

Importance of Common Wall Lizards in the Transmission Dynamics of Tick-Borne Pathogens in the Northern Apennine Mountains, Italy

Laura Tomassone¹  · L. A. Ceballos^{1,2} · C. Ragagli³ · E. Martello¹ · R. De Sousa⁴ · M. C. Stella¹ · A. Mannelli¹

Received: 20 February 2017 / Accepted: 5 May 2017
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Abstract During the investigations on ticks and tick-borne pathogens (TBP) range expansion in the Northern Apennines, we captured 107 *Podarcis muralis* lizards. Sixty-eight animals were infested by immature *Ixodes ricinus*, *Haemaphysalis sulcata* and *H. punctata*. *Borrelia burgdorferi* s.l. was detected in 3.7% of *I. ricinus* larvae and 8.0% of nymphs. Together with the species-specific *B. lusitaniae*, we identified *B. garinii*, *B. afzelii* and *B. valaisiana*. *Rickettsia* spp. (18.1% larvae, 12.0% nymphs), namely *R. monacensis*, *R. helvetica* and *R. hoogstraalii*, were also found in *I. ricinus*. *R. hoogstraalii* was detected in *H. sulcata* nymphs as well, while the two *H. punctata* did not harbour any bacteria. One out of 16 lizard tail tissues was positive to *R. helvetica*. Our results support the hypothesis that lizards are involved in the epidemiological cycles of TBP. The heterogeneity of *B. burgdorferi* genospecies mirrors previous findings in questing ticks in the area, and their finding in attached *I. ricinus* larvae suggests that lizards may contribute to the maintenance of different genospecies. The rickettsiae are

new findings in the study area, and *R. helvetica* infection in a tail tissue indicates a systemic infection. *R. hoogstraalii* is reported for the first time in *I. ricinus* ticks. Lizards seem to favour the bacterial exchange among different tick species, with possible public health consequences.

Keywords *Podarcis muralis* · Northern Apennines · Ixodid ticks · Zoonoses · *Borrelia burgdorferi* s.l. · SFG *Rickettsia*

Introduction

Like other small vertebrates, lizards are suitable hosts for the immature stages of different tick species across Europe and the Mediterranean basin, including *Ixodes ricinus*, the major vector of tick-borne diseases (TBD) in Europe [45] (Table 1).

Recently, studies have investigated the possible role of lizards as reservoir of TBD agents. The infection by *Borrelia burgdorferi* s.l. and *Rickettsia* spp. in tissues and attached ticks was shown in several lizard species (Table 2). Lizards are considered reservoir of *Borrelia lusitaniae* [42], and some authors also suggest that they may be reservoir of Spotted Fever Group (SFG) rickettsiae, *R. helvetica* and *R. monacensis* in particular [7, 21, 55]. Interestingly, multiple pathogens (*B. burgdorferi* s.l., SFG rickettsiae, *Anaplasma phagocytophilum*) have been shown to co-infect immature *I. ricinus* ticks feeding on lizards [14, 56].

In the Tuscan-Emilian Apennine National Park, Italy, lizards are among the small vertebrate species inhabiting dry and sunny rocky habitats. Our previous studies showed the existence of a complex vertebrate-tick-microbial community in the area. Indeed, *I. ricinus*, which recently colonized the territory, coexists with *I. trianguliceps*, *Dermacentor marginatus*, *Haemaphysalis sulcata* and *H. punctata* [30, 40]. A focus of transmission of *Rickettsia slovacica* and

Electronic supplementary material The online version of this article (doi:10.1007/s00248-017-0994-y) contains supplementary material, which is available to authorized users.

✉ Laura Tomassone
laura.tomassone@unito.it

¹ Dipartimento di Scienze Veterinarie, University of Turin, Largo Braccini 2, Grugliasco Turin, Italy

² Present address: Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Turin, Italy

³ UTCB Lucca- Comando Unità per la Tutela Forestale Ambientale e Agroalimentare Carabinieri, Lucca, Italy

⁴ Centro de estudos de Vetores e Doenças Infecciosas, Instituto Nacional de Saude Dr. Ricardo Jorge, Águas de Moura, Portugal

Table 1 Bibliographic reports of ixodid tick species feeding on lizards in Europe and Northern Africa

Tick species	Lizard species	Country	
<i>Ixodes ricinus</i>	<i>Lacerta agilis</i>	Germany [42], Netherlands [55], Hungary [15], Poland [11, 14, 18], Romania and Slovakia [27]	
	<i>Lacerta viridis</i>	Hungary [15], Slovakia [56]	
	<i>Lacerta bilineata</i>	Italy [48]	
	<i>Lacerta schreiberi</i>	Portugal [21, 35], Spain [21]	
	<i>Podarcis taurica</i>	Hungary [15]	
	<i>Podarcis muralis</i>	Germany [42], Italy [1; this study]	
	<i>Podarcis hispanica</i>	Portugal [35]	
	<i>Podarcis vaucheri</i>	Algeria [52]	
	<i>Timon lepidus</i>	Portugal [35]	
	<i>Teira dugesii</i>	Portugal [7]	
	<i>Psammodromus algirus</i>	Algeria [52], Portugal [35], Spain [29]; Tunisia [10]	
	<i>Timon pater</i>	Algeria [52]	
	<i>Dermacentor marginatus</i>	<i>Lacerta viridis</i>	Slovakia [56]
	<i>Haemaphysalis sulcata</i>	<i>Apathya cappadocica</i>	Turkey [20]
		<i>Lacerta media</i>	Turkey [20]
<i>Psammodromus algirus</i>		Spain [29]	
<i>Podarcis muralis</i>		This study	
<i>Haemaphysalis punctata</i>	<i>Psammodromus algirus</i>	Spain [29]	
	<i>Podarcis muralis</i>	This study	

R. raoultii is present [49], involving wild boars [50] and small rodents [31]. Moreover, *B. burgdorferi* s.l. infects questing *I. ricinus*, and tissues and ticks from small rodents [32, 40].

Due to the variety of tick species and TBD agents in the area, and that previous studies of our group in a close park had shown lizards' involvement in the maintenance of *B. lusitaniae* [1], we investigated if lizards play a role in the maintenance of ticks and transmitted pathogens. We present here the results of the evaluation of tick infestation and infection by *B. burgdorferi* s.l. and *Rickettsia* spp. in attached ticks and lizard tissues.

Materials and Methods

Study Area

The research was carried out on the Tuscan side of the Tuscan-Emilian Apennine National Park, Lucca province, Italy (44°

12' N, 10° 22' E) [40]. *Podarcis muralis* and *Lacerta viridis* (Laurenti 1768) are the two Lacertidae reported in the study area [2].

Lizards capture sites ($n = 12$) were located from 800 to 1600 meters above sea level (m a.s.l.) and were specifically chosen to be an optimal habitat for lizards, having a good sun exposure and abundant refuges. Sites were characterized by different vegetation typologies: open meadows with rocks and bushes; hiking trails with stone walls and tall grass; areas of exposed rocks and mixed deciduous woods dominated by hop hornbeam (*Ostrya carpinifolia*) and Turkey oaks (*Quercus cerris*); and, in the upper part of the study area, gravelly soil areas with scarce vegetation at the border of beech (*Fagus sylvatica*) woods (Online Resource 1).

Lizard and Tick Sampling

Lizards were captured by a noose affixed to a stick during six sampling sessions in spring and summer (April–August) from 2011 to 2013. Animals were identified by species, age class (adult, young) and sex, according to Vanni and Nistri [57]. Attached ticks were removed with forceps and stored in 70% ethanol, and were identified by species by using keys from Manilla [28]. In the case the lizard tail detached via tail fracture (a natural escape mechanism in lizards), it was stored in 70% ethanol. Afterwards, each lizard was released in its capture site. Animal capture and sampling protocols were approved by the Commission for Bioethics and Animal Welfare of the University of Turin.

Laboratory Analyses

DNA from ticks was extracted by using the DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany), while DNA extraction from tail tissues was carried out with MagCore HF16 Automated DNA/RNA purification System and MagCore genomic DNA tissue kit (RBC Bioscience, New Taipei City, Taiwan). Negative controls (distilled water) were added during the extraction to verify for possible cross-contaminations.

Tested ticks included all attached *I. ricinus* larvae and nymphs, all *H. punctata*, and a sample of *H. sulcata* nymphs, the size of which was determined in order to detect the presence/absence of *Rickettsia* spp. infection (considering a 95% confidence level and 20% expected prevalence).

All DNA tick and tissue samples were analysed for *B. burgdorferi* s.l. and *Rickettsia* spp. The infection by *B. burgdorferi* s.l. was studied by a PCR protocol targeting the intergenic spacer (IGS) region as previously described [44]. Detection of *Rickettsia* spp. in ticks was performed by targeting the citrate synthase (*gltA*) [22], *OmpA* [41] and *OmpB* genes [46]. *Rickettsia* spp. detection in lizard tissues was performed by a nested-PCR targeting the *OmpB* gene [4].

Table 2 Bibliographic reports of the infection by *B. burgdorferi* s.l. and *Rickettsia* spp. in lizard tissues and attached *I. ricinus* in Europe and Northern Africa

Lizard species	Pathogens infecting lizards tissues	Pathogens infecting attached <i>Ixodes ricinus</i>	Reference
<i>Lacerta agilis</i>	<i>B. lusitaniae</i>	<i>B. lusitaniae</i> , <i>B. burgdorferi</i> s.s., <i>B. burgdorferi</i> s.l.	[14]
	<i>B. lusitaniae</i>	Negative to <i>B. burgdorferi</i> s.l.	[15]
	N.I.	<i>B. afzelii</i> , <i>B. garinii</i> , <i>B. burgdorferi</i> s.s.	[18]
	N.I.	<i>B. lusitaniae</i> , <i>B. valaisiana</i>	[27]
	N.I.	<i>B. afzelii</i> , <i>B. burgdorferi</i> s.s., <i>R. helvetica</i>	[55]
<i>Lacerta schreiberi</i>	N.I.	<i>B. lusitaniae</i>	[42]
	Negative to <i>B. burgdorferi</i> s.l.	<i>R. monacensis</i> , <i>R. helvetica</i>	[21]
<i>Lacerta viridis</i>	<i>B. lusitaniae</i>	<i>B. lusitaniae</i> , <i>B. afzelii</i> , <i>B. burgdorferi</i> s.s.	[15]
<i>Podarcis muralis</i>	<i>B. lusitaniae</i>	<i>B. lusitaniae</i>	[1]
	N.I.	<i>B. lusitaniae</i> , <i>B. valaisiana</i>	[42]
<i>Podarcis hispanica</i>	<i>R. helvetica</i>	<i>B. lusitaniae</i> , <i>B. afzelii</i> , <i>B. valaisiana</i> , <i>B. garinii</i> , <i>R. monacensis</i> , <i>R. helvetica</i> ,	This study
	Negative to <i>B. burgdorferi</i> s.l.	<i>R. hoogstraalii</i>	
	Negative to <i>B. burgdorferi</i> s.l.	<i>B. lusitaniae</i>	[35]
<i>Podarcis taurica</i>	<i>B. lusitaniae</i>	<i>B. lusitaniae</i> , <i>B. afzelii</i> , <i>B. burgdorferi</i> s.s.	[15]
<i>Psammotromus algirus</i>	<i>B. lusitaniae</i>	<i>B. lusitaniae</i>	[10]
	<i>B. lusitaniae</i>	<i>B. lusitaniae</i>	[35]
<i>Teira dugesii</i>	<i>B. lusitaniae</i> , <i>R. helvetica</i> , <i>R. monacensis</i>	<i>B. lusitaniae</i> , <i>R. helvetica</i> , <i>R. monacensis</i>	[7]
<i>Timon lepidus</i>	Negative to <i>B. burgdorferi</i> s.l.	<i>B. lusitaniae</i>	[35]

N.I. not investigated

In all PCR reactions, 2.5 µl of DNA sample was tested. In each PCR run, distilled water was added as negative control; DNA from *B. afzelii* (Nancy strain) and *R. conorii* (Malish strain) were used as positive controls. The efficiency of the extraction protocol was verified in PCR-negative samples: for tick extracts, by a *16S* rDNA PCR [6]; and for tail tissue extracts, by a *cytB* gene PCR [23].

Positive amplicons were purified with the ExoSAP-IT PCR Clean-up Kit (GE Healthcare, Chalfont, UK) and sent to an external service (Macrogen, Amsterdam, The Netherlands) for automatic sequencing. Sequences were analysed and edited by using DNASTAR Lasergene software (Madison, WI, USA), and we used BLAST to identify similarities to known sequences (<http://blast.ncbi.nlm.nih.gov/blast.cgi>).

To confirm *B. burgdorferi* s.l. genospecies identification, we performed an in silico restriction fragment length polymorphism analysis and a 'virtual hybridization' [47].

Statistical Analysis

Prevalence and 95% exact binomial confidence intervals (CI) of infestation by immature *I. ricinus* and *H. sulcata* were calculated (BINOMIAL option, PROC FREQ, SAS Institute

1999). Prevalence of infestation by ticks, in young and adult lizards and between sexes, and between lizards and small rodents captured in the same area [31], was compared by Fisher exact test; a two-tailed significance level of $\alpha = 0.05$ was adopted. Mean numbers of ticks per host and 95%CI as well as negative binomial dispersion parameters (k) were obtained by intercept-only generalized linear models (GLM) with PROC GENMOD in the SAS system. Negative binomial error (log link) was used to take into account aggregated distribution of ticks among hosts [24]. The degree of coinfection by *I. ricinus* larvae and nymphs, and by *I. ricinus* and *H. sulcata*, on the lizards, was tested by the Kappa coefficient (AGREE option, FREQ procedure, SAS Institute 1999). McNemar's chi-square for non-independent observations was calculated to compare the probabilities of infestation by tick species and stages.

Prevalence of infection by *B. burgdorferi* s.l. and *Rickettsia* spp. was calculated by species/stage of attached tick and in lizard tissues. To take into account for correlation arising from collecting *I. ricinus* larvae from the same individuals, we used Generalized Estimating Equations (GEE) with repeated measures [9]; this was not applied to nymphs, since few specimens were tested.

Due to the low number of capture sites, it was not possible to compare tick infestation among vegetation typologies.

Results

Lizard Capture and Infestation by Ticks

We captured 107 *Podarcis muralis* lizards in nine study sites, located in the whole altitudinal range, and collected 16 tails following spontaneous caudal autotomy. Sixty-eight animals (63.6%; 95%CI 53.7, 72.6) were infested by ticks. Ticks were exclusively attached in the axillary region.

Adult lizards were significantly more infested than young animals ($p = 0.02$), while no differences were recorded between sexes ($p = 0.2$). The number of infested animals was significantly higher in April–May (78.3%) than in June (54.3%) and August (50.0%) ($p = 0.02$).

I. ricinus parasitized 45 lizards (145 larvae, 25 nymphs), while *H. sulcata* infested 37 lizards (119 larvae, 107 nymphs); *H. punctata* (2 larvae) were collected on two lizards.

I. ricinus larvae infested 34.6% lizards, with a mean number of 1.4 specimens per lizard, and showed an aggregated distribution (negative binomial parameter $k = 0.21$; Table 3). They were collected from May to August. Nymphs were collected from April and were absent in August; they parasitized 14.0% of lizards (Table 3). Coinfestations by *I. ricinus* larvae and nymphs occurred in seven animals, captured in May–June; the Kappa coefficient (0.087; 95%CI –0.8, 0.25) indicated no evidence of coinfection by the two tick stages beyond chance expectation. Prevalence of infection by larvae was significantly larger than nymphs' prevalence ($p < 0.001$). Infestation prevalence by *I. ricinus* larvae in lizards was significantly lower ($p < 0.001$) than the infestation prevalence of *Apodemus* spp. mice in the area (54.4%), while nymphs infestation was significantly higher ($p < 0.001$; 3.7% in mice) [31].

H. sulcata larvae were collected on 13.1% of the animals, in May and August only. Nymphs infested 24.3% of lizards (Table 3); they were present in all months, with a higher number of infested lizards in April–May. Only three lizards (two

captured in August, one in May) were simultaneously infested by both stages.

Coinfection by *I. ricinus* and *H. sulcata* occurred in 14 animals; there was no evidence of coinfection by the two species beyond chance expectation (Kappa coefficient –0.06; 95%CI –0.25, 0.12). The prevalence of infection by *I. ricinus* and *H. sulcata* on lizards was not significantly different ($p = 0.28$). Eleven of the coinfecting lizards were captured in the same study site, located at 800 m a.s.l. and characterized by mixed oak wood. In this site, *I. ricinus*, *H. sulcata* and *H. punctata* were simultaneously collected also by dragging in August 2013 (unpublished data).

The two *H. punctata* larvae were collected on two lizards, one was simultaneously infested by *H. sulcata* ($n = 13$ larvae), and the other by *H. sulcata* and *I. ricinus* (22 and 1 larvae, respectively).

Ten out of the 16 lizards, which tails detached, were infested by ticks; six animals by *H. sulcata* only, and four by both *I. ricinus* and *H. sulcata*.

Infection by TBD Agents in Ticks and Tissues from Lizards

B. burgdorferi s.l. was detected in 3.5% *I. ricinus* larvae and in 8% nymphs (Table 4). *B. lusitaniae* and *B. valaisiana* were infecting one nymph and one larva each; *B. garinii* and *B. afzelii* were detected in two larvae. It was not possible to identify the genospecies in one positive larva. The obtained sequences were 100% identical to those previously detected in questing ticks in the study area [40]. The seven positive ticks were collected from six lizards, since one lizard hosted one larva and one nymph, both positive to *B. lusitaniae*. They were captured in three study sites at 800–1145 m a.s.l.

I. ricinus were also infected by *Rickettsia* spp. (18.1% larvae and 24.3% nymphs), namely *R. monacensis*, *R. helvetica* and *R. hoogstraalii*. *R. hoogstraalii* was detected in *H. sulcata* nymphs as well (Table 4). *R. monacensis gltA* and *OmpA* sequences and *R. helvetica gltA* sequence were 100% similar to reference sequences deposited in GenBank (KU310588, LN794217). We could amplify DNA fragments of *R. hoogstraalii* encoding for *gltA* and *OmpB* genes, but not

Table 3 Infestation of *P. muralis* lizards by immature *I. ricinus* and *H. sulcata*, Tuscan-Emilian National Park, Italy, 2011–2013

Tick species	<i>Ixodes ricinus</i>		<i>Haemaphysalis sulcata</i>	
	Larvae	Nymphs	Larvae	Nymphs
No. infested hosts; % prevalence of infestation (95%CI)	37; 34.6 (25.6–44.4)	15; 14.0 (8.1–22.1)	14; 13.1 (7.3–21.0)	26; 24.3 (16.5–33.5)
Mean no. ticks/captured host (95%CI)	1.4 (0.87–2.1)	0.23 (0.13–0.42)	1.1 (0.4–2.8)	1.0 (0.6–1.7)
Mean no. ticks/infested host (95%CI)	3.9 (2.9–5.3)	1.7 (1.1–2.4)	8.5 (6.0–11.9)	4.1 (3.0–5.6)
k (95%CI)	0.21(0.13–0.33)	0.18 (0.07–0.51)	0.04 (0.02–0.08)	0.13 (0.07–0.22)

k negative binomial dispersion parameter

Table 4 Prevalence of *B. burgdorferi* s.l and SFG *Rickettsiae* in ticks feeding on *P. muralis* lizards in the Tuscan-Emilian National Park, Italy, 2011–2013

Tick species	<i>Ixodes ricinus</i>		<i>Haemaphysalis sulcata</i>
	Larvae (142)	Nymphs (25)	Nymphs (14)
% prevalence of <i>B. burgdorferi</i> s.l. (95%CI); genospecies (no. positive ticks)	3.7 (1.5–8.9); <i>B. lusitaniae</i> (1), <i>B. valaisiana</i> (1), <i>B. garinii</i> (1), <i>B. afzelii</i> (1), nd (1)	8.0 (1.0–26.0); <i>B. lusitaniae</i> (1), <i>B. valaisiana</i> (1)	0 (0.0–23.2)
% prevalence of <i>Rickettsia</i> spp. (95%CI); species (no. positive ticks)	18.1 (10.9–28.7); <i>R. monacensis</i> (11); <i>R. helvetica</i> (5); <i>R. hoogstraalii</i> (6), nd (3)	12.0 (2.5–31.2); <i>R. monacensis</i> (2); nd (1)	21.4 (4.7–50.8); <i>R. hoogstraalii</i> (3)

95%CI for *I. ricinus* larvae were calculated using GEE with repeated measures; exact binomial 95%CI are given for *I. ricinus* and *H. sulcata* nymphs

the *OmpA* gene, as reported by other authors [3, 36]. Our *gltA* sequences, from both *I. ricinus* and *H. sulcata* (GenBank Accession No. KY418024, KY418025), showed 100% similarity to the *Rickettsia* endosymbiont of *H. punctata* isolate Hae69 from Spain (EU303311) and 99% to the endosymbiont of *H. sulcata* from Croatia (DQ081187); these endosymbionts have been subsequently classified as *R. hoogstraalii* by Duh et al. [13]. The *OmpB* gene (GenBank Accession No. KY418026) had 99% similarity to *R. hoogstraalii* from soft ticks in the USA (EF629536).

Rickettsia spp.-positive ticks ($n = 31$) were collected from 17 lizards, that had from one to seven positive ticks attached. These animals were captured in five different sites, three of which were the same in which *B. burgdorferi* s.l.-positive ticks were detected; the two additional sites were at higher altitude (1270 and 1440 m a.s.l.).

Coinfection by *B. afzelii* and *R. monacensis* was observed in one *I. ricinus* larva.

The two *H. punctata* larvae did not harbour any bacteria.

We did not detect *B. burgdorferi* s.l. in tail tissues, while one of the tails was positive to *Rickettsia* spp. (6.25%; 95%CI 0.16–30.2). The *OmpB* sequence (GenBank Accession No. KY434315) was 99% similar to *R. helvetica* from questing *I. ricinus* in Germany (HQ232251). The positive tissue belonged to a lizard captured in an oak wood site at 1145 m a.s.l., which was infested by 6 *I. ricinus* (negative to PCR) and 9 *H. sulcata* (not tested by PCR) larvae at the moment it was captured.

Discussion

The detection of *B. burgdorferi* s.l. and SFG *Rickettsiae* in attached ticks, and of *R. helvetica* in a tail tissue, supports the hypothesis that lizards are involved in the transmission cycle of tick-borne pathogens in the Tuscan-Emilian Apennine National Park, where they serve as feeding hosts for *I. ricinus* and *H. sulcata* immatures mainly.

I. ricinus immatures also infest small rodents in our study area [31], but we observed that lizards are better hosts for nymphs and are significantly more infested, compared to mice. This finding confirms the results of a previous study in a close hilly area in Tuscany [1]. Contrarily to this older study, we registered an overall lower *I. ricinus* infestation prevalence in lizards, lower mean numbers of ticks per lizard, and we detected a higher *I. ricinus* aggregation. These differences may be due to the recent spread of *I. ricinus* in the Northern Apennines [40], with a consequent lower tick burden, and to the major environmental variability and harsher climatic conditions in this mountain area, which could lead to a more heterogeneous frequency of questing ticks.

Also, *H. sulcata* were abundant and aggregated on lizards. *H. sulcata* is a xerophilic tick species present in the Mediterranean basin, but it is abundant in the park area, where adults feed on mouflons (*Ovis orientalis musimon*) [40]. Although its immatures are recognized parasites of reptiles [58], scarce bibliographic findings on lizards are available (Table 1).

Rodents and lizards are reported as hosts for *H. punctata* immatures by Walker et al. [58], but we found just two attached specimens on lizards and none on small rodents [30, 31], although this tick species is widespread in the Northern Apennines [40]. We can thus hypothesise that they preferentially feed on birds, that are also reported as preferential hosts for *H. punctata* immatures [5], or other small mammals species in the study area. The two larvae we collected on lizards were not infected by TBD agents. However, previous studies showed *H. punctata* infection by *B. burgdorferi* s.l. [54]. It would thus be interesting to further investigate *H. punctata* infection by the pathogens that cause TBD cases in the Park area [49, 51]. Likewise, lizards do not appear to be attractive hosts for *D. marginatus* immatures, although we abundantly collected this tick species by dragging and on small rodents [30, 31, 40]. This may be due to its nidicolous habits, that make immatures preferentially live in small rodents nests; nevertheless, *D. marginatus* was reported to infest lizards by other authors [56].

B. burgdorferi infection prevalence in attached nymphs, and the heterogeneity of genospecies, mirrors previous findings in questing ticks in the area [40]. *B. lusitaniae* was detected in one *I. ricinus* nymph and one larva; however, other immatures were infected by *B. valaisiana*, *B. garinii* and *B. afzelii*. *B. afzelii* had been already reported in *I. ricinus* larvae feeding on lizards in Hungary [15] and Slovakia [27]. Since transovarial transmission of *B. burgdorferi* s.l. is unlikely [43], the finding of genospecies other than *B. lusitaniae* in attached larvae may be explained by the involvement of lizards in their maintenance (systemic infection), by a precedent interrupted blood-meal taken on an infected reservoir host, or by the cofeeding transmission among larvae and nymphs feeding in close proximity [17]. This last hypothesis is countered by the low coinfection by *I. ricinus* nymphs and larvae observed on our lizards; all these possible explanations deserve further investigations anyway.

No lizard tails were infected by *B. lusitaniae*, contrarily to what was observed in *P. muralis* tissues in a close study area [1]. However, we tested a small number of tissue samples.

Tick immatures were also infected by SFG rickettsiae. We identified *R. helvetica*, *R. monacensis* and *R. hoogstraalii*, which are added to *R. slovacica* and *R. raoultii*, the two other species that have a natural focus of transmission in our study area, associated to *D. marginatus* [30]. We detected *R. helvetica* in few attached *I. ricinus* larvae, as previously reported in studies on lizards in mountain areas of the Iberian Peninsula [21] and Slovakia [56], Madeira island [7] and the Netherlands [55]. The fact that *R. helvetica* was also identified in a tail tissue and that we observed ticks exclusively feeding in the axillary region indicates a disseminated infection. This is in agreement with the hypothesis that lizards may act as amplifiers of this rickettsia, which is considered a potential pathogen for humans [53].

De Sousa et al. [7] hypothesise that lizards may also be reservoirs of *R. monacensis*, the agent of spotted fever rickettsioses [38]. As reported in previous studies in Spain and Portugal, *R. monacensis* was the dominant rickettsia species in lizard ticks and infected attached *I. ricinus* larvae and nymphs [7, 21].

Surprisingly, we detected a third rickettsial species in *I. ricinus* larvae, *R. hoogstraalii*, that we also identified in attached *H. sulcata*. This rickettsia is documented for the first time in Italy. *R. hoogstraalii* has been originally detected in *H. sulcata* from sheep and goats in Croatia, and it is closely related to *Rickettsia felis* [12]. Duh et al. [13] showed that it causes a cytopathic effect in Vero cells and different arthropod cell lines, but its pathogenicity in vertebrate hosts is unknown. Other reports from Europe refer to infection in *Haemaphysalis* spp. ticks: *H. punctata* and *H. sulcata* in Spain [29, 39], *H. punctata* in Cyprus [3] and *H. parva* in Turkey [20, 36]. In other continents, *R. hoogstraalii* was associated to soft ticks [8, 19, 33, 37]. Our finding of this organism not only in

H. sulcata but also in *I. ricinus* could suggest a spillover of the rickettsia into *I. ricinus*, determined either by the intake of rickettsemic bloodmeals from lizards or by the cofeeding of the two tick species [59]. This same hypothesis was made by Marquez [29] in Spain, who observed *R. hoogstraalii* in both *H. sulcata* and *H. punctata* sharing their feeding hosts, *P. algirus* lizards in particular.

Such bacterial exchange could have consequences on ticks as vectors of diseases, due to the varying interactions that bacteria can have in the tick microbiome [16]. Vaclav et al. [56] studied the coinfection by *Anaplasma* spp., *Rickettsia* spp. and *B. lusitaniae* in *I. ricinus* attached on green lizards in Central Europe and concluded that the risk of tick infection with one pathogen may be dependent of the other pathogens. In particular, the authors showed positive interactions between *Rickettsia* spp. and *B. lusitaniae* that could have important public health consequences, since the simultaneous transmission of multiple pathogens was shown to alter host susceptibility and immune response, and increase the severity of clinical signs [25]. On the other hand, infections by rickettsial endosymbionts may preclude secondary infections with pathogenic rickettsiae [16, 26, 34, 59]. Further studies are needed to evaluate the possible pathogenicity of *R. hoogstraalii* to mammals; however, its infection in *I. ricinus* could have public health consequences, either favouring or precluding the infection with other agents of TBD.

In conclusion, our investigation showed the implication of another vertebrate host (lizard) in the maintenance of ticks and tick-borne bacteria in the study area, and the presence of rickettsial agents that had not been discovered in previous studies. This underlines, once again, the high complexity of tick-borne diseases systems. To tackle such complexity and control the emergence of TBD, we need to unravel the interactions in bacterial–vector–vertebrate communities both from an ecological and a metagenomic point of view.

Acknowledgments The UTCB Lucca (ex *Corpo Forestale dello Stato, Ufficio Territoriale per la Biodiversità*) provided accommodation, animal handling facilities and valuable help; a special thanks to Cecilia Ambrogi and to Maurizio Pieroni, who helped with fieldwork. Eliana C. Guillemi, INTA, Argentina, helped with labwork.

The study was funded by the University of Torino. The work of L. Tomassone and R. da Sousa was done under the frame of EurNegVec COST Action TD1303.

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflicts of interest.

References

- Amore G, Tomassone L, Grego E, Ragagli C, Bertolotti L, Nebbia P, et al (2007) *Borrelia lusitaniae* in immature *Ixodes ricinus* (Acari: Ixodidae) feeding on common wall lizards in Tuscany, central Italy. *J Med Entomol* 44:303–307

2. Bonani S, Bruni A, Cappelli F, Dondini G, Olivari S, Perilli E, Vergari S (2002) Faggete dell'Appennino settentrionale: habitat e vertebrati. Quaderni conservazione habitat, Ed. Arcari, Mantova
3. Chochlaklis D, Ioannou I, Sandalakis V, Dimitriou T, Kassinis N, Papadopoulos B, et al (2012) Spotted fever group Rickettsiae in ticks in Cyprus. *Microb Ecol* 63:314–323. doi:10.1007/s00248-011-9926-4
4. Choi YJ, Lee SH, Park KH, Koh YS, Lee KH, Baik HS, et al (2005) Evaluation of PCR-based assay for diagnosis of spotted fever group rickettsiosis in human serum samples. *Clin Diagn Lab Immunol* 12: 759–763
5. Cringoli G, Iori A, Rinaldi L, Veneziano V, Genchi C (2005) Zecche. Rolando Editore, Napoli
6. D'Oliveira C, van der Weide M, Jacquet P, Jongejan F (1997) Detection of *Theileria annulata* by the PCR in ticks (Acari: Ixodidae) collected from cattle in Mauritania. *Exp Appl Acarol* 21:279–291
7. De Sousa R, Lopes de Carvalho I, Santos AS, Bernardes C, Milhano N, Jesus J, et al (2012) Role of the lizard *Teira dugesii* as a potential host for *Ixodes ricinus* tick-borne pathogens. *Appl Environ Microbiol* 78:3767–3769. doi: 10.1128/AEM.07945-11
8. Dietrich M, Lebarbenchon C, Jaeger A, Le Rouzic C, Bastien M, Lagadec E, et al (2014) *Rickettsia* spp. in seabird ticks from western Indian Ocean islands, 2011–2012. *Emerg Infect Dis* 20:838–842. doi:10.3201/eid2005.131088
9. Diggle P, Heagerty P, Liang KY, Zeger S (2002) Analysis of longitudinal data. Oxford University Press, New York
10. Dsouli N, Younsi-Kabachii H, Postic D, Nouira S, Gern L, Bouattour A (2006) Reservoir role of lizard *Psammotromus algirus* in transmission cycle of *Borrelia burgdorferi* sensu lato (Spirochaetaceae) in Tunisia. *J Med Entomol* 43:737–742
11. Dudek K, Skórka P, Sajkowska ZA, Ekner-Grzyb A, Dudek M, Tryjanowski P (2016) Distribution pattern and number of ticks on lizards. *Ticks Tick Borne Dis* 7:172–179. doi:10.1016/j.ttbdis.2015.10.014
12. Duh D, Punda-Polić V, Trilar T, Petrovec M, Bradarić N, Avsic-Zupanc T (2006) Molecular identification of *Rickettsia felis*-like bacteria in *Haemaphysalis sulcata* ticks collected from domestic animals in southern Croatia. *Ann N Y Acad Sci* 1078:347–351
13. Duh D, Punda-Polic V, Avsic-Zupanc T, Bouyer D, Walker DH, Popov VL, et al (2010) *Rickettsia hoogstraalii* sp. nov., isolated from hard- and soft-bodied ticks. *Int J Syst Evol Microbiol* 60: 977–984. doi: 10.1099/ijs.0.011049-0
14. Ekner A, Dudek K, Sajkowska Z, Majláthová V, Majláth I, Tryjanowski P (2011) *Anaplasmataceae* and *Borrelia burgdorferi* sensu lato in the sand lizard *Lacerta agilis* and co-infection of these bacteria in hosted *Ixodes ricinus* ticks. *Parasit Vectors* 4:182. doi: 10.1186/1756-3305-4-182
15. Földvári G, Rigó K, Majláthová V, Majláth I, Farkas R, Pet'ko B (2009) Detection of *Borrelia burgdorferi* sensu lato in lizards and their ticks from Hungary. *Vector Borne Zoonotic Dis* 9:331–336. doi:10.1089/vbz.2009.0021
16. Gall CA, Reif KE, Scoles GA, Mason KL, Mousel M, Noh SM, Brayton KA (2016) The bacterial microbiome of *Dermacentor andersoni* ticks influences pathogen susceptibility. *ISME J* 10: 1846–1855. doi:10.1038/ismej.2015.266
17. Gern L, Rais O (1996) Efficient transmission of *Borrelia burgdorferi* between cofeeding *Ixodes ricinus* ticks (Acari: Ixodidae). *J Med Entomol* 33:189–192
18. Gryczyńska-Sięmiątkowska A, Siedlecka A, Stańczyk J, Barkowska M (2007) Infestation of sand lizards (*Lacerta agilis*) resident in the northeastern Poland by *Ixodes ricinus* (L.) ticks and their infection with *Borrelia burgdorferi* sensu lato. *Acta Parasit* 52:165. doi:10.2478/s11686-007-0015-2
19. Kawabata H, Ando S, Kishimoto T, Kurane I, Takano A, Nogami S, et al (2006) First detection of *Rickettsia* in soft-bodied ticks associated with seabirds, Japan. *Microbiol Immunol* 50:403–406
20. Keskin A, Bursali A, Keskin A, Tekin S (2016) Molecular detection of spotted fever group rickettsiae in ticks removed from humans in Turkey. *Ticks Tick Borne Dis* 7:951–953. doi:10.1016/j.ttbdis.2016.04.015
21. Kubelová M, Papoušek I, Bělohávek T, de Bellocq JG, Baird SJ, Šíroky P (2015) Spotted fever group rickettsiae detected in immature stages of ticks parasitizing on Iberian endemic lizard *Lacerta schreiberi* Bedriaga, 1878. *Ticks Tick Borne Dis* 6:711–714. doi: 10.1016/j.ttbdis.2015.06.003
22. Labruna MB, Whitworth T, Horta MC, Bouyer DH, JW MB, Pinter A, et al (2004) Rickettsia species infecting *Amblyomma cooperi* ticks from an area in the state of São Paulo, Brazil, where Brazilian spotted fever is endemic. *J Clin Microbiol* 42:90–98
23. Lee JH, Hassan H, Hill G, Cupp EW, Higazi TB, Mitchell CJ, et al (2002) Identification of mosquito avian-derived bloodmeals by polymerase chain reaction-heteroduplex analysis. *Am J Trop Med Hyg* 66:599–604
24. Littell RC, Milliken GA, Stroup WW, Wolfinger RD, Schabenberger O (2006) SAS for mixed models, 2nd ed. SAS@Institute, Cary
25. Lommano E, Bertaiola L, Dupasquier C, Gern L (2012) Infections and coinfections of questing *Ixodes ricinus* ticks by emerging zoonotic pathogens in western Switzerland. *Appl Environ Microbiol* 78:4606–4612. doi:10.1128/AEM.07961-11
26. Macaluso KR, Sonenshine DE, Ceraul SM, Azad AF (2002) Rickettsial infection in *Dermacentor variabilis* (Acari: Ixodidae) inhibits transovarial transmission of a second *Rickettsia*. *J Med Entomol* 39:809–813
27. Majláthová V, Majláth I, Hromada M, Tryjanowski P, Bona M, Antczak M, et al (2008) The role of the sand lizard (*Lacerta agilis*) in the transmission cycle of *Borrelia burgdorferi* sensu lato. *Int J Med Microbiol* 298:161–167. doi:10.1016/j.ijmm.2008.03.005
28. Manilla G (1998) Acari, Ixodida (Fauna d'Italia 36). Edizioni Calderoni, Bologna
29. Márquez FJ (2008) Spotted fever group *Rickettsia* in ticks from southeastern Spain natural parks. *Exp Appl Acarol* 45:185–194. doi:10.1007/s10493-008-9181-7
30. Martello E, Selmi M, Ragagli C, Ambrogi C, Stella MC, Mannelli A, Tomassone L (2013) *Rickettsia slovacica* in immature *Dermacentor marginatus* and tissues from *Apodemus* spp. in the northern Apennines, Italy. *Ticks Tick Borne Dis* 4:518–521. doi:10.1016/j.ttbdis.2013.07.002
31. Martello E, Mannelli A, Ragagli C, Ambrogi C, Selmi M, Ceballos LA, Tomassone L (2014a) Range expansion of *Ixodes ricinus* to higher altitude, and co-infestation of small rodents with *Dermacentor marginatus* in the northern Apennines, Italy. *Ticks Tick Borne Dis* 5:970–974. doi:10.1016/j.ttbdis.2014.07.021
32. Martello E, Mannelli A, Ragagli C, Selmi M, Ambrogi C, Grego E, et al (2014b) Use of small rodents for the surveillance of agents and vectors of tick-borne zoonoses in the northern Apennines, Italy. Proceedings of the 1st conference on neglected vectors and vector-borne diseases (EurNegVec). *Parasit Vectors* 7(Suppl 1):O36
33. Mattila JT, Burkhardt NY, Hutcheson HJ, Munderloh UG, Kurtti TJ (2007) Isolation of cell lines and a rickettsial endosymbiont from the soft tick *Carios capensis* (Acari: Argasidae: Ornithodorinae). *J Med Entomol* 44:1091–1101
34. Niebyski ML, Peacock MG, Fischer ER, Porcella SF, Schwan TG (1997) Characterization of an endosymbiont infecting wood ticks, *Dermacentor andersoni*, as a member of the genus *Francisella*. *Appl Environ Microbiol* 63:3933–3940
35. Norte AC, Alves da Silva A, Alves J, da Silva LP, Nuncio MS, Escudero R, et al (2015) The importance of lizards and small

- mammals as reservoirs for *Borrelia lusitaniae* in Portugal. Environ Microbiol Rep 7:188–193. doi: [10.1111/1758-2229.12218](https://doi.org/10.1111/1758-2229.12218)
36. Orkun Ö, Karaer Z, Çakmak A, Nalbantoğlu S (2014) Spotted fever group rickettsiae in ticks in Turkey. Ticks Tick Borne Dis 5:213–218. doi: [10.1016/j.ttbdis.2012.11.018](https://doi.org/10.1016/j.ttbdis.2012.11.018)
 37. Pader V, Nikitorowicz Buniak J, Abdissa A, Adamu H, Tolosa T, Gashaw A, et al (2012) Candidatus *Rickettsia hoogstraalii* in Ethiopian *Argas persicus* ticks. Ticks Tick Borne Dis 3:338–345. doi: [10.1016/j.ttbdis.2012.10.021](https://doi.org/10.1016/j.ttbdis.2012.10.021)
 38. Parola P, Paddock CD, Socolovschi C, Labruna MB, Mediannikov O, Kernif T, et al (2013) Update on tick-borne rickettsioses around the world: a geographic approach. Clin Microbiol Rev 26:657–702. doi: [10.1128/CMR.00032-13](https://doi.org/10.1128/CMR.00032-13)
 39. Portillo A, Santibáñez P, Santibáñez S, Pérez-Martínez L, Oteo JA (2008) Detection of *Rickettsia* spp. in *Haemaphysalis* ticks collected in La Rioja, Spain. Vector Borne Zoonotic Dis 8:653–658. doi: [10.1089/vbz.2007.0272](https://doi.org/10.1089/vbz.2007.0272)
 40. Ragagli C, Mannelli A, Ambrogi C, Bisanzio D, Ceballos LA, Grego E, et al (2016) Presence of host-seeking *Ixodes ricinus* and their infection with *Borrelia burgdorferi* sensu lato in the northern Apennines, Italy. Exp Appl Acarol 69:167–178. doi: [10.1007/s10493-016-0030-9](https://doi.org/10.1007/s10493-016-0030-9)
 41. Regnery RL, Spruill CL, Plikaytis BD (1991) Genotypic identification of rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. J Bacteriol 173:1576–1589
 42. Richter D, Matuschka FR (2006) Perpetuation of the Lyme disease spirochete *Borrelia lusitaniae* by lizards. Appl Environ Microbiol 72:4627–4662
 43. Richter D, Debski A, Hubalek Z, Matuschka FR (2012) Absence of Lyme disease spirochetes in larval *Ixodes ricinus* ticks. Vector Borne Zoonotic Dis 12:21–27. doi: [10.1089/vbz.2011.0668](https://doi.org/10.1089/vbz.2011.0668)
 44. Rijpkema SG, Molkenboer MJ, Schouls LM, Jongejan F, Schellekens JF (1995) Simultaneous detection and genotyping of three genomic groups of *Borrelia burgdorferi* sensu lato in Dutch *Ixodes ricinus* ticks by characterization of the amplified intergenic spacer region between 5S and 23S rRNA genes. J Clin Microbiol 33:3091–3095
 45. Rizzoli A, Silaghi C, Obiegala A, Rudolf I, Hubálek Z, Földvári G, Plantard O, Vayssier-Taussat M, Bonnet S, Spitalská E, Kazimirová M (2014) *Ixodes ricinus* and its transmitted pathogens in urban and peri-urban areas in Europe: new hazards and relevance for public health. Front Public Health 2:251. doi: [10.3389/fpubh.2014.00251](https://doi.org/10.3389/fpubh.2014.00251)
 46. Roux V, Raoult D (2000) Phylogenetic analysis of members of the genus *Rickettsia* using the gene encoding the outer membrane protein rOmpB (*ompB*). Int J Syst Evol Microbiol 50:1449–1455
 47. Rudenko N, Golovchenko M, Grubhoffer L, Oliver Jr JH (2009) *Borrelia carolinensis* sp. nov., a new (14th) member of the *Borrelia burgdorferi* sensu lato complex from the southeastern region of the United States. J Clin Microbiol 47:134–141. doi: [10.1128/JCM.01183-08](https://doi.org/10.1128/JCM.01183-08)
 48. Scali S, Manfredi MT, Guidali F (2001) *Lacerta bilineata* (Reptilia, Lacertidae) as a host of *Ixodes ricinus* (Acari, Ixodidae) in a protected area of northern Italy. Parasitologia 43:165–168
 49. Selmi M, Bertolotti L, Tomassone L, Mannelli A (2008) *Rickettsia slovaca* in *Dermacentor marginatus* and tick-borne lymphadenopathy, Tuscany, Italy. Emerg Infect Dis 14:817–820. doi: [10.3201/eid1405.070976](https://doi.org/10.3201/eid1405.070976)
 50. Selmi M, Martello E, Bertolotti L, Bisanzio D, Tomassone L (2009) *Rickettsia slovaca* and *Rickettsia raoultii* in *Dermacentor marginatus* ticks collected on wild boars in Tuscany, Italy. J Med Entomol 46:1490–1493
 51. Selmi M, Bertolotti L, Bisanzio D, Mattei R, Pagani A, Mazzatenta C, Mannelli A (2010) *Ixodes ricinus* as vector of human tick borne zoonoses in Tuscany. International Conference EDEN 2010, Emerging Vector-borne Diseases in a Changing European Environment, Montpellier, France: 6
 52. Soualah-Alila H, Bouslama Z, Amr Z, Bani Hani R (2015) Tick infestations (Acari: Ixodidae) on three lizard species from Seraidi (Annaba District), northeastern Algeria. Exp Appl Acarol 67:159–163. doi: [10.1007/s10493-015-9932-1](https://doi.org/10.1007/s10493-015-9932-1)
 53. Sprong H, Wielinga PR, Fonville M, Reusken C, Brandenburg AH, Borgsteede F, et al (2009) *Ixodes ricinus* ticks are reservoir hosts for *Rickettsia helvetica* and potentially carry flea-borne *Rickettsia* species. Parasit Vectors 2:41. doi: [10.1186/1756-3305-2-41](https://doi.org/10.1186/1756-3305-2-41)
 54. Tälleklint L (1996) Lyme borreliosis spirochetes in *Ixodes ricinus* and *Haemaphysalis punctata* ticks (Acari: Ixodidae) on three islands in the Baltic Sea. Exp Appl Acarol 20:467. doi: [10.1007/BF00053310](https://doi.org/10.1007/BF00053310)
 55. Tijssse-Klasen E, Fonville M, Reimerink JH, Spitzen-van der Sluijs A, Sprong H (2010) Role of sand lizards in the ecology of Lyme and other tick-borne diseases in the Netherlands. Parasit Vectors 3:42. doi: [10.1186/1756-3305-3-42](https://doi.org/10.1186/1756-3305-3-42)
 56. Václav R, Ficová M, Prokop P, Betáková T (2011) Associations between coinfection prevalence of *Borrelia lusitaniae*, *Anaplasma* sp., and *Rickettsia* sp. in hard ticks feeding on reptile hosts. Microb Ecol 61:245–253. doi: [10.1007/s00248-010-9736-0](https://doi.org/10.1007/s00248-010-9736-0)
 57. Vanni S, Nistri A (2006) Atlante degli Anfibi e dei Rettili della Toscana. Ed. Museo di Storia Naturale dell'Università degli Studi di Firenze Sezione di Zoologia “La Specola”, Regione Toscana Giunta Regionale Assessorato all'Ambiente, Firenze, Italy
 58. Walker AR, Bouattour A, Camicas JL, Estrada-Peña A, Horak IG, Latif AA, et al (2003) Ticks of domestic animals in Africa: a guide to identification of species. Bioscience Reports, Edinburgh ISBN: **0-9545173-0-X**
 59. Wright CL, Sonenshine DE, Gaff HD, Hynes WL (2015) *Rickettsia parkeri* transmission to *Amblyomma americanum* by cofeeding with *Amblyomma maculatum* (Acari: Ixodidae) and potential for spillover. J Med Entomol 52:1090–1095. doi: [10.1093/jme/tjv086](https://doi.org/10.1093/jme/tjv086)