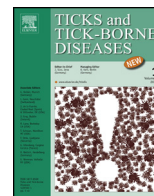




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Original article

High prevalence of *Borrelia burgdorferi* s.l. in the European red squirrel *Sciurus vulgaris* in France



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ABSTRACT

The European red squirrel (*Sciurus vulgaris*) has long been suspected to be a reservoir host of the agents of Lyme borreliosis, in particular *B. burgdorferi* sensu stricto (s.s.). However, very few data support this hypothesis. Hereafter, we investigated the infections with *B. burgdorferi* genospecies in road-killed red squirrels collected across France. We also characterized the diversity of hard tick species collected from a subsample of hosts. DNA of *B. burgdorferi* genospecies were detected and identified from PCR products in ear biopsies using reverse line blot hybridization. Variation in prevalence was investigated according to biogeographic areas (Mediterranean, Atlantic, Continental, and Alpine), season, sex, relative age, and body mass from 273 squirrels collected 2003–2010. Among the 746 identified tick specimens, no adult was observed, 63% were nymphs, and 37% were larvae all belonging to the species *Ixodes ricinus* except one nymph identified as *I. trianguliceps*. Overall, no squirrels of Mediterranean origin and no unweaned juveniles were found infested by hard ticks. Only season explained variation in *I. ricinus* abundance on squirrels, with more ticks present in spring to summer than in autumn to winter. Squirrels of Mediterranean origin ($n = 20$) were not found infected with *B. burgdorferi* sensu lato (s.l.), which is almost certainly related to the low occurrence of *I. ricinus* in this region. Based on individuals analyzed in the other regions of France, 11.5% ($n = 26$) unweaned young harboured *B. burgdorferi* s.l., which indicates that infection occurred already in the nest. In adults ($n = 227$), the prevalence of infection with *B. burgdorferi* s.l. was 27.3%, with 18.9% *B. burgdorferi* s.s., 11.9% *B. afzelii*, and 3.5% *B. garinii*. The season and the body mass, sex, and geographic origin of adults had no effect on the frequency of infection. Infection prevalence of *S. vulgaris* is among the highest found in rodents in Europe, particularly for *B. burgdorferi* s.s. supporting the hypothesis that sciurids are particularly suitable hosts for this genospecies.

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Introduction

Lyme borreliosis is the most widespread vector-borne zoonotic disease in the temperate countries of North America, Europe, and Asia (Rizzoli et al., 2011). This disease is caused by several genospecies of *Borrelia burgdorferi* sensu lato (Kurtenbach et al., 2006; Stanek and Reiter, 2011) and transmitted by hard ticks, in Europe primarily by *Ixodes ricinus*. The maintenance of *B. burgdorferi* s.l. requires the presence of vertebrates as reservoir hosts, among

which rodents and birds dominate (Gern, 2009). In Europe, the ecology of *B. burgdorferi* s.l. has been extensively studied (Piesman and Gern, 2004; Rizzoli et al., 2011). However, potential reservoir hosts have still to be identified, as well as the geographic distribution of the various genospecies (Humair and Gern, 2000; Gern and Humair, 2002; Derdáková and Lenčáková, 2005).

There are at least 7 genospecies of *B. burgdorferi* s.l. reported for Europe (Piesman and Gern, 2004; Gern, 2009), 5 of which are human pathogenic: *B. afzelii*, *B. bavariensis*, *B. burgdorferi* sensu stricto (s.s.), *B. garinii*, and *B. spielmanii* (Stanek and Reiter, 2011). In mammals, the most common genospecies is *B. afzelii* and to a much lesser extent *B. burgdorferi* s.s. and *B. bavariensis* (Gern and Humair, 2002; Huegli et al., 2002; Margos et al., 2009). In North America, *B. burgdorferi* s.s. is the dominant genospecies (Hanincová et al., 2006; Kurtenbach et al., 2006; LoGiudice et al., 2003; Rudenko et al., 2009), and the most competent reservoir hosts are mice

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Peromyscus spp. and Eastern chipmunks *Tamias striatus* (Mather et al., 1989; Slajchert et al., 1997; but see Brisson et al., 2008). In Europe, the identity of the reservoir hosts for *B. burgdorferi* s.s. is poorly documented (Baranton and de Martino, 2009). In contrast, it is well known that *B. garinii* preferentially infects birds and *B. afzelii* rodents (Gern, 2009; but see Franke et al., 2010) or insectivores (i.e., hedgehogs *Erinaceus* spp.; Skuballa et al., 2012). The European red squirrel *Sciurus vulgaris* is the only native arboreal sciurid species in western Europe (Gurnell, 1987). Although widely distributed across the European countries, the study of the red squirrel is difficult because it is vulnerable to many threats affecting its habitat, and it may disappear locally (Shar et al., 2008). Evidence of its reservoir competence for Lyme borreliosis agents is very poor. Indeed, we are aware of only 6 red squirrels tested for *B. burgdorferi* s.l. infection, 4 of which harboured *B. burgdorferi* s.s., 2 harboured *B. afzelii* including one coinfection with *B. burgdorferi* s.s., and one harboured *B. garinii* (Humair and Gern, 1998). *B. burgdorferi* s.s. and *B. afzelii* were also found to be transmitted to *I. ricinus* by squirrels (Humair and Gern, 1998). More evidence suggesting that *B. burgdorferi* s.s. may preferentially infect sciurids in Europe is that this genospecies dominates the diversity in an introduced squirrel originating from Korea (Chapuis et al., 2011; Pisanu et al., 2013), the Siberian chipmunk *Tamias (Eutamias) sibiricus barberi* in France (Marsot et al., 2011, 2013).

The main aim of this study was to investigate the potential for the European red squirrel to act as a reservoir host for *B. burgdorferi* s.l. To do so, we characterized the species diversity of hard ticks and of *B. burgdorferi* genospecies from *S. vulgaris* collected across the entire French mainland. We analyzed how infestation with ticks, and how infection prevalence of Lyme borreliosis agents varied according to geographic area, season, sex, age, and body mass. We aimed to confirm an expected high prevalence of *B. burgdorferi* s.s. in *S. vulgaris* and to illustrate how the identified genospecies of *B. burgdorferi* s.l. were distributed in these hosts both at regional and host individual scales.

Materials and methods

Material collection

From 2003 to 2010, a total of 273 squirrels found freshly dead, most of them due to road traffic across the entire mainland of France, was analyzed. These animals were equally distributed across seasons of the year, with an average of 20 ± 3 (SE) individuals sampled per month from January to December. These animals were collected by many collaborators, including citizens, nature preservation and veterinary associations, and the network of mammalogists of the National Forest Office. All animals were collected by fully authorized persons by the French Ministry of Ecology (Derogation no. 08/550/DEROG). Animals were individually placed in a plastic bag and frozen for delayed analyses. The location was determined using the latitude and longitude of the centroid associated with the administrative limits of the villages where a given squirrel was collected. Each individual was allocated to one of the 4 broad biogeographic areas defined according to climatic gradients (Fig. 1): western Atlantic, east Continental, Mediterranean, or Alpine (Inventaire national du Patrimoine naturel, 2012). The sex of each squirrel was noted, and full body mass was used as a proxy for age: an unweaned juvenile weighs less than 150 g, and adults are above this weight, including subadults (see Lurz et al., 2005; Wauters et al., 1993).

Hard ticks were counted on the entire body of a subsample of 237 squirrels ($n=215$ adults and 22 juveniles) under close focus binoculars (6–12 \times magnification). A sample of ticks was collected ($n=842$) for identification to the species level according to the key

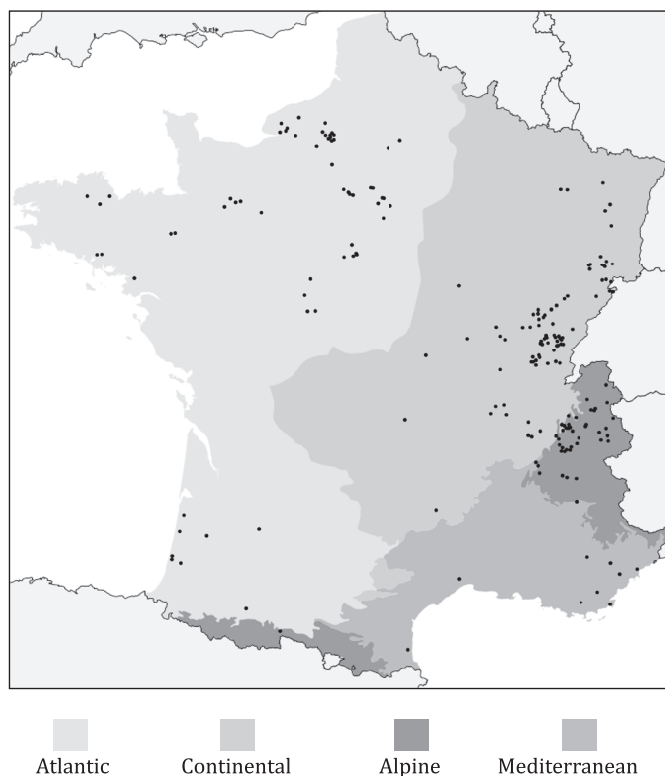


Fig. 1. The distribution of the European red squirrel, *Sciurus vulgaris*, collected in the different biogeographic regions in France.

provided by Pérez-Eid (2007). Unfortunately, 96 ticks (mainly larvae) could not be determined because of their state of degradation and were not included in the analysis.

PCR analyses

DNA from one ear biopsy of squirrel was extracted using NucleoSpin® Tissue kit (Machery-Nagel, Düren, Germany). *B. burgdorferi* s.l. genospecies were detected in the DNA extracts by PCR with B5S-Bor and 23S-Bor primers that amplify the variable spacer region between 2 repeated copies of the 23S and 5S ribosomal gene (Rijpkema et al., 1995). Positive controls were included in each PCR using isolates of *B. burgdorferi* s.s., *B. garinii*, *B. afzelii*, *B. lusitaniae*, and *B. valaisiana*. Distilled water was used as a blank. PCR products were identified by reverse line blot hybridization (RLBH) using 7 different oligonucleotide probes: *B. burgdorferi* s.l., *B. burgdorferi* s.s., *B. garinii*, *B. afzelii*, *B. lusitaniae*, and *B. valaisiana* as described in previous studies (Gern et al., 2010; Poupon et al., 2006; Rijpkema et al., 1995). Because samples infected with some strains of *B. afzelii* may cross react with the *B. lusitaniae* probe (Gern et al., 2010), samples that hybridized on *B. afzelii* and *B. lusitaniae* probes were considered infected with *B. afzelii* only. To differentiate infection with *B. bavariensis* from infection with *B. garinii*, a fragment of the housekeeping gene RPLB was amplified with a semi-nested PCR adapted from Margos et al. (2008). For the first PCR, primers RPLB outF (5'-TGGGTATTAAGACTTATAAGC-3') and RPLB outR (5'-GCTGTCCCAAGGAGAYAC-3') were used. The second PCR were performed with the first PCR products using the primers RPLB InF (5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGCGCTATAAGACGACTTTATC-3') and InR (5'-GCTGTCCCAAGGAGAYAC-3'). Then, PCR products were purified and

sequenced using Sanger DNA Sequencing (Beckman Coulter Genomics, Takeley, United Kingdom).

Statistical analyses

The variation in prevalence of the different genospecies of *B. burgdorferi* s.l. was analyzed according to geographic area, sex, and body mass of adults, including all possible combination of 2-way interactions using generalized linear models with binomial errors and logit link (Venables and Ripley, 2002). We also added a season variable in the models, divided into 2 levels: a spring–summer period that included individuals collected from March to August and an autumn–winter period for those collected from September to February. The same combination of factors was applied to investigate the variation in *I. ricinus* loads using generalized linear models with negative binomial errors and a log link (Venables and Ripley, 2002). As a part of data exploration (Zuur et al., 2009, 2010, 2012), we checked for the absence of (i) spatial and temporal autocorrelation that may have arisen from data collected closely in space and time, (ii) overdispersion in binomial models, and (iii) zero-inflation in negative binomial models. Models were selected using differences in Akaike Information Criterion corrected for small sample size less than 2 units (Symmonds and Moussali, 2011). Analyses were conducted using the software R 2.15.2 (R Core Team, 2012) with the ‘ncf’ package (Bjornstad, 2009) for spatial autocorrelation, the ‘MASS’ package (Venables and Ripley, 2002) for binomial or negative binomial GLMs, the ‘glm-ADMB’ package (Skaug et al., 2013) for zero-inflated negative binomial GLMs, and the ‘MuMIn’ package (Barton, 2012) for model selection. The means are always followed by ±1SE.

Results

Ixodid ticks

Among the 237 red squirrels examined for hard tick species, none of the 22 juveniles were found infested. Additionally, none of the 15 adults examined from the Mediterranean region was found infested (Table 1). Among the remaining 200 squirrels, 112 had no ticks, and 88 harboured on average 15 ± 5 (range: 1–415) ticks. Only season significantly explained tick load variation (p = 0.036; Table 2) on 191 individuals for which month of collection was known (Table 2). Individuals collected in the spring-to-summer period (n = 127) were infested with 21 ± 4 ticks/host on average, whereas infestation on those squirrels collected in the autumn-to-winter period was 4 times lower (5 ± 1, n = 64). Tick load did not significantly vary with body mass according to geographic origin or sex (Table 2). Among the 746 identified tick specimens, no adult was observed, 470 were nymphs, and 275 were larvae belonging to the species *I. ricinus*. One nymph was identified as *I. trianguliceps*.

Lyme borreliosis agents

Of the 273 squirrels examined (Table 1), none of the 20 individuals (18 adults and 2 juveniles) collected in the Mediterranean region was infested with *B. burgdorferi* s.l. Additionally, 3 of the other 26 juveniles (11.5%) collected in the remaining mainland were found infested with *B. burgdorferi* s.l., 2 with *B. burgdorferi* s.s., and one with *B. afzelii*. Considering the 227 remaining adults collected in the Atlantic, Continental, and Alpine regions, 65 (27.3%) were infested with *B. burgdorferi* s.l. Three genospecies were identified (Table 1): *B. burgdorferi* s.s. in 45 squirrels (18.9%), *B. afzelii* in 28 (11.9%), and *B. garinii* in 8 squirrels (3.5%). One squirrel was found to be coinfecting with all 3 genospecies, 2 with *B. burgdorferi* s.s. and *B. garinii*, and 12 with *B. burgdorferi* s.s. and *B. afzelii*. The

Table 1 Details of the age- and sex-related body mass of red squirrels *Sciurus vulgaris* according to biogeographic regions, the corresponding number of hosts infected with genospecies of *Borrelia burgdorferi* s.l., and mean abundance of *Ixodes ricinus*. Individual body masses in italics.

Region	Age	Sex	n	Body mass	Borrelia burgdorferi sensu lato (s.l.)		Borrelia burgdorferi sensu stricto (s.s.)			I. ricinus			
					B. burgdorferi sensu lato (s.l.)		B. burgdorferi sensu stricto (s.s.)			I. ricinus			
					B. burgdorferi sensu lato (s.l.)	B. burgdorferi sensu stricto (s.s.)	s.s. + afzelii	s.s. + garinii	s.s. + garinii + afzelii	afzelii	garinii	n	Mean ± SE
Alpine	Juv	♀	0	-	1	1	1	1	1	1	1	0	-
	Ad	♂	0	-	1	1	1	1	1	1	1	0	-
Atlantic	Juv	♀	17	307 ± 12	5	2	1	1	1	2	1	13	5 ± 3
		♂	19	314 ± 6	3	1	1	1	1	1	1	20	4 ± 2
	Ad	♀	2	62; 144	0	0	0	0	0	0	0	2	0
		♂	34	57; 101	0	5	1	1	1	2	1	30	33 ± 15
Continental	Juv	♀	48	318 ± 9	15	6	5	1	1	3	1	44	32 ± 11
		♂	11	290 ± 7	1	1	1	1	1	1	1	7	0
	Ad	♀	11	72 ± 5	2	1	1	1	1	1	1	10	0
		♂	51	71 ± 7	12	4	3	1	1	2	1	45	9 ± 3
Mediterranean	Juv	♀	58	328 ± 7	18	10	3	1	1	5	1	48	4 ± 2
		♂	1	319 ± 6	0	0	0	0	0	0	0	0	0
	Ad	♀	1	143	0	0	0	0	0	0	0	1	0
		♂	10	116	0	0	0	0	0	0	0	9	0
Total	Ad	♀	8	303 ± 15	0	0	0	0	0	0	0	6	0
		♂	273	277 ± 18	65	30	12	2	1	15	5	237	0

Table 2
The selection of models with a difference in AICc of less than 2 units, exploring the variations in the abundance of *Ixodes ricinus* and in the prevalence of 2 genospecies of *Borrelia burgdorferi* s.l., in red squirrels. *K*, number of model parameters; *-LL*, log-likelihood; *w_i*, weight-of-evidence of the models; $\beta \pm SE$ averaged estimate of coefficients; LCI and UCI, lower and upper 95% confidence interval of the coefficient estimate.

Sources of variation	<i>K</i>	$\Delta AICc$	<i>-LL</i>	<i>w_i</i>	$\beta \pm SE$	LCI	UCI	<i>p</i>
<i>I. ricinus</i>								
Season + Body mass*Geography	8	0.00	477.96	0.32				
Season + Geography + Body mass*Sex	8	0.23	478.07	0.29				
Season + Geography + Body mass	6	0.39	480.32	0.26				
Season + Geography + Body mass + Body mass*Geography + Body mass*Sex	10	1.84	476.97	0.13				
Season_(spring-summer)					0.98 ± 0.47	0.06	1.89	0.036
Geography _(Atlantic)					4.62 ± 4.81	-4.83	14.08	0.198
Body mass					0.02 ± 0.02	-0.01	0.05	0.197
Sex _(male)					4.51 ± 2.43	-0.29	9.31	0.066
Body mass*Geography _(Atlantic)					-0.02 ± 0.02	-0.05	0.11	0.199
Body mass*Sex _(male)					-0.01 ± 0.01	-0.03	<0.01	0.059
<i>B. burgdorferi</i> s.s.								
Season	2	0.00	105.75	0.35				
Body mass + Season	3	1.22	105.33	0.19				
Season + Sex	3	1.81	105.63	0.14				
Season _(spring-summer)					-0.08 ± 0.37	-0.81	0.65	0.829
Body mass					<0.01 ± <0.01	-0.01	0.01	0.486
Sex _(male)					-0.38 ± 1.67	-3.66	2.89	0.919
<i>B. afzelii</i>								
Season	2	0.00	81.39	0.15				
Null	1	0.82	82.81	0.10				
Body mass + Season	3	0.99	80.85	0.09				
Body mass	2	1.38	82.08	0.08				
Season + Body mass*Geography	7	1.56	76.93	0.07				
Body mass*Geography	6	1.67	78.06	0.07				
Season + Sex	3	1.81	81.26	0.06				
Intercept					-2.45 ± 3.44	-9.22	4.32	0.478

prevalence of *B. burgdorferi* s.s. and *B. afzelii* in adults did not vary according to geographic origin, season, sex, or body mass (Table 2).

Discussion

We have shown that *S. vulgaris* is a particularly suitable host for *B. burgdorferi* s.l., which has, to date, only been inferred from a small number of observations (Humair and Gern, 1998). Together with the high infestation load of ticks, particularly nymphs, this provides strong evidence that the red squirrel may be an important reservoir host of Lyme borreliosis.

The quantitative analysis made to investigate tick load variations and Lyme borreliosis agents may have been biased because of a lack of (i) host habitat characterization, and (ii) an uneven spatial and seasonal sampling. Indeed, several squirrels were sampled from urban areas where the lack of ungulates (i.e., roe deer *Capreolus capreolus*) has occasioned an absence of infesting stages of *I. ricinus*. Additionally, autumn and winter seasons may have reduced the hosts' exposure to ticks. Finally, tick abundance must be interpreted with caution, as time delay between squirrel recovery and death may have caused ticks to leave from their dead host.

Nevertheless, the high infection prevalence of 27.3% in squirrels is in accordance with those reported for *B. burgdorferi* s.s. in North American squirrels, which show a prevalence of approximately 30% (i.e., *S. griseus* in California: 30%, *n* = 222, Salkeld et al., 2008; Lane et al., 2005; Nieto et al., 2010; Salkeld and Lane, 2010; *T. striatus* in Illinois: 26%, *n* = 130, Slajchert et al., 1997; see also Hanincová et al., 2006; LoGiudice et al., 2003). In France, the introduced Siberian chipmunk *T. (E.) s. barberi* has been found to be infected at a prevalence of approximately 20% (i.e., 24%, *n* = 188 adults; 17%, *n* = 335 including juveniles; see Marsot et al., 2011). Such a prevalence is indeed much higher than those usually reported in the literature for *B. burgdorferi* s.s. in western Europe, where the prevalence rarely reaches values above 5% in native terrestrial small rodents

(Hanincová et al., 2003; Humair et al., 1999; Kurtenbach et al., 1998; Marsot et al., 2011; Michalik et al., 2005).

The diversity of *Borrelia* genospecies found in our study is in agreement with that found locally in a small number of *S. vulgaris* in Switzerland (Humair and Gern, 1998) and in the introduced Siberian chipmunk in France (Marsot et al., 2011): *B. burgdorferi* s.s. dominates the spirochaete community infecting these hosts, followed by *B. afzelii* and *B. garinii*, which are less common. The very low prevalence of *B. garinii* indicates occasional acquisition. This situation is consistent with a lack of suitability of rodent hosts for these subtypes, which preferentially infect ground-frequenting birds (Kurtenbach et al., 1998; Gern, 2009). The *B. afzelii* infection prevalence in red squirrels in the present study (10%) is closest to the prevalence observed in wood mice for this genospecies (Humair et al., 1999; Hanincová et al., 2003; Marsot et al., 2011). The grey squirrel (*Sciurus carolinensis*) introduced in the United Kingdom has been found to be a reservoir for *B. afzelii* (Craine et al., 1997). Wood mice are considered more resistant than bank voles to *B. afzelii* infection (Kurtenbach et al., 1994), and such differences have also been detected in varying susceptibility for different strains within *B. burgdorferi* s.s. according to rodent host species (Derdáková et al., 2004; Slajchert et al., 1997). Therefore, the low prevalence of *B. afzelii* observed in red squirrels here may be due to a low susceptibility to infection.

The absence of ticks and infection with *B. burgdorferi* genospecies on squirrels originating from Mediterranean areas is in accordance with the low occurrence of *I. ricinus* in most dry habitats encountered on this particular bioclimatic region (Gilot and Pérez-Eid, 1998; Medlock et al., 2013; Pérez-Eid, 2007). We are aware, however, that we observed only a small number of individuals from this area (*n* = 20) and may have missed individuals with ticks. Indeed, a small number of localities in southern France may have a low level of acarological risk of Lyme borreliosis (Estrada-Peña et al., 2011) because of a locally favourable wetland habitat for *I. ricinus*. Further investigations of infestation with hard ticks

and infections with *B. burgdorferi* genospecies in sciurids in the Mediterranean habitats are required.

A small percentage of young squirrels were found infected with *B. burgdorferi* s.l. These results are in accordance with the reduced space use of unweaned young squirrels weighing less than 150 g (Wauters et al., 1993). Accordingly, these squirrels were infected with approximately half the number of *B. burgdorferi* s.l. than subadults and adults. The fact that juvenile squirrels are infected with *B. burgdorferi* s.l. may also indicate that these individuals become infected by *I. ricinus* nymphs carried to the nest in the course of rearing activities by their mothers.

Conclusion

The overall prevalence of *B. burgdorferi* s.l. (27.3%) found in red squirrels collected in France (except in the Mediterranean region), is among the highest prevalence found in rodent populations in Europe. Our results add compelling evidence for a major role of the European red squirrel in hosting an important diversity of pathogenic agents of Lyme borreliosis in western Europe (Humair and Gern, 1998), among which the rarest genospecies *B. burgdorferi* s.s. dominates. This result is notable when compared with other rodent species, which are almost entirely infected with *B. afzelii* and to lesser extent with *B. garinii* (Rizzoli et al., 2011). We emphasized that biogeographic origin linked to the presence/absence of *I. ricinus*, along with host age-related behaviour in exposure, contributed to the observed *B. burgdorferi* s.l. prevalence in European red squirrels in France. It should be noted that the ratio of *I. ricinus* stages on red squirrels was in favour of nymphs, as has already been noted by Humair and Gern (1998). Such a situation and the low densities that characterize red squirrel populations in Europe (Lurz et al., 2005) indicate a reduced role for this sciurid in disseminating Lyme borreliosis agents to the rest of the host community compared with small mammals and ground-feeding passerine birds.

Conflict of interest

The authors declare no conflict of interest.

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