Original article

# Lyme borreliosis agents and the genetics and sex of their vector, *Ixodes ricinus*

Thierry De Meeûs [a],*, Yvan Lorimier [b], François Renaud [a]

[a] *Centre d'Etude sur le Polymorphisme des Micro-Organismes (CEPM) UMR CNRS-IRD 9926, Equipe Evolution des Systèmes Symbiotiques (ESS), Unite Mixte de Recherches, 911 Avenue d'Agropolis, B.P. 64501, 34394 Montpellier cedex 5, France*
[b] *Institut de Zoologie, 11, rue Emile-Argand, 2007 Neuchâtel, Switzerland*

## Abstract

The tick *Ixodes ricinus* is responsible for the transmission and maintenance of a wide variety of pathogenic organisms in the Northern Hemisphere, among which Lyme disease represents a major threat to humans. Despite numerous studies, the epidemiology of the different bacterial species responsible for this disease remains unclear. Recent evidence for a sex-biased genetic structure of its European vector leads us to analyse the consequences of this pattern on *Borrelia* transmission. Here we show that male and female ticks are not equivalently infected by *Borrelia burgdorferi*, that *Borrelia afzelii* affects tick migration capabilities, especially for the most vagile sex (i.e., male) and that Lyme borreliosis agents are consequently vectorised in a much more complex way than usually thought. Such results change the epidemiological perception of Lyme borreliosis and suggest new co-evolutionary pathways between the ticks and the borrelia.
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## 1. Introduction

Tick-borne diseases make up the overwhelming majority of human vector-borne infections in the temperate zones of the Northern Hemisphere [1], among which Lyme borreliosis has major public health and economic effects [2]. To date, many questions remain unanswered regarding the epidemiology of this disease [1] and the variety of its clinical manifestations [3]. Sex-biased dispersal was recently demonstrated for *Ixodes ricinus* [4], the European vector of Lyme disease [1], associated with a weak geographic genetic differentiation of this tick on the scale of Switzerland. This was interpreted as a consequence of sex-specific host preferences: immature (larvae and nymphs) male ticks being more prone to attach to birds, and/or immature female being more prone to attach to small mammals [4]. *Borrelia* isolates showing less than 70% homology to each other in DNA–DNA reassociation analysis do belong to different genospe-

cies [5], and different genospecies of *Borrelia burgdorferi* sensu lato are currently recognised [6]. Only three genospecies are responsible for Lyme disease in humans, and each of these presents different clinical manifestations in humans [3] and different reservoir hosts: red squirrels, for *B. burgdorferi* sensu stricto (Bbss); *Apodemus* mice, *Clethrionomys* voles and red squirrels, for *B. afzelii* (Bba); and birds, for *B. garinii* (Bbg) [6]. Thus, because *I. ricinus* female larvae and nymphs may feed preferentially on small mammals and because *Borrelia* species are host specific, it is possible that *I. ricinus* females and males are not identically infected.

To test this prediction, we analysed 858 ticks sampled in eight sites in Switzerland over two years (1995–1996). The infection pattern of these ticks was investigated in relation to geography, sampling year, sex and genetic variation (assessed with microsatellite loci) of the ticks. We found that Bbss is found more often in female ticks than in male ticks and that Bba occurs more often in local ticks than in immigrant ticks. Such features have evolutionary implications in terms of *Borrelia* and ticks' local adaptation dynamics. As a consequence, the epidemiology of Lyme borreliosis agents could be re-examined in the light of such results.

* Corresponding author.
Tel.: +33-467-41-63-10; fax: +33-467-41-62-99.
*E-mail address:* demeeus@mpl.ird.fr (T. De Meeûs).

## 2. Materials and methods

### 2.1. Sampling

Unfed questing adult ticks were sampled during the springs of 1995 (76 females, 88 males) and 1996 (393 females, 301 males) in the eight sites of Switzerland already presented in [4]. Ticks were collected by hand from the undergrowth in deciduous forest habitats. All specimens were preserved in 70% ethanol. Individuals were longitudinally cut in two halves. One half was used for microsatellite characterisation (all individuals in 1995, 320 females and 198 males in 1996, see [4] for more information), and the other for bacteriological analysis (all 858 ticks).

### 2.2. Borrelia characterisation

Specific primers were used for detecting the presence of *B. burgdorferi* sensu lato in ticks and for identifying which genospecies was present [7]. Three genospecies could be identified, *B. burgdorferi* sensu stricto (Bbss, 34 ticks infected), *B. afzelii* (Bba, 46 ticks) and *B. garini* (Bbg, three ticks). 38 ticks contained *B. burgdorferi* that could not be determined (Bbundet). Bbundet and Bbg were not considered for the statistical analysis. Fig. 1 presents the detail of tick infections by year, site and sex of the ticks. The raw data are available on-line at http://cepm/cepm/SiteWebESS/GB/deMeeus/TdeMeeusGB.html/RawDataMeeus.html.

### 2.3. Co-occurrence of Borrelia species in individual ticks

The co-occurrences of the different species of *Borrelia* were tested by the same randomisation procedure as the one used in Fstat V 2.9.3.2 (updated from [8]) for testing linkage disequilibrium between pairs of loci. It is thus a test of paired associations. This procedure uses the log-likelihood ratio *G*-statistic, so that overall sample statistics can be obtained by summing the *G*-statistic overall samples. The association between the three genospecies (Bbss, Bba and Bbg) could be tested in one sample (Neuchâtel females in 1996), where the exact probability of obtaining a dataset as rare or rarer than the observed one was computed.

### 2.4. Modelling Borrelia presence in ticks

The factors (sample, year and tick sex) that may influence *Borrelia* presence in a tick were explored by generalised linear modelling using S-Plus 2000 professional release 2 (Mathsoft Inc.). As an exact binomial distribution was not expected, we calculated quasi-likelihood estimates with a logit link and a $\mu(1 - \mu)$ variance function (as appropriate for a logistic regression). Quasi-likelihood estimation allows one to estimate regression relationships without full knowledge of the response variable's error distribution [9]. We retained the minimum model after dropping terms in turn, using the Akaike Information Criterion (AIC) [9]. The com-
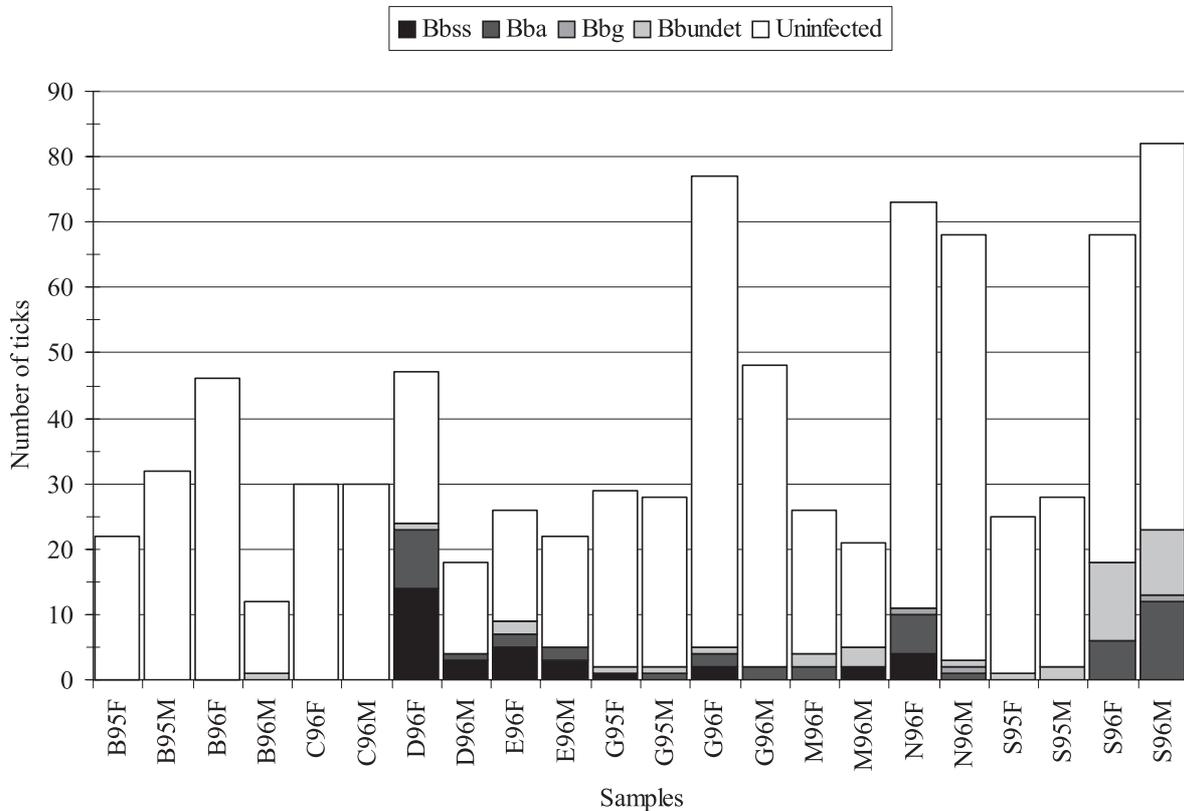


Fig. 1. Number of *I. ricinus* infected by the different *B. burgdorferi* genospecies in the different female (F) and male (M) tick samples in years 1995 (95) and 1996 (96). B = Bern, C = Monte Ceneri, D = Dorenaz, E = Eclepens, G = Gorges du Trient, M = Montmollin, N = Neuchâtel, S = Staadswald; see [4] for sample locations.

plete model to begin with was always of the form: *Borrelia presence =site + year + sex + site:year + site:sex + year:sex + site:year:sex* (:for interaction between factors).

### 2.5. Genetic structure of infected and non-infected ticks

As some ticks were genotyped for a previous study [4], the genotypic data were included in order to check for a possible influence of the tick's genetics on *Borrelia* presence. In particular, biased genetic structure between infected and non-infected ticks was tested using a specific randomisation procedure [10–13] available in Fstat V 2.9.3.2. Under the null hypothesis that uninfected and infected individuals disperse equally, $\theta$ (Weir and Cockerham's $F_{st}$ estimator [14]), used as a statistic, does not depend on the variable *sex*. Letting $\theta_I$ and $\theta_U$ be the estimates of $F_{st}$ (differentiation) between samples for infected and uninfected individuals, respectively, the test proceeds as follows: (i) the difference between $\theta_I$ and $\theta_U$, say $\Delta\theta_{Obs}$, is first calculated; (ii) an infection status is randomly assigned to each of the multilocus genotypes (keeping the genotypes in their original sample and the infected/uninfected ratio in each sample constant); (iii) the difference between $\theta_I$ and $\theta_U$ is recomputed for the randomised data set, say $\Delta\theta_{Rand1}$; (iv) steps (ii–iii) are repeated 10 000 times. The probability that dispersal is unbiased by infection status is then estimated as the proportion of times where $\Delta\theta_{Randi}$ obtained from the randomised data sets is larger or equal to the statistic obtained from the observed data set ($\Delta\theta_{Obs}$). If $\Delta\theta$ is computed as an absolute value then the test is bilateral, otherwise it is unilateral. The parameter tested, $F_{st}$ (genetic differentiation between samples), is the most powerful in most conditions [12]. As genetic differences exist between female and male ticks and between years [4], the data were split into as many subsamples as necessary to obtain homogeneous subsamples (e.g. females from Bern 1995, males from Bern 1995, females from Bern 1996, males from Bern 1996). Then, overall loci tests were undertaken for each *Borrelia* genospecies. For the X-linked IR08 locus [4], males were coded as homozygous, or the dataset was analysed without IR08 (with identical results). Females and males were also analysed in separate files to confirm the pattern observed, and for which we computed unilateral probabilities.

## 3. Results

### 3.1. Tick infection

The number of infected ticks varied considerably across our Swiss samples (prevalence between 0 and 0.29, see Fig. 1). Across all samples, 106 ticks were infected, with a prevalence of 0.04, 0.05 and 0.003 for Bbss, Bba and Bbg, respectively. There is a positive association between the different *Borrelia* genospecies. The different *P* values obtained over all samples are $P < 0.001$ for the association

between Bbss and Bba, $P = 0.053$ for the association between Bbss and Bbg, $P = 0.093$ between Bba and Bbg, and finally, the exact probability of obtaining the association observed between the three genospecies in female ticks from Bern in 1996 is $P = 0.005$.

### 3.2. Modelling Borrelia presence in ticks

For Bbss, the minimum model retained was *Bbss =site + sex* ($R^2 = 0.31$, $P < 0.0001$ and $P = 0.012$ for *site* and *sex*, respectively). For Bba, the minimum model retained was *Bba =site + year* ($R^2 = 0.12$, $P < 0.0001$ and $P = 0.002$ for *site* and *year*, respectively). Site was thus a highly significant factor for Bbss and Bba. Some sites are likely more favourable than others due to the presence of reservoir hosts. An increase in the prevalence of Bba was observed from 1995 to 1996 (0.01–0.07) and, more importantly, Bbss was found more often infecting female ticks than male ticks (mean prevalence of 0.06 and 0.02 for females and males, respectively).

### 3.3. Genetic structure of infected and non-infected ticks

For Bbss tick infections, genetic differentiation was not significantly different ($P = 0.51$) between infected and non-infected ticks. The between-samples genetic differentiation was significantly higher ($P = 0.002$) for ticks infected with Bba ($\theta = 0.076$) than for ticks uninfected by this genospecies ($\theta = 0.002$). Thus, ticks infected by Bba are more philopatric (disperse less) than uninfected ticks. For year 1996 alone $P = 0.007$ and for the dataset without locus IR8, $P = 0.003$. Finally, when males and females are analysed separately, the signal stays in the same direction in each sex but is stronger for males ($\theta = -0.005$ for uninfected and $\theta = 0.14$ for infected males, $P = 0.01$) than for females ($\theta = 0.004$ for uninfected and $\theta = 0.046$ for infected females, $P = 0.05$). Fig. 2 presents the different values of $\theta$ obtained for uninfected and infected female and male ticks. Note that splitting the data greatly increases the sampling variance of $\theta$, in particular for infected males (only 14 males are infected with Bba). Fig. 3 shows the result obtained for each of the five loci when
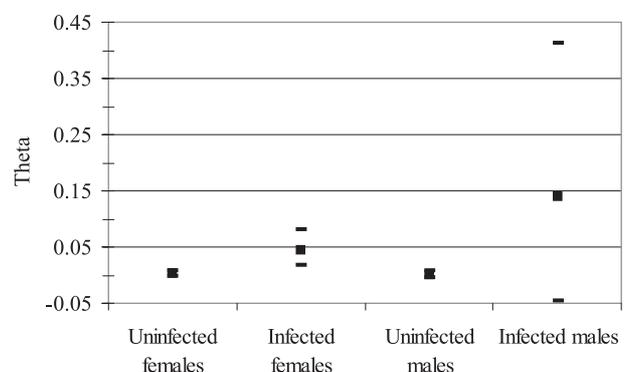


Fig. 2. The different values of $\theta$ ($F_{st}$ estimator) obtained for uninfected and infected female and male ticks. The 95% confidence intervals were obtained after bootstrapping over loci.
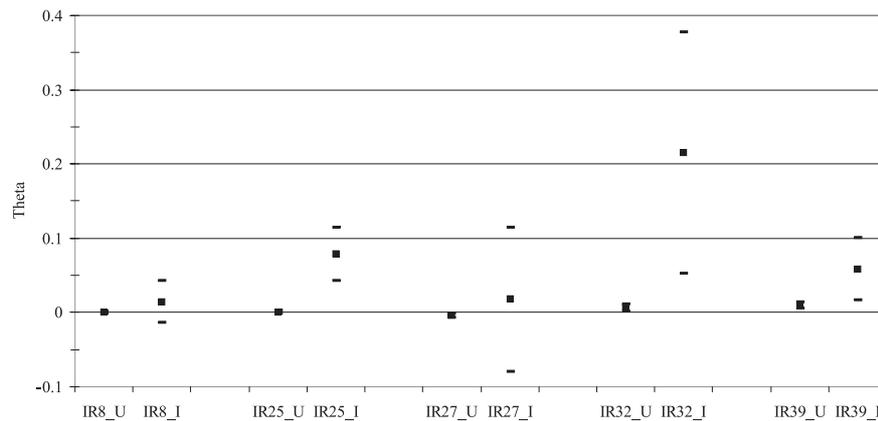
Fig. 3. The different values of $\theta$ ($F_{st}$ estimator) obtained for uninfected (U) and infected (I) ticks for each of the five microsatellite loci (IR8, IR25, IR27, IR32 and IR39). The 95% confidence intervals were obtained after jack-knifing over populations.

comparing uninfected and infected ticks. Thus, the results observed at each locus all converge to the same conclusion of a higher differentiation between infected ticks.

## 4. Discussion

It is worth noticing at this stage that, because ticks were sampled before any contact with the final host, and because transovarial transmission is very rare [6,15], all observations reported here are consequences of larval and nymphal activities.

Recent work has shown that *I. ricinus* females are more philopatric (disperse less) than males [4]. Such a pattern is detectable only for very intense biases (i.e., a very low migration rate for the philopatric sex) [12]. This suggests that female immature ticks should be more prone to infection by *B. burgdorferi* genospecies from less mobile hosts such as small mammals, i.e., Bbss and Bba [6], and above all, females should be very rarely infected by birds' *Borrelia* (Bbg). Unfortunately, not enough Bbg could be found in our dataset for that prediction to be accurately tested, as only two males (Neuchâtel 1996 and Staadswald 1996) and one female (Neuchâtel 1996, also infected by Bbss and Bba) were found positive for this genospecies. However, Bbss was found more often infecting females than males of *I. ricinus*, while Bba was found equally well in both sex. Perhaps, then, female specificity is stronger for squirrels (Bbss and Bba host) than for voles and field mice (Bba hosts), and/or perhaps the exact specificity of borrelia is not completely elucidated. Field tests on these hosts, on the sex of the larvae and nymphs they harbour and the *Borrelia* genospecies by which these ticks are infected would be required for these results to be fully explained.

The genetic differentiation between samples was found more pronounced for ticks infected with Bba than for uninfected ticks. Several hypothesis may be advanced to interpret this unexpected result.

Pleiotropic selection hypothesis (PSH). As microsatellite markers are supposedly non-coding sequences (i.e., neutral)

and are all responding in the same direction, we must admit here that there are some selective processes that make some genome-wide genotypes more sensible to Bba infection and pleiotropically less mobile, especially for male ticks. This seems very unlikely and a neighbour-joining tree construction of individual ticks (available on request to the corresponding author) did not show any tendency for uninfected or infected ticks to group in single branches, as would have been expected in such a case.

Cryptic species hypothesis (CSH). A complex of two cryptic tick species, one being more receptive to Bba and less vagile (especially males) than the second, could lead to the observed result. However, splitting the data into infected and uninfected ticks did not result in any decrease of the $F_{is}$ compared with the global dataset, and no linkage disequilibrium could be evidenced in any of these populations [4], as would have been expected in such a case. This would also haveled to some structure in the neighbour-joining tree made for the PSH. Moreover, the CSH is hard to reconcile with the sex-biased dispersal that was observed in that species [4].

Spirochete local adaptation hypothesis (SLAH). Resident ticks are easier to infect by resident Bba, and thus most of the time, infected ticks are genetically more different from one site to another than uninfected ticks. This hypothesis is not supported by Bba prevalence observed on *I. ricinus* females (strongly philopatric [4]), which are not different from the prevalence observed in males. Local adaptation of the pathogen also supposes that it migrates much more than the host [16,17] and, because of the weakness of population differentiation of ticks [4,18] and the strong epidemiological structure of *B. burgdorferi* [19], this is the reverse that would have been expected here.

Migration-infection survival hypothesis (MISH). Survival is severely compromised for migrating ticks when infected by Bba. This could occur whether the tick is infected before (foreign infection) or after migration (local infection) and will statistically affect more greatly the most vagile sex (i.e., males), as is actually observed. We failed to find any evidence of an effect of *B. burgdorferi* sensu lato on tick

survival in the literature, but as nobody has ever studied tick's dispersal survival in itself, we cannot reject this hypothesis.

Dispersal manipulation hypothesis (DMH). The spirochaete modifies tick dispersal capabilities, and this phenomenon statistically affects the most vagile sex more (i.e., males), as is actually observed. For the same reason as for MISH, we cannot reject this hypothesis. Ticks do not migrate by themselves, but while attached to a host [20]. Bba is specific to small rodents [6], which have a limited ability to disperse ticks outside of their territories [20,21]. Promoting tick specificity toward these small rodents would represent an adaptive host manipulation on the part of the spirochaete.

The problem with MISH and DMH is that we have to assume a strong effect of infection (infected ticks almost do not migrate at all, or nearly all migrators die when infected) and above all, a very low effective local population size to reach the observed $F_{st}$. For instance, using Eq. (10) of [22] on sex-biased dispersal, a subpopulation size as low as $N = 12$ is required to obtain an $F_{st}$ of 0.07 and 0.006 for the less mobile (migration rate $m = 0$) and most mobile ($m = 0.7$) sex, respectively. Unfortunately, our genetic markers cannot enable us to properly estimate effective population sizes, due to the huge amount of null alleles [4]. Further studies, such as those on hosts captured in the field and examined for ticks and *Borrelia*, will thus be needed.

The co-occurrence of *Borrelia* genospecies within individual ticks is surprising and might come from the existence of resistant and sensible individuals in tick populations, whatever the sensibility mechanism may be (acquired or innate). However, because the different genospecies display different specificities, there is thus a potential conflict of interest for transmission to the vertebrate host.

Finally we demonstrated that female ticks are more often infected by Bbss than male ticks and thus transmit this pathogenic agent more often. Moreover, dispersal capabilities of ticks are apparently affected by Bba infection in a sex-specific pattern, male dispersal being more affected than female dispersal. *B. burgdorferi* sensu lato is probably detrimental to its tick vector [19,23–26], and our results strongly suggest that Bba has the ability to alter the population genetics of its host tick by modifying tick dispersal capabilities, especially for males. This is consistent with the weak geographic and sex-biased population genetic structure for *I. ricinus* [4,18], together with the pronounced epidemiological structure of *B. burgdorferi* [19]. Adult females feed mostly on big mammals (e.g. deer) [20] that may transport ticks far away (adult male ticks do not feed). However, these hosts are not, or poorly competent for Lyme borreliosis transmission [1,27]. Transovarial transmission being rare [6], big mammals and adult ticks probably account little for *Borrelia* migration patterns. It is thus probable that infected and uninfected immature ticks (larvae and nymphs) show different behaviour, and that the different genospecies of *B. burgdorferi*, known to induce different clinical manifestations [3], are heterogeneously vectorised by *I. ricinus*, as a function of sex of the ticks and of the genospecies of the *Borrelia*. This suggests a role of this pathogenic agent in local adaptation dynamics and in pairing patterns of its vector, *I. ricinus*. The co-evolutionary relationships between ticks, Lyme disease agents and vertebrate hosts should thus reflect these phenomena. Furthermore, the new information advanced here should help in the attempt to formalise the epidemiology of Lyme borreliosis [28,29], and the methods presented here proved to be powerful tools that may be more widely applied and should offer new research perspectives on the study of vector-borne diseases and host parasite interactions.

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