict and mitigate against the future risks that TBPs pose, it is crucial to have detailed knowledge on the hosts that support tick reproduction in nature.

1. Introduction

More than half of human pathogens are zoonotic, i.e. transmitted from other animal species to humans, and the majority of these have their origin in wildlife host species (Jones et al., 2008). Increasingly relevant are vector-borne zoonotic infections, of which the most important in the Northern hemisphere are caused by tick-borne pathogens (TBPs) (Jones et al., 2008; Rizzoli et al., 2011; Medlock et al., 2013; Sykes and Makiello 2017; Petrulionienė et al., 2020). Ticks are obligate parasites that require blood meals from vertebrate hosts for development and reproduction (Randolph, 2004; Estrada-Peña and De La Fuente, 2014). Consequently, the hosts affect the abundance of the ticks and contribute to TBPs circulation, ultimately affecting the risks that TBPs pose to humans (Randolph, 2004).

The tick *Ixodes ricinus* is the most important vector of TBPs in Europe (Jore et al., 2011; Jaenson et al., 2012; Medlock et al., 2013; Laaksonen et al., 2017, 2018; Kjær et al., 2019). *Ixodes ricinus* quests for hosts on the

vegetation, with larvae, nymphs and adult females feeding mostly on small-, medium- and large-sized animals, respectively (Randolph, 2004; Francischetti et al., 2009; Mannelli et al., 2012; Estrada-Peña and De La Fuente, 2014). Females require sufficiently large blood meals, typically from a medium- or large-sized animal, to reproduce. Hence, the availability of these “breeding hosts” is an important factor determining tick abundance. However, it remains poorly known which of the potential hosts actually serve as breeding hosts for the females in different ecosystems (Kilpatrick et al., 2017).

Identifying the hosts of the female ticks is challenging, because after feeding the tick detaches from the host, produces eggs, and dies. So far, the breeding host potential of different vertebrate species has been directly examined based on tick infestation levels on wild-captured or hunted individuals (Tälleklint and Jaenson, 1997). Alternatively, host competence has also been examined by holding wild-captured animals in captivity and measuring the engorgement and detachment rates and subsequent moulting success of the ticks (LoGiudice et al., 2003;

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Keesing et al., 2009; Brunner et al., 2011). Due to high temporal and individual-level variation in tick infestation rates (Tälleklint and Jaenson, 1997, Cayol et al., 2017), high numbers of individuals per host species need to be examined to obtain accurate estimates. Consequently, it is challenging to examine the role of different vertebrate species as hosts for ticks. Although traditional DNA based blood meal analysis can detect transstadial traces of hosts DNA from nymphs and adults, enabling the identification of hosts on which the larvae and nymphs were feeding on (Humair et al., 2007; Moránmora' et al., 2007; Collini et al., 2015; Kim et al., 2021; Goethert et al., 2021; Sormunen et al., 2023), such methods do not detect transovarial (i.e. over-generational) traces of host DNA (Gómez-Díaz and Figuerola, 2010; Kim et al., 2021). Thus, such blood meal analyses cannot be used to identify breeding hosts of ticks. However, the identification of breeding hosts is pivotal in estimating the role of different host species in driving tick reproduction and tick abundance in different ecosystems.

In this study, we introduce a method that enables sampling larval ticks from the field to identify the breeding host on a trophic level (i.e. the host their mother fed on) by using stable isotope analysis (SIA). As 'you are what you eat' is the fundamental principle in SI ecology (Fry, 2006), the host animals are expected to differ in their SI values reflecting their diet. SI values of nitrogen ($\delta^{15}\text{N}$) and carbon ($\delta^{13}\text{C}$) tend to increase in a predictable manner in the food web, and hence, can be used to separate consumers based on their diet source (Fry, 2006). Consequently, the SI value of tick larvae reflects the SI value of the breeding host, which the female, i.e. the mother, fed on. Based on this principal, we have established a database, consisting stable isotope data from different potential host species by collecting blood samples from a variety of hosts, and larval samples from *I. ricinus* females that have fed on known hosts. By using the SI marker measurements from larvae collected from vegetation, we can determine the trophic group of the mother's host species.

2. Material and methods

2.1. Study system and establishing reference database

In our study system in Finland the expected breeding hosts of *I. ricinus* are the roe deer (*Capreolus capreolus*), which is important host for *I. ricinus* in Europe (Vor et al., 2010; Medlock et al., 2013; Hofmeester et al., 2016; Mysterud et al., 2021), introduced white-tailed deer (*Odocoileus virginianus*), which is an important host for *Ixodes* ticks in North America (Eisen and Stafford, 2021), and moose (*Alces alces*) (Qviller et al., 2013; Hofmeester et al., 2016; Fabri et al., 2021). In addition, medium-sized animals, such as raccoon dog (*Nyctereutes procyonoides*), red fox (*Vulpes vulpes*), badger (*Meles meles*), hedgehog (*Erinaceus europaeus*), brown hare (*Lepus europaeus*) and mountain hare (*Lepus timidus*) are wildlife hosts that are known to provide blood meals for adult female *I. ricinus* (Jaenson & Tälleklint, 1996, Tälleklint and Jaenson, 1997, Hofmeester et al., 2016, Wodecka et al., 2016, Jahfari et al., 2017, Mysterud et al., 2021) Also, dogs (*Canis lupus familiaris*) and other domestic animals may support tick populations (Shaw et al., 2001; Nijhof et al., 2007; Jore et al., 2014).

To create a database for the stable isotope values (nitrogen and carbon), we utilised blood samples from a variety of potential tick breeding host species, including wild and domestic mammals (Table 1). All blood samples were collected and provided by voluntary hunters or by the Finnish Food Authority (governmental authority responsible for monitoring the health and wellbeing of wild and domestic animals in Finland). Samples from hunted individuals were collected directly after killing, while the samples from the Finnish Food Authority were collected during the autopsy, after unspecified time after the death. Samples were collected from several municipalities across Finland (see deposited data). The blood samples were stored at $-20\text{ }^{\circ}\text{C}$, until the preparation of the samples for SIA.

To acquire reference larvae, engorged females were collected

Table 1

List of host species included in the database, with number of samples per species. "Tick" refers to *Ixodes ricinus* larval tick samples and/or eggs, produced by the engorged *I. ricinus* females with a known host. Tick samples from hedgehog included both larvae and egg samples (separately). Samples from roe deer and moose contained only unhatched eggs. "The number of tick hosts" refers to the number of host individuals the engorged ticks were collected from. There were several *I. ricinus* females per one host individual in hedgehog, raccoon dog and sheep. Except for one raccoon dog and a mountain hare, the blood samples and ticks were collected from different host individuals.

Host species	Sample type		Number of tick hosts
	Blood	Tick	
Badger (<i>Meles meles</i>)	4	0	0
Brown bear (<i>Ursus arctos</i>)	2	0	0
Cat (<i>Felis catus</i>)	0	4	4
Dog (<i>Canis lupus familiaris</i>)	20	4	4
Fox (<i>V. vulpes</i>)	8	0	0
Hedgehog (<i>Erinaceus europaeus</i>)	3	9 (2 eggs)	3
Lynx (<i>Lynx lynx</i>)	5	0	0
Mink (<i>Neovison vison</i>)	4	0	0
Pine marten (<i>Martes martes</i>)	2	0	0
Raccoon dog (<i>Nyctereutes procyonoides</i>)	14	5	1
Squirrel (<i>Sciurus vulgaris</i>)	1	0	0
Roe deer (<i>Capreolus capreolus</i>)	8	1 (eggs)	1
European hare (<i>Lepus europaeus</i>)	2	0	0
Mountain hare (<i>Lepus timidus</i>)	3	1	1
Moose (<i>Alces alces</i>)	8	1 (eggs)	1
White-tailed deer (<i>Odocoileus virginianus</i>)	19	1	1
Sheep (<i>Ovis aries</i>)	0	6	3

directly from hosts (Table 1), kept at room temperature in a resealable plastic bag with a piece of moist paper tissue to prevent drying, until the female tick laid eggs, and the eggs hatched into larvae. The engorged *I. ricinus* females were provided by hunters, pet owners or were collected by researchers (ticks from hedgehogs). The engorged *I. ricinus* females originated from Central Finland (Jyväskylä; $n = 11$, and Luhanka; $n = 2$) and from an archipelago in southern Finland (Porvoo, $n = 19$). Beside two samples (a raccoon dog and a mountain hare), the blood and the engorged ticks were from different host individuals.

After the eggs had hatched into larvae, samples for the SIA analysis were collected (Fig. 1). Tick larvae samples were collected by wiping the inside of the plastic bag with a white cotton cloth, from which they were collected with nano tape (Fig. 1a). The adhesion of nano tape is achieved without chemical adhesives, and it leave no residue on the substrate. Thus, the protocol is comparable with the method used to collect larval ticks from the field with fabric and tape (see "Collecting larvae from the vegetation"). Thereafter, the sample tapes were stored at $-20\text{ }^{\circ}\text{C}$, until further procedures. To prepare the samples for the SIA, the tick larvae were first detached from the tape by soaking the tape in a small amount of distilled water, and then scraping the larvae into the water with a help of a small laboratory spatula (Fig. 1b). The larvae were collected from the water by using a plastic loop (Fig. 1c), and placed into a plastic centrifuge tube containing 0.5 ml of distilled water.

In total, the database consists of 103 blood samples, from 15 different host species and 32 tick larvae samples with known mothers (Table 1). Additionally, we included 4 samples of tick eggs (from known mothers), that failed to hatch, to the reference tick data (Table 1). Seventeen of the largest tick larvae samples were divided into replicate samples for the SIA, and their mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were used in the data analyses.

2.2. Ethical statement

All blood samples have been obtained from dead individuals. Blood samples were provided by licenced hunters, who have hunted the animals following species specific rules and regulations in Finland. Dead animals sent to Finnish Food Authority were sampled for blood during

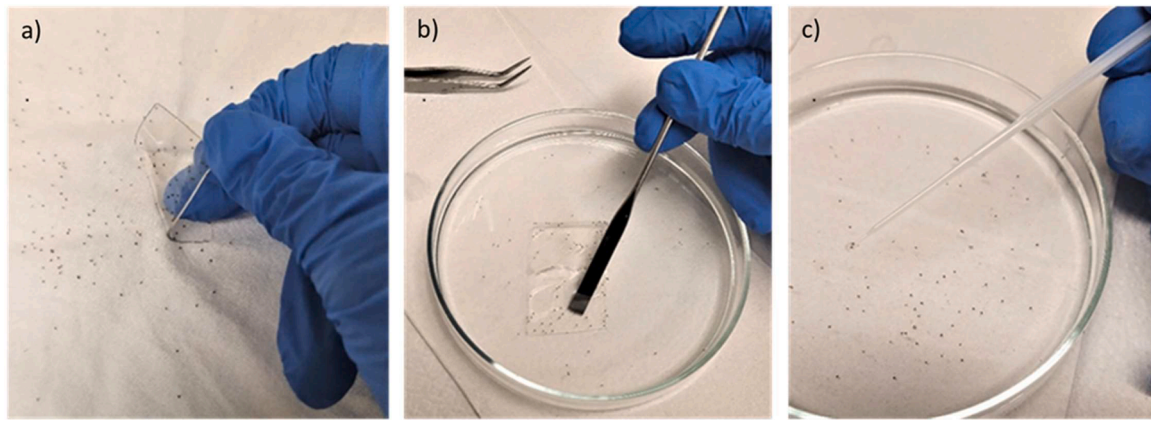


Fig. 1. Sampling of the *Ixodes ricinus* larval ticks. The tick larvae were sampled from flannel fabric using nano tape (a), washing of the nano tape with distilled water to detach the ticks (b), and collecting the larval ticks from the water to a microtube with an inoculation loop (c).

autopsy. The ticks were collected from the hunted individuals or from pets with tweezers. Thus, no animal experimentation permissions have been required, except for three hedgehogs that were caught by hand and from which ticks were removed with tweezers. Permission to capture, handle and remove ticks from hedgehogs was provided by Central Finland ELY center: KESELY/737/2021.

2.3. Collecting larvae from the vegetation

Larval ticks were collected from the vegetation using a dragging method: A cotton flannel sheet (1m²) was dragged along vegetation and checked every 10 m. Aggregates of more than 20 larval ticks, if located in a small, within 15 × 15 cm area on the sheet, were sampled for SIA. At least 20 larvae per aggregate were collected from the sheet using nano tape. If larva did not form a clear aggregate, but were spread across larger area on the sheet, a sample was not collected. The protocol aimed to ensure that each sample of the field collected larvae was originated from one female tick. The sheet was cleared from all ticks before continuing the dragging. The tape with the larvae was placed in a sealed plastic bag and stored at −20 °C until further processing. Larval tick collections were carried out in an archipelago in southern Finland (Porvoo, *n* = 45 samples), and in urban forests in Central Finland (Jyväskylä, *n* = 6 samples).

2.4. Stable isotope analysis

To remove the water from the samples, both blood and tick larvae samples were freeze dried (Christ Alpha 1–2LD Plus, Martin Christ, Germany). Further, 0.6 mg of each sample (in tick larvae 0.6 mg per sample, or the total mass of the sample if <0.6 mg) were measured into a tin cup and used for the SIA. Stable isotopes of carbon and nitrogen were analyzed at the University of Jyväskylä, Finland, using a Thermo Finnigan DELTAplusAdvantage continuous flow stable isotope-ratio mass spectrometry (CF-SIRMS) connected to a Carlo Erba Flash EA1112 elemental analyzer. Results are expressed using the standard δ notation as parts per thousand (‰) difference from the international standards. The reference materials used were internal standards of known relation to the international standards of Vienna Pee Dee belemnite (for carbon) and atmospheric N₂ (for nitrogen). Precision was always better than 0.16‰ for carbon and 0.21‰ for nitrogen based on the standard deviation of replicates of the standards run repeatedly after every 5 samples in each sequence.

2.5. Statistical analyses

Based on the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values received from the stable isotope analyses, host blood samples were categorized into two distinguishable

groups based on the diet of the species: herbivores and carni-omnivores (Fig. 2). Group “Herbivore” included samples from roe deer, European hare, mountain hare, moose, and white-tailed deer, and group “Carni-omnivore” included samples from badger, bear, dog, fox, hedgehog, lynx, mink, pine marten, raccoon dog and squirrel (Fig. 2). For the diet groups of the tick larvae host (see below), sheep was included to the “Herbivore” and cat for the “Carni-omnivore” group, as listed in Table 1. These two groups were used in the statistical data analyses.

To test for the effect of the host species diet on the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (separately) in their blood, and on the tick larvae (whose mother consumed the blood), we used Linear mixed-effects models (LMM) [R package lme4 (Bates et al., 2015)] with the isotope δ -values as response variables, diet group (“Herbivore” vs “Carni-omnivore”) as fixed factor and the host species and origin (Origin = municipality the sample is collected from) as random factors. As there was no information of the geographic origin of the dog blood samples, all dog samples had the same value (RV) in the random effect.

2.5.1. MixSIAR analyses

The proportions of the blood sources (“Herbivore” hosts vs “Carni-omnivore” hosts) exploited by the ticks (reference ticks and field-collected ticks, separately) were assessed using the Bayesian stable isotope mixing model MixSIAR ver. 3.1.12 (Stock et al., 2018). Values for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ from the ticks (consumer data), and from the blood samples (sources data) were included in the model with a trophic discrimination factor (TDF, i.e., consumer-source difference, Δ). For the reference data, we ran two separate models to estimate which provides a better estimate of the used host groups in comparison to the known hosts of the reference ticks; model 1 was carried out without random factor (with process and residual error structure), and in model 2 the tick samples’ IDs (running number) was added as random factor (process error only). Adding ID as the random factor produces individual diet estimation for each sample, showing in which group (herbivore or carni-omnivore) the model categorized each tick sample. Median value 0.5 was used as the threshold value between the diet groups. The field collected tick data was analyzed only with model without random factors (process and residual error structure), which performed better with the reference data (see results). All models were run with “long” Markov Chain Monte Carlo (MCMC) option (Number of chains= 3, Chain length =300,000, Burn-in= 200,000, Thinning= 100). Based on the Gelman-Rubin and the Geweke diagnostics all models converged properly.

TDF was calculated using the equation: $\Delta = \delta_c - \delta_f$, where δ_c refers to the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the tick larvae (consumer) and δ_f to the blood (food source) (Fry, 2006). As the blood samples and ticks were mostly collected from different host individuals, we were not able to calculate TDFs for individual tick-host pairs. Instead, we calculated TDFs for the

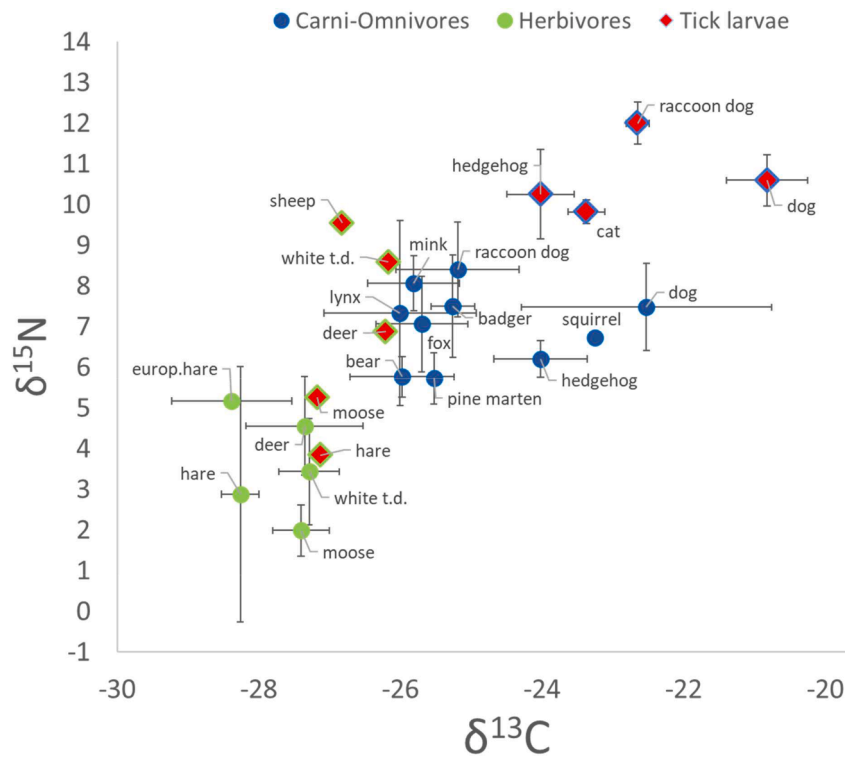


Fig. 2. The mean isotope δ -values (\pm SD) for host blood (blue and green circles) and reference *Ixodes ricinus* tick larvae samples of known host species (Red diamonds). The color of the frame refers to the trophic group of the original host; green = herbivore, blue = carnio-omnivore). The species names next to the tick samples refer to the host species the tick mother fed on.

species we had both blood and larval tick samples in our database (even if from different host individual) (Table 1). In case of more than one sample per host species, we used their average as a representative value for that particular species and tissue (blood or tick larvae). Finally, the TDFs used in our study were generated by combining the species specific TDFs and calculating their mean and SD. Thus, the used TDF (\pm SD) between our tick larvae and blood samples was 1.14 (\pm 0.84) for $\delta^{13}\text{C}$, and 3.26 (\pm 1.36) for $\delta^{15}\text{N}$ (Table A1). All the data analyses were carried out with R 4.2.1. (R Core Team, 2022).

3. Results

3.1. Isotopes of the reference blood and ticks

The host blood samples formed two distinguishable groups based on the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the diet of the species; herbivores and carnio-omnivores (Fig. 2). Further, the tick larvae samples of known origin (reference ticks) seemed to reflect the diet of their breeding host (Fig 2). The diet of the host (herbivore vs carnio-omnivore) had significant effect on the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in both, reference blood and reference tick larvae (Table 2); in both sample types the herbivores had lower values in

$\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ than carnio-omnivores.

3.2. Isotopes of reference and field collected ticks in relation to host groups

The reference larvae and field collected larval samples showed a great variation in the carbon and nitrogen isotope values (Fig. 3). After correcting for TDF ($\Delta \delta^{13}\text{C} = 1.14 (\pm 0.84)$, $\Delta \delta^{15}\text{N} = 3.26 (\pm 1.36)$), reference tick isotope values fell visually into the correct host categories, as ca. 70% of reference ticks' mothers were collected from carnio-omnivores and ca. 30% from herbivores (Fig. 3). Especially carbon isotope values seemed to separate two groups of ticks feeding either on herbivore or carnio-omnivore host groups. Similarly, nitrogen isotope values in the reference larvae showed great variation but some larval ticks known to feed on herbivorous hosts had overlapping $\delta^{15}\text{N}$ values with those of carnio-omnivorous hosts. Carbon and nitrogen isotope values of the field collected tick larvae seemed to reflect mostly the values of herbivorous hosts, especially for carbon isotopes, although when observed visually, many individuals fell between two host groups, especially in relation to nitrogen isotope values (Fig. 3).

Table 2

The effect of the host species diet (herbivore vs carnio-omnivore) on the values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in their blood, and on the *Ixodes ricinus* tick larvae (whose mother consumed the blood of a known host), tested with LMM.

Tissue	Response	Fixed effect	Estimate	SE	t-value	df	p-value
Blood	$\delta^{13}\text{C}$ (‰)	Intercept	-24.92	0.25	-101.70	59	<0.001
		Trophic group (herbivore)	-2.73	0.37	-7.33	13	<0.001
	$\delta^{15}\text{N}$ (‰)	Intercept	7.40	0.30	24.33	59	<0.001
		Trophic group (herbivore)	-3.54	0.50	-7.14	13	<0.001
Tick larvae	$\delta^{13}\text{C}$ (‰)	Intercept	-22.69	0.43	-53.08	20	<0.001
		Trophic group (herbivore)	-4.05	0.68	-5.99	8	<0.001
	$\delta^{15}\text{N}$ (‰)	Intercept	10.56	0.60	17.70	20	<0.001
		Trophic group (herbivore)	-3.56	0.95	-3.74	8	0.006

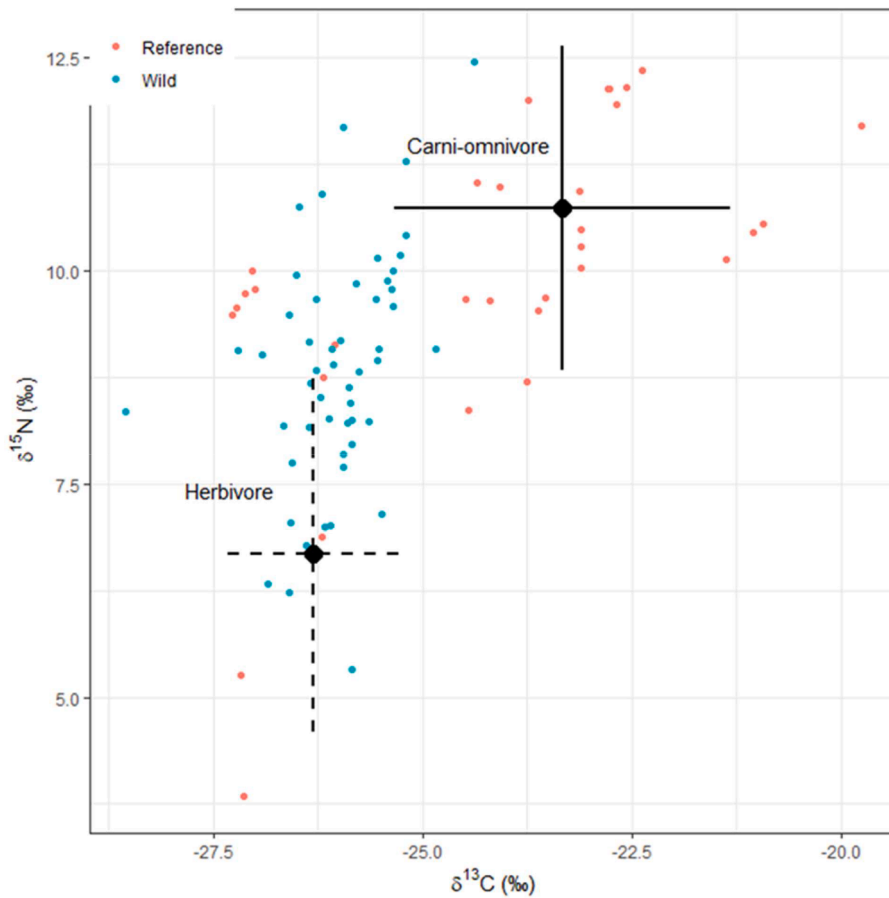


Fig. 3. MixSIAR isospace plots of the *Ixodes ricinus* tick larvae samples. The isospace plots are representing the reference tick larvae samples (red points), and the field captured tick larvae samples (blue points) with the trophic groups of the hosts (herbivore and carni-omnivore) after TDF ($\Delta \delta^{13}\text{C} = 1.14 (\pm 0.84)$, $\Delta \delta^{15}\text{N} = 3.26 (\pm 1.36)$) have been applied to the host blood samples (large filled circles with SD errors).

3.3. MixSIAR models to identify ticks hosts

The observed proportions of the blood sources exploited by the reference ticks was 0.31 for herbivore and 0.69 for carni-omnivore, calculated directly from the reference data (Fig. 4a). Two MixSIAR models were used to estimate the blood sources in the reference data and model 1 reached higher accuracy with lower variance compared to the

model 2 (Fig. 4a, posterior density plots in Appendix, Fig. A1 a and b). Model 1 (model without random factors) produced estimation only for the whole population, while model 2 (individual samples as random factor) additionally produced individual diet estimations for each sample. The individual estimations from model 2 correctly categorized the reference tick larvae samples into the correct diet groups, when median threshold value 0.5 was applied (i.e. the model estimated the diet of the

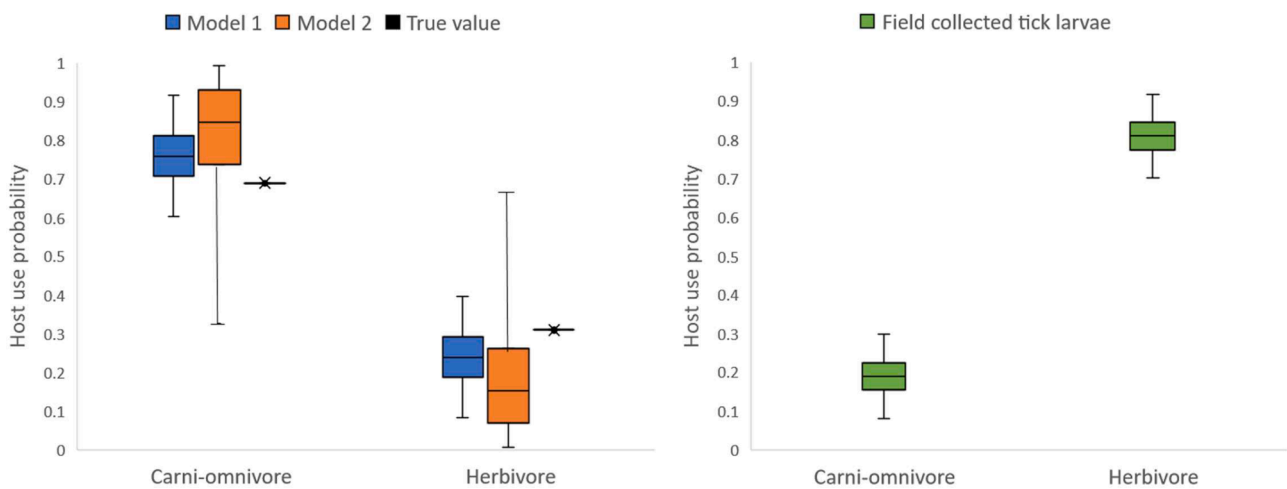


Fig. 4. The MixSIAR model estimations for the proportions (median with upper and lower quartiles, with whiskers representing the 95% Bayesian credible interval) of the blood sources exploited by the (a) reference and (b) field collected *Ixodes ricinus* tick larvae. “True value” in panel (a) refers to the observed value, calculated directly from the reference data.

blood source (host) to consist over 50% of herbivorous or carnivorous food source) (Table A2).

For the field collected tick larvae, the model estimated that a median of 81% (95% CrI 70–92%) of the larval samples were from female ticks that had fed on herbivores (Fig. 4b, MixSIAR posterior density plot in Appendix Fig. A1c).

4. Discussion

In this study, we show that the isotopic signatures of *I. ricinus* tick larvae reflect the diet of the host of the mother tick. We established a reference database containing information on the stable isotope values in the blood of 15 potential breeding hosts species of *I. ricinus*, and in tick larvae from *I. ricinus* females that have fed on known hosts. With this novel method we were able to identify the trophic group of the breeding host, i.e. the host on which the tick mother fed, by utilizing field collected tick larvae. To our knowledge, this is the first analytical method enabling the identification of tick breeding hosts' trophic group through sampling field collected larvae and first study where stable isotope analyses have been applied to detect transovarial traces of a blood meal in ticks.

Our analytical method based on screening larval ticks from the field provides a direct and unbiased method to estimate the role of different host species for tick reproduction in each ecosystem. Earlier methods included correlation analyses between the abundances of hosts (Gilbert et al., 2012; Hofmeester et al., 2017) or examination of tick infestation on wild-captured or hunted individuals (Tälleklint and Jaenson, 1997; LoGiudice et al., 2003; Brunner et al., 2011). However, correlations between host and tick abundance may not reveal causal relationship. In addition, examining species for the tick infestation is very laborious and may lead to biased conclusions of the role of hosts species given insufficient numbers of individuals are examined. For instance, high tick infestation load does not necessarily mean that the host species is important in supporting tick development or reproduction, as the rate of successful feeding varies substantially between host species (LoGiudice et al., 2003; Keesing et al., 2009; Brunner et al., 2011). Moreover, the tick infestation load on host species may vary, for instance, depending on host density, other species in the host community composition, tick abundance, habitat, vegetation, and climate, being potentially very context dependent and requiring ecosystem specific estimations on the role of any specific host species for ticks. Our method provides an excellent direct, unbiased, low effort and relatively low-cost tool to identify the host, albeit only on trophic level, that support the reproduction of ticks in different ecosystems.

Despite the differences in the study aims, with our work focusing on transovarial and earlier studies on transstadial (from previous life stage) traces of blood meals, our results are in line with studies showing SIA's applicability to identify the source of a tick's blood meal on a trophic level (LoGiudice et al., 2018; Kim et al., 2021). For instance, we were able to categorize the breeding hosts (based on the SIA values in blood) into two distinguishable trophic groups; herbivores and carnivores. Similarly, the Bayesian stable isotope mixing model MixSIAR (Stock et al., 2018), which was used for the reference ticks, was able to reliably separate the herbivorous and omnivorous sources of the blood meal with high accuracy. In addition, based on our background information on the mammal community occupying the field sites the larvae samples were collected from, the model's estimation for the field collected tick data is also plausible. Moreover, the TDF calculated from our reference data (blood and ticks) were relatively close to the values found from literature on *I. ricinus* ticks, despite the values we report are the first ones presenting over-generational enrichment, instead of direct consumer-source relationship (Schmidt et al., 2011; Heylen et al., 2019).

To identify the blood meal host of previous life stage on a species level, the traditional DNA based blood meal analysis has proven to be more precise compared to SIA (Kim et al., 2021). DNA markers in

different species are unique, while the diet-derived SI markers often overlap with species from similar ecological niches (Baltensperger et al., 2015; LoGiudice et al., 2018; Heylen et al., 2019; Goethert et al., 2021; Kim et al., 2021; Goethert and Telford III, 2022), making species-level identification difficult, or even impossible. Further, the diet of many omnivorous host species varies between seasons. For example, brown bears eat more vertebrates and insects in spring and summer, while switching to more plant-based diet during autumn (Stenset et al., 2016). For some species, the human activity can strongly affect their diet, e.g. via feeding squirrels and hedgehogs in urban areas. However, the DNA analysis has its limitations too; it is sensitive to contaminations, and most of all, it cannot detect transovarial traces of blood meal because no host DNA is detectable in the offspring (Gómez-Díaz and Figuerola, 2010; Kim et al., 2021). Thus, despite the lack of precision in SIA, it provides a powerful, complementary tool in tick host identification (trophic group). When combined with background information on species composition in a particular ecosystem, rather accurate breeding host estimations can be reached. For example, the field collected tick samples used in our study were mostly from an archipelago (Porvoo, Finland) with very abundant ungulate community (roe deer, white-tailed deer and moose), accompanied by a stable community of mesopredators such as raccoon dogs and foxes. The MixSIAR model estimated that ca. 81% of the breeding hosts were herbivores, and 19% carnivores. The estimation reflects well the observed species composition in the area, as well as the prevailing assumption of deer being one of the main breeding hosts for *Ixodes* ticks in Europe (Vor et al., 2010; Medlock et al., 2013; Hofmeester et al., 2016; Myrsterud et al., 2021).

While the importance of ungulates as breeding hosts for ticks seems undisputed, there are documentations of e.g. islands in Sweden with established tick population, while completely lacking ungulates (Jaenson & Tälleklint, 1996). There, the tick population is maintained by hares. Further, there is growing evidence of many urban sites, such as parks and greenspaces, becoming hot spot areas for ticks (Hansford et al., 2017, 2022; Cayol et al., 2018; Klemola et al., 2019; Sormunen et al., 2020). The host species driving tick reproduction in many of these urban sites is yet to be discovered, for which the SIA method demonstrated here provides an excellent tool. This information is also essential from the aspect of human health, as exposure to ticks, and hence, TBDs, can be particularly high in these sites, compared to less densely populated rural areas.

The method in our study was carried out using only one species, *I. ricinus*, however, the method should be applicable to all parasitic hard tick species. Considering the global abundance and severity of vector-borne zoonotic infections derived by a tick bite, such as Lyme disease, tick-borne encephalitis (TBE) and anaplasmosis, an efficient and reliable method to study the mechanisms behind the complex host-vector-pathogen interactions driving ticks and TBDs is of high relevance. Further, the SIA's ability to detect transovarial traces of a blood meal, as demonstrated here, could potentially be applied also on other invertebrate pathogen vector species, such as mosquitoes (Rasgon, 2008).

Further studies are warranted to improve the accuracy and precision, but also to make the method more user-friendly. For instance, with ticks, each sample should include 20 tick larvae at minimum. To increase the likelihood of all the larvae originating from the same female, the field collected individuals need to be clustered as a clear aggregate on the sheet to be accepted as one sample. Thus, in the future, a stable isotope analysis optimized for smaller sample size would improve the practicality of the method (Langel and Dyckmans, 2014). To improve the reliability, potential spatiotemporal differences in the isotope values present in the host blood within same species or species groups need to be considered and examined. Also measurements for accurate trophic enrichment values are pivotal, as incorrect values can significantly distort the outcome (Bond and Diamond, 2011). Compound-specific stable isotope analysis (CSIA) of macromolecules, such as fatty acids and amino acids, could provide means for dealing with variability and

overlapping values of host species. Unlike in bulk isotope analysis (as used here), specific macromolecules may differ in synthesis pathway or their routing between tick host species and their dietary sources possibly providing more distinct isotope ratios (Komonen et al., 2022). This could allow distinction of tick hosts in species or consumer group level. As a collaborative effort, building up an extensive, global and updating open access database containing information on the blood SI values and species-specific trophic enrichment factors over various taxa and species, and over different geographical locations as well as seasons should be encouraged. This would provide a powerful asset, both for ecological and public health related study questions.

CRedit authorship contribution statement

Saana Sipari: Methodology, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Mikko Kiljunen:** Conceptualization, Data curation, Formal analysis, Writing – review & editing. **Minna Nylund:** Data curation, Writing – review & editing. **Eva R. Kallio:** Conceptualization, Methodology, Data curation, Writing – review & editing.

Declaration of Competing Interest

The authors confirm no conflicts of interests.

Data availability

The raw data and the codes are provided as supplementary material.

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Supplementary materials

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Appendix

Appendix A: Fig. A1 presents the MixSIAR posterior density plots for the reference data models 1 and 2, and for the field collected data. Table A1 presents the trophic discrimination factors for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in larval *Ixodes* ticks calculated from our data, and for reference, all the TDF values reported for *Ixodes* ticks from earlier literature. In Table A2 we have listed all the individual diet estimations (median with 95% Bayesian credible interval) for each sample from the MixSIAR model 2.

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