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Borrelia burgdorferi in a focus of Lyme borreliosis: epizootiologic contribution of small mammals

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Abstract. The contribution of woodmice (*Apodemus sylvaticus),* yellow-necked mice (*Apodemus flavicollis*) and bank voles (*Clethrionomys glareolus*) was compared in a focus of Lyme borreliosis in Switzerland during a 7 months' study. All three species of mice and one species of shrews (*Sorex araneus*) were shown parasitized by infected *Ixodes ricinus* immatures. About 14% of larvae and 50% of nymphs collected on small mammals were infected with *B. burgdorferi.* Spirochetes were isolated from blood of 3 woodmice and one yellow-necked mouse. The infectious status of rodents was estimated by tick xenodiagnosis. Prevalence of infected rodents ranged from 20% to 44%. Mice presented a higher potential infectivity than voles. The prevalence of infected rodents showed a seasonal variation.

The tick-borne disease, Lyme borreliosis, is caused by the spirochete *Borrelia burgdorferi* Johnson, Schmid, Hyde, Steigenwalt et Brenner (Burgdorfer et al. 1982, Johnson et al. 1984). Hard bodied ticks from the *Ixodes ricinus* complex are the main vectors of this pathogen in the Old and the New World (Burgdorfer 1989). In Europe, *I. ricinus* L. is the primary vector of *B. burgdorferi* (Burgdorfer et. al 1983) and recently the hedgehog tick, *Ixodes hexagonus* Leach, has proved to be efficient in transmitting the borrelial agent of Lyme disease (Gern et al. 1991). The prevalence of *B. burgdorferi* infections in unfed nymphal and adult *I. ricinus* in various endemic regions of Switzerland ranges from 10 to 50% (Aeschlimann et al. 1986, Miserez et al. 1990, Péter 1990). The infection in questing larval *I. ricinus* is lower (3.1%) (Zhioua et al. 1988, Miserez et al. 1990), indicating that the transovarial transmission rarely occurs.

Since the discovery of *B. burgdorferi,* epizootiological studies have demonstrated the importance of reservoir hosts in the maintenance of the spirochete. *B. burgdorferi* has been detected in a variety of mammalian and avian species in North America (Anderson 1988, 1989) and the white-footed mouse (*Peromyscus leucopus* Rafinesque) proved to be the most competent reservoir host (Levine et al. 1985, Donahue et al. 1987, Mather et al. 1989). In Europe, *B. burgdorferi* has been observed in three rodent species - woodmouse (*Apodemus sylvaticus* L.) (Aeschlimann et al. 1986), yellow-necked mouse (*Apodemus flavicollis* Melchior) (Aeschlimann et al. 1986, Hovmark et al. 1988), and bank vole (*Clethrionomys glareolus* Schreber) (Hovmark et al. 1988). Additionally, the capacity of woodmice and yellow-necked mice to serve as reservoirs has been demonstrated in a Lyme disease focus in Switzerland (Aeschlimann et al. 1986).

In a 7 months' study, we examined more precisely the infectivity potential of rodents and the infection status of birds for *B. burgdorferi* in the same enzootic site. We report herein data upon small mammals.

MATERIALS AND METHODS

Study area

Field work was conducted at the Staatswald (Ins, Canton of Berne, altitude: 433 m), a forest on the Swiss Plateau. The study site is a humid woodland established on a productive drained marsh area. Many studies have been conducted on tick biology (Mermod et al. 1973, 1974, 1975, Gigon 1985) and on ticks as vectors of pathogens (Gern 1985, Aeschlimann et al. 1986, Zhioua et al. 1988) in this woodland revealed as a typical biotope for *I. ricinus.* Moreover, the Staatswald has proved to be an area where Lyme borreliosis is endemic (Aeschlimann et al. 1986).

Investigation of small mammals and ticks

Small mammals were live-trapped from April through October 1988, at monthly intervals for two nights and two days consecutively in two capture sites. In site A, where seasonal evolution was studied, 100 traps were placed in a grid-like layout (distance between traps 3 m; identical location during each capture session). In site B, 25 traps were placed in line. Animals were baited with carrots, cereal granules (for rodents) and cat food (for shrews).

Captured animals were brought to the laboratory and held separetely in cages over pans of water for a few days until all ticks had dropped from the hosts. Engorged ticks were collected daily and maintained at 95% humidity and room temperature until moulting was completed. The animals were then anestheti-

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zed, weighed, measured, identified to species and sexed; blood samples were collected and xenodiagnosis was used. Finally, the animals were released at the capture site.

Ticks were examined for *B. burgdorferi* by direct immunofluorescence (DI). When moulted, each live tick was dissected and body contents were spread on a glass slide by means of tweezers. Preparations were dried for a few hours at 33 °C, fixed in acetone for 10 minutes and stored at -20 °C. Tick tissues were overlaid with fluorescein isothiocyanate-conjugated antibody (Peacock et al. 1971, Gern et al. 1991) and incubated in humid chambers for 30 minutes at 33 °C. Slides were rinsed with phosphate buffered saline (pH 7.3), air-dried, mounted with buffered glycerol (pH 7.3) and examined for spirochetes with an Olympus epifluorescence microscope at 400 x.

Isolation of *B. burgdorferi*

Varying amounts of whole blood (0.01 to 0.5 ml) from captured animals were inoculated into tubes containing 4 ml BSK II medium (Barbour 1984). Cultures were incubated at 33 °C and examined by darkfield microscopy for spirochetes at fortnightly intervals for the first 2 months and then monthly up to 8 months post inoculation.

Xenodiagnosis

I. ricinus larvae, derived from a spirochete-free colony maintained for many years, were used for xenodiagnosis. Larvae were fed on trapped rodents anaesthetized with Nembutal (1:9; 0.075 ml/g) (Abbott AG) and caged separately over water pans. Detached replete larvae were collected daily, allowed to moult to the nymphal stage and then examined for spirochetes (see above). Rodents trapped in April, and all of the shrews, were not tested by tick xenodiagnosis.

Statistical analysis

Analyses of the proportions of tick-infested animals and spirochete-infected hosts were performed by Fisher's exact test (significant difference: $p \leq 0.05$). Mean numbers of ticks per host were compared by Student *t*-test (significant difference: $p \leq 0.05$). The Kruskal-Wallis and Mann-Whitney U tests were used to compare medians (significant difference: $p \leq 0.05$). The Mann-Whitney U test may only be performed if the result from Kruskal-Wallis test has proved significant.

RESULTS

Examination of small mammals

Three hundred and forty small mammals were collected from April through October 1988 at the Staatswald (sites A and B). Five species were represented: bank voles (*C. glareolus*; n=148), yellow-necked mice (*A. flavicollis;* n=78), woodmice (*A. sylvaticus;* n=73), common shrews (*Sorex araneus* L.; n=36), *Apodemus* sp. (n=4) and short-tailed vole (*Microtus agrestis* L.; n=1).

Of the captured animals, 316 were examined for ticks. *I. ricinus* represented 99.6% (2267/2276) of the ticks collected (Table 1). The remaining 0.4% comprised 2 larval and 7 adult *I. trianguliceps,* removed from *Apodemus* mice and bank voles. Woodmice and yellow-necked mice were significantly more frequently infested with *I. ricinus* than bank voles (Fischer's exact test: respectively p=0.016 and p=0.006). *I. ricinus* larvae were far more abundant than nymphs. The mean number of larvae collected on *A. flavicollis* and *A. sylvaticus* were significantly higher than that on *C. glareolus* (Student *t*-test: respectively $p < 0.001$ and $p < 0.001$; Mann-Whitney U test: respectively $p < 0.001$ and p=0.005). Mice and bank voles were parasitized by *I. ricinus* nymphs, whereas nymphs were not found on shrews and short-tailed vole. Mean numbers of nymphs per host were low compared to mean numbers of larvae, although a significant number of rodents were infested by the nymphal stage: 16.7% (13/78) of *A. flavicollis,* 14.0% (20/143) of *C.glareolus* and 10.0% (7/70) of *A. sylvaticus.*

The number of animals trapped in site A increased gradually from April to September (Fig. 1). Numbers of juvenile bank voles peaked in May-June and in autumn, whereas juvenile mice mainly peaked in autumn, as determined by analyses of weight. The percentage of small mammals parasitized by *I. ricinus* larvae remained relatively high and constant throughout the season, fluctuating between 77.3% and 97%. The seasonal variation in the number of larvae on small mammals is illustrated in the Fig. 2. We calculated medians in order to reduce the

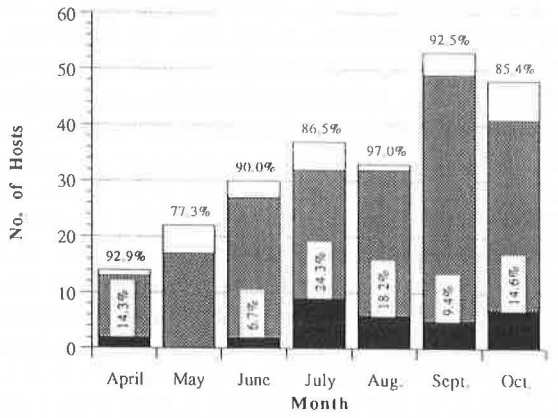

Fig.1. Seasonal distribution of small mammals infested with *Ixodes ricinus* immatures.
Staatswald (site A), Switzerland, April - October 1988
Number of hosts without ticks - white
Number of hosts with larvae only - stippled
Number of hosts with nymphs and larvae - black
Percentages above the columns represent the prevalence of hosts parasitized by larvae and those inside the columns the prevalence of hosts parasitized by nymphs.
Nymphs were always present simultaneously with larvae.

importance of extreme values, which tend to influence means. Medians showed a statistical difference between one another using the Kruskal-Wallis test (p). The Mann-Whitney U test clearly showed that the median number in October was statistically lower than the values from previous months, and the medians in August and September were significantly higher than that in July. Conclusively, larvae attached on rodents were numerous from April to September and the seasonal distribution of larvae presented two peaks: one in May-June and another one in August-September. Rodents were more frequently infested

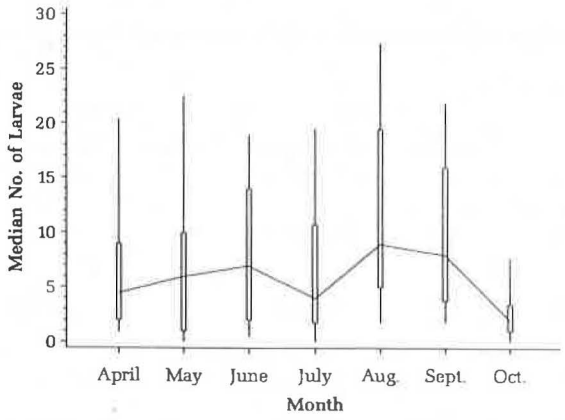

Fig.2. Seasonal distribution of the median number of *Ixodes ricinus* larvae per host.
median number of larvae per host –
delimits the percentiles 25 and 75 □
delimits the percentiles 10 and 90 |

with *I. ricinus* nymphs in July and August (Fig. 1). Hosts were generally parasitized by a single nymph. However, a few individuals harbouring 2 or 3 nymphs were observed in July and the following months. Monthly means of nymphs per host were low: 0.2 (April), 0.0 (May), 0.1 (June), 0.3 (July), 0.2 (August), 0.1 (September) and 0.2 (October).

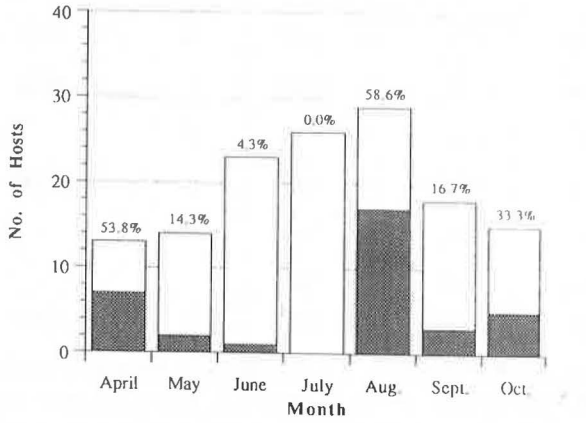

Fig. 3. Seasonal distribution of small mammals parasitized by infected *Ixodes ricinus* larvae.
Number of hosts without infected larvae - white
Number of hosts with infected larvae - stippled
Percentages above the columns represent the prevalence of hosts with infected larvae.

Infection and infectivity potential

About 14% of larval and 50% of nymphal *I. ricinus* were infected with spirochetes (Table 2). Four species of small mammals harboured spirochete-infected *I. ricinus* immatures: yellow-necked mice, woodmice, bank voles and shrews. The proportion of *A. sylvaticus* parasitized by infected *I. ricinus* was statistically higher than that of *A. flavicollis* (p=0.037). The seasonal distribution of small mammals parasitized by infected *I. ricinus* larvae was uneven (Fig. 3). A greater proportion of micromammals captured in August and April was shown to harbour infected ticks than did those captured in June and July. Neither of the *I. tranguliceps* larvae tested (n=2) were found infected with spirochetes, even if the individuals had fed on spirochetemic hosts.

Of the 141 animals examined by xenodiagnosis, 49 (34.8%) were infected and transmitted spirochetes to larval *I. ricinus*. The spirochetes were identified as *B. burgdorferi* using DI with monoclonal antibody H5332. Yellow-necked mice proved to be more frequently infective (24/54; 44.4%) than woodmice (16/43; 37.2%) and bank voles (9/44; 20.5%). Thus, prevalences of infection in hosts were comparable to percentages of hosts with infected ticks (Table 2), except for *A. flavicollis* which appeared to be more infective using xenodiagnosis.

The number of infective rodents, detected by xenodiagnosis, showed seasonal variations that confirmed the seasonal distribution presented in Fig. 3. Infective hosts were more numerous from August.

Spirochetes were isolated from 3 woodmice and one yellow-necked mouse, all of which were captured in August. All isolates, detected 10 days after inoculation of culture medium, were motionless and grew slowly in comparison with other isolates maintained in our laboratory. The four haemocultures were contaminated and could not be purified. Therefore, characterization with poly - and monoclonal antibodies was unsuccessful. However, two of four spirochetemic rodents were shown to be infected by *B. burgdorferi* using xenodiagnosis.

DISCUSSION

The results confirm that the Staatswald woodland is a typical biotope for *I. ricinus* (Mermod et. al. 1973, Gigon 1985). The five micromammalian species trapped *(A. flavicollis, A. sylvaticus, C. glareolus, M. agrestis,* and *S. araneus)* were all parasitized by *I. ricinus* subadults. The larval stage feeds more readily on micromammals than does the nymphal stage (Aeschlimann 1972, Mermod et al. 1973, Gern 1985, Matuschka et al. 1991). According to our observations, 40 times more *I. ricinus* larvae feed on small mammals than do nymphs, whereas rodents infested with larvae are only 6-7 times as numerous as those parasitized by nymphs. The clumped distribution in the biotope and the questing position near to the ground characterizing *I. ricinus* larvae explain the heavy and frequent infestation of rodents and shrews with larvae, in contrast to the random distribution and the higher questing place of nymphs (Mermod et al. 1973, Gigon 1985). Nevertheless, considering the high infection rate of unfed nymphs in the study area (30%) (Aeschlimann et al. 1986), the infestation of rodents with nymphs is sufficiently important to transmit the spirochete to hosts. Both prevalence and intensity of tick infestation of mice and bank voles were comparable to those reported in previous studies in the same area (Mermod et al. 1973, 1974, Gern 1985).

Table 1. Small mammals infested with larval and nymphal *Ixodes ricinus*. Staatswald (sites A & B), Switzerland, April - October 1988.

Host species	Number of hosts examined	Number of hosts infested (%)		Larvae		Nymphs	
				Number	Mean number per host ± S.D.	Number	Mean number per host ± S.D.
Apodemus flavicollis (Yellow-necked mouse)	78	71	**(91.0)**	1049	**13.4±19.8**	26	**0.3±1.2**
Apodemus sylvaticus (Woodmouse)	70	65	**(92.9)**	632	**9.0±10.2**	15	**0.2±0.8**
Apodemus sp.	4	4	**(100)**	18	**4.5±3.0**	0	-
Clethrionomys glareolus (Bank vole)	143	111	**(77.6)**	739	**5.2±6.2**	24	**0.2±0.5**
Microtus agrestis (Short-tailed vole)	1	1	**(100)**	27		0	-
Sorex araneus (Common shrew)	20	16	**(80.0)**	137	**6.9±6.5**	0	-
	316	268	**(84.8)**	2602	**8.2±12.3**	65	**0.2±0.8**

Table 2. Prevalence of small mammals parasitized by spirochete-infected immature *Ixodes ricinus*.

Host species	Number of hosts examined	Number of hosts with infected ticks (%)		Larvae			Nymphs		
				Number examined	Number infected (%)		Number examined	Number infected (%)	
Apodemus flavicollis	50	10	**(20.0)**	596	48	**(8.1)**	10	7	**(70.0)**
Apodemus sylvaticus	39	17	**(43.6)**	378	91	**(24.1)**	3	2	**(66.7)**
Apodemus sp.	2	0	**(0)**	8	0	**(0)**	0		
Clethrionomys glareolus	75	19	**(25.3)**	372	54	**(14.5)**	13	4	**(30.8)**
Microtus agrestis	1	0	**(0)**	23	0	**(0)**	0		
Sorex araneus	16	2	**(12.5)**	109	11	**(10.1)**	0		
	183	268	**(84.8)**	1486	204	**(13.7)**	26	13	**(50.0)**

Three-year long studies (Mermod et al. 1973, 1974, 1975, Gern 1985) have shown that the infestation of rodents by *I. ricinus* ticks is prominent from April to October. Thus, we limited our study to the time of maximal tick activity. In 1988 in the study site, the peak larval activity on small mammals occurred in May-June and more intensively in August-September. A similar bimodal activity pattern has been observed previously (Mermod et al. 1973, 1974, 1975, Gern 1985). The nymphal abundance was maximal in July during the estival depression of larvae; micromammal hosts parasitized by nymphal *I. ricinus* were more numerous at that time than during the previous months. The relative number of hosts infested with nymphs and the mean number of nymphs collected from rodents in April, before the nymphal depression in May, suggest that there was an initial peak of nymphs in April. Larval abundance on small mammals was bimodal and the maximal observed abundance of nymphs preceded the second peak of larvae. Thus, the inverted pattern of seasonal abundance of subadult ticks (nymphs feeding before larvae) on small mammals occurred once, and possibly twice. This inversion is different from that existing in North America with *I. dammini* Spielman, Clifford, Piesman et Corwin: the unimodal peak of abundance of *I. dammini* immatures is seasonally more limited and the inversion is more pronounced, as nymphs occur about 2 months before larvae (Wilson and Spielman 1985).

Borrelial infection was detected in both larval and nymphal *I. ricinus*. The infection rate of engorged larvae was significantly greater than that of starved larvae (3.1%) (Zhioua et al. 1988, Miserez et al. 1990) indicating that rodents (*A. flavicollis, A. sylvaticus, C. glareolus)* are infective for the ticks. This conclusion was supported by the results of xenodiagnosis. Thus, previous observations (Aeschlimann et al. 1986) about the reservoir role of mice are confirmed and the implication of voles is now clarified. However, the status of *S. araneus* is still unclear. Tick xenodiagnosis appears to be a sensitive method for

detecting Lyme borreliosis spirochetes in hosts and the only safe procedure to determine the status of a host as infective host.

The epizootiologic picture of Lyme borreliosis in the Old World shows notable differences from that in the New World. One species, *P. leucopus,* is considered to act as the primary competent reservoir of *B. burgdorferi* in eastern North America (Levine et al. 1985, Donahue et al. 1987, Mather et al. 1989), whereas in Europe several rodent species are infective for ticks. Even if the analogous species, *A. flavicollis* and *A. sylvaticus,* proved to have the greatest infectivity potential in Europe, the contribution of voles, *C. glareolus,* is not negligible in the maintenance of the Lyme disease spirochete. Rodents play a role as amplifying hosts, since one infective rodent can give rise to a large number of infected larvae. This amplifying role, vital to the maintenance of *B. burgdorferi* in nature, appeared to be seasonal in this study. Rodents were mainly infective in late summer after the peak abundance of nymphs. Rodents became infected with *B. burgdorferi* in midsummer, when *I. ricinus* nymphs fed actively on these hosts and, after a prepatent period, were infective to *I. ricinus* larvae during the second larval peak abundance. The decline in numbers of infective rodents in early fall was due to the peak appearance of young *Apodemus* in the rodent population. The increase observed in mid-autumn may be explained by the fact that *I. ricinus* nymphs were still present in sufficient numbers to ensure the infection of young mice. The amplifying role was also important in early spring. Field collected rodents infected with *B. burgdorferi* may remain infective for ticks for 7 months (Donahue et al. 1987) and even for more than 40 months (Gern et al., submitted). Thus, rodents appear to maintain *B. burgdorferi* for life suggesting that the rodents are reservoirs. Small mammals, that acquire *B. burgdorferi* from nymphal tick bites, may harbour spirochetes throughout the winter and be infective for larval *I. ricinus* in the following spring. The decline in infection rates in late spring-early summer was probably due to mortality of older rodents, that have overwintered, and the appearance of new broods (Southern 1964), that were not immediately exposed to infected nymphs.

In conclusion, *B. burgdorferi* appears to be maintained in nature primarily in a transmission cycle involving larvae, nymphs and reservoir hosts. Transovarial transmission occurs, but at a too low level to maintain *B. burgdorferi* solely in a *I. ricinus* population. Nymphs are the "motor" role of the transmission cycle, as they transmit *B. burgdorferi* to the small mammals. One nymphal infectious bite is sufficient to transmit infection to mice (Donahue et al. 1978). Once infected, the rodent reservoir plays a role as an amplifier, as it infects a great number of ticks, and plays a maintaining role, as spirochetes persist in the host for a long period of time. The amplifying role is seasonal and this seasonality is probably influenced by the dynamics of populations of larvae, nymphs and rodents in the focus. Further investigations are needed to prove this assumption. Additionally, the infectivity potential of other hosts for *I. ricinus* immatures should be studied in order to complete the epizootiological picture of *B. burgdorferi* maintenance in a focus of Lyme borreliosis.

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