

# Seasonality of *Ixodes ricinus* Ticks on Vegetation and on Rodents and *Borrelia burgdorferi* sensu lato Genospecies Diversity in Two Lyme Borreliosis–Endemic Areas in Switzerland

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## Abstract

We compared *Ixodes ricinus* questing density, the infestation of rodents by immature stages, and the diversity of *Borrelia burgdorferi* sensu lato (sl) in questing ticks and ticks collected from rodents in two Lyme borreliosis (LB)-endemic areas in Switzerland (Portes-Rouges [PR] and Staatswald [SW]) from 2003 to 2005. There were variations in the seasonal pattern of questing tick densities among years. Questing nymphs were globally more abundant at PR than at SW, but the proportion of rodents infested by immature ticks was similar (59.4% and 61%, respectively). Questing tick activity lasted from February to November with a strong decline in June. The seasonal pattern of ticks infesting rodents was different. Ticks infested rodents without decline in summer, suggesting that the risk of being bitten by ticks remains high during the summer. Rodents from SW showed the highest infestation levels ( $10 \pm 21.6$  for larvae and  $0.54 \pm 1.65$  for nymphs). The proportion of rodents infested simultaneously by larvae and nymphs (co-feeding ticks) was higher at SW (28%) than at PR (11%). *Apodemus flavicollis* was the species the most frequently infested by co-feeding ticks, and *Myodes glareolus* was the most infective rodent species as measured by xenodiagnosis. At PR, the prevalence of *B. burgdorferi* sl in questing ticks was higher (17.8% for nymphs and 32.4% for adults) than at SW (10.4% for nymphs and 24.8% for adults), with *B. afzelii* as the dominant species, but *B. garinii*, *B. burgdorferi* sensu stricto, and *B. valaisiana* were also detected. Rodents transmitted only *B. afzelii* (at PR and at SW) and *B. bavariensis* (at SW) to ticks, and no mixed infection by additional genospecies was observed in co-feeding ticks. This implies that co-feeding transmission does not contribute to genospecies diversity. However, persistent infections in rodents and co-feeding transmission contribute to the perpetuation of *B. afzelii* in nature.

**Key Words:** *Borrelia burgdorferi* sensu lato—Co-feeding transmission—Rodents.

## Introduction

IN EUROPE, LYME BORRELIOSIS (LB) IS CAUSED BY DIFFERENT *BORRELLIA* SPECIES belonging to the *B. burgdorferi* sensu lato (sl) complex. These infectious agents are maintained in natural zoonotic cycles involving wild vertebrates and ticks, which are obligate blood-feeding arthropods. In Europe, *Ixodes ricinus* is the main vector of *B. burgdorferi* sl (Piesman and Gern 2004). Both biotic (host species, density and behavior, and vegetation structure), and abiotic factors (local climatic conditions), play a role in modeling the seasonal activity of ticks through the years (Randolph et al. 2002; Randolph 2004; Perret et al. 2004). This seasonal pattern of the density of

*I. ricinus* questing ticks was reported to be variable between years, and to vary from a unimodal pattern with maximum density in spring to a bimodal pattern reaching its maximum density in spring and in autumn. In Switzerland, both bimodal and unimodal patterns were shown to occur (Perret et al. 2000; Jouda et al. 2004a, 2004b; Burri et al. 2007; Morán Cadenas et al. 2007a).

At least nine *Borrelia* species, *B. burgdorferi* sensu stricto (ss), *B. garinii*, *B. afzelii*, *B. valaisiana*, *B. lusitaniae*, *B. spielmanii*, *B. bissettii*, *B. bavariensis*, and *B. finlandensis* have been identified in *I. ricinus* (Hanincova et al. 2003b; Rauter and Hartung 2005; Richter et al. 2006; Hulinska et al. 2007; Margos et al. 2009; Casjens et al. 2011).

Studies on the ecology of LB have demonstrated that the efficient persistence of *Borrelia* pathogens in endemic areas requires the involvement of reservoir hosts. Small-sized mammals, *Apodemus* mice, and *Myodes* (*Clethrionomys*) voles in particular, are often infested by immature *I. ricinus* stages, and have been described as competent reservoirs for *B. burgdorferi* s.l., as are other mammals, like dormice, hedgehogs, rats, squirrels, hares, ground-frequenting birds like passerines and pheasants, and lizards (Piesman and Gern 2004). Specific associations between hosts, ticks, and *Borrelia* species are observed in nature. For example, *I. ricinus* ticks can acquire *B. afzelii* and *B. bavariensis* after feeding on rodents (Humair et al. 1995, 1999; Huegli et al. 2002; Margos et al. 2009), and *B. afzelii* and *B. burgdorferi* s.s. are associated with red squirrels (Humair and Gern 1998). In contrast to *B. afzelii*, which is adapted to rodents, most variants of *B. garinii* and *B. valaisiana* present a resistance to the avian complement and are considered bird-associated *Borrelia* ecotypes (Olsén et al. 1995; Kurtenbach et al. 1998; Humair et al. 1998). These preferential associations affect the genospecies distribution in an area in relation to the composition of the local fauna.

Larvae become infected if at their first blood meal they encounter a *Borrelia* reservoir host, since transovarial transmission of *B. burgdorferi* s.l. is a rare phenomenon (Bellet-Edimo et al. 2005). Nymphs from this cohort of larvae will be infected because of the maintenance and the transfer of the pathogen at the following stages. *Borrelia* may also pass directly from infected (usually nymphs) to uninfected ticks (usually larvae) when ticks are feeding together on vertebrate hosts before spirochetes have disseminated in the host and produced a systemic infection. This way of transmission is called co-feeding (Gern and Rais 1996).

Here we compared and analyzed seasonal patterns of questing tick activity and infestation of rodents by immature stages in two LB-endemic areas (Staatswald [SW] and Portes-Rouges [PR]) located on the Swiss Plateau. We particularly focused on the simultaneous presence of *I. ricinus* larvae and nymphs co-feeding on rodents. Co-feeding transmission of LB spirochetes has been described in experimental models (Gern and Rais 1996), but very little information is available on this transmission pathway under natural conditions. Finally, we examined the distribution of *B. burgdorferi* s.l. genospecies in questing ticks, ticks that detached from rodents, and xenodiagnostic ticks.

## Materials and Methods

### Collection of ticks and rodents

The present study took place at two different sites on the Swiss Plateau. The first site, SW (Ins, Canton Bern, 433 m elevation, 46°92'N, 07°07'E) is a mixed forest (*Pruno-Fraxinetum*) established on a marl. The second site, PR (Bois de l'Hôpital, Neuchâtel, Canton Neuchâtel, 47°01'N, 06°56'E), is a mixed forest (*Rhamno-Quercetum*) established on a thin soil layer on a rather stony ground (limestone) layer, and is under the climatic influence of the lake of Neuchâtel.

*I. ricinus* ticks were collected by flagging low vegetation on a monthly basis from March 2003 to November 2005, within the same week at both sites. Contacts between the flag and the litter layer could occur only during winter months, when low vegetation was less dense. Sampling was not undertaken under windy, snowy, or rainy conditions. The 1-m<sup>2</sup> flag was

examined every 15 m over 360 m at SW and 1049 m at PR along the same transects. Ticks were identified for species, stage, and sex, and maintained at relative humidity close to 95% at room temperature until processing for isolation of spirochetes (within 7–10 days). Questing tick density was expressed as the number of ticks per 100 m<sup>2</sup>. An annual value for tick density called cumulated tick density (CTD) was obtained by integrating the linearly interpolated curve of the measured questing tick densities over 1 year (Eisen et al. 2003; Jouada et al. 2004a; Burri et al. 2007; Morán Cadenas et al. 2007a).

Small mammals were live-trapped monthly from March 2003 to November 2005 during the same week of questing tick collection, using 50 wooden box traps placed every 5 m along the questing tick collection transects. Traps were placed between 4 and 7 PM, and collected the next morning between 7 and 9 AM. The traps were baited with grains, pieces of apple, and during winter months straw was added. Captured individuals were brought into the laboratory and caged individually over a pan of water until the feeding ticks dropped off. Engorged ticks were collected, counted, and maintained as described above until the molt was completed.

### Xenodiagnosis

About 1 week after capture, rodents were identified for species and sexed. A subset of rodents that had been captured in spring, summer, fall, and winter were submitted to xenodiagnosis 3–4 days after all field-derived ticks detached. About 100 xenodiagnostic *I. ricinus* larvae, derived from our laboratory colony and free of spirochetal infection, were placed on the heads of the rodents and allowed to engorge until repletion. Replete ticks were collected daily from each host in a pan of water and maintained under the same conditions as those described for field-derived ticks, until they molted. Finally, the rodents were released at the exact trapping site, around 20 days after capture. Ticks collected from rodents and xenodiagnostic ticks were examined for *Borrelia* after their molt.

### Isolation of spirochetes from *I. ricinus*

Questing ticks, ticks collected on rodents, and xenodiagnostic ticks were briefly soaked in 70% ethanol and individually squashed with sterilized forceps in tubes containing modified BSK-II medium (Sinsky and Piesman 1989). The culture tubes were screened by darkfield microscopy for spirochetes after 7 and 14 days of incubation at 34°C, and 1.5 mL of positive spirochete subcultures were washed by centrifuging and double washing with 1 mL of PBS-MgCl<sub>2</sub>. Each pellet was suspended in 50 µL of ultra-filtered water and DNA was extracted after incubation at 100°C for 10 min, according to the procedure of Postic and associates (1994). Thermolysates were stored at –20°C until use for polymerase chain reaction (PCR). Cultures that were contaminated were not further investigated and appear as untyped *Borrelia*.

### Identification of *Borrelia* species

PCR and restriction fragment length polymorphism (RFLP), as described by Postic and colleagues (1994), were used to identify the *Borrelia* species. Briefly, the variable spacer region between two repeated genes encoding for

ribosomal 23S and 5S was amplified using primers P1 (5'-CTGCGAAGTTCGCGGGAGA-3') and P2 (3'-TCCTAGG CATTACCATA-3') in a Tgradient Thermocycler 96 (Whatman Biometra, Göttingen, Germany). Negative and positive controls were included in each PCR. In negative controls, water replaced DNA samples. PCR products were separated by electrophoresis in a 1% agarose gel and stained with ethidium bromide. Positive PCR products were digested overnight with *MseI* restriction endonuclease at 37°C, and then separated by electrophoresis in a 16% acrylamide gel for 1 h 30 min at 120 V. Digested DNA was stained with ethidium bromide.

#### Statistical analysis

Rodent species distribution at both sites was compared by Fisher's exact test, tick infestation levels on rodents was compared with an analysis of variance (ANOVA) with logarithmic transformation, and correlations between numbers of larvae and nymphs feeding together on individual hosts were determined with Spearman's rank correlation. The Pearson's chi-square test was used to compare the proportion of rodents infested by nymphs, and the prevalence of infection in ticks, and finally the distribution of *B. burgdorferi* s.l. species was analyzed with the goodness of fit test. Statistical results were considered significant if the *p* value was less than 0.05. All statistical analyses were performed using S-Plus 6.2 for Windows.

## Results

### Questing ticks

All questing ticks collected by flagging low vegetation at the two sampling sites belonged to *I. ricinus*. A total of 614 *I. ricinus* larvae, 9101 nymphs, and 1147 adults were obtained at PR, and 62 larvae, 1712 nymphs, and 540 adults at SW. Questing nymphs were globally more abundant at PR than at SW for the 3-year period, with the highest CTD and the highest peak tick density (PTD) reached in 2003, with 12,054 nymphs per 100 m<sup>2</sup>/year and 163 nymphs per 100 m<sup>2</sup>, respectively (Fig. 1). Questing adults showed the highest CTD and PTD at SW in 2005, with 1882 adults per 100 m<sup>2</sup>/year and 29 adults per 100 m<sup>2</sup>, respectively. There were variations in the seasonal pattern of questing tick densities among years at both sites, and questing tick activity lasted from February to November at PR, and from March to October at SW, with a strong decline in June at both sites (Fig. 1).

### Infestation of rodents by ticks

During this study, 632 rodents of three species (*A. flavicollis*, *A. sylvaticus*, and *M. glareolus*) and two *Sorex araneus* shrews were captured over 3150 trap nights (Table 1). Shrews were not examined for tick infestation because they were dead when the traps were checked. Rodents were less frequently trapped at PR (0.11 rodent/trap night; *n* = 165 rodents) than at SW (0.30 rodent/trap night; *n* = 467). Over the 3-year study at PR each of the three rodent species represented one-third of all captured animals, whereas at SW *M. glareolus* was clearly the dominant species (55%), followed by *A. flavicollis* (35%) (Fisher's exact test, *p* < 0.001).

A total of 5808 *I. ricinus* ticks (5524 larvae [95.1%] and 284 nymphs [4.9%]) detached from captured rodents (Table 1).

The seasonal pattern of ticks infesting rodents was different from the seasonal pattern of questing tick activity (Figs. 1 and 2). Ticks were observed on rodents from February–March to November without a decline in summer (Fig. 2). At the two sites, the proportion of rodents infested by immature ticks was similar (PR: 59.4% and SW: 61%; Table 1). However, rodents from SW showed the highest infestation levels, reaching  $10 \pm 21.6$  for larvae and  $0.54 \pm 1.65$  for nymphs. Each rodent species was infested by significantly more larvae at SW than at PR (ANOVA with logarithmic transformation, *p* = 0.033 for *M. glareolus*, *p* < 0.001 for *A. sylvaticus*, and *p* < 0.001 for *A. flavicollis*), and nymphs represented 5.1% of ticks infesting rodents at SW compared to 3.8% at PR (Table 1). At PR, the larval infestation level among the three rodent species did not show any significant difference, whereas at SW, *M. glareolus* displayed a significantly lower level of infestation by larvae than the two *Apodemus* species (ANOVA with logarithmic transformation, *p* < 0.001). At both sites, the bank vole was the rodent species showing the lowest infestation load (Table 1).

At both sites, a significant positive correlation was observed between the numbers of larvae and nymphs feeding together (co-feeding ticks) on individual hosts (Spearman's rank correlation, *p* < 0.001). Considering each site, the proportion of individuals infested by at least one nymph did not vary among years (Pearson's chi-square test, SW: *p* = 0.062, PR: *p* = 0.91). The probability of finding feeding nymphs on a host increased with larval infestation, particularly at SW (Fig. 3). The proportion of rodents concurrently infested by larvae and nymphs was higher at SW (19.5%, 91/467; *M. glareolus*: 13.7%, 35/255; *A. flavicollis*: 29.6%, 48/162; *A. sylvaticus*: 16%, 8/50) than at PR (9.09%, 15/165; *M. glareolus*: 5.6%, 3/54; *A. flavicollis*: 8.5%, 5/59; *A. sylvaticus*: 13.5%, 7/52). Considering only March to September (the months with the highest tick infestations), there were clearly more rodents with co-feeding ticks at SW (88/310, 28%) than at PR (13/113, 12%) (Pearson's chi-square test, *p* < 0.001), and the proportion of rodents infested by nymphs was also significantly higher at SW (91/310, 29%) than at PR (15/113, 13%) (Pearson's chi-square test, *p* < 0.001). *A. flavicollis* was the species most frequently infested by co-feeding ticks considering both sites (Pearson's chi-square test, *p* = 0.002).

### Borrelia infection in questing ticks

A total of 638 *Borrelia* isolates were obtained from 2073 *I. ricinus* nymphs and 1055 adults collected by flagging vegetation at both sites (Table 2). More ticks were infected at PR (22.3%, 478/2148) than at SW (16.3%, 160/980), but the difference was not significant. However, at each site, adult ticks were significantly more infected than nymphs (Pearson's chi-square test, PR: *p* < 0.001, SW: *p* = 0.005; Table 2). Four *Borrelia* species, *B. afzelii*, *B. garinii*, *B. burgdorferi* ss, and *B. valaisiana*, were identified in questing ticks at both sites. Mixed infections were observed in 23 questing ticks (3.6%; *B. afzelii* and *B. burgdorferi* ss in 6 nymphs and 6 adults, *B. garinii* and *B. valaisiana* in 2 nymphs and 4 adults, *B. garinii* and *B. afzelii* in 2 nymphs and 2 adults, and *B. garinii* and *B. burgdorferi* ss in 1 adult).

Species distribution was not uniform at the two sites and in tick stages (Goodness of fit test, *p* ≤ 0.001), but the *Borrelia* species distribution in nymphs and in adults was not

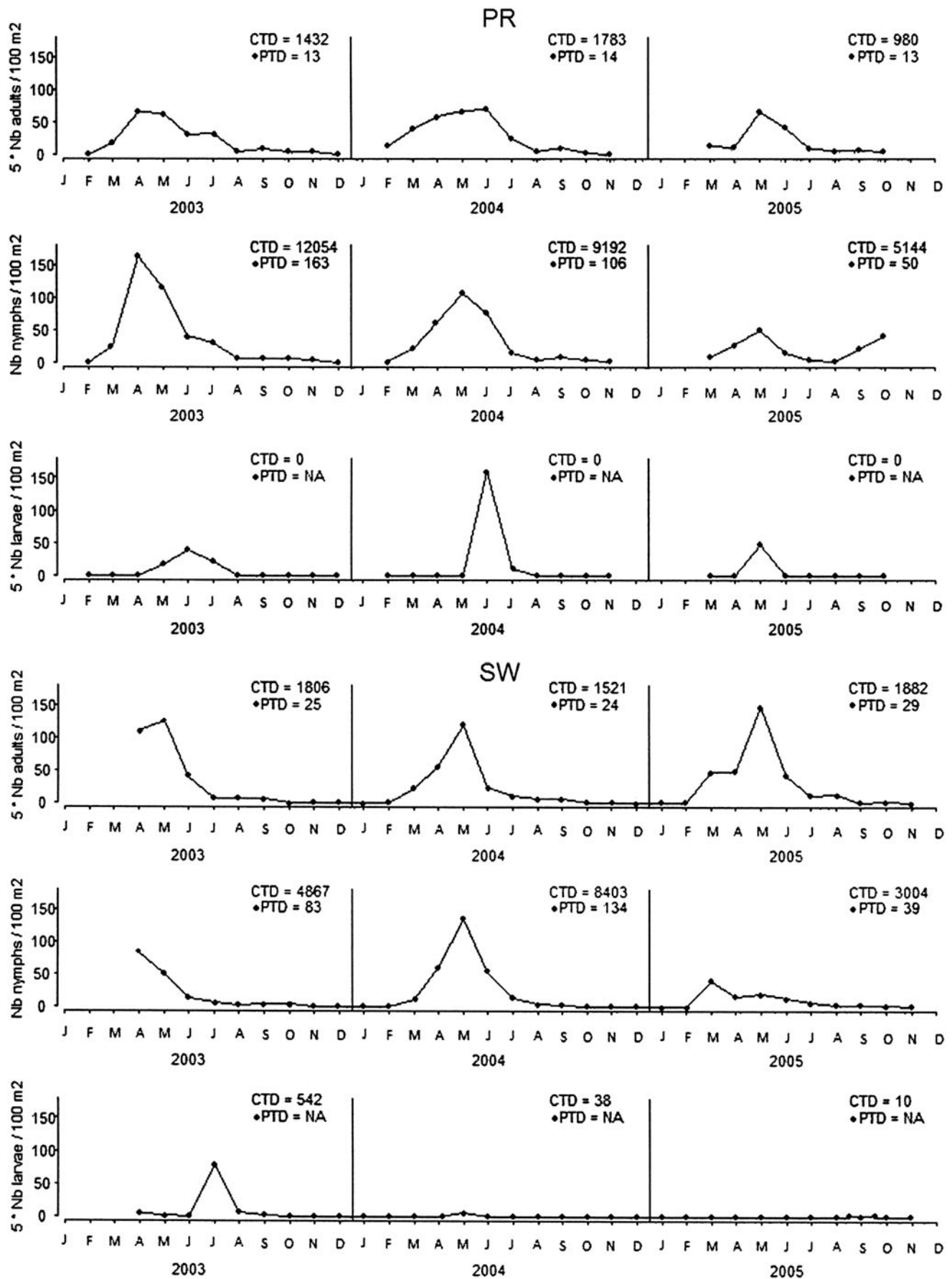


FIG. 1. Seasonal pattern of *Ixodes ricinus* questing tick density at Portes-Rouges (PR) and Staatswald (SW; CTD, cumulated tick density; PTD, peak tick density).

TABLE 1. INFESTATION OF SMALL MAMMALS WITH LARVAL AND NYMPHAL *IXODES RICINUS* AT PORTES-ROUGES (PR) AND STAATSWALD (SW)

Host species	No infested/examined hosts	PR			
		Larvae		Nymphs	
		No	Mean no per host ± SD	No	Mean no per host ± SD
<i>A. flavicollis</i>	36/59 (61%)	453	7.7 ± 15	11	0.19 ± 0.66
<i>A. sylvaticus</i>	30/52 (58%)	238	4.6 ± 7	20	0.38 ± 1.03
<i>M. glareolus</i>	32/54 (59%)	166	3.1 ± 5.1	3	0.06 ± 0.23
Total	98/165 (59.4%)	857	5.2 ± 10.4	34	0.21 ± 0.72

Host species	No infested/examined hosts	SW			
		Larvae		Nymphs	
		No	Mean no per host ± SD	No	Mean no per host ± SD
<i>A. flavicollis</i>	112/162 (69%)	2786	17.2 ± 30.2	139	0.86 ± 2.16
<i>A. sylvaticus</i>	24/50 (48%)	495	9.9 ± 25.6	26	0.52 ± 2.04
<i>M. glareolus</i>	149/255 (58%)	1386	5.4 ± 10.1	85	0.33 ± 1.06
Total	285/467 (61%)	4667	10 ± 21.6	250	0.54 ± 1.65

SD, standard deviation.

different between PR and SW. *B. afzelii* was the dominant species in questing *I. ricinus* nymphs and adults. *B. afzelii* and *B. burgdorferi* ss were more frequently detected in questing nymphs at PR than at SW (PR: 63.7%, SW: 58.3%), and *B. burgdorferi* ss was significantly more frequently detected in nymphs from PR (16.1%) than from SW (5%, Pearson’s chi-square test,  $p=0.04$ ). If we compare the species distribution at each site between questing nymphs and adults, we observe a significant difference at both PR (Pearson’s chi-square test,  $p<0.001$ ) and SW ( $p=0.011$ ). *B. afzelii* was significantly more frequent than *B. garinii* but only in nymphs (Pearson’s chi-square test,  $p=0.003$  for PR, and  $p<0.001$  for SW).

**Borrelia infection in ticks naturally infesting rodents**

A total of 296 *Borrelia* isolates were obtained from 1074 molted ticks (28.4%) that detached from 130 rodents (PR:  $n=56$ ; SW:  $n=74$ ; Table 3). *B. afzelii* was the dominant species ( $n=278$ , 94%), and *B. bavariensis* (identified by reverse line blotting according to Gern et al. 2010, data not shown) was observed once at SW. No infection with more than one *Borrelia* species was detected in molted ticks that engorged on rodents. The infection prevalence in ticks that detached from rodents trapped at PR reached 28.9% (114/395), and 39.3% (22/56) of rodents were infested by infected ticks, whereas at SW more than 50% (55.4%, 41/74) of rodents were infested by infected ticks with an infection prevalence in ticks similar to that observed at PR (26.8%, 182/679; Table 3). The rodent species the most frequently infested by infected ticks was *A. sylvaticus* (47.6%, 10/21) at PR and *A. flavicollis* (65%, 26/40) at SW. Infection rates of larvae (examined as nymphs) that detached from rodents varied according to rodent species, from 18.6% to 37.6% at PR, and from 21.6% to 31.9% at SW (Table 3).

To examine the effect of co-feeding ticks (nymphs and larvae feeding simultaneously on rodents) on the percentage of *Borrelia*-infected larvae, we compared the infection of larvae

feeding on hosts infested by co-feeding ticks with that of larvae feeding on hosts infested by larvae only. At PR a significantly higher infection rate (44%, 62/141) was observed among larvae feeding on hosts infested by both immature stages than on hosts infested by larvae only (18.8%, 42/223; Pearson’s chi-square test,  $p<0.001$ ). The difference at SW was not significant (115/459, 25.1% versus 26/149, 17.4%, Pearson’s chi-square test,  $p=0.07$ ). If we compared the infection rate of larvae feeding on *Apodemus* spp. or on *M. glareolus* at both sites, according to the infestation pattern of rodents (with co-feeding nymphs versus without), the difference was significant at PR for *Apodemus* spp. (51.7%, 62/120 versus 15.4%, 25/162; Pearson’s chi-square test,  $p\leq 0.001$ ), and for *M. glareolus* at SW (44.2%, 42/95 versus 15.5%, 11/71; Pearson’s chi-square test,  $p\leq 0.001$ ). However, the difference was not significant for *Apodemus* spp. at SW (20.1%, 73/364 versus 19.2%, 15/78), and a comparison was not possible for *M. glareolus* at PR because none of the captured voles was simultaneously infested by larvae and nymphs.

**Borrelia infection in xenodiagnostic ticks**

A xenodiagnosis performed on a subset of captured rodents (PR:  $n=23$ ; SW:  $n=38$ ) showed that *B. afzelii* (180/209, 86.1%) was clearly the dominant species transmitted from rodents to ticks (Table 4). *B. bavariensis* (6/209, 2.9%) was also observed in xenodiagnostic ticks, but only in ticks from rodents captured at SW (Table 4). More than 60% of rodents captured at both sites (PR: 69.6%, 16/23; SW: 60.5%, 23/38) transmitted spirochetes to 28.1% (81/288) of xenodiagnostic ticks at PR, and 20.8% (128/616) at SW. Rodent infectivity for xenodiagnostic ticks varied between sites and rodent species. At both sites, *M. glareolus* was the most infectious rodent species since 77% of them transmitted infection to 39% of xenodiagnostic ticks. In contrast, *A. sylvaticus* was the less infectious species, and its infectivity was particularly low at SW, where only 28.6% of them transmitted spirochetes to only 6.7% of ticks (Table 4).

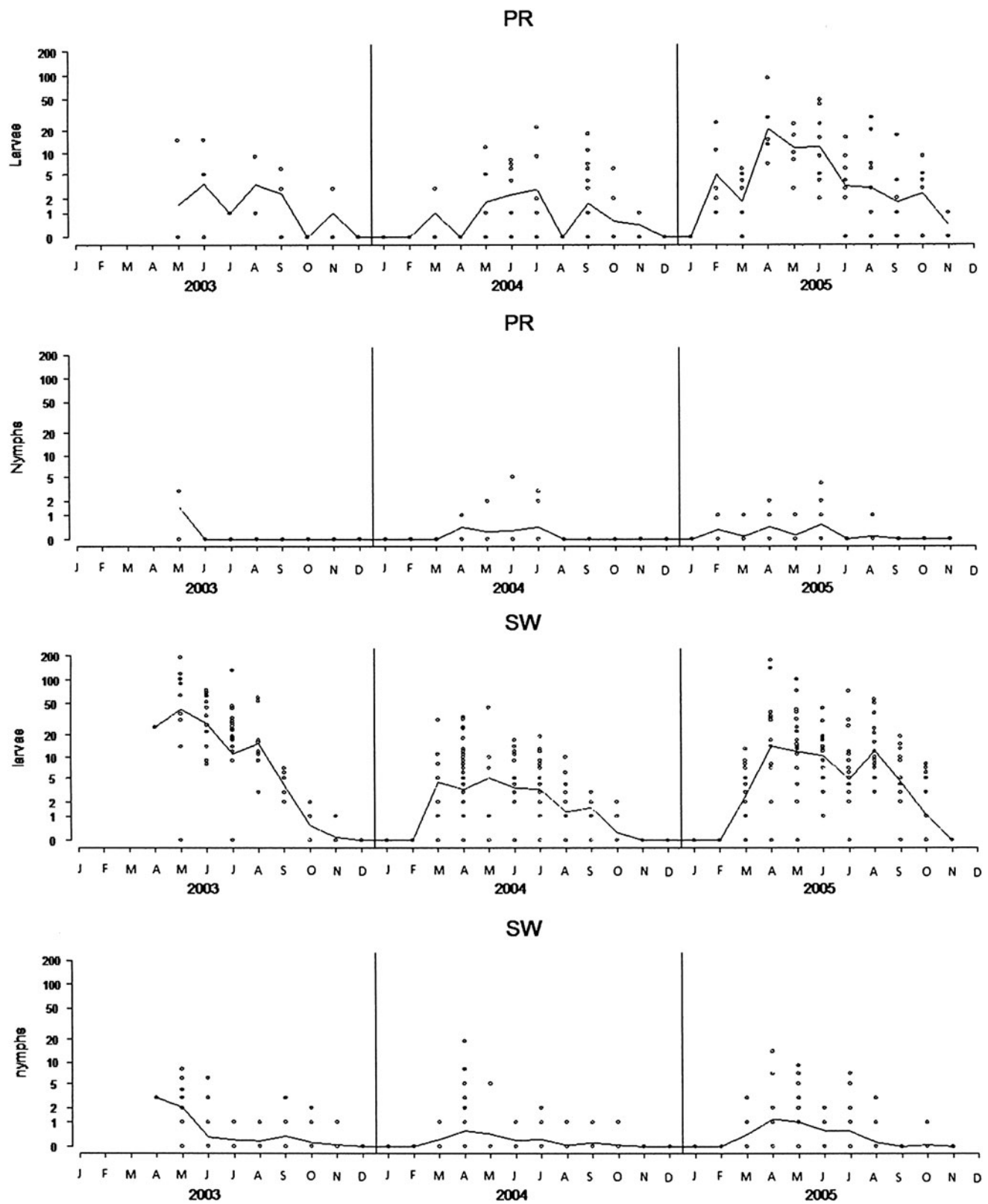


FIG. 2. Annual distribution of the median number of *I. ricinus* immature stages per rodent at Staatswald (SW) and Portes-Rouges (PR). Each circle indicates an individual rodent.

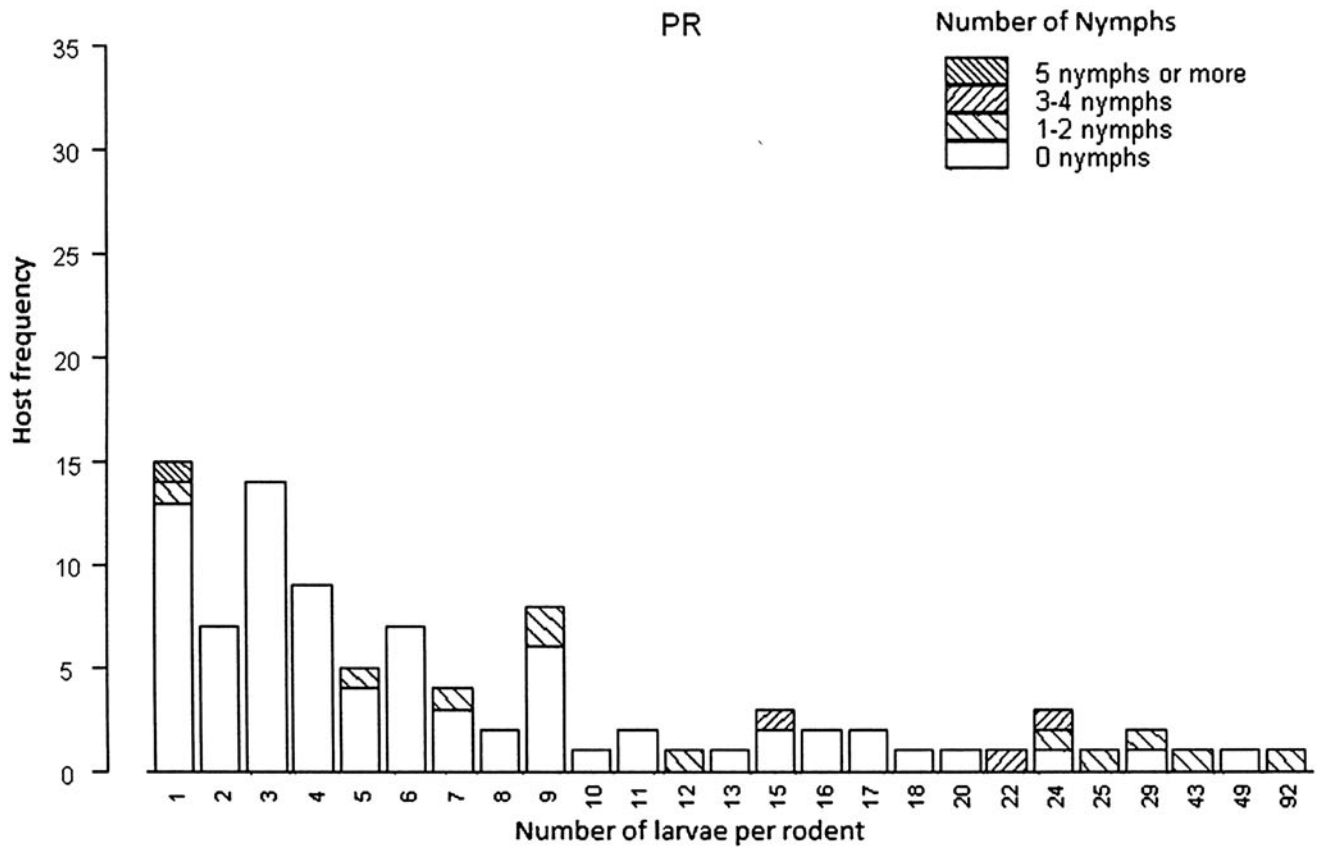
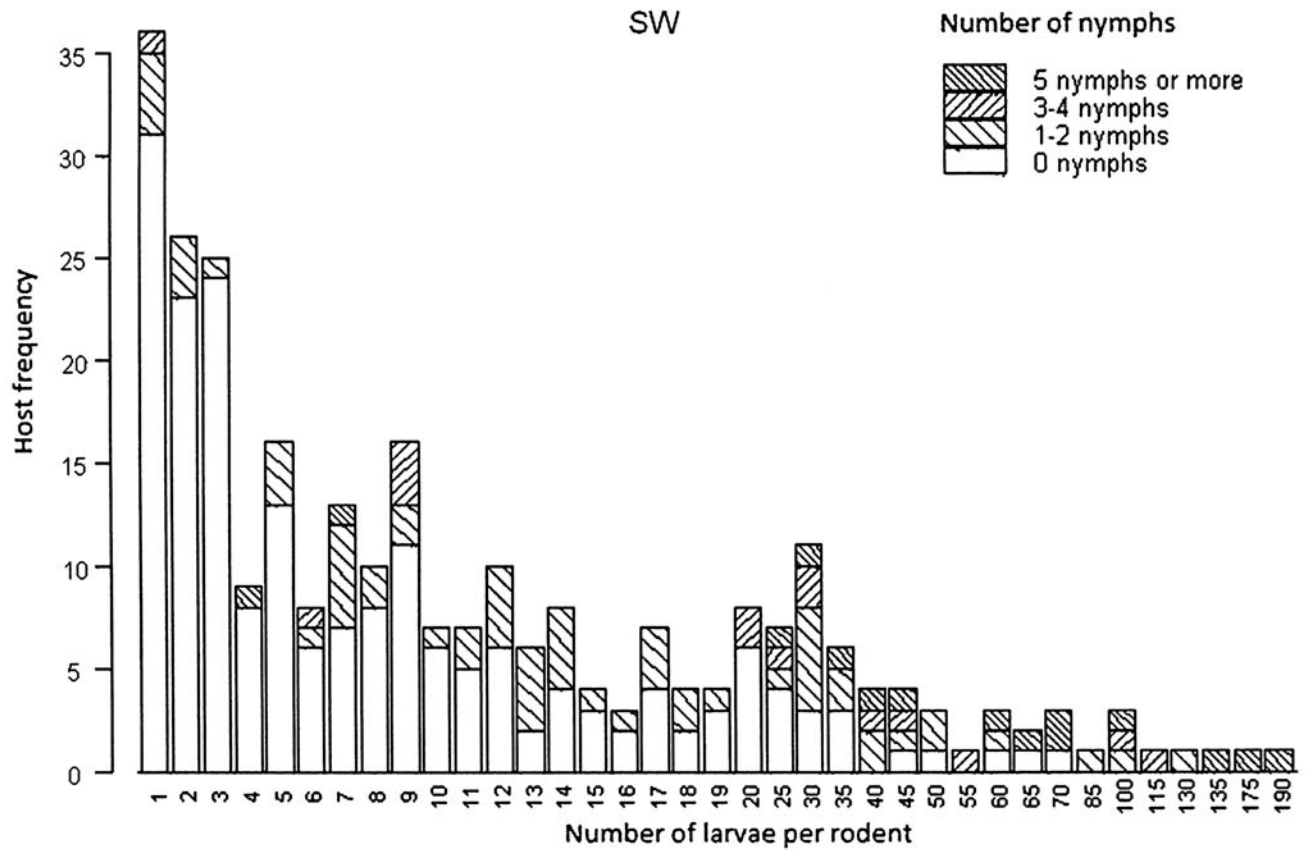


FIG. 3. Coincident aggregated frequency distributions of *I. ricinus* larvae and nymphs on rodents.

TABLE 2. DISTRIBUTION OF *BORRELIA* SPECIES IN QUESTING *Ixodes ricinus* TICKS COLLECTED AT PORTES-ROUGES (PR) AND STAATSWALD (SW)

Site	Stage	No infected/tested ticks (%)	Distribution of <i>Borrelia</i> species (%)					
			BA	BG	BB	BV	Bsp	Mixed
PR	N	267/1497 (17.8)	127 (47.6)	62 (23.2)	43 (16.1)	11 (4.1)	18 (6.8)	6 (2.2)
	A	211/651 (32.4)	71 (33.6)	62 (29.4)	31 (15.2)	28 (13.3)	6 (2.8)	13 (6.2)
	Total	478/2148 (22.3)	198 (41.4)	124 (25.9)	74 (15.5)	39 (8.2)	24 (5)	19 (4)
SW	N	60/576 (10.4)	32 (53.3)	11 (18.3)	3 (5)	3 (5)	10 (16.7)	1 (1.7)
	A	100/404 (24.8)	31 (31)	27 (27)	19 (19)	11 (11)	9 (9)	3 (3)
	Total	160/980 (16.3)	63 (39.4)	38 (23.8)	22 (13.8)	14 (8.7)	19 (11.9)	4 (2.5)

N, nymphs; A, adults; BA, *Borrelia afzelii*; BG, *Borrelia garinii*; BB, *Borrelia burgdorferi* sensu stricto; BV, *Borrelia valaisiana*; Bsp, untyped *Borrelia*.

## Discussion

During this 3-year study, we focused our attention on several parameters related to questing ticks and to infestation of small-sized mammals (*A. flavicollis*, *A. sylvaticus*, and *M. glareolus*) described as reservoirs for *B. burgdorferi* sl in two LB endemic areas located on the Swiss Plateau.

### Questing ticks and ticks naturally infesting rodents

In Switzerland, the seasonal pattern of questing tick density over time (phenology) presents both bimodal (peaks in spring and autumn) and unimodal (peak in spring) patterns (Perret et al. 2000; Jouda et al. 2004a, 2004b; Burri et al. 2007; Morán Cadenas et al. 2007a). In the present study, both profiles were observed. Variability in tick density (CTD) between years was noticed as previously reported in temporal studies on tick density in Europe (Randolph et al. 2002, 2004; Jouda et al. 2004a; Morán Cadenas et al. 2007a; Perret et al. 2000).

Overall, questing nymph density was higher at PR than at SW, contrasting with the intensity of rodent infestation, that was higher at SW. This might be partly explained by differences in vegetation type between the two study sites. In fact, at SW dense vegetation, brambles, fallen trees and branches were present along the sampling transect, differing from the more homogeneous vegetation found at PR. In the inhomogeneous habitat at SW the number of sub-adult questing ticks could have been underestimated because the flag did not penetrate to the leaf-litter surface or to the lowest vegetation

level (Carroll and Schmidtman 1992; Vassallo et al. 1992), which could have biased our data (Burri et al. 2011). Another possible explanation could be that immature ticks have different questing behavior in different areas, for example, questing closer to the ground as suggested by Kurtenbach and associates (2006). This would lead to an increase of contacts between immature tick stages and rodents, and to a decrease in the success of collection of questing ticks by flagging vegetation as observed in the present study.

At both sites, a discrepancy was observed between the seasonal activity patterns of questing ticks and of ticks infesting rodents. In fact, immature ticks feeding on rodents were frequently detected from February to November at both sites, whereas questing tick populations were drastically decreasing in summer. The seasonal pattern of tick infestations of rodents shows that ticks were in fact active for a much greater part of the year than was indicated by flagging the vegetation. According to these observations, monitoring of questing tick activity by flagging vegetation alone does not appear to be a good indicator for the seasonal risk of tick bites in humans. This is corroborated by recent observations in Switzerland describing a contrast between seasonal questing tick activity and the seasonal pattern of tick bites seen in humans, which mostly occur when questing tick density has declined during the summer (Huegli et al. 2009; Federal Office for Public Health, 2010). It remains to be elucidated whether some questing ticks quest lower on vegetation in summer, but are still picked up by humans

TABLE 3. INFECTION PREVALENCE AND GENOSPECIES DISTRIBUTION OF *BORRELIA BURGDORFERI* SENSU LATO IN *I. RICINUS* FEEDING ON DIFFERENT RODENT SPECIES CAPTURED AT PORTES-ROUGES (PR) AND STAATSWALD (SW)

Host species	Site	Hosts		Nymphs			Adults			
		No with infected ticks / tested (%)	No infected/ tested (%)	BA	Bbav	Bsp	No infected/ tested (%)	BA	Bbav	Bsp
<i>A. flavicollis</i>	PR	5/17 (29.4)	43/157 (27.4)	42	0	1	2/2 (100)	1	0	1
	SW	26/40 (65)	80/370 (21.6)	71	1	8	21/47 (44.7)	18	0	3
<i>A. sylvaticus</i>	PR	10/21 (47.6)	47/125 (37.6)	45	0	2	3/6 (50)	3	0	0
	SW	2/6 (33.3)	16/72 (22.2)	16	0	0	9/12 (75)	9	0	0
<i>M. glareolus</i>	PR	7/18 (38.9)	19/102 (18.6)	17	0	2	0/3	0	0	0
	SW	13/28 (46.4)	53/166 (31.9)	53	0	0	3/12 (25)	3	0	0
Total	PR	22/56 (39.3)	109/384 (28.4)	104	0	5	5/11 (45.5)	4	0	1
	SW	41/74 (55.4)	149/608 (24.5)	140	1	8	33/71 (46.5)	30	0	3

BA, *Borrelia afzelii*; Bbav, *Borrelia bavariensis*; Bsp, untyped *Borrelia*.



TABLE 4. INFECTION PREVALENCE AND GENOSPECIES DISTRIBUTION OF *BORRELIA BURGDORFERI* SENSU LATO IN XENODIAGNOSTIC *IXODES RICINUS* TICKS FED ON DIFFERENT RODENT SPECIES CAPTURED AT PORTES-ROUGES (PR) AND STAATSWALD (SW)

Host species	Site	Hosts		Xenodiagnostic ticks		
		No with infected ticks/tested (%)	No infected/tested (%)	BA	Bbav	Bsp
<i>A. flavicollis</i>	PR	4/6 (66.7)	23/75 (30.7)	19	0	4
	SW	14/23 (60.9)	83/389 (21.3)	67	6	10
<i>A. sylvaticus</i>	PR	9/12 (75)	31/157 (19.7)	26	0	5
	SW	2/7 (28.6)	8/120 (6.7)	7	0	1
<i>M. glareolus</i>	PR	3/5 (60)	27/56 (48.2)	26	0	1
	SW	7/8 (87.5)	37/107 (34.6)	35	0	2
Total	PR	16/23 (69.6)	81/288 (28.1)	71	0	10
	SW	23/38 (60.5)	128/616 (20.8)	109	6	13

BA, *Borrelia afzelii*; Bbav, *Borrelia bavariensis*; Bsp, untyped *Borrelia*.

during their activity in tick biotopes. This behavior of *I. ricinus* ticks would be in accord with a study showing that nymphs quest lower on vegetation when desiccation risks are high (Randolph and Storey 1999).

Rodent trapping success was higher at SW (0.30 rodents/trap night) than at PR (0.11 rodents/trap night). The proportion of hosts infested by ticks was similar at both sites, but the infestation load was higher at SW than at PR. On average, more larvae were attached to *Apodemus* mice than to *Myodes* voles, as previously described by other authors (Humair et al. 1993; Kurtenbach et al. 1995; Gray et al. 1999; Hanincova et al. 2003a; Michalik et al. 2005). Nevertheless xenodiagnoses demonstrated that *M. glareolus* was the most infective rodent species at both sites, which corroborates results obtained by Humair and colleagues (1999), although these authors did not screen ticks for *Borrelia* infection after the molt as we did. This is interesting because it is known that ticks fed on voles do not feed or molt successfully due to acquired resistance to ticks (Dizij and Kurtenbach 1995). Nevertheless, our results and those reported by Kurtenbach and associates (1994) show that when ticks attached to voles are analyzed after their molt, vole contribution to the infection of ticks appears to be higher than mouse contribution.

If we compare our data with those reported by Humair and associates (1993) at SW in the early nineties, the larval infestation loads on rodents recorded between the two periods are similar. Nevertheless, during the present study higher infestation levels by nymphs were observed ( $0.54 \pm 1.65$  nymphs/host compared to  $0.2 \pm 0.8$  nymphs/host in the Humair study). Similarly, *Borrelia*-infected ticks were infesting a higher proportion of rodents in the present study (*M. glareolus*: 46.4%, *A. sylvaticus*: 33.3%, and *A. flavicollis*: 65%), compared to data from the Humair study (*M. glareolus*: 25% and *A. flavicollis*: 20%), except for *A. sylvaticus* (43%). Apparently the situation changed at SW, as rodents harbor more nymphs and they carry more infected ticks than they did two decades ago.

The lower tick infestation rate of rodents trapped at PR compared to SW might be explained by differences in questing tick behavior as mentioned above, and by results from previous studies at PR demonstrating the importance of red squirrels (*Sciurus vulgaris*) as hosts for immature ticks (Humair and Gern 1998; Morán Cadenas et al. 2007b). This sug-

gests that at PR sub-adult ticks feed more frequently on squirrels than on rodents. All the more so since a higher prevalence of squirrel-associated species, *B. burgdorferi* ss and *B. afzelii*, was detected in questing nymphs at PR than at SW. However, additional environmental conditions at both sites that were not investigated in the present study might influence the infestation of rodents by immature tick stages.

#### *Borrelia* genospecies

Four *Borrelia* genospecies were detected in questing ticks (*B. afzelii*, *B. burgdorferi* ss, *B. garinii*, and *B. valaisiana*): this large diversity of *Borrelia* genospecies reflects the diversity of hosts (rodents, birds, and medium and large mammals) playing a role at both sites in the tick developmental cycle and in the maintenance of *B. burgdorferi* sl. However, only two genospecies (*B. afzelii* and *B. bavariensis*) were identified in field-derived ticks attached to rodents as well as in xenodiagnostic ticks. This illustrates once again the host specificity displayed by *Borrelia* species, and confirms that rodents transmit *B. afzelii* and *B. bavariensis* to feeding ticks (Hu et al. 1997; Huegli et al. 2002). This contrasts with results obtained by Richter and associates (1999), who observed *B. afzelii*, *B. garinii*, and *B. burgdorferi* ss in ticks that fed on rodents. The most striking feature of these results is the high prevalence of *B. garinii* in *Borrelia*-infected xenodiagnostic ticks that fed on field-derived *Rattus norvegicus* and *A. flavicollis* (52% and 47%, respectively). It remains to be elucidated whether currently these DNA samples would not be identified as *B. bavariensis*, formerly *B. garinii* OspA serotype 4. Interestingly, *B. bavariensis* was observed only at SW, and this observation substantiates the presence of this *Borrelia* species at that site (Hu et al. 2001), although *B. bavariensis* has recently been detected in questing ticks at PR (unpublished data). Our results corroborate the first description of rodents, more specifically *Apodemus* mice, as reservoirs for *B. bavariensis* (Huegli et al. 2002). Currently, *Apodemus* species are the only identified reservoir host for *B. bavariensis*, a *Borrelia* species frequently identified in patients with neuroborreliosis.

#### Co-feeding transmission

In many host-parasite systems, parasites are spatially distributed in clusters on the vertebrate hosts (Shaw et al. 1998).

When infected (usually nymphs) and uninfected (usually larvae) ticks feed simultaneously in close proximity, pathogens may directly pass from infected to uninfected ticks, even in the absence of a systemic infection in hosts. This transmission pathway, which was previously described for Thogoto (Jones et al. 1987) and tick-borne encephalitis (TBE) (Alekseev and Chunikhin 1990; Labuda et al. 1992) viruses, has also been reported for LB spirochetes (Gern and Rais 1996; Hu et al. 2003). During our study, a significant difference was observed between the two sites with regard to the proportion of rodents simultaneously infested by larvae and nymphs. In fact, at SW many more of the hosts (28%) were infested with co-feeding larvae and nymphs compared to PR (12%).

Although co-feeding transmission has been described for LB spirochetes in the laboratory (Gern and Rais 1996; Hu et al. 2003), its role in nature is not well known for *B. burgdorferi* s.l. We observed that at PR larvae feeding simultaneously with nymphs on captured rodents were significantly more often infected than larvae feeding without nymphs. In particular, this was the case for *Apodemus* mice at this site. In contrast, at SW, there was no significant difference when all rodent species were considered. But when each species was examined separately a significant difference was observed for larvae feeding on *M. glareolus*, when they were co-feeding with nymphs, but not on *Apodemus* spp. mice. How can we explain this? First, at SW more larvae infested rodents than at PR, and the percentage of hosts infested by nymphs (29%) was significantly higher than at PR (13%). This indicates that at SW, a higher percentage of hosts were probably already systemically infected, if one assumes that after the bite of infected nymphs the rodents, in particular *Apodemus* mice, remain infectious throughout their lives (Gern et al. 1994). Moreover, it has been reported that the infectivity for ticks of systemically-infected rodents increases with successive infestations by larvae (Gern et al. 1994). Hence, most larvae attached to *Apodemus* mice at SW were probably acquiring infection from rodents that were already infected, even when they were feeding with nymphs. This may explain why at SW larvae attached to *Apodemus* mice did not display a difference in infection rate when they were feeding alone or simultaneously with nymphs. The situation was different for *M. glareolus* at SW or *Apodemus* mice at PR; their infestation level was lower than for *Apodemus* at SW. In fact, at PR *Apodemus* mice had rare contacts with nymphs (globally only 13% of rodents carried nymphs), and at both sites *M. glareolus* displayed the lowest infestation level, with the lowest mean numbers of larvae and of nymphs. The consequence is that persistent or systemic infections were probably less frequent among these individuals. Larvae that were feeding with nymphs on these rodents had a higher likelihood to feed on hosts that were not systemically infected compared to larvae feeding on *Apodemus* mice at SW. In this situation the effect of co-feeding transmission on the larval infection rate is more visible without the “background” created by infection due to persistent or systemic infection. This indicates that the perpetuation of *B. afzelii* depends both on persistent infection in rodents and on co-feeding transmission on hosts, and is modulated by the frequency of contacts between hosts and both larvae and nymphs, which varies among sites.

Here, although more than 28% of rodents at one site were infested by co-feeding larvae and nymphs, no infection by

more than one *Borrelia* species was detected in ticks that fed on these rodents. In fact, only *B. afzelii* was identified in co-feeding ticks. This is interesting because it has been shown previously in the laboratory that other *Borrelia* species can be transmitted by co-feeding ticks, like *B. burgdorferi* ss (Gern and Rais 1996; Hu et al. 2003), *B. garinii*, and *B. valaisiana* (Hu et al. 2003). This indicates that conditions in nature create a situation where only *B. afzelii* is transmitted, probably because of host complement (Kurtenbach et al. 1998). Our observation also suggests that co-feeding transmission on rodents does not contribute to the presence of infections by more than one *Borrelia* species in questing ticks as we observed here, and does not participate in maintaining *Borrelia* genospecies diversity. Nevertheless, we recently described the role of co-feeding in maintaining *Borrelia* diversity at the intra-specific level (Pérez et al. 2011). In fact, we observed that co-feeding transmission contributed in nature to promoting and maintaining *B. afzelii* OspC genotype diversity within local tick populations.

Since co-feeding transmission of TBE virus (TBEV) is important for the maintenance of TBEV in endemic areas (Randolph et al. 1999), it is interesting to note that SW, the site with the highest proportion of hosts infested by co-feeding ticks, has been known as a TBE-endemic area since 1984 (Wyler and Matile 1984), whereas TBE cases have never been reported at PR. As an interesting side note, a prospective study recently suggested that the proportion of rodents with co-feeding ticks might be one of the factors that distinguish a TBEV focus from a non-TBEV focus (Burri et al. 2011).

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#### Author Disclosure Statement

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