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Importance of common wall lizards in the transmission dynamics of tick-borne pathogens in the Northern Apennine mountains, Italy

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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.

Key words. *Podarcis muralis*, Northern Apennines, Ixodid ticks, zoonoses, *Borrelia burgdorferi* s.l., SFG *Rickettsiae*

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 ies (Table 2). Lizards are considered reservoir of *Borrelia lusitaniae* [42], and some authors also suggest they may be reservoir of Spotted Fever Group (SFG) rickettsiae, *R. helvetica* and *R. monacensis* in particular [6, 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*, that recently colonised the territory, coexists with *I. trianguliceps*, *Dermacentor marginatus, Haemaphysalis sulcata* and *H. punctata* [30, 40]. A focus of transmission of *Rickettsia slovaca* and *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 to1600 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 analyzed 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].
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 [9]; 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 analyzed 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 $\alpha=0.05$ 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 PROCGENMOD 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 coinfestation 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. were 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). Coinfestation by *I. ricinus* larvae and nymphs occurred in 7 animals, captured in May-June; the Kappa coefficient (0.087; 95%CI: –0.8, 0.25) indicated no evidence of coinfestation 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 3 lizards (two captured in August, one in May) were simultaneously infested by both stages.

Coinfestation by *I. ricinus* and *H. sulcata* occurred in 14 animals; there was no evidence of coinfestation 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 coinfested 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 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, support 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 hypothesize 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 on 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 coinfestation 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. slovaca* 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 [6] 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*. [6] hypothesize 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 [6, 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 [7, 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.

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Conflict of interest

The authors declare no conflicts of interest.

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

Tick species	Lizard species	Country
Ixodes ricinus	*Lacerta agilis*	Germany [42], Netherlands [55], Hungary [15], Poland [11, 14, 18], Romania and Slovakia [27]
	Lacerta viridis	Hungary [15], Slovakia [56]
	Lacerta bilineata	Italy [48]
	Lacerta schreiberi	Portugal [21, 35], Spain [21]
	Podarcis taurica	Hungary [15]
	Podarcis muralis	Germany [42], Italy [1; this study]
	Podarcis hispanica	Portugal [35]
	Podarcis vaucheri	Algeria [52]
	Timon lepidus	Portugal [35]
	Teira dugesii	Portugal [6]
	Psammodromus algirus	Algeria [52], Portugal [35], Spain [29]; Tunisia [10]
	Timon pater	Algeria [52]
Dermacentor marginatus	*Lacerta viridis*	Slovakia [56]
Haemaphysalis sulcata	*Apathya cappadocica*	Turkey [20]
	Lacerta media	Turkey [20]
	Psammodromus algirus	Spain [29]
	Podarcis muralis	This study
Haemaphysalis punctata	*Psammodromus algirus*	Spain [29]
	Podarcis muralis	This study

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. N.I.= not investigated.

Lizard species	Pathogens infecting lizards tissues	Pathogens infecting attached *Ixodes ricinus*	Reference
Lacerta agilis	*B.lusitaniae*	*B.lusitaniae, B.burgdorferi s.s., B.burgdorferi s.l.*	[14]
	B.lusitaniae	Negative to *B.burgdorferi s.l.*	[15]
	N.I.	*B.afzelii, B.garinii, B.burgdorferi s.s.*	[18]
	N.I.	*B.lusitaniae, B.valaisiana*	[27]
	N.I.	*B.afzelii, B.burgdorferi s.s., R.helvetica*	[55]
	N.I.	*B.lusitaniae*	[42]
Lacerta schreiberi	N.I.	*R.monacensis, R.helvetica*	[21]
	Negative to *B.burgdorferi s.l.*	*B.lusitaniae*	[35]
Lacerta viridis	*B.lusitaniae*	*B.lusitaniae, B.afzelii, B.burgdorferi s.s.*	[15]
Podarcis muralis	*B.lusitaniae*	*B.lusitaniae*	[1]
	N.I.	*B.lusitaniae, B.valaisiana*	[42]
	R.helvetica Negative to *B.burgdorferi s.l.*	*B lusitaniae, B.afzelii, B.valaisiana, B.garinii, R.monacensis, R.helvetica, R.hoogstraalii*	This study
Podarcis hispanica	Negative to *B.burgdorferi s.l.*	*B.lusitaniae*	[35]
Podarcis taurica	*B.lusitaniae*	*B.lusitaniae, B.afzelii, B.burgdorferi s.s.*	[15]
Psammodromus algirus	*B.lusitaniae*	*B.lusitaniae*	[10]
	B.lusitaniae	*B.lusitaniae*	[35]
Teira dugesii	*B.lusitaniae, R.helvetica, R.monacensis*	*B.lusitaniae, R.helvetica, R.monacensis*	[6]
Timon lepidus	Negative to *B.burgdorferi s.l.*	*B.lusitaniae*	[35]

Table 3. Infestation of *P. muralis* lizards by immature *I. ricinus* and *H. sulcata*, Tuscan-Emilian National Park, Italy, 2011 - 2013. k=negative binomial dispersion parameter.

Tick species	*Ixodes ricinus*		*Haemaphysalis sulcata*	
Ticks stage	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)

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. 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.

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

Online Resource 1. Examples of lizard capture sites in the Tuscan-Emilian Apennine National Park, Italy: (a) open meadows with rocks and bushes, (b) hiking trails with rocks and tall grass, (c) mixed deciduous woods with exposed rocks, (d) gravelly soil areas, at the altitudinal limit of the tree vegetation.