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HOST MICROBE INTERACTIONS

Associations Between Coinfection Prevalence of *Borrelia lusitaniae*, *Anaplasma* sp., and *Rickettsia* sp. in Hard Ticks Feeding on Reptile Hosts

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Abstract An increasing number of studies reveal that ticks and their hosts are infected with multiple pathogens, suggesting that coinfection might be frequent for both vectors and wild reservoir hosts. Whereas the examination of associations between coinfecting pathogen agents in natural host–vector–pathogen systems is a prerequisite for a better understanding of disease maintenance and transmission, the associations between pathogens within vectors or hosts are seldom explicitly examined. We examined the prevalence of pathogen agents and the patterns of associations between them under natural conditions, using a previously unexamined host–vector–pathogen system—green lizards *Lacerta viridis*, hard ticks *Ixodes ricinus*, and *Borrelia*, *Anaplasma*, and *Rickettsia* pathogens. We found that immature ticks infesting a temperate lizard species in Central Europe were infected with multiple pathogens. Considering *I. ricinus* nymphs and larvae, the prevalence of *Anaplasma*, *Borrelia*, and *Rickettsia* was 13.1% and 8.7%, 12.8% and 1.3%, and 4.5% and 2.7%, respectively. The patterns of pathogen prevalence and observed coinfection rates suggest that the risk of tick infection with one pathogen is not independent of other pathogens. Our results indicate that *Anaplasma* can play a role in suppressing the transmission of *Borrelia* to tick vectors. Overall, however, positive effects of *Borrelia* on *Anaplasma* seem to prevail as judged by higher-than-expected *Borrelia–Anaplasma* coinfection rates.

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Introduction

An increasing number of studies report that ticks and their vertebrate hosts are infected with a mixture of pathogens, suggesting that coinfection might be the rule rather than the exception for both vectors and wild reservoir hosts [5, 14, 45]. Because direct or indirect relationships within a community of pathogens inevitably affect their fitness, coinfection is expected to result in synergic or antagonistic effects between the members of a pathogen community [26]. Indeed, some pathogens can evade the host's immune system more easily if they are transmitted by vectors simultaneously with other infections [52]. Hosts coinfected with different pathogens may show more severe symptoms of diseases [22], and host coinfection status can modulate the transmission of pathogens to vectors [47].

Ginsberg [14] has shown that if tick populations are large and individual hosts are in their life infested by multiple ticks, the probability of host exposure to a pathogen is close to 100% even if the proportion of infected vectors is small. While the probability of infection could further increase with vector coinfection, this might not be the case if there are negative interactions between coinfecting pathogens [47]. The order of infection also can affect the risk of infection because some pathogens can be more immunosuppressive than others and thus modulate the probability of consecutive infections [19]. Therefore, it is important whether coinfection occurs simultaneously (true coinfection) or sequentially because the two scenarios can

have different clinical and epidemiological outcomes [5, 26]. Whereas the examination of associations between coinfecting pathogens in natural host–vector–pathogen systems is a prerequisite for better insight into disease ecology, only a few studies have explicitly examined the associations between pathogens in wild vector or host populations [11, 32].

Reptiles represent an excellent group to study the epidemiology of various pathogens because of their long life span, site faithfulness, seasonal nature of immunity, and distinct modes of reproduction [43] and immune responses [23]. Moreover, lizards, including green lizards *Lacerta viridis*, are locally important hosts for the early life stages of hard ticks [7, 49]. Though lizards have been long considered as non-competent hosts for *Borrelia* spirochetes, recent experimental and empiric work strongly suggests that lizards can be competent reservoirs of a potentially pathogenic *Borrelia* genospecies, *Borrelia lusitaniae* [2, 9, 12, 29, 42]. In contrast to *Borrelia* spirochetes, little is known about the prevalence and ecology of other tick-borne pathogens associated with reptile hosts. We examined the patterns of associations between three different pathogen agents under natural conditions, using a previously unexamined host–vector–pathogen system. Specifically, we used the green lizard *L. viridis*, which serves as a vertebrate host for hard ticks, the hematophagous ectoparasites—known vectors of several important pathogens, as well as the three selected pathogens: *Borrelia burgdorferi* sensu lato, *Anaplasma* sp., and *Rickettsia* sp.

Materials and Methods

Study System and Area

The European green lizard is a 35–40-cm (total length) long lizard, inhabiting warm and dry areas of central Europe and the Balkans. The breeding season of green lizards begins after hibernation in April and lasts until early June when females lay about seven to 20 eggs. The lizard is one of the largest European lacertids and has a relatively long lifespan (>9 years). *Ixodes ricinus* (Acari: Ixodidae) is an opportunistic hematophagous ectoparasite species of hard ticks, commonly infesting reptiles [4]. *Borrelia* spirochetes are bacteria transmitted by ticks and causing inflammatory disorders [36]. The members of *Anaplasmataceae* are tick-borne obligatory intracellular bacterial parasites attacking neutrophils [13]. Similar to *Anaplasma*, *Rickettsiae* are obligatory intracellular bacterial parasites, but they are transmitted by different types of hematophagous arthropods (ticks, mites, lice, and fleas), which can, due to transovarian transmission of bacteria, serve as both vectors and hosts [31]. The study site (48°57′ N, 20°44′ E, ~200 to 400 m above sea level) in the Slovak Karst National Park was ~10 ha large, characterized by warm temperate climate, open habitat and broad-leaved scrub vegetation (*Quercus petraea*, *Carpinus betulus*, *Cornus mas*, *Acer campestre*, *Crataegus monogyna*, and *Prunus mahaleb*).

Sample Collection

In 2007, we captured free-living green lizards by slipnoosing between 6 April and 11 May. During four 3–4-day-long trips, we trapped a total of 89 individuals. Each captured lizard was sexed according to throat coloration, head shape, the size of femoral pores, the shape of the ventral side of tail base (male tail base has two bulges because it contains the paired hemipenes), and bite marks on the head and belly (males bite the belly of females during copulation; head bite marks usually come from territorial disputes between males). All lizards were marked with a marker pen in the field and stored in individual containers. Later in the same day, we removed with forceps all the ticks feeding on lizards. In addition, we obtained a tissue sample by clipping the tip of one toe on the rear leg from each lizard. Both ticks and toe tips were stored in 93% ethanol. A drop of blood obtained following toe clipping was used for blood smears (not analyzed here). All lizards were released exactly at the point of capture the morning after their capture. The life stage and species of collected ticks was determined using microscope and standard identification keys [8].

DNA Extraction and Detection of *Borrelia*, *Ricketsia*, and *Anaplasma* by PCR

DNA extraction from samples was based on the method using an anion chelating resin (Chelex 100® Resin, Bio-Rad Laboratories, CA, USA, [38]). Briefly, tick was transferred into a 1.5-ml microcentrifuge tube containing 100 μl of pure water, 0.005 g Chelex 100 resin, and 6 μl proteinase K (10 μg/ml). The mixture was homogenized and vortexed 2 min at room temperature. The homogenized sample was then centrifuged at 13,000×*g* for 1 min and incubated at 96°C for 35 min. After vortexing and centrifugation at 13,000×*g* for 1 min, the sample was incubated at 96°C for 15 min. The mixture was then centrifuged at 13,000×*g* for 3 min, and the supernatant was used as a template in PCR essays.

2xPCR Master Mix (Fermentas) was used for all PCR reactions. Primers specific for all *Borrelia* species, together with PCR conditions, have been described previously by Kurtenbach et al. [24]. Rickettsial DNA was detected by PCR as described previously by using primers Rp877p and Rp1258r, which amplify a 396-bp fragment of the citrate

synthase gene (*gltA*) of *Rickettsia* [44]. The DNA extracted from the ticks was also screened with primers EHR16SR and EHR16SD, which amplify a 345-bp fragment of the 16S rRNA gene of bacteria within the family Anaplasmataceae, including the genera *Anaplasma*, *Ehrlichia*, *Neorickettsia*, and *Wolbachia* [37]. All specific PCR products were separated by electrophoresis in 2% agarose gels and visualized by staining with ethidium bromide. *Anaplasma* and *Rickettsia* PCR products were sequenced for further determination using a commercial sequencing service (BITCET).

Reverse Line Blot Hybridization

The biotinylated spacer fragment of the *Borrelia* PCR was hybridized with six *B. burgdorferi* genospecies-specific oligonucleotide probes: *B. burgdorferi* sensu lato, *B. burgdorferi* sensu stricto, *Borrelia valaisiana*, *Borrelia garinii*, *Borrelia afzelii*, and *B. lusitaniae*. Biotinylated *Rickettsia* PCR products were examined using the genospecies-specific oligonucleotide probes to *Rickettsia slovaca* and *Rickettsia helvetica* as described by Jado et al. [21].

The reverse line blotting primers and technique has been in detail described elsewhere [30, 50]. Briefly, solutions with 100 pmol of five different amino-linked oligonucleotide probes were coupled covalently to an activated Biodyne C membrane in a line pattern by using a miniblotter. After binding of the oligonucleotide probes, the membrane was taken from the miniblotter, washed in 2× SSPE (360 mM NaCl, 20 mM $Na_2HPO4 \times H_2O$, 2 mM EDTA) with 0.1% sodium dodecyl sulfate (SDS) at RT, and again placed in the miniblotter with the oligonucleotide lines perpendicular to the slots. Ten microliters of the biotin-labeled PCR product was diluted in 150 ml of 2× SSPE–0.1% SDS, denatured for 10 min at 99°C, and cooled rapidly on ice. The slots of the miniblotter were filled with the denatured PCR product, and hybridization was performed for 1 h at 45°C. The membrane was removed from the miniblotter and was washed twice for 10 min each time in 2× SSPE–0.1% SDS at 40°C. Subsequently, the membrane was incubated for 30 min at 40°C with streptavidin-horse radish peroxidase (Novagen) diluted 1:4,000 in 2× SSPE–0.5% SDS and was washed twice for 10 min in 2× SSPE–0.5% SDS. Hybridization was visualized by labeling with TMB Stabilized Substrate for Horseradish Peroxidase (Promega).

PCR and Restriction Fragment Length Polymorphism Analysis

All samples testing positive for *Borrelia* were further characterized by 5S–23S rRNA integric spacer restriction fragment length polymorphism (RFLP) analysis. Primers and conditions have been described elsewhere [20, 39]. PCR products were digested with *Mse*I and *Dra*I, according to manufacturer instructions (Fermentas, Merck). Digested DNA was electrophoresed through 16% polyacrylamide gels and stained with ethidium bromide; 100 bp DNA ladder (Promega) was used as a molecular size marker.

Data Analyses

Generalized linear mixed models (GLMM) were used to examine variation in the occurrence of pathogens, where lizard identity, and the interaction of lizard identity and the capture date were used as random factors. The residual random effect was included in models as an overdispersion parameter [28]. Parameter estimates and 95% confidence limits were calculated with SAS (SAS 2002 Institute, Cary, NC, USA) using the PROC GLIMMIX procedure. Binomial distribution and the logit link function were applied. The Satterthwaite method, which can give non-integer values, was used to calculate degrees of freedom [28]. The significance of fixed effects was based on the *F* statistics. The interaction terms involving relevant pathogen pairs (i.e., *Borrelia* × *Rickettsia*, *Borrelia* × *Anaplasma*, or *Anaplasma* × *Rickettsia*, respectively) were included in GLMM. Concerning the removal of interaction terms from models and the correct interpretation of main effects, we followed the recommendations of Brambor et al. [6]. Briefly, instead of showing the statistics for the main effects as average (unconditional), to assess the effect of constitutive terms in models including interactions, we show and interpret their effects as conditional [40]. The *G* test was used to test the differences between observed and expected coinfection rates. Expected coinfection rates were calculated as the product of the prevalence of each pathogen individually [32].

Results

PCR Detection of *Borrelia*, *Anaplasma*, and *Ricketsia* DNAs in Ticks and Lizards

Various PCRs were applied to DNA extracts from 799 *Ixodes ricinus* and 25 *Dermacentor marginatus* ticks collected from 89 green lizards. Adult ticks were not found on the lizards. The most abundant pathogen detected in the ticks was *Anaplasma* sp. (Table 1). The further sequencing of *Anaplasma* sp. PCR products revealed 93–96% homology with uncultured *Anaplasma* sp. clone HLA344 16S ribosomal RNA gene (GU075704).

The prevalence of *Borrelia* in *I. ricinus* was only slightly lower compared with *Anaplasma* sp. (Table 1). All

Table 1 Prevalence of *Anaplasma* sp., *Borrelia lusitaniae*, and *Rickettsia* sp. in immature *Ixodes ricinus* and *Dermacentor marginatus* ticks feeding on green lizards *Lacerta viridis*

Tick species	*Anaplasma* sp.		*Borrelia lusitaniae*		*Rickettsia* sp.		No. nymphs/ larvae
	No. (%) positive nymphs	No. (%) positive larvae	No. (%) positive nymphs	No. (%) positive larvae	No. (%) positive nymphs	No. (%) positive larvae	
Ixodes ricinus	85 (13.1%)	13 (8.7%)	83 (12.8%)	2 (1.3%)	29 (4.5%)	4 (2.7%)	649/150
Dermacentor marginatus	1 (7%)	1 (10%)	1 (7%)	0 (0%)	1 (7%)	0 (0%)	15/10

Adult ticks of neither species were found on green lizards

Borrelia-positive samples were subjected to further genotyping analyses.

DNA of *Rickettsia* was detected in 33 ticks. Sequence analysis of *Rickettsia* sp. PCR products identified 99–100% homology with *Rickettsia* sp. 362 23S ribosomal gene (DQ139797.1) and 93% homology with *Rickettsia massiliae* MTU5 (CP000683.1). One *Rickettsia* sp. PCR product was determined as *R. helvetica* by reverse line blot (RLB).

Seventeen nymphs contained both *Borrelia* and *Anaplasma*, ten nymphs contained *Borrelia* and *Rickettsia*, and four nymphs contained *Anaplasma* and *Rickettsia*. We have also observed triple infection with all three pathogens for four nymphs (Fig. 1).

DNA extracted from 25 *D. marginatus* ticks tested positive for *Anaplasma* sp., *B. burgdorferi* s.l., and *Rickettsia* sp. (Table 1).

As for the host, 47.19% (42/89), 51.69% (46/89), and 22.47% (20/89) of different green lizards carried at least one feeding tick testing positive for *B. lusitaniae*, *Anaplasma* sp., or *Rickettsia* sp., respectively. However, examining the toe tips of 89 lizards, we detected neither *B. burgdorferi* s.l., nor *Anaplasma* sp. and *Rickettsia* sp. in any lizard toe tissue sample.

Identification of *Borrelia* Using RLB and RFLP Analyses

RLB was designed to differentiate *Borrelia* genospecies. To assess the specificity of the assay, control samples were used in the RLB assay under stringent hybridization conditions. All our samples tested were positive for *B. burgdorferi* s.l. and *B. lusitaniae*. Cross-hybridization between different genospecies did not occur.

All samples were also analyzed by PCR-RFLP methods of rrf(5S)–rrl(23S) rRNA *B. burgdorferi* s.l. PCR amlicons. RFLP analysis of the amplified products yielded two different patterns. The most abundant was the pattern similar to that of *B. lusitaniae* PotiBI, pattern E [10]. Only three investigated samples showed a slightly different pattern with one extra restriction site. The similar pattern was described for the sample GT163, pattern F + E, determined also as *B. lusitaniae* [10]. Overall, all detected Borreliae belonged to the genospecies *B. lusitaniae*.

Tick-Vector Infection Patterns

The rates of *I. ricinus* single infection versus coinfection differed significantly between the three types of pathogens

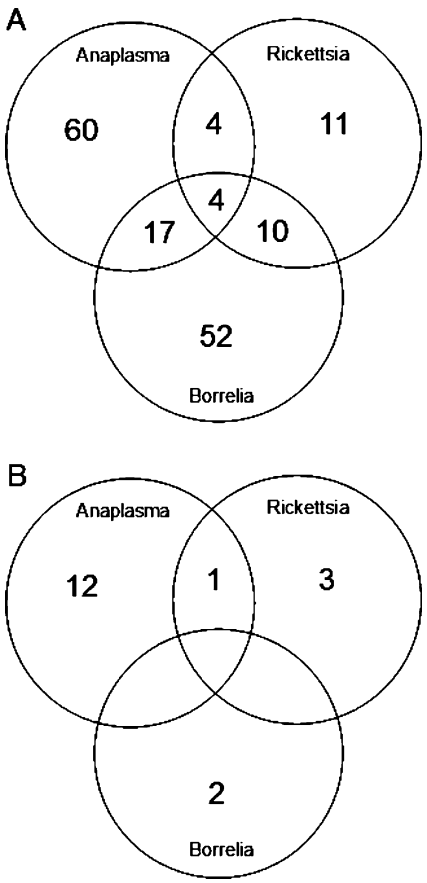

Figure 1 Schematic illustration of *I. ricinus* infection patterns. The values correspond to the number of *I. ricinus* ticks infected with different combinations of three pathogens: *B. lusitaniae*, *Anaplasma* sp., and *Rickettsia* sp. Shown are infection patterns in **a** 649 nymphs and **b** 150 larvae

(G_2=10.34, p<0.05). This was due to *Rickettsia* (Rc), which, compared with other pathogens (i.e., *B. lusitaniae* (Bl) and *Anaplasma* sp. (An)), occurred in ticks as coinfection more often than single infection (Fig. 1). In addition, the nymphal *I. ricinus* coinfection rates of (1) Bl with An, (2) Bl with An and Rc, and (3) Bl with Rc were significantly higher than expected based on single tick infection rates (Table 2).

Patterns of Within-Tick Infection in *I. ricinus*

The probability of Bl infection did not differ between dates and host sexes (GLMM, date: $F_{3,81.44}$=0.70, p>0.05; sex: $F_{1,132.9}$=0.56, p>0.05), but it was significantly higher for tick nymphs than larvae ($F_{1,747.7}$=14.62, p<0.05). The effect on the occurrence of Bl infection of An tick infection was conditional on Rc, but not the other way around. Namely, if ticks were infected with Rc, the likelihood of Bl occurrence was higher regardless of An coinfection status (An × Rc: $F_{1,785.5}$=1.21, p>0.05; effect of Rc occurrence sliced by An occurrence, An absent: $F_{1,735}$=30.87, p<0.05; An present: $F_{1,783.8}$=5.03, p<0.05; Fig. 2). In contrast, if ticks were infected with An, the likelihood of Bl tick infection was lower when An was infecting ticks alone compared with cases when An-infected ticks were coinfected with Rc (effect of An occurrence sliced by Rc occurrence, Rc absent: $F_{1,788.8}$=14.12, p<0.05; Rc present: $F_{1,782.3}$=0.06, p>0.05; Fig. 2).

Trapping date, host sex, and tick life stage did not significantly predict the occurrence of An tick infection (GLMM, date: $F_{3,107.6}$=1.75, p>0.05; host sex: $F_{1,92.82}$= 0.49, p>0.05; tick stage: $F_{1,735.8}$=0.99, p>0.05). The likelihood of An tick infection was significantly higher if ticks were infected with Bl, Rc, or both pathogens compared with cases when ticks were not infected with the two pathogens (Bl × Rc: $F_{1,767.4}$=1.54, p>0.05; effect of Bl occurrence sliced by Rc occurrence, Rc absent: $F_{1,714.6}$=13.37, p<0.05, Rc present: $F_{1,772.3}$=0.02, p>0.05; effect of Rc occurrence sliced by Bl occurrence, Bl absent: $F_{1,772.1}$=5.95, p<0.05, Rc present: $F_{1,755.5}$=0.18, p>0.05; Fig. 3).

The probability of Rc tick infection did not differ between host sexes, trapping date, and tick life stage (GLMM, date: $F_{3,134.8}$=2.09, p>0. 05; host sex: $F_{1,136.5}$=0.02, p>0.05; tick stage: $F_{1,774.9}$=0.26, p>0.05). Considering An infection status, the probability of Rc infection was analogous to that

Table 2 Observed and expected coinfection prevalence of *Anaplasma* sp. (An), *Borrelia lusitaniae* (Bl), and *Rickettsia* sp. (Rc) for *Ixodes ricinus* ticks feeding on green lizards *Lacerta viridis*

Coinfection type	Samples	Observed coinfected (%)	Expected coinfected (%)	G	p
An × Bl					
Larvae	150	0.00	0.05	0.00	1.000
Nymphs	649	2.62	0.74	6.15	0.008[a]
Bl × Rc					
Larvae	150	0.00	0.01	0.00	1.000
Nymphs	649	1.54	0.14	7.41	0.003[a]
An × Rc					
Larvae	150	0.67	0.16	1.39	0.238
Nymphs	649	0.62	0.15	1.93	0.164
Bl × An × Rc					
Larvae	150	0.00	0.00	0.00	1.000
Nymphs	649	0.62	0.01	5.56	0.018[a]

[a] Statistically significant at $p \leq 0.05$

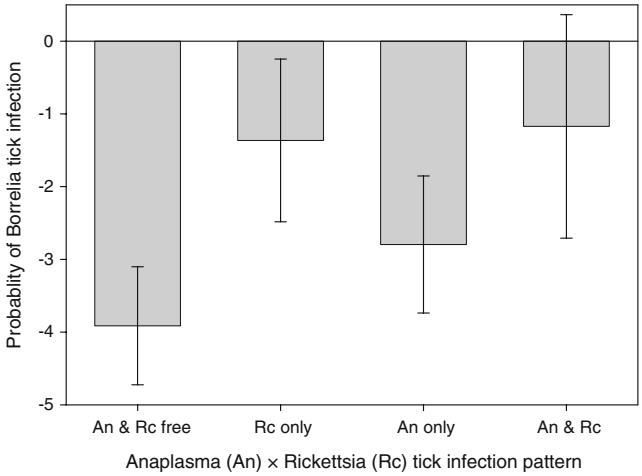

Figure 2 Probability of *B. lusitaniae* infection in *I. ricinus* as a function of *Anaplasma* and *Rickettsia* coinfection prevalence. Displayed are the estimates of least-squares means and 95% confidence limits obtained by GLMM

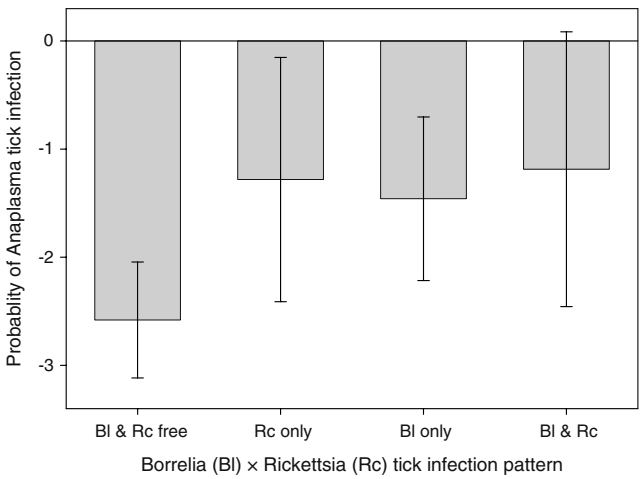

Figure 3 Probability of *Anaplasma* infection in *I. ricinus* as a function of *Borrelia* and *Rickettsia* coinfection prevalence. Displayed are the estimates of least-squares means and 95% confidence limits obtained by GLMM

of Bl infection. Particularly, if ticks were infected with An, the likelihood of Rc tick infection was lower if An was infecting ticks alone compared with cases when An-ticks were coinfected with Bl (Bl × An: $F_{1,788.4}$=7.08, p<0.05; effect of An occurrence sliced by Bl occurrence, Bl absent: $F_{1,787.5}$=14.77, p<0.05; Bl present: $F_{1,790}$=0.21, p>0.05; Fig. 4). In turn, if Bl was detected in ticks, the probability of Rc infection was higher regardless of An infection status (effect of Bl occurrence sliced by An occurrence, An absent: $F_{1,776.4}$=62.91, p<0.05; An present: $F_{1,789.3}$=4.37, p<0.05; Fig. 4).

Discussion

To our knowledge, this is the first study reporting *Anaplasma* in ticks feeding on a scaled reptile (Reptilia: Squamata) from Central Europe. *Anaplasma* was recently detected in ticks infesting western fence lizards (*Sceloporus occidentalis*) and northern alligator lizards (*Elgaria coerulea*) from North America [33], sand lizards *Lacerta agilis* from West Europe [48], and savannah monitors *Varanus exanthematicus* from West Africa [34]. In addition, this work is one of the few studies reporting *Rickettsiae* in ticks from reptile hosts [15, 41, 51]. Our study is first to show not only that hard ticks feeding on green lizards are infected with both Borreliae and *Anaplasma* (see [32] for other host–vector systems) but also that there are distinct patterns of pathogen coinfection prevalence in *I. ricinus* collected from lizards.

Our work has revealed that ticks feeding on green lizards are not infrequently infected with *Borrelia* spirochetes, *Anaplasma* sp., and *Rickettsia* sp. (Table 1).

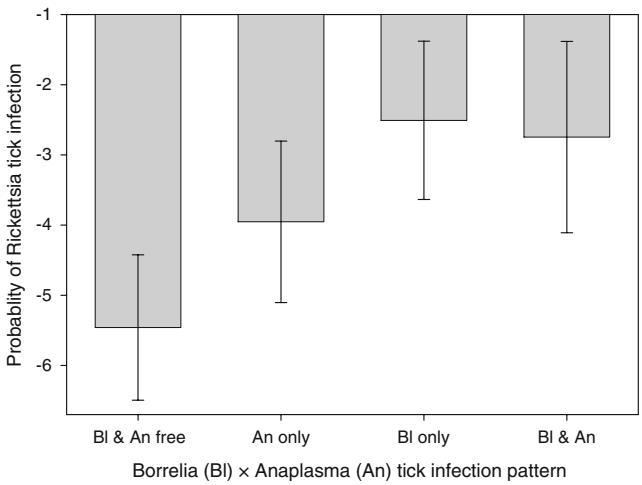

Figure 4 Probability of *Rickettsia* infection in *I. ricinus* as a function of *Borrelia* and *Anaplasma* tick coinfection prevalence. Displayed are the estimates of least-squares means and 95% confidence limits obtained by GLMM

Our study corroborates the recent results on the strong association of *B. lusitaniae* genospecies with lizard hosts [2, 12, 29, 42]. Similar to the results by Richter and Matuschka [42], who examined *B. lusitaniae* infection of *I. ricinus* ticks infesting *L. agilis*, we did not detect any other *Borrelia* genospecies than *B. lusitaniae*. Yet, using RFLP analysis, we detected in ticks two patterns of *B. lusitaniae*—pattern E and pattern F + E.

Our result on 47% of lizards being infested with at least one tick infected with *B. lusitaniae* lies between 66% reported by Richter and Matuschka [42] and 29% reported by Majláthová et al. [29]. The discrepancy between this and the latter study, though both being conducted on the same lizard species and in the same geographic region, could be due to the differences in the period of tick collection. While Majláthová et al. [29] collected ticks from lizards mostly outside the lizards' breeding season (May–September), our study was conducted in the spring during the period of the most intensive sexual activity of lizards (April–May), when the immune system is compromised by sex hormones and lower ambient temperature and lymphoid organs are not yet well developed after winter hibernation [35].

The nature of our study makes it impossible to confirm that the infected ticks acquired Borreliae or other pathogens from lizards on which they fed. We collected the tip of one toe from each lizard for molecular analysis. However, we did not detect Borreliae in any of these samples. This result adds to the negative results on the use of keratinized tissue parts (tail tips) of lizards [2, 29]. Nevertheless, the fact that no tick was infected with other *Borrelia* genospecies than *B. lusitaniae*, even though in Slovakia the majority of questing ticks and the ticks feeding on rodent or avian hosts are infected with other *Borrelia* genospecies [16–18, 25, 29] (Table 3), strongly suggests that the examined ticks obtained Borreliae from lizards. Further work needs to focus on other tissues and serological analysis to detect *Borrelia* in lizard hosts and validate that *Borrelia* can be transmitted from green lizards to ticks.

Despite the wide confidence limits and relatively few ticks coinfected with multiple pathogens, our study has revealed that the prevalence of both *B. lusitaniae* and *Rickettsia* in ticks feeding on green lizards was significantly lower if the ticks infected with *Anaplasma* were not coinfected with *Rickettsia* or *B. lusitaniae*, respectively. In contrast, the probability of *Anaplasma* tick infection increased if the ticks were infected with *B. lusitaniae* and/or *Rickettsia*. Therefore, it appears that there is an important negative effect (direct or indirect) of *Anaplasma* on *B. lusitaniae* and *Rickettsia* prevalence, a positive impact of *B. lusitaniae* and *Rickettsia* on *Anaplasma* prevalence, and positive interactions between *B. lusitaniae* and *Rickettsia*.

Table 3 Patterns of host infestation by ticks and tick infection for different vertebrate hosts in the study region

Vertebrate host	Tick vector	Hosts infested by ticks (%)[a]	Average tick load	Hosts infested by infected ticks (%)[b]	Infected ticks (%)	Detected pathogens infecting ticks	Reference
Lizards							
Lacerta viridis	*Ixodes ricinus*	88	2.8	29	16.6	*Borrelia lusitaniae*, *B. afzelii*, *B. garinii*, *B. burgdorferi* s.s., *B. valaisiana*	[29]
Lacerta viridis	*Ixodes ricinus*	93	8.3	71.9	22.0	*B. lusitaniae*, *Anaplasma* sp., *Rickettsia* sp.	This study
Lacerta viridis	*Dermacentor marginatus*	13	0.3	4.2	16.0	*B. burgdorferi* sensu lato, *Anaplasma* sp., *Rickettsia* sp.	This study
Birds							
Turdus merula	*Ixodes ricinus*	84	17.4	50/76.9	35.9	*B. garinii*, *B. valaisiana*, *B. afzelii*, *B. burgdorferi* sensu stricto	[46]
Turdus philomelos	*Ixodes ricinus*	81	11.7	50/63.6	38.1	*B. garinii*, *B. valaisiana*, *B. afzelii*	[46]
Mammals[c]							
Apodemus flavicollis	*Ixodes ricinus*	73/14	5.2	24.6/46.2	12.7	*B. afzelii*, *B. valaisiana*, *B. burgdorferi* s.s.	[17]
Clethrionomys glareolus	*Ixodes ricinus*	62/11	2.5	27.9/41.7	30.3	*B. afzelii*	[17]

[a] Percentages of infested mammal hosts refer to hosts infested by larvae/nymphs

[b] Percentages of bird and mammal hosts infested by infected ticks refer to hosts infested by larvae/nymphs

[c] Results for mammals are from Western Slovakia

Only a few studies report negative relationships between different vector-borne pathogens [1, 30], and most of these works report the associations between different strains of the same pathogen such as *Anaplasma* [14]. There is currently no convincing evidence that the interactions between *B. burgdorferi* and *Anaplasma phagocytophilum* may take place within vectors [26, 27]. In contrast, Levin [26] has demonstrated that such interactions, likely brought about by host immune responses, can occur within the host. The author found that if white-footed mice *Peromyscus leucopus* were primarily infected with *Anaplasma* and then secondarily with *Borrelia*, fewer xenodiagnostic *Ixodes scapularis* ticks acquired infection with *Borrelia* compared with the ticks feeding on mice infected with *Borrelia* only. An analogous result for *Anaplasma* tick infection was obtained if mice were first infected with *Borrelia* and then with *Anaplasma*. The infection pattern of tick larvae (a measure of host spirochete infection, e.g., [3]), with larvae being predominantly infected with *Anaplasma* only, suggests that the negative associations between *Anaplasma* and *Borrelia* and *Rickettsia* tick prevalence resulted from their interactions within tick hosts rather than ticks. Taken together, our work accords with the suggestion by Levin [26] in that *Anaplasma* infection may suppress the development of *Borrelia* spirochetes and suggests that *B. lusitaniae*, and also *Rickettsiae*, do not benefit from coinfection with *Anaplasma*.

Existing body of work does not clearly suggest that the rates of observed tick coinfections with *Borrelia* and *Anaplasma* are higher than expected [32]. Our study showed that tick *Borrelia–Anaplasma*, and also *Borrelia–Rickettsia* and *Borrelia–Anaplasma–Rickettsia* coinfection rates, is higher than expected based on single infection rates. Thus, while within-tick infection patterns have revealed a negative impact of *Anaplasma* on *Borrelia* prevalence, the results on observed coinfection rates suggest that the positive effects of *Borrelia* on *Anaplasma* prevalence in ticks prevail in our study system. In addition, our results on the negative associations between *Anaplasma* and *Borrelia* prevalence, as well as the heterogeneity of results on vector *Borrelia–Anaplasma* coinfection rates [32], lend support to the idea that in nature, a substantial number of *Borrelia–Anaplasma* coinfections result from interactions between different pathogens and/or sequential (transstadial) rather than simultaneous vector infection [26, 50].

To conclude, we show that ticks infesting a free-living temperate lizard species in a Central European region were infected with multiple pathogens with some of them previously found only in other vertebrates. The patterns of pathogen prevalence and observed coinfection rates suggest that the risk of tick infection with one pathogen is not independent of other pathogens. Overall, positive effects of *Borrelia* on *Anaplasma* seem to prevail as judged by higher-than-expected *Borrelia–Anaplasma* coinfection rates. Further laboratory studies are necessary to prove if *Anaplasma* can play a role in suppressing the transmission of *Borrelia* from the host to tick vectors and thus mediate its circulation in nature.

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