Niche Partitioning of *Borrelia burgdorferi* and *Borrelia miyamotoi* in the Same Tick Vector and Mammalian Reservoir Species

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Abstract

The Lyme borreliosis agent *Borrelia burgdorferi* and the relapsing fever group species *Borrelia miyamotoi* co-occur in the United States. We used species-specific, quantitative polymerase chain reaction to study both species in the blood and skin of *Peromyscus leucopus* mice and host-seeking *Ixodes scapularis* nymphs at a Connecticut site. Bacteremias with *B. burgdorferi* or *B. miyamotoi* were most prevalent during periods of greatest activity for nymphs or larvae, respectively. Whereas *B. burgdorferi* was 30-fold more frequent than *B. miyamotoi* in skin biopsies and mice had higher densities of *B. burgdorferi* densities in the skin than in the blood, *B. miyamotoi* densities were higher in blood than skin. In a survey of host-seeking nymphs in 11 northern states, infection prevalences for *B. burgdorferi* and *B. miyamotoi* averaged ~0.20 and ~0.02, respectively. Co-infections of *P. leucopus* or *I. scapularis* with both *B. burgdorferi* and *B. miyamotoi* were neither more nor less common than random expectations.

INTRODUCTION

The spirochete genus *Borrelia* comprises three major groups of species.[1] The first group includes several agents of relapsing fever (RF), such as *B. duttonii* and *B. hermsii*. These species, with the exception of *B. recurrentis*, are transmitted between vertebrates by soft (argasid) ticks.[2] The species of the second group are transmitted by hard (ixodid) ticks of the genus *Ixodes* and include agents of Lyme borreliosis (LB), such as *B. burgdorferi* and *B. afzelii*, as well as species, such as *B. bissettii*, that have not been associated with human disease. [3] The species in the third group are closer to the RF species on the basis of DNA sequences,

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but like the LB organisms, use ixodid rather than argasid ticks as vectors. This group includes *B. theileri*, which is transmitted by *Rhipicephalus* ticks and causes infections of cattle and other large livestock,[4] and *B. lonestari*,[5,6] which is transmitted by *Amblyomma americanum* and infects deer.[7] Another species in this third group is *B. miyamotoi*, which is transmitted by *Ixodes* species, has rodents as reservoirs, and occurs in Asia, Europe, and North America.[8–12] Because *B. miyamotoi*, *B. lonestari*, and *B. theileri* have proven difficult or impossible to cultivate outside of experimental animals, [5,12,13] understanding the biology of this group of spirochetes has advanced slowly.

Where they occur, *B. miyamotoi* and a LB species, such as *B. burgdorferi* in North America or *B. afzelii* in Europe, share an *Ixodes* sp. tick as a vector and at least one mammalian reservoir. In the eastern United States, *B. burgdorferi* and *B. miyamotoi* infect the white-footed mouse (*Peromyscus leucopus*) and are transmitted between vertebrates by *I. scapularis*.[12,14,15] Infection prevalences in questing nymphal ticks have ranged between 0.20 and 0.50 for *B. burgdorferi* and 0.01 to 0.05 for *B. miyamotoi* by polymerase chain reaction (PCR). [15,16] Both species infect the ticks through horizontal transmission from a reservoir host. Unlike *B. burgdorferi*,[17–19] but like several RF *Borrelia* species,[2] *B. miyamotoi* is vertically transmitted from infected females to their offspring. [12] Similarly to RF *Borrelia* species, *B. miyamotoi* achieves high densities in the blood of rodents. [14,20]

These earlier observations suggested to us that *B. burgdorferi* and *B. miyamotoi* use different strategies for maintenance and dissemination in the same reservoir host and vector species. To further assess similarities and differences between *B. burgdorferi* and *B. miyamotoi* in the same host and vector populations, we compared the prevalences, burdens, and temporal dynamics of their infections in *P. leucopus* and sympatric questing ticks from a study site in Connecticut. Previous studies of the site showed that most *P. leucopus* became infected with *B. burgdorferi* during the course of the transmission season and that the prevalence of *B. burgdorferi* infection of questing nymphs averaged 0.36. [15] In the same study, we also documented the occurrence of *B. miyamotoi* in both *P. leucopus* and *I. scapularis*. The null hypothesis was that *B. burgdorferi* and *B. miyamotoi* were maintained independently in environments where they co-existed. To evaluate this, we determined the frequencies of each type of infection in *P. leucopus* and ticks at the Connecticut field site and, additionally, in questing *I. scapularis* nymphs that had been collected in several other locations across the northeastern, mid-Atlantic, and north-central regions of the United States.

MATERIALS AND METHODS

Field sites and mouse trapping

There were two parts of the study. In the first part, animal trapping and tick collecting were carried out at a field site at a 1,400-ha private, mixed hardwood forest in southern Connecticut (41.351° N, 72.777° W), as described previously. [15] Hereafter, this is called the "Connecticut field site." (The second part is described below.) All trapping and handling procedures were approved by the Yale University Institutional Animal Care and Utilization Committee (Study Protocol 07596). From 1998 to 2003, periodic sampling at our field site found nymphal infection prevalence to vary from 0.23 to 0.50 and an average density at the peak nymphal host-seeking period at the beginning of June to range from 0.16 to 0.55 nymphs/m^2 (J.I.T., unpublished data). Estimates of deer density have ranged from 25 to 120 deer/km. [2,21] The data reported here were from control treatment sites of an experimental study in 2001 to examine the effects of field vaccination of *P. leucopus*.[15] Three 2.15-ha plots were distributed among each of three sites that had similar landscape features and were separated by 100–1,500 m. After blood and tissue samples had been obtained, trapped mice were injected with the study's control immunogen, *Schistosoma* glutathione-*S*-transferase, and monophosphoryl lipid A and synthetic trehalose dicorynomycolate (Ribi Adjuvant System; Corixa, Seattle, WA). In

our previous studies, there was no discernible effect of immunization with the control antigen and adjuvant on *B. burgdorferi* transmission and infection dynamics under laboratory or field conditions. [15,22]

Animals were trapped from June to early September 2001. At each site, mice were trapped for 2–4 nights per trapping period, with a total of four trapping periods per site and ~3 weeks between consecutive trapping periods. Traps were placed 12-m apart and in arrays of 12×12. Aluminum live traps (H.B. Sherman Traps, Tallahassee, FL) were baited with crimped oats. Traps were set between 3:00 and 6:00 PM and checked between 8:00 and 9:00 AM the following day. Individual mice captured for the first time each trapping period were (1) anesthetized with ketamine at a dose of 150 mg/kg, (2) received a uniquely numbered metal ear tag on initial capture, (3) examined for sex, pelage, mass, and number of attached larval and nymphal ticks, (4) bled of $\leq$ 150 μL from the retroorbital sinus, and (5) subjected to 2-mm ear punch biopsy as described by Sinsky and Piesman. [23] Pelage was scored as 1 for juvenile, 2 for sub-adult, and 3 for adult. Mass was determined with a Pesola 100-g scale. On recovery from anesthesia, mice were rehydrated by provision of an apple slice and released at the point of capture. Serum was separated from erythrocytes, leukocytes, platelets, fibrin, and spirochetes by centrifugation at 10,000*g* for 6 minutes [24] and was stored at −20°C until the assays were performed. The blood pellets and biopsy tissues were stored at −70°C until DNA extraction. Mice recaptured within the same trapping period were only weighed and examined for ticks; mice recaptured during subsequent trapping periods were processed as described. [15]

Tick collections

As part of the first part of the study, we sampled grids at the Connecticut field site for host-seeking nymphal *I. scapularis* ticks by drag sampling [25] from late May through August 2002 with the aims to collect ticks in proportion to their relative abundance on grids and to maximize sample sizes for analysis of infection with *B. burgdorferi*. We pulled a standard 1-m^2 white corduroy drag cloth across the leaf litter for 132-m transects within each grid, stopping every 12 m to collect ticks. After dragging transects, we further sampled in high tick density areas. Ticks were immersed in 70% ethanol for storage at 4°C until processing within 3 months. A preliminary study showed that storage of laboratory-infected *I. scapularis* nymphs with *B. burgdorferi* in 70% ethanol at 4°C for 3 months did not decrease the yield of PCR product.

In the second part of the study, host-seeking ticks were collected between mid-May and late August in the years 2004 through 2007 as part of study described by Gatewood and others. [16] Collection sites were selected according to a stratified random selection procedure described by Diuk-Wasser and others. [26] From that study, we used DNA extracts from ticks at the 46 sites at which at least 20 *I. scapularis* nymphs were collected over the course of the survey. These were in nine states of the northeastern and mid-Atlantic regions (CT, MA, MD, ME, NJ, NY, PA, RI, and VA), which were $\leq$ 80° W, and six states of the north-central region (IA, IL, IN, MN, and WI), which were > 80° W. The first part's Connecticut field site was not included in this survey. Each site was visited repeatedly at approximately even intervals throughout the summer months, with a median of five visits during the season. At each visit, host-seeking ticks were collected from vegetation using a 1-m^2 drag cloth over five 200-m transects, for a total of 1,000 m^2 that were sampled per visit. The cloth was inspected for ticks every 20 m, and nymphal ticks were preserved in transect-specific vials of 70% ethanol. Larvae were either placed in the vial or collected using adhesive tape and stored in plastic bags.

DNA extraction and quantitative PCR

From blood and skin biopsies from the Connecticut site, DNA was extracted using the Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA) as described. [27] For ticks collected at the Connecticut site, DNA was extracted from individual nymphal *I. scapularis* following Beati

and Keirans for the 2002 collection. [28] For ticks collected at several locations in 2004–2007 collections, total DNA was extracted using ammonium hydroxide (NH_4OH). For this procedure, ticks were incubated for 2 h in 5 μL of 1.4 mol/L NH_4OH at 22°C and crushed with a plastic pipette tip. To this was added 95 μL distilled H_2O before a second incubation at 95°C for 30 minutes. Material was centrifuged to separate tick debris from DNA solution, and the supernatant was transferred to clean vials containing 1 μL of 100 mmol/L EDTA and stored at −20°C.

In addition to the DNA extracts from blood, tissue, and ticks described above, two other sets of DNA samples, which were extracted from ticks by the method of Beati and Keirans, [28] were available for examination: (1) flat nymphs that were derived from engorged larvae removed from captured mammals at the Connecticut field site, as described by Hanincova and others, [29] and (2) flat larvae and nymphs of laboratory-reared *P. leucopus*, which were infected with *B. miyamotoi*, as described. [12]

DNA extracts were subjected to quantitative multiplex real-time PCR (qPCR), as described, [15,16,30] with two probes hybridizing to a region of the 16S rDNA that differed between *B. burgdorferi* and *B. miyamotoi*. Results were expressed as the number of spirochete cells per tick or volume of blood or tissue. Forward and reverse primers were, respectively, 5′ GCTGTAAACGATGCACACTTGGT and 5′GGCGGCACACTTAACACGTTAG. The corresponding dye-labeled probes were 6FAM-TTCGGTACTA ACTTTTAGTTAA and VIC-CGGTACTAACCTTTCGAT TA with 3′ ends modified with a minor groove binding protein (Applied Biosystems, Foster City, CA). The reaction was performed in 25-μL volume in single tubes or wells at a final concentration of 900 nmol/L for each primer and 200 nmol/L for each probe. [15] The final concentration of EDTA was < 0.1 mmol/L. The PCR conditions were 50°C for 2 minutes and 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 63°C for 60 seconds on an Applied Biosystems 7300 Real-Time PCR apparatus for the 2002 and 2004 samples and a Rotor-Gene RG-3000 apparatus (Corbett Research, San Francisco, CA) for the 2005–2007 samples. The DNA extractions, PCR reaction preparations, and analysis of the products were carried out in three separated laboratory rooms. To monitor for contamination, negative controls were included with all DNA extraction and PCR procedures.

DNA standards were the same for each experiment: strain B31 (ATCC 35210) for *B. burgdorferi* and strain HS1 (ATCC 35209) of *B. hermsii* for the uncultivable *B. miyamotoi*. [15] *B. hermsii* and *B. miyamotoi* have identical sequences for the regions of the primers and probe. *Borrelia* species cells were grown in BSK II medium at 34°C and harvested as described. [31] With DNA standards, the qPCR assays with each probe set was linear with a $R^2 \geq 0.99$ over a range of $1–10^6$ spirochetes per reaction. The linear regression coefficients (95% confidence intervals [CIs]) for C_t values on log-transformed cell counts were −3.31 (−3.40 to −3.28) for *B. burgdorferi* and −3.40 (−3.52 to −3.28) for the *B. hermsii* surrogate ($P > 0.05$). Samples with estimated spirochete counts of less than one per tick or biopsy specimen were considered negative.

The identities of the *Borrelia* species in 100 random samples scored by qPCR as *B. burgdorferi* were confirmed by PCR of the 16S–23S intergenic spacer region (IGR) with species-specific primers. [8,15,32] Random samples scored as *B. miyamotoi* or *B. burgdorferi* by qPCR were confirmed by direct sequencing of the IGR on a CEQ 8000 capillary sequencer (Beckman Coulter, Fullerton, CA).

Statistical analysis

Poisson and logistic regression analyses, goodness-of-fit tests, and non-parametric analyses were performed with LogXact or StatXact v. 6 (Cytel Software, Cambridge, MA) using exact criteria or with Monte Carlo simulation (10,000 replicates). Differences in proportions were

assessed with Confidence Interval Analysis v. 2.1.2 (University of Southampton). Spirochete counts generally followed log-normal distributions and, accordingly, were log-transformed first for calculations of mean and 95% CIs and for performance of *t* tests, ANOVA, or Kruskal-Wallis tests using SYSTAT v. 11 (SYSTAT Software, Inc., Chicago, IL). The antilogs of the means and the asymmetric CIs are given. Odds ratios (ORs) and means are accompanied by 95% CI in parentheses or brackets. Significance tests were two-tailed.

RESULTS

Borrelia infection of blood

Overall, 69 (0.12) of 556 blood specimens from captured mice had *B. burgdorferi*, and 36 (0.06) had *B. miyamotoi* by qPCR (Table 1). The three collecting sites at the Connecticut field site were similar in terms of the prevalences of the two species in the blood of *P. leucopus*. Although *B. burgdorferi* was twice as prevalent as *B. miyamotoi* among captured mice over the sampling period, *B. miyamotoi* reached densities in the blood that were ~5-fold higher than for *B. burgdorferi*. The counts of each species in the blood followed a log-normal distribution with peaks at ~100 and 1,000 spirochetes/mL for *B. burgdorferi* and *B. miyamotoi*, respectively (Figure 1A and B).

No mice captured during the first trapping period had detectable *B. burgdorferi* or *B. miyamotoi* in the blood. Thereafter, both *Borrelia* species were detected in mice, but they differed in the timing in their peaks of prevalence (Figure 2). *B. burgdorferi* bacteremia was most frequent during the second trapping period and declined in prevalence with succeeding trapping periods. In contrast, the frequency of *B. miyamotoi* bacteremia increased in prevalence through Period 4 (Kruskal-Wallis, $P = 0.001$). We did not detect an association between sex and infection by trapping period for either *B. burgdorferi* (OR, 1.2 [0.64–2.1]) or *B. miyamotoi* (OR, 1.9 [0.85–4.0]).

To assess a possible effect of vaccination with the adjuvant and control antigen, we matched by day of capture 120 mice that had not previously been vaccinated with 120 mice that been vaccinated on a prior capture but captured on the same day. The mean and median days of capture were 213 and 207, respectively, for both matched sets. There were 40 males in the non-vaccinated group and 43 males in the vaccinated group (OR, 1.1 [0.7–1.1]). Vaccinated mice were significantly larger than non-vaccinated mice in the matched sets: the mean weights (g) were 20.5 (19.8–21.1) and 18.2 (17.6–18.8), respectively ($P < 10^{-5}$). The estimated age by pelage score was also generally higher in the vaccinated group: the mean pelage scores were 2.5 (2.4–2.6) for non-vaccinated mice and 2.8 (2.7–2.9) for vaccinated mice ($P < 10^{-5}$). Receipt of the adjuvant, however, did not seem to subsequently protect the mice against infection: 26 vaccinated mice versus 13 non-vaccinated were infected with *B. burgdorferi* (OR, 2.3 [1.1–4.7]). For 68 mice that were infected with *B. burgdorferi*, mean spirochete counts in the blood were 67 (37–120) and 46 (33–64) for 22 non-vaccinated and 46 vaccinated mice, respectively ($P = 0.24$). There was no apparent effect of vaccination on frequency of *B. miyamotoi* infection for the set matched by day of capture: 7 of 120 non-vaccinated and 6 of 120 of vaccinated mice were infected (OR, 0.8 [0.3–2.6]).

B. burgdorferi bacteremia was associated with infestation with nymphs but not with larvae for the set of 539 captures for which data on infestations were complete (Figure 2). The mean number of larvae per captured mouse for trapping periods 1, 2, 3, and 4 were 2.6, 1.0, 12.4, and 8.1, respectively. The corresponding values for nymphs were 1.4, 0.4, 0.1, and 0.02. Of 42 mice infested with nymphs alone, 10 (0.24) were bacteremic, whereas of 497 mice with no ticks, with larvae only, or with larvae as well as nymphs, only 56 (0.11) were bacteremic with *B. burgdorferi* (OR, 2.5 [1.2–5.3]; $P = 0.03$). In contrast, *B. miyamotoi* bacteremia was associated with infestation with larvae but not with nymphs (Figure 2). Of 301 mice with larvae

alone, 25 (0.08) had *B. miyamotoi* in the blood, whereas of 238 with no ticks, with nymphs alone, or with nymphs as well as larvae, only 10 (0.04) were bacteremic (OR, 2.1 [0.97–7.1]; $P = 0.05$).

Among 167 mice that were captured at least twice, the prevalence of infection in the blood increased with the succeeding capture for both *B. burgdorferi* (OR, 1.5 [1.1–1.9]) and *B. miyamotoi* (OR, 2.2 [1.5–3.1]). With respect to *B. burgdorferi* in the blood, mice changed from negative at the first capture to positive at a second one as follows: 18 of 83 (0.22) within 3 weeks, 8 of 47 (0.17) within 4 weeks, 10 of 26 (0.39) within 5 weeks, and 2 of 9 within 7 weeks (0.22). Thus, at least 25% of recaptured mice were bacteremic with *B. burgdorferi* at a successive capture. With *B. miyamotoi*, the frequencies of conversion from PCR negative to positive were the following: 16 of 70 (0.23) within 3 weeks, 5 of 36 (0.14) within 4 weeks, and 4 of 9 (0.44) within 6 weeks. The negative-to-positive conversion frequency for *B. miyamotoi* was similar at 0.22. Twenty-one (0.75) of 28 mice that had *B. burgdorferi* in the blood and 6 (0.67) of 9 mice that had *B. miyamotoi* in the blood had undetectable spirochetes in the blood when resampled subsequently within 3 weeks. On the basis of the conversion rates for PCR positivity in the blood, the weighted mean incidence for each species was ~0.1 cases/ mouse/wk during the period of study.

Borrelia infection of the skin

During captures later in the summer, skin biopsies of the ears, as well as blood samples, were obtained from 86 mice (Table 1). Of these mice, 65 (0.76) had skin biopsies that were positive for *B. burgdorferi* by PCR, and 9 (0.11) mice simultaneously had *B. burgdorferi* in the blood; there were no mice with *B. burgdorferi* in the blood but not in the skin. Opposite results were found with *B. miyamotoi*: only 2 (0.02) mice had a positive skin biopsy for *B. miyamotoi*, whereas 10 (0.12), including the two with positive skin biopsies, had *B. miyamotoi* in the blood. Taking the blood and tissue results together (Table 1), we observed that *B. burgdorferi* was more commonly present in skin than one would expect on the basis of the relative frequencies of *B. burgdorferi* and *B. miyamotoi* in the blood (OR, 17.0 [4.0–149]; $P < 10^{-6}$). When stratified by trapping period, the ear tissue infection with *B. burgdorferi* was associated with mass of the captured mouse (OR, 1.3 [1.1–1.7]; $P = 0.01$) but not with sex (OR, 2.3 [0.52–12.0]) or pelage (OR, 1.1 [0.34–4.0]).

B. burgdorferi and *B. miyamotoi* also differed in their cell densities in infected skin (Table 1). There were ~40-fold more *B. burgdorferi* in infected tissue than was found for *B. miyamotoi* in an equivalent volume of skin. Similar to the infection in the blood, the *B. burgdorferi* counts in the skin were log-normal in distribution with a peak at ~1,000 cells/mg of tissue (Figure 1C). Extrapolating the values to a gram of skin tissue, which approximates a milliliter of blood, we estimated that there were ~640,000 *B. burgdorferi*/g of tissue. This compares with ~50 *B. burgdorferi* cells in a comparable volume of blood.

Borrelia infection in sympatric *I. scapularis* ticks

At the Connecticut field site, we collected 689 questing nymphs the year after the *P. leucopus* trapping. These nymphs represented the generation that fed on *P. leucopus* or other hosts as larvae the previous year. By qPCR we identified *B. burgdorferi* in 244 (0.35) nymphs and *B. miyamotoi* in 38 (0.055) nymphs (Table 1). The median counts per tick were 6,130 for *B. burgdorferi* and 317 for *B. miyamotoi*, but the distributions of counts per infected tick differed between species. The counts of *B. burgdorferi* in individual ticks followed a log-normal distribution with an extended tail toward lower burdens (Figure 3). In contrast, the burdens of *B. miyamotoi* cells in infected ticks were bimodal in distribution. Of 38 nymphs infected with *B. miyamotoi*, 20 (0.53) had < 100 spirochetes, which compared with only 19 (0.08) of 244 nymphs with < 100 *B. burgdorferi* spirochetes (OR, 13.2 [5.5–31.2]; $P < 0.0001$).

However, among ticks with $\geq$ 100 spirochetes, *B. miyamotoi* had higher mean numbers than did *B. burgdorferi*: 15,171 (9,616–23,993) versus 6,412 (5,521–7,447), respectively (Table 2).

We subsequently extended this analysis by determining infection prevalences and spirochete burdens in 7,205 questing *I. scapularis* nymphs collected during 2004–2007. Overall, 1,477 (0.21) of ticks had detectable *B. burgdorferi*, and 139 (0.019) ticks, ~10-fold lower, had *B. miyamotoi*. The results by site as defined by geographic coordinates are summarized in Table 2. Although each of the 46 sites in this survey had some ticks infected with *B. burgdorferi*, 13 (0.28) of the sites did not have detectable *B. miyamotoi*. This may be attributable to under-determination; at 9 of these 13 sites sample sizes of ticks were between 22 and 97. There were no collection sites at which *B. miyamotoi* was found but not *B. burgdorferi*. At 19 sites at which both species were present and at least 100 ticks were collected, there was no correlation between the prevalences of *B. burgdorferi* and *B. miyamotoi* ($R^2 = -0.04$; $P = 0.59$).

Figure 4 shows the distributions of log-transformed counts of *B. burgdorferi* or *B. miyamotoi* per infected tick. The results of this broader study were similar to the findings at the Connecticut site, namely an extended tail to the left for an otherwise log-normal distribution for counts of *B. burgdorferi* and a bimodal distribution for *B. miyamotoi*. The mean counts per infected tick were 3,155 (2,825–3,516) for *B. burgdorferi* and 4,246 (2,360–7,621) for *B. miyamotoi* (Kruskal-Wallis, $P = 0.002$), and the medians were 4,898 and 12,735, respectively. With a cut-off point of 300 ($\log_{10} = 2.48$) spirochetes for dividing *B. miyamotoi* counts into the two parts reflected by the histogram (Figure 4), the mean for the first, lower value distribution was 32 (22–47), whereas the mean for the second was 28,576 (19,815–41,115).

We next studied the extent to which infected *I. scapularis* housed or reared under laboratory conditions resembled those in natural environments. For this, we first examined DNA extracts from 1,146 ticks that were originally obtained as engorged larvae on captured mammals at the Connecticut field site in 2004. [29] After repletion, the ticks were maintained in the laboratory until they molted. Eight (0.007) of the nymphs were infected with *B. miyamotoi*. These were originally obtained from one Eastern chipmunk (*Tamias striatus*), one Eastern gray squirrel (*Sciurus carolinensis*), three raccoons (*Procyon lotor*), and three pine voles (*Microtus pinetorum*). The mean number of *B. miyamotoi* per infected nymph was 2141 (4,66–19,841).

We performed qPCR on DNA extracts from 19 nymphs and 47 larvae that were the progeny of an *I. scapularis* female infected with *B. miyamotoi* in a tick-rearing facility. [12] Larvae were fed on uninfected laboratory white-footed mice to repletion, allowed to molt, and were assayed as flat nymphs. By qPCR, 36 (0.77) larvae and 16 (0.84) nymphs were infected with *B. miyamotoi* (OR, 1.3 [0.28–8.6]; $P = 0.99$). Figure 5 shows the distributions of counts of *B. miyamotoi* in infected larvae and nymphs in the laboratory. There was a tendency for a bimodal distribution of the counts, but it was less pronounced than what was observed in field-collected ticks. The mean counts were 2,993 (1,637–5,472) for larvae and 5,167 (2,142–12,463) for nymphs (ANOVA, $P = 0.32$; Kruskal-Wallis, $P = 0.27$). The number of *B. miyamotoi* in nymphs that had been infected vertically rather than horizontally was within the 95% CI of what was observed in the field-collected nymphs (see above and Figure 4).

Co-infections

At the Connecticut field site, 5 (0.009) of 556 mouse blood samples were infected with both *Borrelia* species. By Poisson regression analysis, the mice infected with *B. burgdorferi* were at no greater risk of infection with *B. miyamotoi* compared with non-infected mice (OR = 1.1 [CI 0.35–2.8]; $P = 0.93$). Nine mice that had *B. burgdorferi* either in the blood or skin also had *B. miyamotoi* in either the blood or skin, but this was not significantly more or less than expected, under the assumption of independent transmission of each species (OR, 0.96 [0.21–6.1]). The following year there were 5 (0.007) co-infections with *B. burgdorferi* and *B.*

miyamotoi in 689 questing nymphs. This was less than the 13 predicted co-infections under random expectations (OR = 0.28 [CI 0.09–0.72]; *P* = 0.004). Among the 1,146 ticks recovered as engorged larvae from several species of mammals at this field site and allowed to molt, 226 (0.20) were infected with *B. burgdorferi* and 10 (0.9%) were infected with *B. miyamotoi*. No co-infections were observed when the expectation was 2 (Poisson, *P* = 0.13).

The study of the frequency of co-infections was expanded to samples from a broad survey of field sites across the northeastern, mid-Atlantic, and north-central United States. [16] Of the 7,205 nymphs, 1,477 (0.20) overall were infected with *B. burgdorferi*, 139 (0.019) were infected with *B. miyamotoi*, and 20 (0.003) were infected with both species. The OR for a co-infection was 0.65 (0.38–1.07; *P* = 0.08) by year of collection. There was no apparent effect of the collecting site location on co-infection frequency either at the level of state (OR = 1.01 [0.91–1.04]) or whether the site was east or west of 80° W longitude (OR = 1.15 [0.77–1.70]). In the ticks co-infected with *B. miyamotoi* and *B. burgdorferi*, the mean spirochete count of *B. miyamotoi* per tick was 21,974, a value more typical of the second peak of counts of this species in ticks (Figure 4).

We also examined the subset of five sites (numbers 4, 11, 12, 35, and 44 of Table 2), at which > 400 questing nymphs were collected. These sites each had sample sizes that were comparable to the 689 nymphs collected at the Connecticut site in 2002. Of the 2,579 ticks collected, 470 (0.18) were infected with *B. burgdorferi*, 45 (0.017) were infected with *B. miyamotoi*, and 6 (0.002) were co-infected. Stratified by site, co-infection was not more or less frequent than expected (OR = 0.70 [0.24–1.73]). Thus, in the expanded study of multiple locations, there was no evidence of either positive or negative associations between *B. burgdorferi* and *B. miyamotoi* infection of nymphs.

It was possible that we underestimated the number of co-infections, because the minority member in the mixture was not fully represented as the consequence of interference or other PCR artifact. In other words, when one species was in great excess with respect to another, the PCR assay for the less numerous species may have been falsely negative or underestimated its true count. To assess the magnitude of this putative effect, we took DNA extracts of several ticks with various counts of *B. miyamotoi* and to these added different amounts of *B. burgdorferi* DNA, so that the ratios of one species to the other varied over the range of ~1:1,000 to 1,000:1. Figure 6 shows the results for measurement of *B. miyamotoi* in the presence of different amounts of *B. burgdorferi* (A) and for *B. burgdorferi* in presence of *B. miyamotoi* (B). *B. miyamotoi* was undercounted when its numbers were ~100 or less and *B. burgdorferi* was in excess by 100- or 1,000-fold. There was less of an effect of *B. miyamotoi* on the *B. burgdorferi* in the same reaction. There was a false-negative reaction only when *B. burgdorferi* had a count of ≤ 10 and *B. miyamotoi* was in 1,000-fold excess.

We next performed a permutation study with 100 replicates in which the results of the 7,205 tick extracts of the second part of the study were randomized with respect to qPCR results for *B. miyamotoi* and *B. burgdorferi* (Table S1 of Supplementary Materials can be found online at www.ajtmh.org). In each replicate, there would be a total of 1,477 positive results for *B. burgdorferi* and 139 positive results for *B. miyamotoi*, as before (Table 2), but these were no longer linked, and co-infections occurred under random expectations. We also counted the mixtures in this simulation in which either *B. burgdorferi* or *B. miyamotoi* would have been in 100-fold or greater excess. This provided an estimate of the number of co-infections that might have gone undetected.

After 100 replicates, there was a mean of 28.1 co-infections with a central 95% range of 20.0–37.0. The mean number of mixed infections in which *B. miyamotoi* was the minority partner by 100 or greater fold was 4.3 (1.0–7.0); the corresponding values for *B. burgdorferi* were 3.8

(1.0–7.5). If we assume that, in both these circumstances, co-infections went undetected, the expected mean number of co-infections from this simulation under random conditions was 20 (13–28), which was the experimental observation from the survey.

DISCUSSION

Ecologic specialization, or niche differentiation, is hypothesized to be a mechanism for species co-existence. [33] From that broad perspective, co-existence of *B. burgdorferi* and *B. miyamotoi* in the same arthropod and mammalian species presents an interesting case for study. To that end, we compared the population and transmission dynamics of *B. miyamotoi* and *B. burgdorferi* between a local population of *I. scapularis* vectors and *P. leucopus* reservoirs in a site in southern Connecticut. To explore the generality of trends, we extended the study to other areas across northeastern and north-central United States where host-seeking *I. scapularis* nymphs are common. [15,16]

Before comparing infections by these two species in more detail, we first consider the following: (1) the possibility that a third (or fourth) *Borrelia* species was present at the study sites and in both the tick vector and vertebrate host, (2) the co-occurrence of two LB *Borrelia* species in Europe and how this differs from what we report here, and (3) the possible contributions of other vertebrate hosts to the maintenance of *B. miyamotoi*.

1. In the extended area of *B. burgdorferi* transmission in North America, three other *Borrelia* species besides *B. miyamotoi* conceivably could co-occur with *B. burgdorferi: B. andersonii*, which comprise a natural cycle involving *I. dentatus* and rabbits, [34,35] *B. bissettii*, whose cycle in the central United States involves *I. spinipalpis* ticks and woodrats, [36] and "*B. davisii*," which we found in the blood of *P. leucopus* at the Connecticut field site but not in any *I. scapularis* ticks at the same site. [14] Given the mismatches with the probes, *B. andersonii* and "*B. davisii*" would not have been detected by qPCR, but, at the same time, neither species would be expected to be found in *I. scapularis* in these locations. *B. bissettii* would be detected by the LB species group probe, but this species was identified only once, or 0.2%, out of 430 *I. scapularis* extracts that were positive for *B. burgdorferi* and further characterized by sequencing for the 11 state survey described here (J. Bunikis and A. G. Barbour, unpublished data). Given these findings and the circumstances of the study, we concluded that only *B. burgdorferi* and *B. miyamotoi* were to be expected in *I. scapularis* and *P. leucopus*.

2. In Europe, co-occurrence of two or more *Borrelia* species of LB agents in the same ticks is well documented. [37–39] However, there are important differences between that phenomenon from what we report here on the co-existence of *B. burgdorferi* and *B. miyamotoi*. For one, co-existing *B. garinii* and *B. afzelii* tend to differ in their ranges of preferred vertebrate hosts [40] and not so much in their behavior in their vertebrate hosts of choice. Namely, both *B. garinii* and *B. afzelii*, like *B. burgdorferi*, are characterized by a transient, low-density bacteremia followed by persistence in the skin. [41–43] Furthermore, *B. afzelii* and *B. garinii* have similar burdens in *I. ricinus* ticks. [30,32] Unlike the case of *B. miyamotoi*, vertical transmission, if it occurs at all, is inefficient. [17,19]

3. Under both experimental and natural conditions, *B. burgdorferi* infects many species of vertebrates, several of which serve as competent reservoirs. [44] In this respect *B. burgdorferi* is a "generalist" pathogen. [45] Less is known of the host range for *B. miyamotoi*. Most vectors for the RF group of species are nidicolous and, not surprisingly, relapsing fever agents are often specialists for the mammalian species that inhabit the burrows or nests infested with the ticks. [2] On the other hand, *B.*

miyamotoi uses a vector that seeks out hosts and successfully feeds on a variety of vertebrate species. Plausibly, there are other reservoirs for *B. miyamotoi* besides *P. leucopus*. In support of this suggestion was the report of Krampitz [46] in 1986 of a "European hard tick spirochete," which, in retrospect, was probably *B. miyamotoi*. This uncultivable organism had two characteristics that were atypical for LB species: transovarial transmission and high peak densities in the blood of infected mammals. Stanek and others [47] described a *Borrelia* species from *I. ricinus* ticks with similar characteristics in laboratory animals and more recently confirmed this to be *B. miyamotoi* (G. Stanek, personal communication). The potential range for reservoir hosts for this species includes voles, gerbils, and rabbits, [46,47] in addition to mice. [12,14] There are also reports that *B. miyamotoi* infects large mammals, such as deer and cattle [48,49] (G. Hickling, personal communication). Although we cannot rule out an important role for another vertebrate species for maintenance of *B. miyamotoi*, this study focuses on the reservoir *P. leucopus*.

Infection of *P. leucopus*

Mice were captured and re-captured at the Connecticut field site over the course of a transmission season. The study was carried out on mice on control grids from a vaccination study. [15] Re-captured mice would have received the control antigen and adjuvant 3–6 weeks previously. Prior vaccination was associated with increased frequency of *B. burgdorferi* but not *B. miyamotoi* in the blood. Co-variant with the higher frequency of *B. burgdorferi* infections were the generally larger sizes and greater ages of the vaccinated group when matched by day of capture to unvaccinated animals. The higher infection prevalence may be attributable to the greater cumulative likelihood of infection for older mice, as noted by Bunikis and others. [27] In any case, there was no evidence that prior vaccination affected the course of infection; among infected mice, burdens of *B. burgdorferi* were the same for vaccinated and non-vaccinated mice. Although we cannot rule out a confounding effect of control vaccination, we had not previously noted discernible effects of this control vaccination on *B. burgdorferi* infection of *P. leucopus*, [15,22] and we think it unlikely that there was a differential effect of the vaccination with the negative control antigen on *B. miyamotoi* and *B. burgdorferi* dynamics.

Under these experimental conditions, the findings on infection prevalence and spirochete burdens in blood samples and ear biopsies indicated that *B. burgdorferi* produces a transient, low-density bacteremia and a more persistent, higher density infection of the skin. In contrast, *B. miyamotoi* was in higher densities in the blood among bacteremic animals, but in lesser amounts in the skin than *B. burgdorferi*. From the data on recaptured mice, the duration of bacteremia for *B. miyamotoi* seems to be similar to that *B. burgdorferi*. However, with its lower burdens in skin, the opportunity for horizontal transmission of *B. miyamotoi* may be restricted to the period of bacteremia. In contrast, for *B. burgdorferi* transmission *P. leucopus* remain infectious for larvae and nymphs long after spirochetes have been cleared from the blood. [41, 50] A narrower window for transmission of *B. miyamotoi* by an infected mammal may be one explanation for the lower overall prevalence of this species in ticks.

Notwithstanding these differences, the seasonal dynamics of infection of the blood of reservoir mice with *B. burgdorferi* and *B. miyamotoi* correlated with the activity patterns of, respectively, nymphal and larval ticks at the Connecticut study site. The prevalence of *B. burgdorferi* infection of the blood of the mice waned with decreasing activity of the nymphs during the second half of the summer. Meanwhile, the increase in the prevalence of *B. miyamotoi* infection of the mice coincided with peak activity of larvae (Figure 2). This is consistent with transmission from larvae that had been vertically infected with *B. miyamtoi*, as discussed below.

The apparent seasonality of *B. burgdorferi* bacteremia conceivably could be attributable to an increasing proportion of non-susceptibles in the population of mice as they become immune.

However, our previous study of the Connecticut field site indicated that *P. leucopus* have serial infections, as would be expected for a pathogen for which immunity is largely strain specific. [27] A given mouse may be protected against re-infection by the same strain, but it would remain at risk of infection by other strains throughout the year.

Infection of *I. scapularis*

Borrelia miyamotoi infects *I. scapularis* ticks in the northeastern United States, [12,15,51] *I. pacificus* in California, [11] and *I. ricinus* in Sweden, [9,30] Germany, [52] and the Czech Republic. [53] At these diverse locations, the prevalence of infection in nymphs was usually 0.5–3% and seldom > 4%. In all these surveys, at least one LB species co-existed with *B. miyamotoi* in ticks at the collecting sites and usually in ratios of ~10:1 for infection prevalences. In the survey of host-seeking *I. scapularis* nymphs in 11 states, we observed similar results. Mean prevalences for 46 sites over the 4-year period of study were 0.19 for *B. burgdorferi* and 0.019 for *B. miyamotoi* overall. The failure to record *B. miyamotoi* at some locations is plausibly attributable to undersampling; at sites with detectable *B. burgdorferi* but not *B. miyamotoi*, the sample sizes of collected ticks were lower than the overall average.

The differential in prevalences between the two *Borrelia* species seems to be even greater in adult ticks. Prevalences of *B. burgdorferi* in adult *I. scapularis* are generally ~2-fold higher than in nymphs, which is attributable to the cumulative risk of infection with each feeding. [54,55] In contrast, prevalences of *B. miyamotoi* were about the same in adults and nymphs among *I. pacificus* populations in the far-western United States [11] and adult *I. scapularis* populations in the north-central United States (S. Hamer and J. Tsao, unpublished data).

Among infected nymphs, the distributions of counts of *B. burgdorferi* spirochetes in the nymphs were similar at the Connecticut field site and the collecting at other locations in the larger survey (Figures 3 and 4). The majority of infected questing nymphs had between 1,000 and 10,000 *B. burgdorferi*; the mean was 3,155 for the large survey. Using a qPCR procedure with primers for the *recA* gene of *B. burgdorferi* but without a probe, Wang and others [56] reported a mean of 1,964 spirochetes per tick among 91 infected nymphal *I. scapularis* from the northeastern United States. We had previously found a mean of 2,240 spirochetes per infected nymph using a reverse transcriptase-PCR assay for ribosomal RNA. [57] The PCR-derived values for *B. burgdorferi* burdens in *I. scapularis* were similar to previous findings using antigen detection; Brunet and others [58] observed that infected nymphs in Massachusetts had ~2,000 spirochetes, and Burkot and others [59] reported that infected adults from New York had ~5,000 spirochetes per tick.

B. miyamotoi was notable for the bimodal distribution of its counts in questing nymphs at both the Connecticut site (Figure 3) and other locations (Figure 4). This distribution was not observed for blood and tissue counts of *B. miyamotoi*. When we first observed this phenomenon in the ticks, we asked whether the peak with lower counts represented spirochete burdens in congenitally infected ticks. This may still be the explanation for ticks under natural conditions, but the subsequent study of laboratory-reared *I. scapularis* infected vertically with *B. miyamotoi* showed a log-normal distribution with only one discernible peak, and there was no difference between larvae and nymphs (Figure 5). Thus, the relationship between *B. miyamotoi* burdens in tick and the likelihood of transmission at its next feeding remains to be determined.

Co-infections

Infections of a *P. leucopus* mouse with both *B. miyamotoi* and *B. burgdorferi* were not more or less common than expected on the basis of the frequencies of the individual species at the Connecticut site. At the Connecticut field site, co-infections of questing *I. scapularis* nymphs

were less common than expected for that year, but this evidence of a negative association was not confirmed either in the study of ticks removed from mammals at the same field site or when a much larger sample of > 7,000 ticks from the 4-year, 11-state survey was examined and stratified by year and location. In the later analysis, the 95% CIs for the ORs were narrow enough to exclude strong or moderate interactions between the two *Borrelia* species.

In experiments in which the ratios of one species to the other in the multiplex qPCR were varied over a wide range, there was reduced detection of the minor population at extreme ratios. Thus, it is possible that we did not identify some co-infections when one species was in much lower numbers than the other in a given tick. However, when we assumed that co-infections with ratios of ≥ 100 between the species' counts were under-determined, the multiple-replicate simulation under random expectations yielded a mean number for co-infections that was the same that we observed in the multi-state survey. Although we cannot rule out a weak association, either positive or negative, between *B. miyamotoi* and *B. burgdorferi* in ticks and/ or vertebrates, the preponderance of evidence supports the conclusion that the infections are largely independent. Our thesis is that this independence is attributable to the different niches that *B. miyamotoi* and *B. burgdorferi* exploit.

Transovarial transmission of *B. miyamotoi*

Scoles and others [12] reported that vertical transmission of *B. miyamotoi* in the laboratory was common and found filial infection prevalences among larvae of 0.06 to 0.73. In this study, which used a sensitive qPCR, filial infection frequency in larvae was 0.77. Transovarial transmission of *B. burgdorferi* in *I. scapularis* is either nonexistent or rare. [17–19] The infection of unfed larvae is *prima facie* evidence for a different niche for *B. miyamotoi* from that of *B. burgdorferi* in ticks. This, along with the evidence of a different niche for the spirochetes in *P. leucopus* distinguishes *B. miyamotoi* from *B. burgdorferi* and serve to separate their fates.

When the distribution of *B. miyamotoi* spirochetes in the tissues of ticks is studied, we predict that this species' cells will be more widely distributed in unfed ticks than is the case for *B. burgdorferi*, which is predominantly found in the midgut of flat nymphs. [60,61] Supporting evidence was the earlier observation by Lane and Burgdorfer [62] that transovarially transmitted spirochetes in *I. pacificus* were distributed throughout the larva's tissues. These spirochetes were probably *B. miyamotoi*, because they were not bound by a monoclonal antibody that was specific for *B. burgdorferi*[63] and *B. miyamotoi* is now known to occur in these ticks. [11]

Although many of the details of the life cycle of *B. burgdorferi* and related species remain to be characterized, the basic features of the natural history of this microorganism are understood. [3] This is not the case for *B. miyamotoi*, which is representative of a newly described group of species that resemble relapsing fever species in some ways and Lyme borreliosis species in others. Like *B. hermsii* and most other relapsing fever species,[2] *B. miyamotoi* enhances its fitness by the strategy of transovarial transmission. The association between bacteremia with *B. miyamotoi* and larval activity was evidence for this (Figure 2). However, with filial infection frequencies of < 0.80, vertical transmission alone will likely not suffice for maintenance in the environment for more than a few generations. [64] Horizontal amplification of *B. miyamotoi* occurs in vertebrates, as has been documented with *P. leucopus* in the laboratory [12] and under natural conditions by this study. However, the limited empiric findings to date suggest that this mode of transmission seems to be less efficient for *B. miyamotoi* than for *B. burgdorferi*. Better understanding of how *B. miyamotoi* is maintained enzootically calls for further clarification of the relative importance of vertical and horizontal transmission by this species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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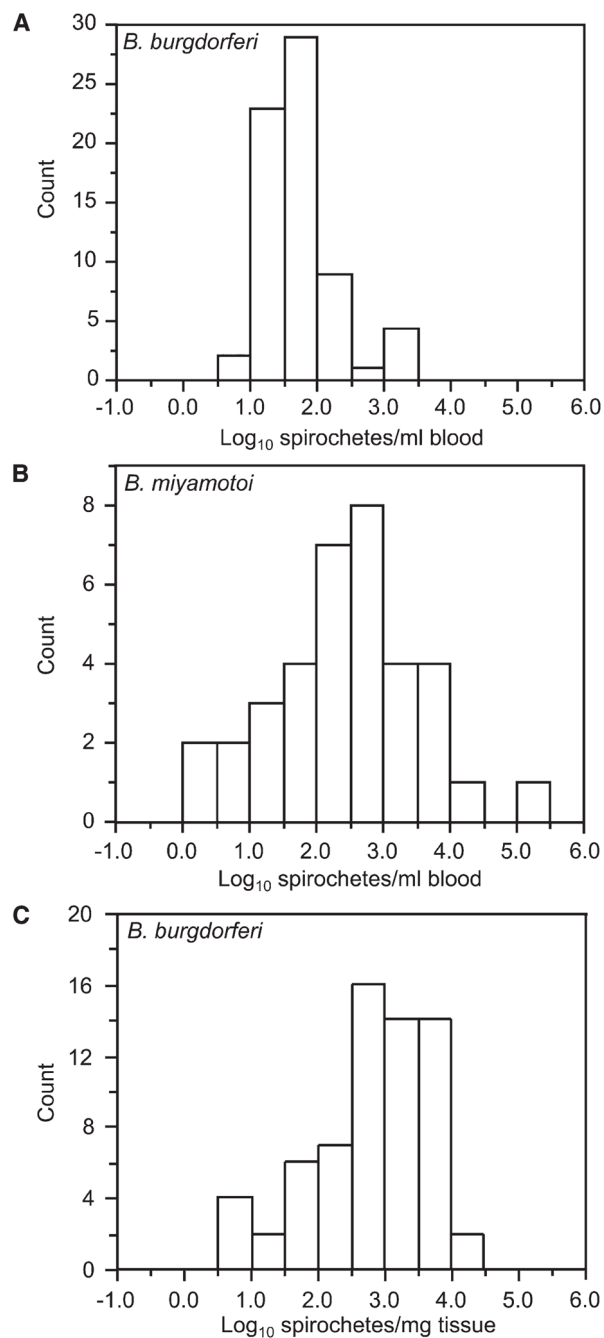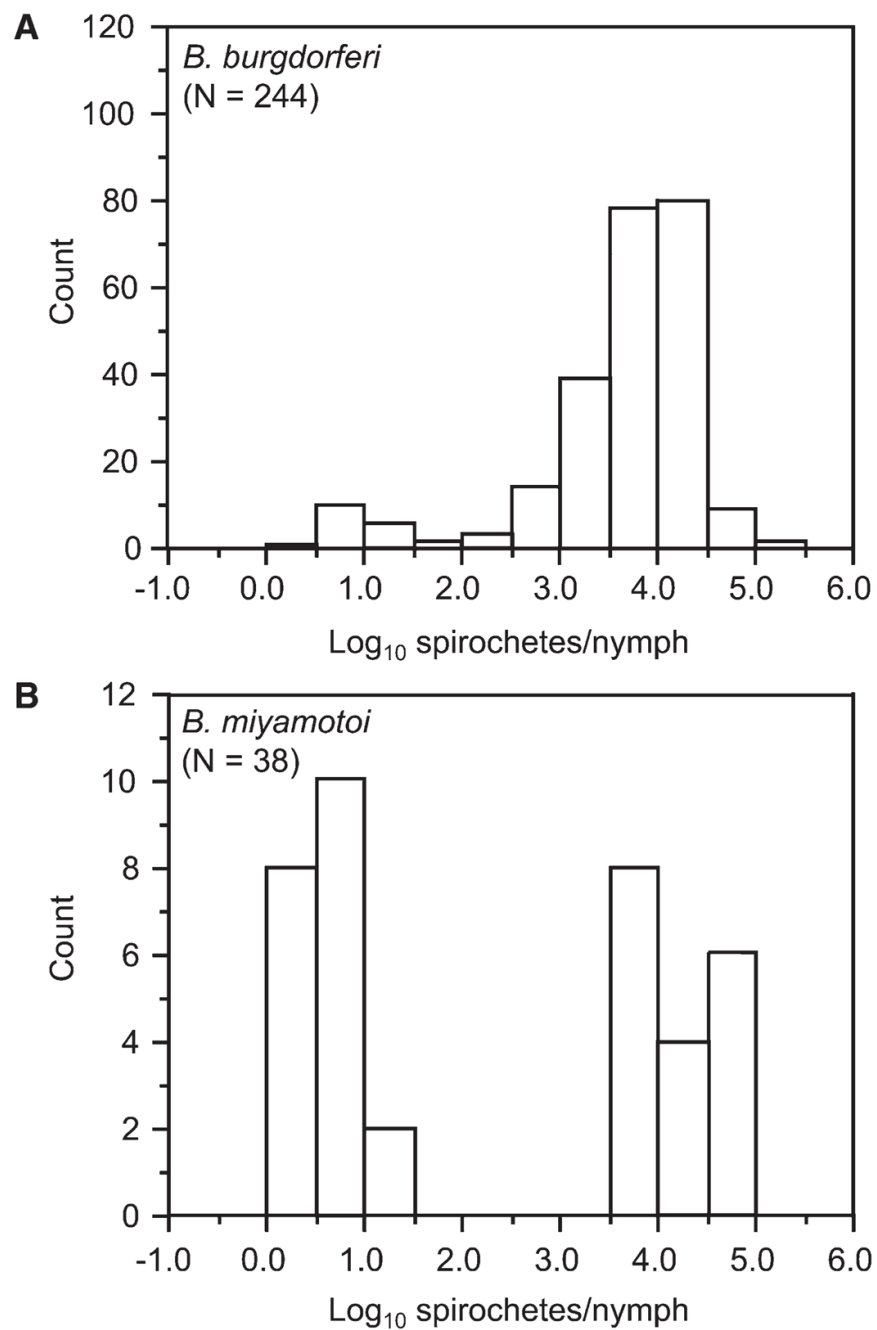
Figure 1.
Frequency histograms of quantitative PCR counts spirochetes of *B. burgdorferi* (**A**) and *B. miyamotoi* (**B**) in the blood and of *B. burgdorferi* (**C**) in the ear biopsy tissue of *P. leucopus* mice at Connecticut field site.
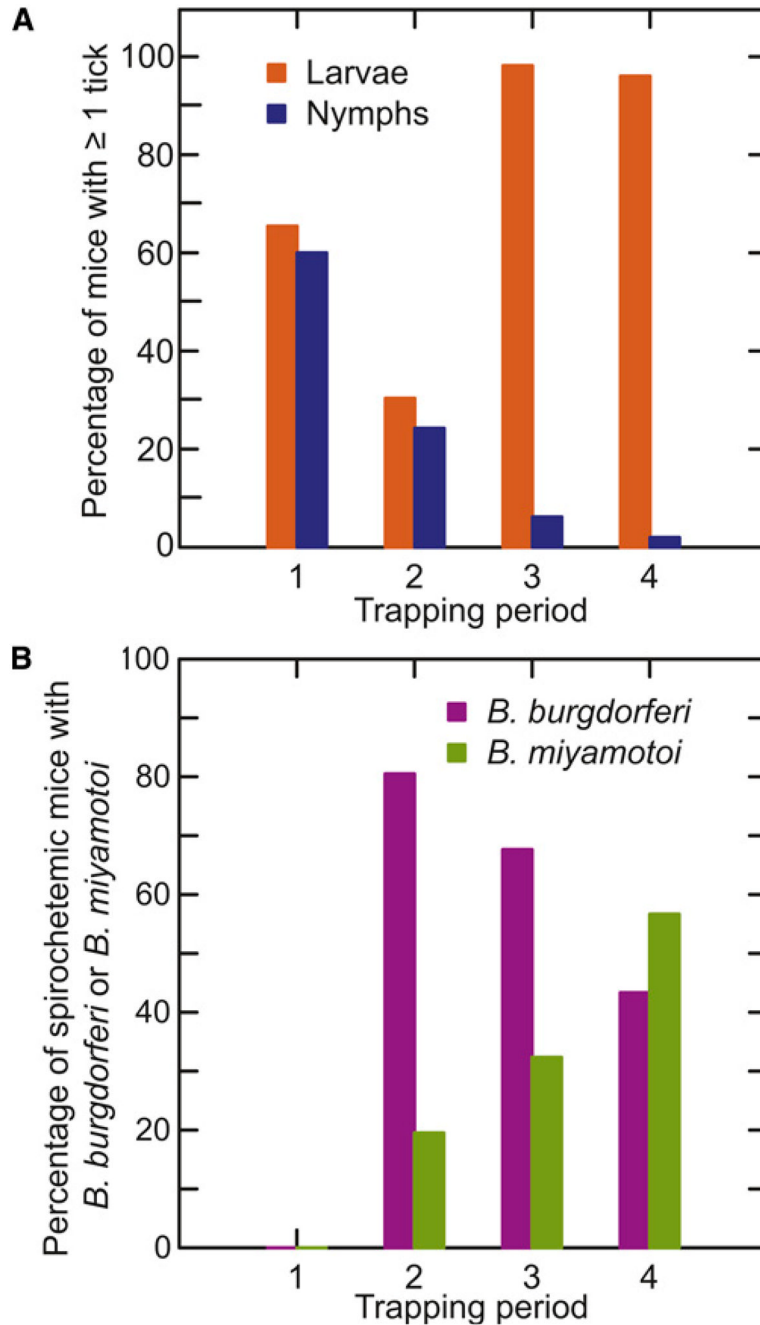
Figure 2.
Tick infestations and infections of the blood of *P. leucopus* by trapping period. **A**, Prevalence of *I. scapularis* larvae and nymph infestations. **B**, Prevalence of *B. burgdorferi* and *B. miyamotoi* bacteremia as detected by species-specific quantitative PCR. The number of captured mice for trapping periods 1, 2, 3, and 4 were 110, 170, 162, and 101, respectively. This figure appears in color at www.ajtmh.org.

Figure 3.
Frequency histograms of quantitative PCR counts of spirochetes of *B. burgdorferi* (**A**) or *B. miyamotoi* (**B**) in infected host-seeking *I. scapularis* nymphs at Connecticut field site.
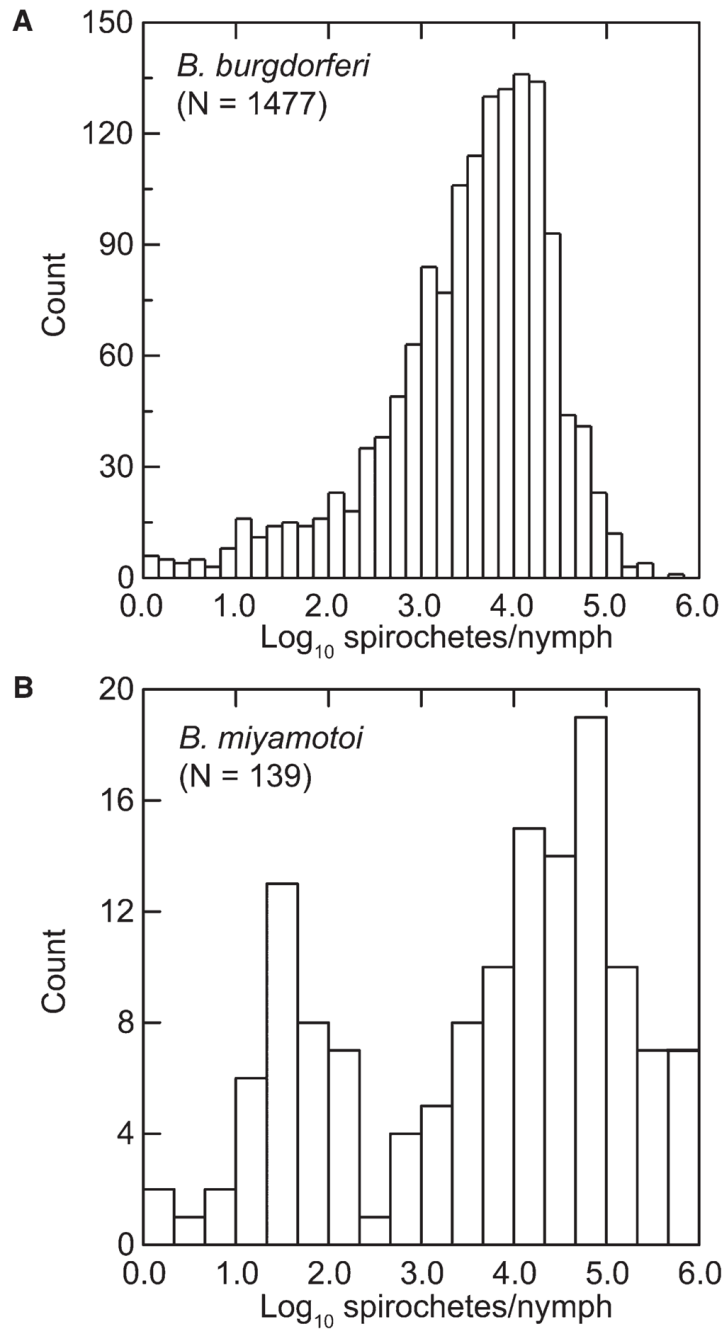
Figure 4.
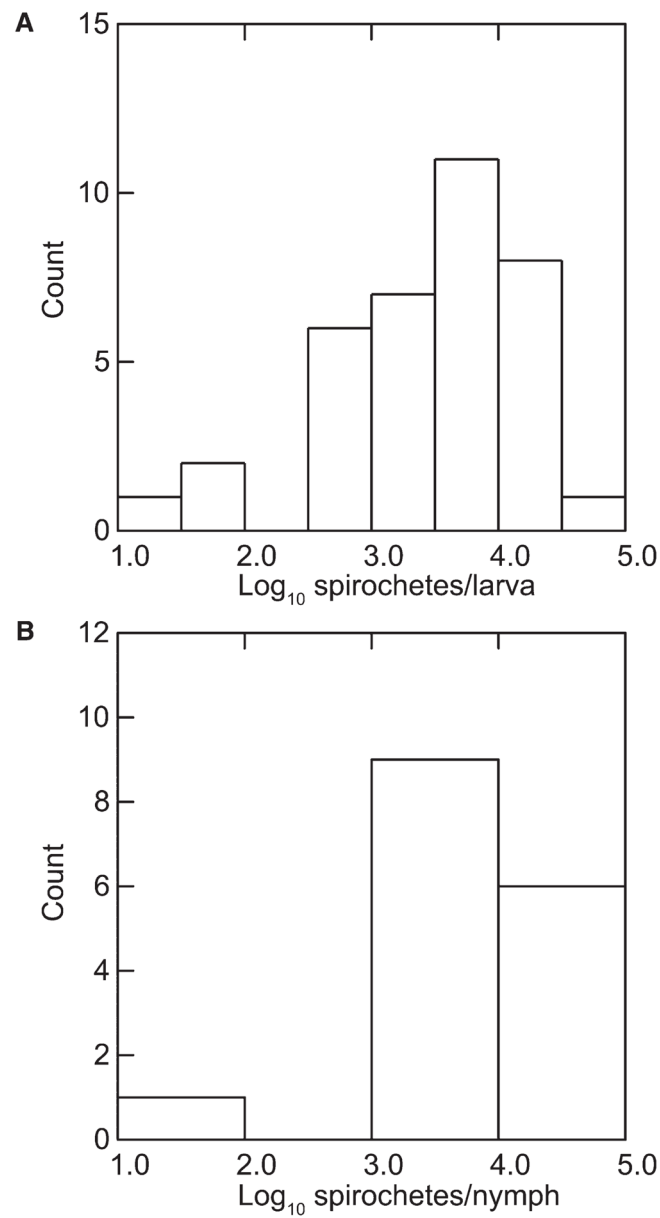Frequency histograms of quantitative PCR counts of spirochetes of *B. burgdorferi* (**A**) or *B. miyamotoi* (**B**) in infected host-seeking *I. scapularis* nymphs at 46 sites in the northeastern and north-central United States.

Figure 5.
Frequency histograms of quantitative PCR counts of spirochetes of *B. miyamotoi* in infected
larvae (**A**) and nymphs (**B**) of laboratory-reared *I. scapularis*.
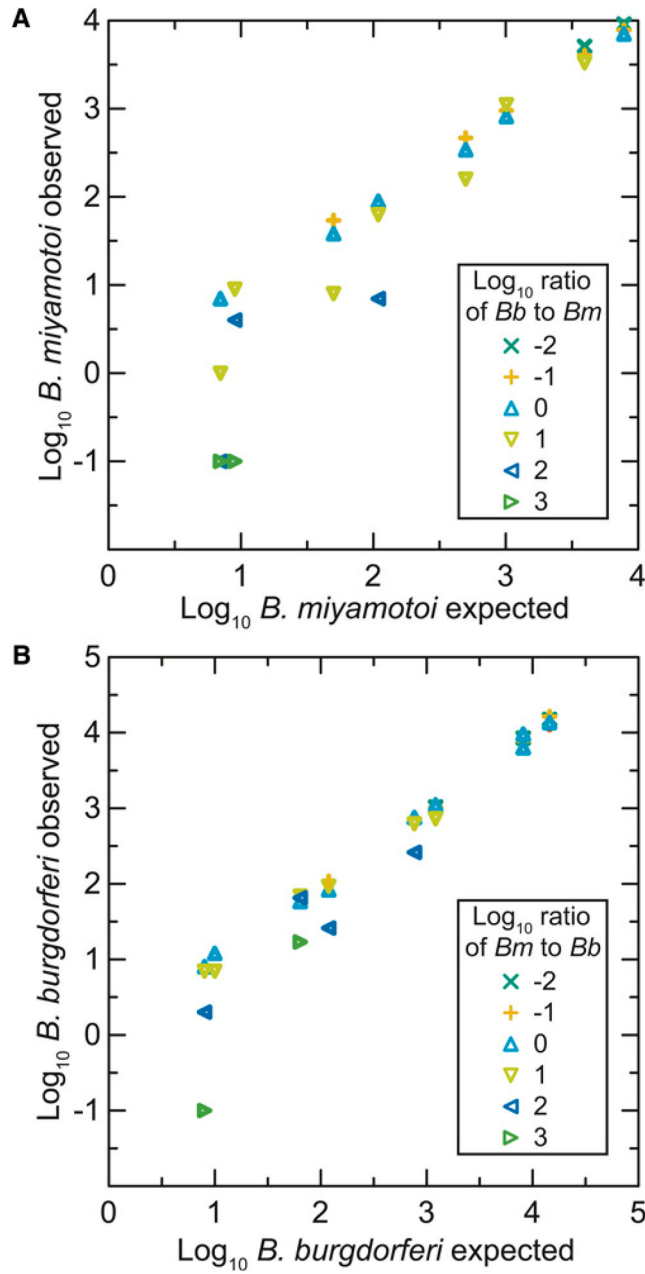
Figure 6.
Standard curves of quantitative PCR of *B. miyamotoi* (**A**) and *B. burgdorferi* (**B**) DNA in mixtures at various ratios. To DNA extracts of *I. scapularis*, nymphs with different amounts of *B. miyamotoi* were added equal volume aliquots of *B. burgdorferi* DNA in different concentrations. The NH_4OH method of extraction of DNA from ticks was used as described in the Materials and Methods section. This figure appears in color at www.ajtmh.org.

Table 1

Prevalence and burdens of *B. burgdorferi* (*Bb*) and *B. miyamotoi* (*Bm*) in *P. leucopus* mice and *I. scapularis* nymphs at the Connecticut field site

Source	Sample size	Bb positive [*]	Bm positive [*]	Co-infection	Difference in proportion	Mean number of spirochetes per milligram of tissue, milliliter of blood, or tick [*]	
						Bb	Bm
Blood	556	69 (0.12)[†]	36 (0.07)[†]	5 (0.009)[†]	0.06 (0.03–0.09)[‡]	50 (40–63)[‡]	251 (126–631)[‡]
Ear tissue	86	65 (0.76)[†]	2 (0.02)[†]	1 (0.012)[†]	0.73 (0.62–0.81)[‡]	640 (398–1,023)[‡]	15 (10–22)[‡]
Nymphs	689	244 (0.35)[†]	38 (0.06)[†]	3 (0.004)[†]	0.30 (0.26–0.34)[‡]	6,412 (5,521–7,447)[‡]	15,171 (9,616–23,993)[‡]

[*] Determined by quantitative PCR with specific probes for *Bb* and *Bm*.

[†] Proportion.

[‡] 95% confidence interval.

Table 2

Prevalences of *B. burgdorferi* and *B. miyamotoi* in *I. scapularis* nymphs by location

Site	State	Degrees north	Degrees west	Number of nymphs	Prevalence of *B. burgdorferi*	Prevalence of *B. miyamotoi*
1	CT	41.476	72.340	159	0.138	0.019
2	CT	42.030	73.433	122	0.221	0.000
3	MA	42.314	71.956	159	0.138	0.025
4	MD	39.479	75.988	474	0.177	0.021
5	ME	44.367	68.209	43	0.233	0.000
6	ME	43.568	70.241	145	0.497	0.007
7	NJ	40.160	74.134	74	0.135	0.027
8	NJ	41.277	74.698	35	0.229	0.000
9	NY	40.866	72.941	57	0.193	0.105
10	NY	41.036	72.227	71	0.310	0.000
11	NY	40.762	73.155	427	0.206	0.016
12	NY	41.127	73.732	688	0.228	0.015
13	NY	43.179	75.721	77	0.026	0.013
14	PA	40.207	75.787	32	0.094	0.031
15	PA	40.076	76.892	100	0.310	0.010
16	PA	41.201	78.506	97	0.196	0.000
17	PA	42.163	80.091	68	0.250	0.029
18	RI	41.582	71.716	94	0.234	0.011
19	VA	38.768	77.409	173	0.173	0.035
20	IA	42.876	91.769	29	0.172	0.000
21	IA	43.176	91.241	60	0.200	0.017
22	IL	41.978	89.369	88	0.136	0.011
23	IL	40.140	87.738	27	0.037	0.000
24	IL	41.170	89.295	67	0.149	0.030
25	IN	41.123	86.593	109	0.101	0.055
26	MI	45.419	87.807	31	0.129	0.000
27	MI	42.334	86.307	213	0.146	0.009
28	MN	46.168	92.841	268	0.410	0.037
29	MN	46.757	94.622	247	0.320	0.008

Site	State	Degrees north	Degrees west	Number of nymphs	Prevalence of *B. burgdorferi*	Prevalence of *B. miyamotoi*
30	MN	47.243	95.207	141	0.213	0.021
31	MN	46.656	92.348	47	0.191	0.043
32	MN	47.157	94.875	129	0.333	0.000
33	MN	45.319	95.024	22	0.091	0.000
34	MN	45.949	92.576	111	0.333	0.027
35	WI	44.749	89.849	506	0.184	0.020
36	WI	44.276	90.668	148	0.088	0.020
37	WI	45.181	91.166	138	0.217	0.022
38	WI	46.367	90.626	104	0.154	0.000
39	WI	45.182	89.742	49	0.184	0.082
40	WI	44.323	89.244	263	0.240	0.015
41	WI	44.944	91.780	181	0.199	0.022
42	WI	43.665	87.720	47	0.064	0.000
43	WI	44.961	91.284	184	0.147	0.027
44	WI	43.564	89.810	492	0.102	0.016
45	WI	43.705	90.567	287	0.209	0.038
46	WI	45.013	92.703	130	0.177	0.000
Mean/median				157/110	0.194/0.188	0.019/0.016