Multistrain Infections with Lyme Borreliosis Pathogens in the Tick Vector

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ABSTRACT Mixed or multiple-strain infections are common in vector-borne diseases and have important implications for the epidemiology of these pathogens. Previous studies have mainly focused on interactions between pathogen strains in the vertebrate host, but little is known about what happens in the arthropod vector. *Borrelia afzelii* and *Borrelia garinii* are two species of spirochete bacteria that cause Lyme borreliosis in Europe and that share a tick vector, *Ixodes ricinus*. Each of these two tick-borne pathogens consists of multiple strains that are often differentiated using the highly polymorphic *ospC* gene. For each *Borrelia* species, we studied the frequencies and abundances of the *ospC* strains in a wild population of *I. ricinus* ticks that had been sampled from the same field site over a period of 3 years. We used quantitative PCR (qPCR) and 454 sequencing to estimate the spirochete load and the strain diversity within each tick. For *B. afzelii*, there was a negative relationship between the two most common *ospC* strains, suggesting the presence of competitive interactions in the vertebrate host and possibly the tick vector. The flat relationship between total spirochete abundance and strain richness in the nymphal tick indicates that the mean abundance per strain decreases as the number of strains in the tick increases. Strains with the highest spirochete load in the nymphal tick were the most common strains in the tick population. The spirochete abundance in the nymphal tick appears to be an important life history trait that explains why some strains are more common than others in nature.

IMPORTANCE Lyme borreliosis is the most common vector-borne disease in the Northern Hemisphere and is caused by spirochete bacteria that belong to the *Borrelia burgdorferi sensu lato* species complex. These tick-borne pathogens are transmitted among vertebrate hosts by hard ticks of the genus *Ixodes*. Each *Borrelia* species can be further subdivided into genetically distinct strains. Multiple-strain infections are common in both the vertebrate host and the tick vector and can result in competitive interactions. To date, few studies on multiple-strain vector-borne pathogens have investigated patterns of cooccurrence and abundance in the arthropod vector. We demonstrate that the abundance of a given strain in the tick vector is negatively affected by the presence of coinfecting strains. In addition, our study suggests that the spirochete abundance in the tick is an important life history trait that can explain why some strains are more common in nature than others.

KEYWORDS *Borrelia afzelii*, *Borrelia garinii*, *Ixodes ricinus*, mixed infection, multiple-strain infection, *ospC*, pathogen strain, tick, vector-borne disease

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Genetically diverse pathogens often establish mixed infections in their hosts that consist of multiple strains or genotypes of the same pathogen species (1–3). Mixed infections represent a major problem for public health because they complicate the development of treatments and vaccines (1, 4). Some pathogen strains are more

infectious or do more harm to certain host species (5, 6). The strain composition of the mixed infection can therefore greatly influence disease pathology (7). In addition, competitive interactions between pathogen strains can influence the evolution of virulence (8), transmission (9), or infectivity (10).

In vector-borne diseases, mixed-strain infections are common and occur in both the vertebrate reservoir host and the arthropod vector (11). Studies on interactions between strains of vector-borne pathogens typically focus on the vertebrate reservoir host (2, 12–17), and there are relatively few studies that have investigated the importance of interstrain interactions in the arthropod vector (18–22). Manipulative experiments that compare pathogen performance in vectors that have fed on hosts infected with single or multiple strains are best for establishing causality (18–22). However, field studies that characterize the patterns of cooccurrence and abundance of multiple-strain vector-borne pathogens in the arthropod vector can indicate which pathogen strains are likely to have negative (or positive) interactions with each other in nature. Such field studies have made significant contributions to our understanding of these important infectious diseases (23–31).

Borrelia burgdorferi sensu lato is a complex of spirochete bacteria that causes Lyme borreliosis (LB), the most common vector-borne disease in Europe and North America (32). In Europe, the two most common etiological agents of LB are *Borrelia afzelii* and *Borrelia garinii*. These two *Borrelia* genospecies are sympatric in distribution but are adapted to different classes of vertebrate reservoir hosts: rodents and birds, respectively (33–35). Their principal vector is the castor bean tick, *Ixodes ricinus*, which has three obligate blood-feeding stages: larva, nymph, and adult. Larval ticks acquire spirochetes while feeding on infected reservoir hosts and subsequently molt into infected nymphs that transmit the pathogen to the next generation of reservoir hosts the following year. Vertical transmission of *B. burgdorferi sensu lato* is absent or rare in *Ixodes* ticks (36, 37). Due to their adaptation to different reservoir hosts, *B. afzelii* and *B. garinii* rarely coinfect the same tick (27–29, 38).

LB pathogens establish mixed-strain infections in both the vertebrate host (23, 30, 31, 39) and the tick vector (23, 24, 40–42). Strains are often defined by the highly polymorphic, single-locus *ospC* gene (5, 24, 30, 31, 40, 42–44). This gene codes for outer surface protein C (OspC), which is a critical virulence factor for the infection of the vertebrate host (45, 46). The *ospC* alleles can be clustered into *ospC* major groups (oMGs), which are more than 8% divergent in DNA sequence from other such clusters (5, 24, 42–44). We have previously shown that genetic divergence among sequences belonging to the same oMG is <2% and that there are no intermediately divergent (2 to 8%) *ospC* sequences (24). Thus, one advantage of this strain-typing system is that minor sequencing errors do not create new oMGs or result in the misclassification of the *ospC* sequences to the wrong oMG. Each of the three most important LB pathogens, *B. afzelii*, *B. garinii*, and *B. burgdorferi sensu stricto*, has approximately 20 different oMGs (24, 43). In the present study, we used the *ospC*-typing system to study mixed-strain infections within each of two *Borrelia* species, as others have done previously (24, 30, 31, 40, 47).

The first aim of the present study was to characterize the pattern of cooccurrence between *ospC* strains in the tick vector in two independent LB pathogens: *B. afzelii* and *B. garinii*. The second aim was to test whether the abundance of a given strain in the nymphal tick was negatively or positively influenced by the presence of other coinfecting strains. The third aim of the study was to test whether the strain-specific abundance in the nymphal tick influenced its strain-specific frequency in the tick population. For each of the two *Borrelia* genospecies, we determined the frequencies and the abundances of the different oMGs in a local population of *I. ricinus* nymphs over a period of 3 years. For each focal *ospC* strain, we tested whether its presence/absence in the tick was influenced by the other *ospC* strains by analyzing all of the ticks in the data set. Then, for the subset of ticks infected with the focal *ospC* strain, we tested whether the abundance of the focal strain was influenced by the abundances of the other coinfecting strains. We predicted that the abundance of each *ospC* strain

TABLE 1 Frequencies and abundances of the oMGs in the *Borrelia afzelii*-infected *Ixodes ricinus* nymphs are shown

ospC allele	ospC status[a]	F1 (% [no.])[b]	F2 (% [no.])[c]	Abundance[d]	Mean abundance[e]
A9	Native	36.58 (25,108)	67.32 (103)	606,811	85
A10	Native	37.93 (26,035)	64.71 (99)	545,915	161
A2	Native	11.58 (7,950)	32.03 (49)	93,839	31
A7	Native	3.51 (2,408)	30.07 (46)	145,292	34
A14	Native	5.81 (3,988)	20.91 (32)	27,746	22
A1	Native	4.41 (3,029)	20.91 (32)	199,099	39
G13	Exotic	0.05 (33)	13.07 (20)	5,195	7
G7	Exotic	0.04 (26)	10.46 (16)	177,246	7
G2	Exotic	0.03 (19)	10.46 (16)	150	2
G4	Exotic	0.02 (12)	5.23 (8)	9,131	8
G15	Exotic	0.01 (10)	4.58 (7)	335	5
G14	Exotic	0.01 (10)	5.23 (8)	289	2
G6	Exotic	0.00 (3)	1.31 (2)	108	15
Total	Both	100 (68,631)	100 (153)	1,811,156	
Total	Native	99.84 (68,518)	100 (153)	1,618,702	
Total	Exotic	0.16 (113)	35.95 (55)	192,454	

[a]*ospC* status refers to whether the oMGs are native to *B. afzelii* or were derived from *B. garinii*.
[b]Frequency 1 (F1) is the percentage of the oMG in the sample of sequences ($n = 68,631$ sequences). The number in parentheses represents the number of reads that belonged to that particular oMG.
[c]Frequency 2 (F2) is the percentage of the oMG in the sample of infected nymphs ($n = 153$ nymphs). The number in parentheses represents the number of nymphs that were infected with that particular oMG.
[d]Total spirochete load for each strain in all the ticks ($n = 153$ nymphs). This number is calculated as follows: multiply the estimate of the spirochete load (qPCR) by the strain-specific frequencies (454 sequencing) and then sum across all 153 nymphs.
[e]Geometric mean spirochete load per strain in the subsample of ticks infected with that strain (i.e., nymphs that were not infected with that strain were excluded from the calculation).

inside the tick would be negatively affected by the abundances of the coinfecting strains. Finally, we predicted that the *Borrelia ospC* strains that maintain the highest spirochete loads inside the tick vector would be the most common strains in our local population of *I. ricinus* ticks. The present study found evidence of both positive and negative interactions between pairs of *ospC* strains in both *B. afzelii* and *B. garinii*. We also show that the spirochete abundance in the tick vector predicts whether a given strain is common or rare.

RESULTS

ospC major groups of *B. afzelii* and *B. garinii*. Using 454 sequencing, we obtained 68,631 *ospC* gene sequences for the 153 *B. afzelii*-infected nymphs and 24,014 *ospC* gene sequences for the 100 *B. garinii*-infected nymphs. The *ospC* alleles clustered into 13 different oMGs: six belonged to *B. afzelii* (A1, A2, A7, A9, A10, A14) and seven to *B. garinii* (G2, G4, G6, G7, G13, G14, G15). All of these oMGs had been identified in previous studies on *B. afzelii* and *B. garinii* (5, 24, 31, 44). Of the 68,631 reads from the *B. afzelii*-infected nymphs, 113 reads (0.16%) clustered with the oMGs of *B. garinii* (Table 1). Conversely, of the 24,014 reads from the *B. garinii*-infected nymphs, 1,836 reads (7.65%) clustered with the oMGs of *B. afzelii* (Table 2). In what follows, we took the conservative approach and removed these 1,949 reads (113 + 1,836) from the statistical analysis.

Mean spirochete loads of the two *Borrelia* species in the ticks. For the subset of nymphs infected with *B. afzelii* ($n = 153$ nymphs), the mean spirochete load was 1,438 spirochetes per nymph (95% confidence interval [CI], 941 to 2,197 spirochetes per nymph). For the subset of nymphs infected with *B. garinii* ($n = 100$ nymphs), the mean spirochete load was 4,449 spirochetes per nymph (95% CI, 2,419 to 8,183 spirochetes per nymph). Thus, the mean spirochete load in *B. garinii* was three times higher than in *B. afzelii* and this difference was significant (analysis of variance [ANOVA]: $F_{1,245} = 6.217$, $P = 0.013$). The range in nymphal spirochete load was very large in both *B. afzelii* (4 to 2,832,000 spirochetes/nymph) and *B. garinii* (5 to 4,928,000 spirochetes/nymph).

TABLE 2 Frequencies and abundances of the oMGs in the *Borrelia garinii*-infected *Ixodes ricinus* nymphs

ospC allele	*ospC* status[a]	F1 (% [no.])[b]	F2 (% [no.])[c]	Abundance[d]	Mean abundance[e]
G7	Native	21.06 (5,057)	45.00 (45)	1,099,502	730
G13	Native	20.30 (4,875)	38.00 (38)	653,839	699
G2	Native	23.04 (5,532)	36.00 (36)	1,213,210	691
G4	Native	6.85 (1,646)	16.00 (16)	724,520	621
G15	Native	5.84 (1403)	13.00 (13)	58,523	148
G14	Native	6.36 (1,527)	9.00 (9)	18,120	141
G6	Native	8.90 (2,138)	9.00 (9)	528,528	1,431
A10	Exotic	4.45 (1,068)	42.00 (42)	146,648	84
A9	Exotic	2.92 (701)	31.00 (31)	648,460	69
A2	Exotic	0.14 (30)	15.00 (15)	18,713	70
A1	Exotic	0.05 (12)	10.00 (10)	89,942	75
A14	Exotic	0.06 (15)	10.00 (10)	404	3
A7	Exotic	0.04 (10)	9.00 (9)	98,767	441
Total	Both	100 (24,014)	100 (100)	5,299,176	
Total	Native	92.35 (22,178)	97 (97)	4,296,242	
Total	Exotic	7.65 (1,836)	70.30 (71)	1,002,934	

[a]*ospC* status refers to whether the oMGs are native to *B. garinii* or were derived from *B. afzelii*.
[b]Frequency 1 (F1) is the percentage of the oMG in the sample of sequences ($n = 24,014$ sequences). The number in parentheses is the number of reads that belonged to that particular oMG.
[c]Frequency 2 (F2) is the percentage of the oMG in the sample of infected nymphs ($n = 100$ nymphs). The number in parentheses is the number of nymphs that were infected with that particular oMG.
[d]Total spirochete load for each strain in all the ticks ($n = 100$ nymphs). This number is calculated as follows: multiply the estimate of the spirochete load (qPCR) by the strain-specific frequencies (454 sequencing) and then sum across all 100 nymphs.
[e]Geometric mean spirochete load per strain in the subsample of ticks infected with that strain (i.e., nymphs that were not infected with that strain were excluded from the calculation).

Mean strain richness of the two *Borrelia* species in the ticks. For the subset of nymphs infected with *B. afzelii* ($n = 153$), the mean strain richness was 2.85 strains/nymph (95% CI, 2.59 to 3.11 spirochetes per nymph), and 77.1% (118/153) of the nymphs carried more than one *ospC* strain. For the subset of nymphs infected with *B. garinii* ($n = 100$), the mean strain richness was also 2.85 strains/nymph (95% CI, 2.53 to 3.17 spirochetes per nymph), and 77% (77/100) of the nymphs carried more than one *ospC* strain.

Associations between the presence of the *ospC* strains inside the nymphal tick. We used generalized linear models (GLM) with binomial errors to model the prevalence of each focal *ospC* strain as a function of the presence/absence of the coinfecting *ospC* strains. After Bonferroni correction, the global P value of the GLM was statistically significant for *B. afzelii ospC* strains A2 ($P = 0.001$), A9 ($P < 0.001$), and A10 ($P = 0.003$) and for *B. garinii ospC* strain G13 ($P = 0.003$). For each of these four focal *ospC* strains, we inspected the statistical significance and sign of the parameter estimates to determine the effect of each of the coinfecting strains on the prevalence of the focal strain. For *B. afzelii*, strains A1 ($P = 0.012$), A7 ($P = 0.049$), and A9 (0.002) had positive effects on the prevalence of focal strain A2. Strain A2 had a positive effect ($P = 0.001$), whereas strains A1 ($P = 0.048$) and A10 (<0.001) had a negative effect on the prevalence of focal strain A9. Strain A9 had a negative effect ($P = 0.001$) on the prevalence of focal strain A10. For *B. garinii*, strain G7 had a negative effect on the prevalence of focal strain G13 ($P = 0.019$).

Associations between the abundances of the *ospC* strains inside the nymphal tick. We used multiple regression to model the $\log_{10}$-transformed abundance of each focal *ospC* strain as a function of the $\log_{10}(x + 1)$-transformed abundances of the coinfecting *ospC* strains. When all of the nymphs ($n = 253$) were included in the analysis, there was no significant effect of the abundances of the coinfecting *ospC* strains on the abundance of the focal *ospC* strain (data not shown). When the analysis was restricted to a subset of nymphs that had a total abundance between 10^4 and 10^5 spirochetes ($n = 85$ nymphs), there was evidence of negative interactions. After
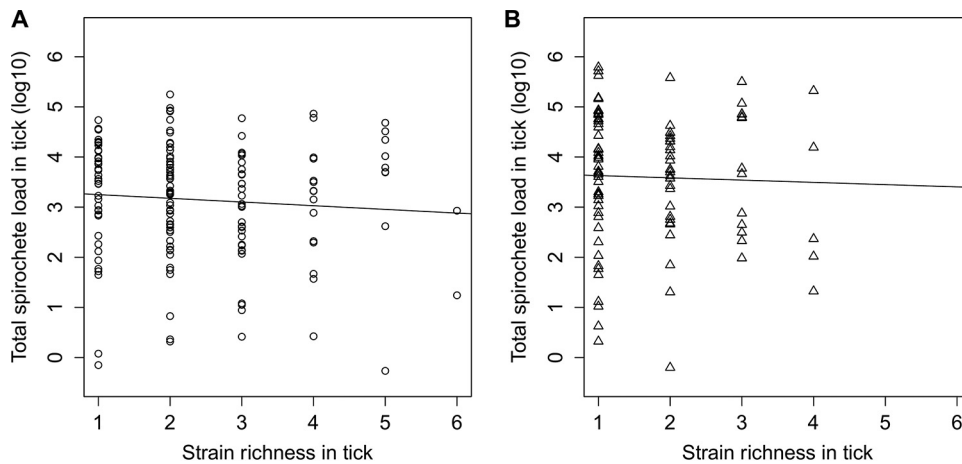
FIG 1 Relationship between the total population size of the *Borrelia* pathogen and the strain richness inside the tick vector. The relationship between the total spirochete load ($\log_{10}$ transformed) in *I. ricinus* nymphs and the strain richness in the nymph are shown for both *B. afzelii* (A) and *B. garinii* (B). The effect of the strain richness on the total spirochete load was not statistically significant (ANCOVA: $P = 0.341$).

Bonferroni correction, the global P value of the multiple regression was statistically significant for *B. afzelii ospC* strain A10 ($P < 0.001$) and *B. garinii ospC* strain G2 ($P = 0.003$). For each of these two focal *ospC* strains, we inspected the statistical significance and sign of the partial regression coefficients to determine the effect of each of the abundances of the coinfecting strains on the abundance of the focal strain. For *B. afzelii*, the abundances of strain A2 ($P < 0.001$) and strain A9 ($P = 0.004$) both had negative effects on the abundance of focal strain A10. For *B. garinii*, the abundances of strain G4 ($P < 0.001$) and G13 ($P < 0.001$) both had negative effects on the abundance of the focal strain G2. After Bonferroni correction, the global P value of the multiple regression was no longer statistically significant for *B. afzelii ospC* strain A9 ($P = 0.033$). However, we point out that inspection of the partial regression coefficients indicated that the abundance of strain A10 had a significant and negative effect on the abundance of focal strain A9 ($P = 0.003$).

Relationship between nymphal spirochete load and nymphal strain richness. We used analysis of covariance (ANCOVA) to model the $\log_{10}$-transformed total spirochete load in the nymphal tick as a function of the *Borrelia* species and strain richness. The interaction between *Borrelia* species and strain richness was not significant (ANCOVA: $F_{1,244} = 0.032$, $P = 0.859$). After the interaction was removed, there was no effect of the strain richness on the $\log_{10}$-transformed total spirochete load in the nymph for the two *Borrelia* genospecies combined (ANCOVA: $F_{1,245} = 0.912$, $P = 0.341$) (Fig. 1). This result suggests that the total spirochete load in the nymph is constant as the number of *ospC* strains increases.

Relationship between infection intensity per strain and strain richness. We used ANOVA to test whether the $\log_{10}$-transformed spirochete load per strain (infection intensity) differed among the four categories of strain richness (1, 2, 3, and 4 to 6 strains). The mean $\log_{10}$-transformed infection intensity per strain decreased with increasing strain richness, and this effect was significant ($F_{3,244} = 6.97$, $P < 0.001$) (Fig. 2). The mean infection intensities for strain richness of 1, 2, 3, and 4 to 6 strains were 2,921 (95% CI, 2,736 to 3,120), 1,031 (95% CI, 970 to 1,096), 520 (95% CI, 458 to 591), and 286 (95% CI, 233 to 352) spirochetes per strain, respectively (Fig. 2). This result shows that for both *Borrelia* species, the mean infection intensity per strain decreases as the strain richness increases in the tick vector.

Relationship between spirochete abundance within the tick and the relative frequency of the oMG strain. We used GLM with binomial errors to model the relative frequency of each *ospC* strain in the population of *I. ricinus* nymphs as a function of the *Borrelia* species and the $\log_{10}$-transformed strain-specific spirochete abundance in
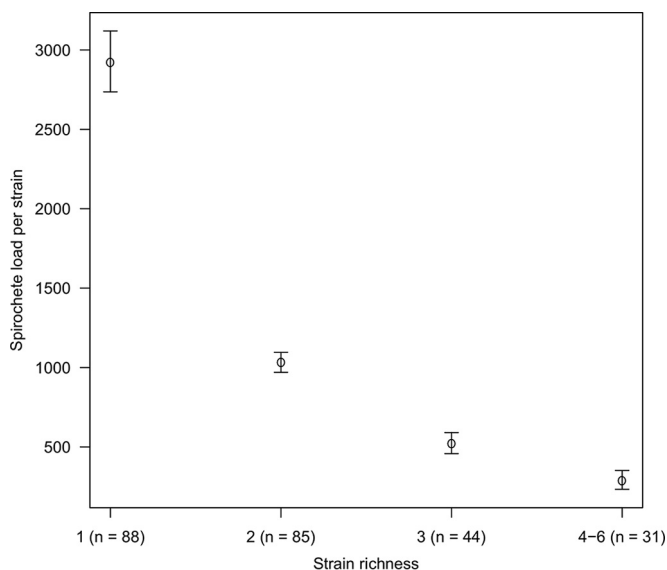
FIG 2 The mean infection intensity per *Borrelia* strain decreases with strain richness in the tick vector. Strain richness in *I. ricinus* nymphs has a negative effect on the mean infection intensity (number of spirochetes per strain in the nymph), and this effect was highly significant (ANOVA: $P < 0.001$). The two *Borrelia* species and the higher categories of strain richness (4 to 6 strains) were combined to increase the power of the analysis. The sample sizes for each strain richness category (number of ticks) are shown in parentheses. The dots and bars show the means and the 95% confidence limits, respectively.

the nymphal tick. The interaction between *Borrelia* species and $\log_{10}$-transformed spirochete abundance in the nymphal tick was not significant (GLM: $F_{1,9} = 1.982$, $P = 0.193$) (Fig. 3). We found a positive and significant effect of the strain-specific spirochete abundance in the nymphal tick on the strain-specific relative frequency in the population (GLM: $F_{1,10} = 10.864$, $P = 0.008$) (Fig. 3). The slope (1.912 ± 0.612) indicated that for each 10-fold increase in spirochete abundance, the odds ratio of the relative frequency (number of nymphs carrying that strain/number of nymphs not carrying that strain) would almost double in the population of *I. ricinus*. There was a significant effect
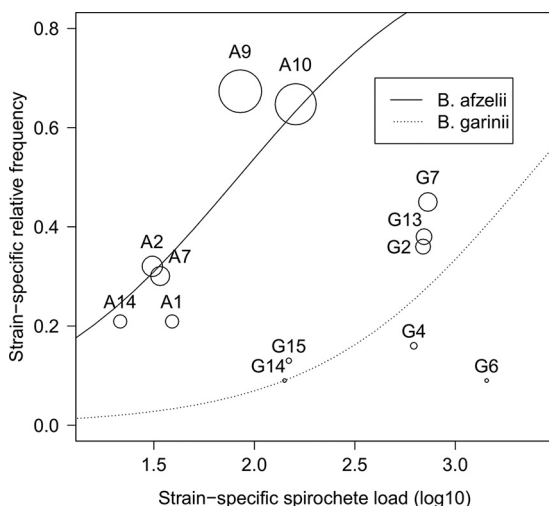
FIG 3 *Borrelia* strains that are common have a higher spirochete load in the tick vector. The relationship between the relative frequencies of each oMG strain in the population of *I. ricinus* nymphal ticks and their mean spirochete loads ($\log_{10}$ transformed) in the nymphal tick are shown for both *B. afzelii* (solid line) and *B. garinii* (dotted line). Each data point is labeled with the name of the oMG. The size of the data point refers to the number of infected nymphs over which the strain-specific spirochete load was calculated. The effect of the strain-specific spirochete load on the strain-specific relative frequency was statistically significant (GLM: $P = 0.008$).

of *Borrelia* species on the mean strain-specific relative frequency (GLM: $F_{1,10}$ = 15.199, P = 0.003) (Fig. 3). For *B. afzelii* and *B. garinii*, a mean spirochete load of 83 spirochetes and 2,291 spirochetes, respectively, was necessary to establish a relative frequency of 50.0% (Fig. 3).

DISCUSSION

The present study found both positive and negative interactions between strains of *B. afzelii* in the tick vector with respect to cooccurrence and abundance (spirochete load). Specifically, we found that the abundances of the two most common *ospC* strains of *B. afzelii*, A9 and A10, had a reciprocal and negative effect on each other. In the present study, the patterns of cooccurrence and coabundance were quantified in the tick vector. We emphasize from the outset that the vertebrate host community plays a critical role in structuring the community of *ospC* strains, which is subsequently acquired by the nymphal tick during the larval blood meal. However, we will also argue that subsequent processes operating inside the tick vector might influence the patterns of strain-specific abundance. Another important result of this study was our finding that the strain-specific spirochete load in the nymphal tick was positively related to the relative frequency of the strain over the 3-year duration of the study (Fig. 3). These observations suggest that the spirochete load in the nymphal tick may be an important fitness trait that explains why some strains of *Borrelia* pathogens are more common than others. Throughout the discussion, we will emphasize that the correlational nature of the data in the present study can be caused by numerous alternative explanations.

Multiple-strain infections were common in *I. ricinus* nymphs for both *Borrelia* species. For the subset of infected nymphs, ~80% were infected with multiple *ospC* strains, and the average nymph carried 2.85 strains. These results are similar to those of our previous study on nymph-derived isolates of *B. afzelii* and *B. garinii*, where the mean *ospC* strain richness was 2.4 to 3.0 strains/isolate and where 79 to 85% of isolates contained multiple strains (24). Studies on *B. burgdorferi sensu stricto* in North America have likewise shown that multiple-strain infections are common in wild nymphs of *Ixodes scapularis* and *Ixodes pacificus* (23, 41, 42, 48, 49). We point out here that the choice of the molecular method can greatly influence the observed level of pathogen strain diversity. For example, a recent study on *B. afzelii ospC* strain diversity using cold single-strand conformational polymorphism (SSCP) analysis found that only 1.5% (2/132) of questing nymphs carried more than one *ospC* strain (40). The use of 454 sequencing in the present and previous studies (24) revealed that the prevalence of multiple *ospC* strain infections in *I. ricinus* nymphs is an order of magnitude higher than what was previously shown (40). Interestingly, laboratory studies have not been able to replicate the high prevalence of multiple strain infections in nymphal ticks after letting them feed as larvae on mice coinfected with two strains (50, 51). The proportion of ticks carrying multiple strain infections was 0.8% (1/129) in the study of Derdakova et al. (50) and 1.5% (7/453) in the study of Devevey et al. (51). Interactions between *Borrelia* strains in the nymphal tick are therefore 50 to 80 times more likely to occur in nature than in the lab. This discrepancy shows that the lab studies are not capturing the *Borrelia* interactions between strains in the tick vector.

The community of *ospC* strains in the nymphal ticks is acquired from the community of vertebrate hosts the previous year during the larval blood meal. Patterns of cooccurrence and coabundance between strains in the vertebrate host therefore play a critical role in structuring those same patterns in the nymphal tick. The present study found positive associations between three pairs of strains: A1 and A2, A2 and A7, and A2 and A9. We have previously shown that *ospC* strains are often positively associated in the tick vector (24). A study on *B. afzelii* in a wild bank vole population in Sweden showed that *ospC* strains are positively associated in the reservoir host (31). One obvious explanation for positive associations between pathogen strains is that *Ixodes* ticks are generalist ectoparasites (52) that feed on a community of vertebrate species that differ in their competence to host and transmit the pathogen (53–56). Host blood meal analysis of questing *I. ricinus* nymphs at our site has shown that larval ticks feed

on *B. afzelii*-competent hosts such as rodents (57, 58) but also on artiodactyls and carnivores (57, 58), which are incompetent for *B. afzelii* (54, 55, 59, 60). If nymphs that have fed (as larvae) on competent and incompetent hosts are analyzed together, the community of pathogen strains will inevitably show aggregation. Even if all the ticks feed on a single species of competent vertebrate host, another explanation for the pattern of positive associations between pathogen strains is the well-known phenomenon of tick aggregation on certain individual hosts (61–63). Hosts that feed many nymphal ticks will collect more strains and will transmit this diverse strain community to feeding larval ticks. In summary, the community of pathogen strains inside the vector will invariably show a pattern of positive associations if vectors feed on a community of vertebrate reservoir hosts that vary in their ability to feed nymphal ticks and/or in their competence to host and transmit the pathogen to larval ticks.

Processes operating in the vertebrate host can also create negative associations between pathogen strains or species in the arthropod vector. The present study found a negative association between strains A9 and A10. Two alternative explanations include the multiple niche polymorphism (MNP) hypothesis and apparent competition mediated by the host immune system. The MNP hypothesis suggests that the different *ospC* strains cycle in different vertebrate host species (23, 64), and there is conflicting evidence for this hypothesis for *B. burgdorferi sensu stricto* in North America (23, 65–67). In Europe, studies on host specialization of *ospC* strains have been restricted to *B. afzelii* and its rodent hosts (30, 40, 68). A study in France found that an invasive species of chipmunk harbored a different set of *ospC* genotypes than the native bank vole (68). In contrast, studies on *B. afzelii* in Switzerland and Sweden found that most or all of the different *ospC* strains were found in a limited set of small mammal species (30, 40). We believe that host specialization was not responsible for creating the negative association between strains A9 and A10 in the vertebrate host because both strains are highly competent in infecting rodent reservoir hosts (see below).

Apparent competition is when the host immune system mediates the interactions between pathogen strains (2, 3, 13). We have recently shown that antibodies developed against one OspC antigen can sometimes protect mice against infection with strains carrying a different oMG allele (69). Such apparent competition in the vertebrate host might produce negative associations between *ospC* strains in the tick vector. A field study on mixed infections in a population of wild rodents found that strains carrying genetically similar oMG alleles were less likely to cooccur in the same host (31). In contrast, we found no evidence for this pattern in our local population of *I. ricinus* nymphs (24). The genetic distance between oMG alleles A9 and A10 (21.7%) is greater than that of almost all other pairs of oMG alleles in *B. afzelii* (93.2% [41/44] of oMG pairs have a genetic distance of <21.7%) (24), suggesting that cross-immunity between these two OspC antigens is unlikely. It is, therefore, also unlikely that apparent competition and cross-immunity between the two OspC antigens created this negative association. In general, the pattern of genetic variation at the *ospC* locus suggests that this polymorphism has evolved to avoid cross-immunity between oMGs and to facilitate superinfection of previously infected hosts (24, 70, 71).

The negative interaction between strains A9 and A10 is interesting because these two are the most common *ospC* strains of *B. afzelii* at our field site, as shown in the present study and in a previous 11-year study at the same site (24). We recently used a laboratory LB system consisting of *Mus musculus* mice and our pathogen-free colony of *I. ricinus* ticks to compare the fitness of six *ospC* strains of *B. afzelii*, including strains A9 and A10 (referred to in that study as A1 and YU, respectively) (72). Estimates of tick-to-host, host-to-tick, and cofeeding transmission were combined into a single estimate of the reproductive number (R_0) for each strain (72). Interestingly, strains A9 and A10 have the highest R_0 values in this laboratory LB system (72), suggesting that both of these strains are highly competent in infecting and transmitting from rodents. A field study of *B. afzelii ospC* strain diversity at two other sites in Switzerland found that strains A9 and A10 (referred to in that study as A1 and YU) were the two most common strains in wild rodents, the ticks that were attached to those rodents, and the questing

nymphs (40). Taken together, these studies suggest that *B. afzelii ospC* strains A9 and A10 are common at multiple sites in Switzerland and that they frequently encounter each other in rodent hosts and the tick vector. The present study suggests that these two strains may compete with each other inside the rodent host to achieve transmission to naive larval ticks. We point out here that from a statistical perspective, the power to detect significant interactions between strains is much higher when the strains are common. Thus, our demonstration of a significant negative association between the two most common *ospC* strains of *B. afzelii* may reflect both biological and statistical realities.

The present study found that the average total spirochete load in the nymphal tick remained constant as the number of *ospC* strains (strain richness) per tick increased (Fig. 1). As a result, the mean infection intensity per strain decreased as strain richness increased in the nymphal tick. Specifically, the mean infection intensity of a nymph infected with a single strain (2,921 spirochetes per strain) was 10 times higher than that of a nymph infected with 4 or more strains (Fig. 2). A recent study on multiple *B. afzelii ospC* strain infections in wild small mammal hosts found the same pattern (i.e., the mean infection intensity per strain decreased as the strain richness increased), and the authors argued that this result indicated the presence of within-host competition (31). A study on multiple-strain infections of *B. burgdorferi sensu stricto* in *I. scapularis* found no significant difference in infection intensities between singly and multiply infected samples, and the authors suggested that this pattern was consistent with competition between coinfecting strains (74). Likewise, we suggest that if *I. ricinus* nymphs limit the total spirochete load, the number of spirochetes per strain will inevitably decrease as strain richness increases. A recent study using genetically tagged strains of *B. burgdorferi sensu stricto* in *I. scapularis* found that strains with the highest spirochete load in the nymphal tick were more likely to be transmitted to the rodent host (21). Thus, the 10-fold reduction in the mean spirochete load per strain observed in the present study (Fig. 2) may have important consequences for the strain-specific probability of nymph-to-host transmission. Future experimental infection studies should test whether the mean infection intensity of a given strain is reduced in coinfected nymphs compared to that in nymphs infected with single strains (50, 51).

The present study also demonstrated that the abundance of certain focal *ospC* strains in the tick vector was negatively influenced by the abundance of coinfecting strains. To avoid retesting the pattern of presence/absence associations between strains, the analyses of abundance were restricted to the subset of nymphs that were infected with the focal *ospC* strain of interest. No negative abundance interactions were observed in the analysis that included all the nymphs ($n = 253$) because the variance in spirochete load among nymphs was too large (10^1 to 10^6 spirochetes/nymph). Negative abundance interactions were only observed when the analysis was restricted to a subset of nymphs ($n = 87$) with similar spirochete loads (10^4 to 10^5 spirochetes/nymph). In *B. afzelii*, the abundance of strain A9 had a negative effect on the abundance of strain A10 and vice versa. Interestingly, these two *ospC* strains of *B. afzelii* also have the highest spirochete loads inside the nymphal tick (Fig. 3). Again, statistical power influenced our ability to detect statistically significant interactions.

The simplest explanation for the negative abundance interactions in the nymphal tick is that these patterns were acquired from the vertebrate host. For example, if strain A9 is twice as abundant as strain A10 in the tissues of the vertebrate reservoir host, the larval tick would acquire a spirochete inoculum with the same 2:1 ratio of strains A9 and A10, and this ratio would be maintained in the nymphal tick. To date, no studies have tested whether the relative abundances of the strains in the tissues of the rodent host are transmitted to and maintained in the tick vector. Two independent studies on *B. afzelii* found that there was no relationship between rodent spirochete load and nymphal spirochete load (69, 75). This result suggests that processes operating in the nymphal tick may also influence the strain-specific spirochete abundance in the tick vector.

The abundance or density of vector-borne pathogens often plays a critical role in

their transmission (15). Previous work on *B. afzelii* has shown that host-to-tick transmission success increases with the spirochete density in the skin of the rodent reservoir host (69, 75). Strains of *B. afzelii* that establish higher spirochete loads in the rodent tissues (*ospC* strain A10) have higher host-to-tick transmission than strains with lower spirochete loads (*ospC* strain A3) (69). Conversely, strain-specific differences in spirochete load inside the nymphal tick might influence the probability of tick-to-host transmission. Infection experiments with different *ospC* strains of *B. afzelii* have shown that some strains (e.g., A10) establish much higher spirochete loads inside the nymphal ticks than other strains (e.g., A3) (69, 73). A recent experimental infection study using genetically tagged strains of *B. burgdorferi sensu stricto* (that were otherwise identical in fitness) showed that strains that were more abundant in the nymphal tick had a higher probability of transmission to the rodent host (21). In the present study, we found that *ospC* strains with a high spirochete load in the nymphal tick had a higher frequency in the population of *I. ricinus* ticks over the 3 years of the study. Taken together, these observations suggest that the spirochete load inside the nymphal tick plays a role in the ability of the strain to persist inside the nymphal tick and to achieve transmission to a susceptible reservoir host.

The mean nymphal spirochete load of *B. garinii* (4,449 spirochetes) was three times higher than that of *B. afzelii* (1,438), as we have reported previously (27). Conversely, the mean spirochete load necessary to establish a relative frequency of 50.0% in the population of nymphal ticks is almost 30 times higher for *B. garinii* (2,291 spirochetes) than for *B. afzelii* (83 spirochetes). These observations suggest that *B. garinii* is better than *B. afzelii* at maintaining a high spirochete load inside the nymphal tick over time and, conversely, that *B. afzelii* is much more infectious than *B. garinii* for the same spirochete load. The two *Borrelia* species cycle in different classes of vertebrate hosts, rodents for *B. afzelii* and birds for *B. garinii* (33–35), and this host specificity is mediated by the vertebrate complement system (34, 76). Previous work has shown that *B. garinii* is generally more susceptible to vertebrate complement than *B. afzelii* (77). We speculate that *B. garinii* is more susceptible to bird complement than *B. afzelii* is to rodent complement, which would require *B. garinii* to maintain a higher spirochete load in the nymphal tick than *B. afzelii* to achieve the same probability of infecting the vertebrate host. In summary, the mean nymphal spirochete loads differ between the two *Borrelia* species and among the *ospC* strains within each *Borrelia* species.

In conclusion, studies on interactions between strains of vector-borne pathogens have typically focused on the vertebrate host at the expense of the arthropod vector. In the present study, we show that the two most common *ospC* strains of *B. afzelii* in our local population of *I. ricinus* ticks, A9 and A10, are negatively associated with each other with respect to occurrence and spirochete abundance in the nymphal tick. This negative association suggests that these two *ospC* strains compete with each other in the vertebrate host and/or the tick vector. The flat relationship between nymphal spirochete abundance and the number of strains inside the tick vector suggests that the spirochete load per strain decreases as strain richness increases. Strains with a higher abundance in the nymphal tick were more common in our population of *I. ricinus* ticks. Thus, the spirochete load in the nymphal tick appears to be an important predictor of strain fitness. The study of mixed-strain infections in the arthropod vector is important for understanding the epidemiology of vector-borne pathogens.

MATERIALS AND METHODS

Tick collection and *Borrelia* detection. *I. ricinus* nymphal ticks were sampled from the Bois de l'Hôpital site in a deciduous forest near Neuchâtel (47°00′55.6″N, 6°94′16.7″E; surface area of 1 ha) over a period of 3 years (2009 to 2011). These ticks were sampled as part of the PhD thesis of Coralie Herrmann, and details of the field sampling and subsequent molecular methods have been described previously (27, 78–81).

Briefly, total DNA from nymphal ticks was extracted using NH_4OH. Ticks were tested for infection with *B. burgdorferi sensu lato* using a qPCR assay that targeted the flagellin gene. The use of standards in this qPCR assay allowed us to estimate the spirochete load in each nymphal tick. Nymphs that tested positive for *Borrelia* infection were processed using a PCR reverse line blot (RLB) assay that targets the 23S-5S spacer gene (82). The RLB allowed us to identify each of the five *B. burgdorferi sensu lato* genospecies

present at our study site: *B. burgdorferi sensu stricto*, *B. afzelii*, *B. garinii*, *B. valaisiana*, and *B. bavariensis*. Of the 7,400 nymphs that were collected, 1,741 tested positive for infection with one or more *Borrelia* genospecies, and 788 and 290 nymphs were singly infected with *B. afzelii* and *B. garinii*, respectively (27).

Sampling strategy for *Borrelia*-infected nymphs. We included only the questing nymphs that, according to the RLB, were singly infected with either *B. afzelii* or *B. garinii*. For each of the two *Borrelia* species, we randomly sampled a maximum of 52 infected nymphs for each of the 3 years of the study. Using this sampling strategy, we obtained 153 infected nymphs for *B. afzelii* (49, 51, and 52 nymphs for 2009, 2010, and 2011, respectively) and 100 infected nymphs for *B. garinii* (12, 42, and 47 nymphs for 2009, 2010, and 2011, respectively).

Characterization of the community of oMG strains via 454 sequencing. For each of the 253 *Borrelia*-infected nymphs, the *ospC* gene (~600 bp) was amplified using the PCR protocol of Bunikis et al. (44). Previous work by Strandh and Råberg (30) has shown that the amplification efficacy of this PCR protocol is the same for the different oMGs. 454 sequencing in the forward direction only was performed on a 454 Roche GS FLX sequencing apparatus and was outsourced to Microsynth AG (Balgach, Switzerland) as previously described in Durand et al. (24). The 454 sequencing run produced 130,948 sequences of the *ospC* gene. After cleaning the data set (24), we retained 92,645 *ospC* gene sequences that were each 585 bp long. For each nymphal tick, the community of oMGs was based on an average of 366 *ospC* gene sequences (geometric mean, 165 reads per tick). A nymph was considered infected with a given *ospC* strain if we recovered a single read for that oMG.

Statistical analyses. All statistical analyses were performed using R (83).

Relative frequencies of the oMG strains. In the present study, we reported the relative frequencies of the oMG strains. The relative frequency is the frequency of each oMG strain in the subset of nymphs infected with that particular *Borrelia* genospecies (see the supplemental material for details).

Calculation of the spirochete abundance for each oMG strain in the mixed-strain infection in the tick. To calculate the abundance of each oMG strain in each tick, we used the same approach as Strandh and Råberg (30). For each tick, we calculated the abundance of each *ospC* strain by multiplying the spirochete load of the tick (as estimated by qPCR) by the frequencies of the *ospC* strains (as estimated by 454 sequencing). For example, the *B. afzelii*-infected tick S5C1N041 had a spirochete load of 456 spirochetes, and we obtained 211 *ospC* gene sequences that belonged to two oMGs, A9 and A10, with the frequencies of 0.839 (177/211) and 0.161 (34/211), respectively. Thus, tick S5C1N041 contained 383 and 73 spirochetes for oMGs A9 and A10, respectively.

Associations between the presence/absence of the *ospC* strains inside the nymphal tick. We used generalized linear models (GLM) with binomial errors to test whether each focal *ospC* strain was positively or negatively associated with the other coinfecting *ospC* strains. Each *Borrelia* genospecies was analyzed separately. To minimize the type I error rate, we first evaluated the global significance of each GLM before examining the slopes of the coinfecting strains. For each focal *ospC* strain, the global significance was determined by comparing the full model (the explanatory variables were the presence/absence of the coinfecting *ospC* strains) with the null model (single intercept) using a log-likelihood ratio test. For *B. afzelii* and *B. garinii*, we analyzed 6 and 7 *ospC* strains, and to correct for multiple comparisons, the statistical significance was set at $0.05/6 = 0.008$ and $0.05/7 = 0.007$.

Associations between the abundances of the *ospC* strains inside the nymphal tick. Competition between strains (or species) occurs when the abundance of a focal strain is negatively affected by the abundances of other strains. We used multiple regression to model the $\log_{10}$-transformed abundance of each focal *ospC* strain as a function of the abundances of the other *ospC* strains. Each *Borrelia* genospecies was analyzed separately. For each focal *ospC* strain, the analysis was restricted to the subset of nymphs that were actually infected with that strain. The explanatory variables were the abundances of the coinfecting strains and because these data included zeros, they were scaled using the $\log_{10}(x + 1)$ transformation. To minimize the type I error rate, we first evaluated the global significance of the multiple regression model before examining the partial regression coefficients of the coinfecting strains. For *B. afzelii* and *B. garinii*, we analyzed 6 and 7 *ospC* strains and to correct for multiple comparisons the statistical significance was set at $0.05/6 = 0.008$ and $0.05/7 = 0.007$.

Relationship between nymphal spirochete load and nymphal strain richness. We used analysis of covariance (ANCOVA) to test the relationship between the $\log_{10}$-transformed total spirochete load in the nymphal tick and the number of *ospC* strains per nymphal tick (strain richness) for the two *Borrelia* species.

Relationship between infection intensity per strain and strain richness. A recent study on *B. afzelii ospC* strains in wild rodent reservoirs found that the mean abundance per strain (referred to as the infection intensity) was lower in mixed infections than in single-strain infections, and this result was interpreted as proof for within-host competition (30). In the present study, we used the same approach to investigate the relationship between the mean spirochete load per strain and strain richness in ticks. If the total spirochete load in the nymphal tick is constant, it suggests that the mean spirochete load per strain decreases as the nymphal tick contains a higher number of strains. For each tick, we calculated the mean number of spirochetes per strain by dividing the total spirochete load by the strain richness, and these infection intensities were $\log_{10}$-transformed to improve normality. We used ANOVA to test whether there was an effect of strain richness on the mean $\log_{10}$-transformed infection intensity per strain. The two *Borrelia* species and the higher categories of strain richness (4 to 6 strains) were combined to increase the power of the analysis.

Relationship between spirochete abundance within the tick and the relative frequency of the oMG strain. We predicted that the oMG strains that are abundant inside the tick (have a high spirochete load) would be more common (found in many ticks) than oMG strains that are less abundant. For each

oMG strain, we calculated the mean $\log_{10}$-transformed spirochete load using the subset of nymphs that carried that particular strain (i.e., nymphs not infected with that strain were excluded). For the set of 13 oMG strains, we used a generalized linear model with quasibinomial errors to model the relative frequency (averaged over the 3 years of the study) as a function of the mean $\log_{10}$-transformed spirochete load and *Borrelia* genospecies. We used quasibinomial errors to correct for the overdispersion in the residuals.

Accession number(s). The *ospC* gene sequence data have been deposited in the Sequence Read Archive under BioProject number PRJNA354775.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02552-16.

TEXT S1, PDF file, 0.1 MB.

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