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Ixodes ricinus Density, and Distribution and Prevalence of *Borrelia burgdorferi* Sensu Lato Infection Along an Altitudinal Gradient

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ABSTRACT In this study, we measured the phenology of *Ixodes ricinus* ticks and their infection with *Borrelia burgdorferi* sensu lato (sl) simultaneously along an altitudinal gradient to assess the impact of climate on the phenology of ticks and on their infection with *B. burgdorferi* sl. From 1999 to 2001, free-living *I. ricinus* ticks were collected monthly by flagging vegetation at three different altitudes (620, 740, and 900 m above sea level) on the slope of a mountain in Chaumont (Neuchâtel, Switzerland). *I. ricinus* ticks were examined for the presence of *B. burgdorferi* sl by using direct fluorescent antibody assay and isolation of spirochetes. *Borrelia* species were characterized by polymerase chain reaction followed by restriction fragment-length polymorphism. Tick density and tick phenology varied with altitude. Although the peak tick density decreased and the onset of ticks was delayed with altitude, the phenology was much more stable among years at the highest altitudes than at the lowest. The prevalence of *B. burgdorferi* infection in nymphs and adults decreased with altitude. The prevalence of infection differed significantly among years, and it was significantly higher in adults (30%) than in nymphs (21%). *B. burgdorferi* infection in adults was positively related with adult density, but this was not observed for nymphs. Five *B. burgdorferi* sl genospecies were successfully isolated: *B. garinii*, *B. burgdorferi* sensu stricto, *B. afzelii*, *B. valaisiana*, and *B. lusitaniae*. Mixed infections were obtained from five of 140 infected ticks. The greatest diversity in *Borrelia* species was observed at the lowest altitude where all five *Borrelia* species were present, whereas at the two highest altitudes, *B. lusitaniae* was not observed.

KEY WORDS *Ixodes ricinus*, tick density, climate, *Borrelia burgdorferi* sensu lato, phenology

IN EUROPE, THE MAIN VECTOR of *Borrelia burgdorferi* sensu lato (sl), a complex of *Borrelia* species that includes the agent of Lyme borreliosis, is *Ixodes ricinus* (Burgdorfer et al. 1983). This tick is broadly distributed from north Africa to Scandinavia and from Ireland to central Russia (Gern and Humair 2002) and has been described up to an altitude of 1,450 m in Switzerland (Cotty et al. 1986). The annual pattern of the questing tick density of this tick changes widely over its geographic distribution (Korenberg 2000) and also in the same area according to year as shown for example in Ireland (Gray 1984), in the United Kingdom (Randolph et al. 2002), and in Switzerland (Perret et al. 2000).

B. burgdorferi sl is genetically heterogeneous. Five different *Borrelia* genospecies have been found associated with *I. ricinus: B. garinii* (Baranton et al. 1992), *B. burgdorferi* ss (Johnson et al. 1984), *B. afzelii* (Canica et al. 1993), *B. valaisiana* (Wang et al. 1997), and *B. lusitaniae* (Le Fleche et al. 1997). In Europe, *B. burgdorferi* sl has been described in 26 countries (Hubalek and Halouzka 1997) with an average prevalence of *Borrelia* infection of 10.8% in nymphs and 17.4% in adults (Hubalek and Halouzka 1998). In Switzerland, *Borrelia* infection in *I. ricinus* ticks collected in various areas varies between 5 and 47.5% (Aeschlimann et al. 1986, Péter et al. 1995).

The impact of climate on *I. ricinus* ticks in the context of global climate change is a matter of debate. Although the distribution of *I. ricinus* has been shown to extend northwards and westwards within Sweden in relation with climate-warming (Tälleklint and Jaenson 1998, Lindgren et al. 2000), the impact of climate changes in central Europe where *I. ricinus* is present may rather affect the tick patterns of seasonal dynamics (Randolph 2001, Randolph et al. 2002). Climate may also affect tick abundance over 1 yr and its evolution over the year. Although tick abundance over 1 yr can be expressed as a single number (cumulative tick density, Eisen et al. (2003), the evolution of questing tick density is a periodic function that we define here as tick phenology. Tick phenology is characterized by annual cyclic events such as onset, peak(s), and fall of questing tick density.

As temperature globally decreases with altitude, studying phenology of ticks within a short horizontal distance on the slope of a mountain is like studying phenology of ticks under different climatic scenarios.

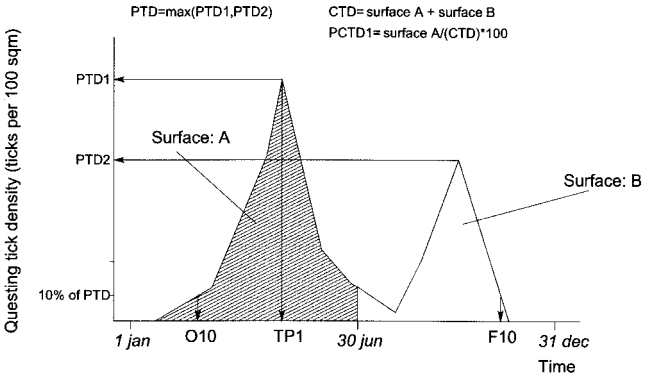

Fig. 1. Descriptive statistics (see text) for questing tick density time plots (TP1, time of the spring peak; PCTD1, proportion of activity in the first half-year.

Therefore, in this study, we measured the phenology of ticks and their infection with *Borrelia* spp. simultaneously along an altitudinal gradient to assess the impact of climate on the phenology of ticks and on their infection with *B. burgdorferi* sl.

Materials and Methods

Collection of Ticks. Free-living ticks were collected every month, during 3 yr from March 1999 to November 2001, by flagging a 1-m^2 cotton cloth on the low vegetation. The surface flagged by month and site was a 150-m^2 transect. The study site is a mixed forest (deciduous dominant) situated on the slope of a mountain (Chaumont) north of Neuchâtel (Switzerland) ranging from 500 to 1000 m in altitude with a southern exposition. Ticks were collected at three different altitudes: Chaumont high (H) (900 m), Chaumont middle (M) (740 m), and Chaumont low (L) (620 m). Different tick developmental stages and sexes were maintained separately in tubes containing grass until species determination and examination for *Borrelia* infection. Tubes were maintained in the laboratory at room temperature and *Borrelia* examination took place 2–3 d after tick collection.

Air temperature was recorded continuously at both ends of the altitudinal gradient by two Meteosuisse automatic weather stations: "Chaumont" located at 1,073 m above sea level, ≈1 km from the Chaumont High sampling location, and "Neuchâtel" at 487 m above sea level, located ≈1 km from Chaumont Low sampling location. Data were published by Wuetrich (2000), Wuetrich and Jornod (2001), and Baniewicz (2002). To estimate the annual average temperature at our sampling sites, we assumed that air temperature decreased linearly as altitude increases along the slope.

Questing tick density was expressed as the number of ticks found per 100 m^2. An annual value for tick density called cumulated tick density (CTD) was obtained by integrating the linearly interpolated curve of questing tick density over 1 yr (Eisen et al. 2003) (Fig. 1). The maximal tick density over 1 yr was called the peak tick density (PTD; Fig. 1). The date of onset of significant questing tick activity in spring was calculated from the questing tick density curve as the date when tick density exceeded 10% of the peak tick density (O10; Fig. 1) (Eisen et al. 2002). Similarly, the end of tick activity was calculated as the last date when tick density decreased below 10% of the peak tick density (F10; Fig. 1). Nymphs and adults were treated separately within all calculations.

We calculated the density of infected ticks (nymphs and adults) per 100 m^2 when tick density was maximal (PTDi) for each year and for each altitude by multiplying PTD by the percentage of infected ticks.

Borrelia Infection and Isolations. Each *I. ricinus* adult tick collected and 10 nymphs were examined every month by direct immunofluorescence (IF) and cultivation. Ticks were dropped in 70% ethanol and cut into two pieces, one-half was examined by IF and the other half was used for *B. burgdorferi* sl isolation as described previously (Gern et al. 1999). To test the effect of cutting ticks into two pieces and of examining only halved tick by IF, 10 additional nymphs were examined each month by IF only.

For IF, one-half or the entire tick was spread on a glass slide, dried overnight at 37°C, and fixed in acetone for 10 min. Fluorescein isothiocyanate-conjugate that was prepared from a pool of Lyme borreliosis patient sera, which detects all *Borrelia* species, was used (Gern et al. 1999). Slides were incubated in humid chambers for 30 min at 37°C and were examined for *Borrelia* by fluorescence microscopy. Spirochetes were counted per one-half tick, and infection degree was classified into three categories: low, 1–50 spirochetes; medium, 50–500 spirochetes; and high, >500 spirochetes.

The other half of the tick was individually transferred into culture tubes containing BSKII medium (Sinsky and Piesman 1989). Culture medium was examined by dark-field microscopy for *Borrelia* after 1 wk of incubation at 34°C and then every week for 1 mo.

Polymerase Chain Reaction (PCR) and Restriction Fragment-Length Polymorphism. *Borrelia* species infecting *I. ricinus* ticks placed in culture tubes were identified by PCR followed by restriction fragment-

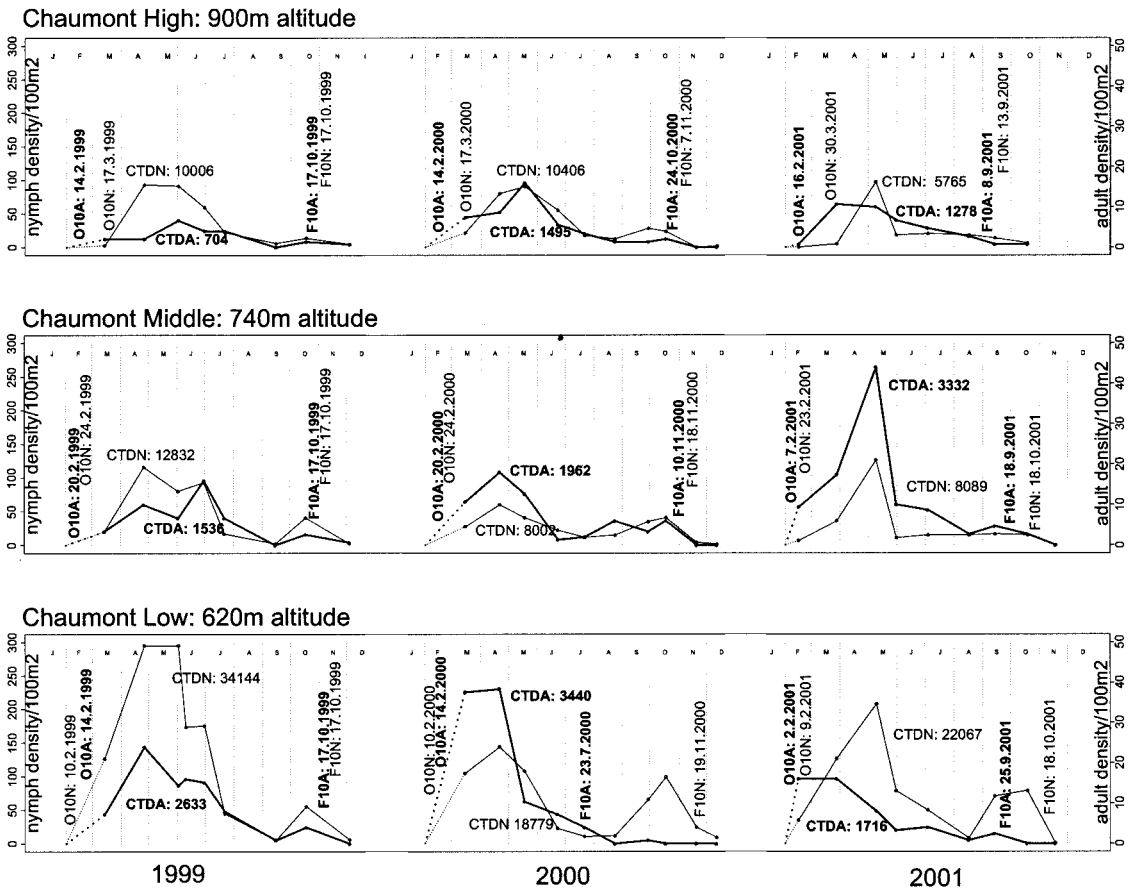

Fig. 2. Questing *I. ricinus* density (thin line, nymphs; bold line, adults) at three altitudes on the Chaumont Mountain from 1999 to 2001. CTD (ticks/100 square meters per year; CTDN, nymphs; CTDA, adults). O10N, nymphs; O10A, adults. See text for description of other abbreviations,

length polymorphism according to Postic et al. (1994). Primers used to amplify the variable spacer region between two repeated genes encoding for ribosomal 23S and 5S were primer 1 (5′-CTGCGAGTTCGCGG-GAGA-3′) and primer 2 (5′-TCCTAGGCATTCAC-CATA-3′). Each amplification reaction was carried out for 35 cycles with the following modification: denaturation was performed for 1 min at 94°C, annealing at 55°C for 1 min, and extension at 72°C for 1 min. Negative and positive controls (DNA from *B. burgdorferi* ss, *B. garinii*, *B. afzelii*, *B. valaisiana*, and *B. lusitaniae*) were included in each run. PCR products were digested with *Mse*I restriction endonuclease.

Statistical Analysis. The relationship between PTD, CTD, O10, F10 (Fig. 1), and altitude (considered as an ordered factor) was evaluated with the Jonckheere test (Siegel and Castellan 1988). The relationship between the variance of maximum tick density and altitude was evaluated by linear regression with altitude expressed in meters above sea level.

The relationship between altitude and tick infection rates with *B. burgdorferi* sl or altitude and the degree of infection in ticks was calculated by logistic regressions. In these regressions, altitude was considered as an ordered factor, whereas the year was considered as a regular factor. Infection rates between nymphs and adults were compared using Fisher's exact test. Logistic regressions and variance analysis were calculated with Splus six from Windows (Insightful Corporation, Seattle, WA). All other statistics were calculated with R for Linux version 0.90.0 (Ikaha and Gentleman 1996).

Results

Questing tick density expressed as the CTD (the integral over 1 yr of the questing tick density) decreased with altitude for both nymphs and adults (Fig. 2; Jonckheere test: $n = 9$, $P = 0.017$ for nymphs and $P = 0.0098$ for adults). The phenology of ticks also varied with altitude (Fig. 2). The onset of tick activity in spring (O10) was inversely related to altitude, especially in nymphs (Jonckheere test: $n = 9$, $P = 0.007$ for nymphs and $P = 0.0590$ for adults), whereas the end of tick activity (F10) was not related to altitude

Table 1. Annual average temperatures recorded in Neuchâtel and at the top of the Chaumont mountain, and calculated values for our sampling sites assuming a linear temperature gradient

Location	Altitude (meters above sea level)	Annual mean temperature (°C)			
		1999	2000	2001	1999–2001
Chaumont*	1,073	6.30	6.80	6.10	6.40
Neuchâtel*	487	10.20	11.10	10.60	10.63
Chaumont High**	900	7.45	8.07	7.43	7.65
Chaumont Middle**	740	8.52	9.24	8.66	8.81
Chaumont Low**	620	9.31	10.12	9.58	9.67

* Recorded values.
** Calculated values assuming a linear temperature gradient.

(Jonckheere test: $n = 9$; $P = 0.85$ for nymphs and $P = 0.25$ for adults). The density of ticks reached a main spring peak in April to May. The time of the peak did not vary with altitude (Jonckheere test: $n = 9, P = 0.18$ for nymphs and $P = 0.13$ for adults). However, the peak tick density decreased with increasing altitude (Jonckheere test: $n = 9, P = 0.0098$ for nymphs and $P = 0.035$ for adults). A noticeable exception occurred in 2001 when the highest density of adults was recorded at the medium altitude. Variance among years of the questing nymph density during the spring peak was linearly and inversely related to altitude (F test, $P = 0.0135$).

A secondary peak of questing nymphs was sometimes observed in autumn. The nymphal density during this autumn peak (PTD2) decreased with increasing altitude (Jonckheere test: $n = 9, P = 0.005$). During 2000, the autumn peak of nymphs at the two lowest altitudes reached 70% of the spring peak (PTD1). Autumn peaks for adult ticks were not very important at any altitude.

Air temperature was recorded at both ends of the altitudinal gradient by automatic weather stations (Table 1). The average annual temperature from 1999 to 2001 decreased by 4.23°C between 487- and 1,073-m altitude. This means a temperature gradient of 0.72°C per 100 m. By assuming a linear temperature gradient along the slope of the Chaumont mountain, we can estimate the average annual temperature at our sampling sites (Table 1). The temperatures recorded in Neuchâtel from 1999 to 2001 were higher than the average annual temperature calculated over the 20th century, which was 9.4°C (Baniewicz 2002). The warmest year of all 3 yr studied was 2000 when the autumn peak of nymphs also reached its maximum value.

Because parts of nymphs were examined for *B. burgdorferi* sl infection by IF alone and other nymphs were examined by IF and isolation, we compared infection rates in both groups of ticks and found no significant difference for ticks examined by IF and culture (130/608, 21%), and for ticks examined by IF alone (96/504, 19%, Fisher's test, $P = 0.369$). Therefore, IF results for all nymphs, examined by IF alone or by IF and culture, were pooled.

A total of 1,619 *I. ricinus* ticks (1,112 nymphs and 507 adults) was examined for *Borrelia* infection (Table 2). The overall prevalence of *B. burgdorferi* sl in these ticks was 24% (382/1619) (IF and/or isolation). Adults were significantly more infected (30%) than nymphs (21%, Fisher's test, $P < 0.0001$). No seasonal variations in the infection prevalence could be found at any altitude (data not shown). Nymphal and adult infection prevalence with *Borrelia* sl decreased with increasing altitude and varied among years (Table 2, logistic regression for nymphs: $t = 2.354$, $P = 0.01$ for altitude, and $t = 4285$, $P < 0.001$ for year; logistic regression for adults: $t = 2.122$, $P = 0.034$ for altitude and $t = 2.307$, $P = 0.022$ for year).

Because in 2001 more adults were found in Chaumont M than at the two other altitudes, we tested whether the adult infection prevalence was related to the adult density. Indeed, we found a significant positive relation between adult density and infection prevalence (logistic regression with monthly density and monthly prevalence: $t = 2.465$, $P = 0.0137$).

During the 3-yr study, the peak tick density of infected ticks (PTDi) varied from 7.4 to 68.6 infected nymphs per 100 m^2 and from 1.4 to 16.7 infected adults per 100 m^2 (Table 3). PTDi for nymphs decreased with increasing altitude (Table 3, variance analysis: df = 2, $F = 14.497$, $P = 0.0146$ (model coefficient of altitude, L = 20.6, M = −6.04, H = −14.6), and slightly varied among years (Table 3, variance analysis: df = 2, $F = 4.737$, $P = 0.0881$), with a higher density of infected nymphs in 2001 than in 1999 and 2000 (Table 3). In

Table 2. Proportion of *B. burgdorferi* infected *I. ricinus* ticks at three different altitudes on the slope of the Chaumont Mountain from 1999 to 2001

Altitudes	Proportion of infected nymphs (%)				Proportion of infected adults (%)			
	1999	2000	2001	Total	1999	2000	2001	Total
H (900 m)	9/109 (8)	24/130 (18)	19/115 (17)	52/354 (15)	2/10 (20)	12/43 (28)	13/55 (24)	27/108 (25)
M (740 m)	17/112 (15)	28/130 (22)	41/145 (28)	86/387 (22)	2/10 (20)	17/60 (28)	64/149 (43)	83/219 (38)
L (620 m)	17/124 (14)	27/108 (25)	46/139 (33)	90/371 (21)	3/9 (33)	21/110 (19)	20/61 (33)	44/180 (24)
Total	43/345 (12)	79/368 (21)	106/399 (27)	228/1112 (21)	7/29 (24)	50/213 (23)	97/265 (37)	154/507 (30)

Table 3. Peak tick density of infected *I. ricinus* nymphs and adults at three different altitudes on the slope of the Chaumont Mountain from 1999 to 2001

Altitudes	1999		2000		2001	
	PTDi Nymphs*	PTDi Adults**	PTDi Nymphs*	PTDi Adults**	PTDi Nymphs*	PTDi Adults**
H (900 m)	7.4	1.4	16.38	4.48	16.49	2.75
M (740 m)	17.4	3.2	13.2	5.04	35.28	16.72
L (620 m)	41.3	7.92	36	7.22	68.64	4.8

* Expressed as the number of infected nymphs per 100 m^2.
** Expressed as the number of infected adults per 100 m^2.

adults, the fluctuations among years and altitudes were not very important (Table 3, variance analysis: df = 2, $F = 0.5023$, $P = 0.638$ for years and df = 2, $F = 0.5023$, $P = 0.446$ for altitudes).

A total of 120 *Borrelia* isolates were obtained from 1,115 ticks (11%) placed in culture tubes. PCR/restriction fragment-length polymorphism revealed five genospecies of *B. burgdorferi* sl. Because half-ticks were examined both by IF and culture, we amplified *Borrelia* DNA from 20 ungrown cultures of 70 ticks, collected in 2001, found infected by IF. These ungrown cultures contained DNA from *B. garinii* ($n = 5$), *B. afzelii* ($n = 11$), *B. valaisiana* ($n = 2$), and *B. burgdorferi* ss ($n = 2$).

Globally, *Borrelia* characterization by restriction fragment-length polymorphism revealed *Borrelia* species in 140 ticks (Table 4): *B. garinii* was the most frequent species in nymphs (61%) and adults (53%) at all three altitudes. *B. lusitaniae* was isolated only from three females (two females had mixed infection, see below) collected at Chaumont L. No *B. lusitaniae* isolates were obtained from nymphs, and no *B. lusitaniae* were obtained at the two highest altitudes.

Mixed infections were identified at Chaumont L ($n = 3$), at Chaumont M ($n = 1$), and at Chaumont H ($n = 1$). Two mixed infections were obtained from nymphs at Chaumont H and L (*B. garinii* and *B. valaisiana*), and three mixed infections were observed in adults: two at Chaumont L (*B. valaisiana* and *B. lusitaniae*, and *B. garinii* and *B. lusitaniae*), and one at Chaumont M (*B. afzelii* and *B. burgdorferi* ss).

Spirochete numbers were estimated in all 348 ticks found infected by IF. Most ticks, 78%, ($n = 271$) were weakly infected (<50 spirochetes), whereas 16% ($n = 55$) were moderately infected (50–500 spirochetes) and 6% ($n = 22$) were highly infected (>500 spirochetes). For further processing, ticks were classified into two groups: weakly infected (<50 spirochetes) and highly infected (50 or more spirochetes). Using this classification, we observed that the degree of infection in ticks was not related to altitude (logistic regression: nymphs: $P = 0.24$ and adults: $P = 0.48$). Isolation success was significantly increased when the number of spirochetes (determined by IF) was high in nymphs: 14 isolates were obtained from 24 highly infected nymphs (58%), whereas only 14 isolates were obtained from 92 weakly infected nymphs (15%, Fisher's test, $P = 0.00005$). The same was not true for adults. Indeed isolation success from weakly infected adults already reached 50% (53/105), whereas isolation success from highly infected adults reached 67% (16/24, Fisher's test, $P = 0.18$).

Discussion

In this study, we examined the effect of altitude on *I. ricinus* density (CTD), phenology, and its infection with *B. burgdorferi* sl. We observed that the onset of tick activity in spring was delayed with increasing altitude. Because temperature decreases with higher altitude, this observation can be explained by delayed development of ticks in spring at higher altitude, and by the delayed activation of already molted individuals. This is consistent with previous observations in a nearby area showing that onset of tick activity varied among years according to temperature in spring (Perret et al. 2000).

Tick density decreased with increasing altitude. This was true for the density over the whole year as well as for the peak tick density in spring. Although the maximum tick density in spring was highly variable among years at lower altitude, it was very stable at the highest altitude. In addition to these results, we observed that the proportion of ticks collected in the second half-year varied among years and decreased with altitude.

A study on the development potential of *I. ricinus* at altitudes ranging from 650 to 1,550 m in Bohemia (Daniel 1993) showed that the establishment of ticks at high altitudes was limited by delayed development of ticks due to colder temperature (especially the number of days below development threshold) and to desiccation. At high altitude, those ticks which managed to complete their development started to be

Table 4. Proportion of *B. genospecies* identified in *I. ricinus* ticks collected in Chaumont

	B. garinii	*B. afzelii*	*B. burgdorferi ss*	*B. valaisiana*	*B. lusitaniae*	Mixed infections
Nymphs	28/46 (61%)	9/46 (20%)	7/46 (15%)	0/46	0/46	2/46 (4%)
Adults	50/94 (53%)	16/94 (17%)	11/94 (12%)	13/94 (14%)	1/94 (1%)	3/94 (3%)
Total	78/140 (56%)	25/140 (18%)	18/140 (13%)	13/140 (9%)	1/140 (1%)	5/140 (4%)

active in late spring or summer only while conditions are the most desiccating. In a recent note, Daniel et al. (2003) showed that *I. ricinus* distribution in Sumava National Park extended toward higher altitudes during the last decades, probably in relation with climate warming. In our observation, the tick population was not only established at 900 m but also was much more stable than at the lower altitudes: the maximum tick density was very stable among years, and no autumn peak occurred. Delays in development associated with lower temperatures in early summer were used by Randolph et al. (2002) to explain the nonoccurrence of autumn peaks of ticks in Great Britain. In our conditions, lower temperature during summer at higher altitude could also have reduced the development speed of spring-fed ticks, especially larvae, thus preventing the occurrence of an autumn peak of nymphs, whereas higher temperature at lower altitudes may allow the occurrence of an autumnal peak. In 2000, for example, which was the warmest of the 3 yr under study, the autumn peak of nymphs reached the highest value. The warmer temperature may have allowed spring–summer-fed ticks to molt and emerge the same year as reported by Chmela (1969).

During the last decade, *I. ricinus* distribution extended northwards and westwards within Sweden (Tälleklint and Jaenson 1998, Lindgren et al. 2000). In continental Europe, *I. ricinus* is broadly distributed, but the question of the evolution of tick phenology within continental Europe under warming climate remains controversial. We might expect more instability in tick phenology. Indeed, increased winter temperature might permit an early onset of tick activity in spring, and increased summer temperature might increase tick developmental speed, inducing an autumn peak of activity (Randolph et al. 2002). However, increased temperature, in spring and summer, inhibits the questing behavior of ticks, as shown in nature and under experimental conditions (Perret et al. 2000, 2003), thus reducing questing tick density. Therefore, we expect more instability in *I. ricinus* phenology under warmer conditions at low altitude, depending not only upon global changes but also upon the synchrony of weather conditions with the tick life cycle, for example, in spring when many ticks quest warmer temperature would reduce questing duration (Perret et al. 2003) and in summer when those ticks molt warmer temperature would enhance their development (Chmela 1969, Campbell 1948 cited in Randolph et al. 2002). At higher altitudes, however, increased temperatures may lead to an increase in tick density through an earlier onset of tick activity in spring and through reduced developmental duration from one instar to the next. Increased temperature might also induce the occurrence of an autumn peak at higher altitudes (Steele and Randolph 1985, Randolph et al. 2002), as long as the host community is not affected by temperature changes and as long as ticks can quest for long enough to grab a host before dying of desiccation and/or energy exhaustion.

The overall prevalence of *B. burgdorferi* sl in *I. ricinus* ticks was 24% with a higher prevalence in adults than in nymphs. This is in line with what is usually reported for Europe (Hubalek and Halouzka 1998). Infection prevalences in nymphs and adults are similar to the prevalences reported by Aeschlimann et al. (1986) in Neuchâtel for ticks collected in 1984 and 1985. This indicates a long-term stability in tick infection prevalence, even if fluctuations among years are possible, as shown in the current study. In nymphs, the lowest prevalence of infection was observed in 1999 and the highest in 2001 at all altitudes. In adults, the fluctuation of the infection prevalence was slightly marked, probably due to the lower number of adults examined.

In the same study, Aeschlimann et al. (1986) compared infection in ticks, all collected the same day, at different locations ranging from 400- to 700-m altitude and found a decrease in prevalence of nymphal tick infection with increasing altitude. Similarly, Rizzoli et al. (2002) have demonstrated that the density of *Borrelia* infected nymphs collected in different locations in Italian Alps, decreased up to an altitude of 1,300 m. Here, we observed that this decrease is stable over time (3 yr). We found that the prevalence of infection in adult ticks was related not only to altitude but also to adult density. This might be due to the low number of adults examined and to the fact that in 2001, most adults were collected at middle altitude. We did not find any seasonal variation in the infection prevalence of ticks at any altitude. Similar results have been reported in Sweden (Mejlon et al. 1993) and in Poland (Wegner et al. 1997).

Studies on spatial variation in density of *I. ricinus* ticks infected with *B. burgdorferi* sl are rather rare. In southern part of Sweden, densities reaching 7.68 infected *I. ricinus* nymphs per 100 m^2 (Mejlon and Jaenson 1993, Tälleklint and Jaenson 1996) have been reported. In our study, when during the year questing tick density reaches its maximum, the risk to encounter an infected tick greatly varies among years and altitudes. The densities of infected nymphs observed in our study at the lowest altitude are particularly high ranging between 36 and 68.6 infected nymphs per 100 m^2 and remain high at 900 m (range, 7.4–16.5).

All five European *Borrelia* species, *B. garinii*, *B. afzelii*, *B. burgdorferi* ss, *B. valaisiana*, and *B. lusitaniae*, have been observed in *I. ricinus* adults in our study. The simultaneous presence of the five *Borrelia* species in an endemic area has never been described in Switzerland. In this country, *B. lusitaniae* was only observed in locations south of the Alps (Jouda et al. 2003). This species is usually not very frequent in endemic areas in Europe and has been reported only in a few countries: Czech Republic, Moldavia, Ukraine (Postic et al. 1997), Slovakia (Gern et al. 1999), Poland (Mizak and Krol 2000), Spain (Escudero et al. 2000, Barral et al. 2002), and France (Richter et al. 2003). However, in some areas this species may be very frequent in ticks like in Portugal and North Africa (Nuncio et al. 1993, Zhioua et al. 1999, De Michelis et al. 2000, Younsi et al. 2001, Sarih et al. 2003). In our study, *B. lusitaniae* was found to be associated with *B. garinii* in one adult and with *B. valaisiana* in another

one. *B. lusitaniae* was not detected in nymphs. The reservoir hosts for *B. lusitaniae* have not yet been identified. However, it is interesting to notice that, in the current study, *B. lusitaniae* was associated with *B. garinii* and *B. valaisiana*, two *Borrelia* species infecting birds (Humair et al. 1998, Kurtenbach et al. 1998, Gern and Humair 2002).

B. garinii was largely predominant, indicating that birds are the main reservoirs at all altitudes (Gern and Humair 2002). The rodent-associated *B. garinii* OspA serotype 4 (Huegli et al. 2002) has not been detected in this forest (unpublished data).

Most *I. ricinus* ticks in Chaumont were infected by <50 spirochetes. In nymphs, the success of isolation was related to degree of infection, whereas in adults, *Borrelia* isolates were equally obtained from ticks infected by few, or many spirochetes. These results may be explained by the difference in the midgut size between adults and nymphs incubated in BSKII medium and also by the heterogeneous distribution of *Borrelia* in ticks (Lebet and Gern 1994).

Global warming has been predicted to increase vectorborne diseases (Epstein 2001). However, some controversy exists. Because variable phenologies and variable *Borrelia* infections of ticks were observed simultaneously at different altitudes, under different temperature conditions, within the same forest at a short distance, this area is suitable to follow the impact of different climatic scenarios on tick phenology. Further surveys of this area may help to understand the evolution of tick density, phenology, and Lyme borreliosis risk induced by global warming.

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