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Borrelia miyamotoi Infections among Wild Rodents Show Age and Month Independence and Correlation with *Ixodes persulcatus* Larval Attachment in Hokkaido, Japan

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Abstract

To clarify how *Borrelia miyamotoi* is maintained in the environment in Hokkaido, we examined *Ixodes persulcatus* for its prevalence among wild rodents and its tick vector by detecting a portion of the borrelial *flaB* gene in rodent urinary bladder and blood samples, and from whole ticks. We compared *B. miyamotoi* infection rates to *Borrelia garinii* and *Borrelia afzelii*, which are human Lyme disease pathogens also carried by wild rodents, and which are transmitted by the same vector tick. Whereas *B. garinii* and *B. afzelii* showed age dependence of infection rates among wild rodents (18.4% and 9.9% among adults and 6.0% and 3.4% among sub-adults, respectively) when looking at urinary bladder samples, *B. miyamotoi* infection rates were not age dependent for either blood (4.2% among adults, and 7.9% among sub-adults) or urinary bladder samples (1.0% among adults, and 1.7% among sub-adults). Moreover, while *B. garinii* and *B. afzelii* infection rates showed increases across months (June, July [$p < 0.05$] and August [$p < 0.01$] had higher rates than in May for adult rodents with *B. garinii*, and July and August had higher rates than in May [$p < 0.01$] for adult rodents with *B. afzelii*), *B. miyamotoi* infection rates did not show significant month dependence. These differences in month and age dependence led us to suspect that *B. miyamotoi* may not develop persistent infections in wild rodents, as *B. garinii* and *B. afzelii* are thought to. Furthermore, we examined the extent of rodent exposure to *I. persulcatus* nymphs and larvae throughout most of the tick's active season (May through September), and determined that *B. miyamotoi* infection rates in sub-adult rodents were correlated with larval burden ($p < 0.01$), suggesting that larvae may be very important in transmission of *B. miyamotoi* to wild rodents.

Key Words: *Borrelia*—vectors—Lyme disease—ticks—*Ixodes persulcatus*—relapsing fever.

Introduction

BORRELIA MIYAMOTOI, A RELAPSING fever group borrelia, was first isolated from hard-bodied ticks in Japan (Fukunaga et al. 1995). Although the pathogenicity of *B. miyamotoi* to humans has long been in question, recent evidence from Russia suggests that it is a human pathogen (Platonov et al. 2011). In Japan, *B. miyamotoi* is carried by the vector tick *Ixodes persulcatus*, as it is in other parts of Asia (Fukunaga et al. 1995, Fomenko 2010). *I. scapularis*, *I. pacificus*, and *I. ricinus* are the vectors in their respective ranges in other parts of the world (Fraenkel et al. 2002, Mun et al. 2006, Barbour et al. 2009).

Vector ticks have been shown to retain *B. miyamotoi* infections trans-stadially, and subsequent generations can be in-

fecting trans-ovarially; however, the infection rates have been noted to decrease between moltings, and not all larvae born from an infected female become infected (Scoles et al. 2001). Assuming that this is representative of *B. miyamotoi* infections of ticks in the environment, at least one reservoir species is necessary for maintenance of the spirochete. However, most studies on *B. miyamotoi* have focused strictly on its relationship with the vector tick, and few have tried to determine the reservoir species or evaluate the relationship of the pathogen with its reservoirs.

To determine how *B. miyamotoi* is maintained in an ecosystem, we examined wild rodents and questing *I. persulcatus* ticks for the presence of the borrelial flagellin gene (*flaB*), and also evaluated the exposure of rodents to *I. persulcatus*

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nymphs and larvae. Because rodents have been implicated as the most important reservoirs of *B. garinii* and *B. afzelii*, which are the pathogens of human Lyme disease in Japan (Nakao et al. 1994, Takano et al. 2011), and *B. miyamotoi*, which shares the same vector, we hypothesized that they share the same reservoirs. Therefore, we compared infection rates of *B. miyamotoi* among wild rodents trapped in Hokkaido, Japan, to the more studied *B. garinii* and *B. afzelii*.

Materials and Methods

Rodent trapping and tick collection

Two study sites were selected in Hokkaido, Japan, one at the base of the Shiretoko peninsula in eastern Hokkaido and the other near Shimizu, in central Hokkaido. Rodent trapping was performed using Sherman traps during 3- to 6-day stints in both locations each month from May through September of 2010 and 2011. Traps were initially placed at 5-meter intervals in two separate grids of 10×10 (200 traps), baited with oats, and were checked every 24h. In July 2011, traps placed in central Hokkaido were reduced to 100 traps on a single grid, and traps placed in eastern Hokkaido were reduced to 40 traps on a single grid (4×10). For the remaining months of 2011 (August and September), only 40 traps on a single grid (4×10) were placed in each location. Trapped rodents were anesthetized with isoflurane in an open-drop system as described elsewhere ("Rodent anesthesia using open-drop exposure to isoflurane" 2010). Following cardiocentesis under anesthesia, rodents were promptly euthanized by cervical dislocation for sampling of the urinary bladder, which has been reported to develop persistent borrelial infections (Masuzawa et al. 1992). Feeding ticks attached to the neck and head were also removed and collected. All samples were stored at -20°C or -80°C until extraction of DNA. All rodent sampling was performed using methods approved by the Animal Care and Use Committee of Hokkaido University (Approval No. JU1105).

Between May and July of 2011, we collected questing nymph and adult ticks by flagging within vegetation with flannel cloth (approximately 1×1.5 meters) in and around the rodent trapping areas. Ticks were removed from the flags, and placed in BD Falcon™ conical tubes (BD Biosciences, San Jose, CA) with 21-gauge air holes along with several fronds of grass. Collected ticks were stored alive in the tubes sealed in plastic bags and refrigerated at 4°C for no more than 2 weeks or frozen at -80°C until DNA extraction.

Rodent and tick species identification and aging/staging

Rodents were identified to species based on morphologic characteristics (Abe 1994, Ohdachi et al. 2009). Individual rodents were also classified as adult (born prior to the sampling season) or sub-adult (born during the sampling season) based on tooth wear with a previously described 9 age-group classification scheme for *Apodemus* spp. (Hikida and Murakami 1980), and a previously described 6 age-group classification scheme for *Myodes* spp. (Abe 1976). *Myodes* spp. rodents were classified as adults if their teeth were designated as five or higher. In the case of *Apodemus* spp., because sub-adults born early in the season but trapped late in the season, often had tooth scores equivalent or higher than adults trap-

ped in May, a graduated scale was implemented to remove early-season born rodents from late-season adult numbers. The lower-end cutoff for the adult designation was set at 4 and above in May, then consecutively raised one level each month until August (5 or higher for June, 6 or higher for July, and 7 or higher for August). Furthermore, because in September, sub-adults were often indistinguishable from adults, we classified all rodents trapped in September as sub-adults so that infection rates would be biased toward the sub-adult group rather than the adult group. All aging classification was performed by a single observer.

In 2011, feeding ticks collected from rodents were identified to species and stage based on described, morphologic differences (Yamaguti et al. 1971), and a tally was kept for each rodent. Questing ticks were also identified to species and stage using the same reference literature, and were then individually processed for DNA extraction.

DNA extraction, PCR, and sequencing

Urinary bladder DNA from mice was extracted using the Wizard® genomic DNA purification kit (Promega, Madison, WI) by the recommended protocol for soft tissue. DNA was extracted from clotted blood samples using a method described previously (Kanai et al. 1994). Questing tick DNA was extracted using the Wizard® genomic DNA purification kit with a modified protocol.

PCR was performed on DNA of rodent urinary bladders and blood, and on questing tick DNA for the presence of the borrelial *flaB* gene according to a previously described method that can detect as few as 10 spirochete cells in a sample (Sato et al. 1997, Takano et al. 2010). Confirmation of positive results was performed by replication. PCR products of positive samples were purified using ExoSAP-IT (GE Healthcare UK, Ltd., Buckinghamshire, UK) prior to direct sequencing on an ABI Prism 3130xl Genetic Analyzer (Life Technologies Co., Grand Island, NY). Sequencing results were compared to reference sequences recorded in GenBank for identification to species (*B. garinii* strain 20047, greater than 98% similarity, and *B. afzelii* strain VS461 and *B. miyamotoi* strain HT31, each greater than 99% similarity; accession numbers D86617, D63365, and D43777, respectively). Mixed sequencing results were resolved by TA cloning using either pGEM®-T Vector System I (Promega) with ECOS™ Competent *E. coli* (Nippon Gene Co., Ltd., Tokyo, Japan) or pCR™4-TOPO® with One Shot® TOP10 Chemically competent *E. coli* (Life Technologies Co.). Eight clones for each mixed sample were selected for re-sequencing and analysis. Once mixed samples were identified to species, they were counted as separate positives for each *Borrelia* sp. unless the mixture was of multiple strains of the same species, in which case, the sample was considered to be a single positive sample.

Results

Rodent species identification and aging

A total of 879 rodents were captured and included in this study. On the basis of aging criteria, 293 and 586 rodents were identified as adults and sub-adults, respectively. Five species were represented: *Apodemus argenteus* ($n=137$: adult=58, sub-adult=79), *A. speciosus* ($n=446$: adult=121, sub-adult=325), *Myodes rex* ($n=12$: adult=3, sub-adult=9), *M. rufocanus*

TABLE 1. PREVALENCE OF *BORRELIA* SPP. IN URINARY BLADDER AND BLOOD SAMPLES AMONG RODENT SPECIES

Rodent species	Urinary bladder					Blood				
	No. tested	<i>B. afzelii</i>	<i>B. garinii</i>	<i>B. miyamotoi</i>	Total	No. tested	<i>B. afzelii</i>	<i>B. garinii</i>	<i>B. miyamotoi</i>	Total
<i>A. argentus</i>	137	1 (0.7%)	5 (3.6%)	1 (0.7%)	7 (5.1%)	87	0 (0%)	0 (0%)	1 (1.1%)	1 (1.1%)
<i>A. speciosus</i>	446	39 (8.7%)	69 (15.5%)	10 (2.2%)	109 (24.4%) ^a	291	1 (0.3%)	0 (0%)	24 (8.2%)	25 (8.6%)
<i>M. rex</i>	12	0 (0%)	0 (0%)	0 (0%)	0 (0%)	9	0 (0%)	0 (0%)	0 (0%)	0 (0%)
<i>M. rufocanus</i>	195	4 (2.0%)	7 (3.6%)	2 (1.0%)	12 (6.1%) ^a	106	0 (0%)	1 (0.9%)	10 (9.4%)	11 (10.4%)
<i>M. rutilus</i>	89	5 (5.6%)	8 (9.0%)	0 (0%)	11 (12.4%) ^a	56	0 (0%)	0 (0%)	3 (5.4%)	3 (0.5%)
Total	879	49 (5.6%)	89 (10.1%)	13 (1.5%)	139 (15.8%) ^a	549	1 (0.2%)	1 (0.2%)	38 (6.9%)	40 (7.3%)

^a*B. afzelii* and *B. garinii* mixed samples present (*A. speciosus*=9, *M. rufocanus*=1, *M. rutilus*=2, Total=12).

(*n*=195: adult=62, sub-adult=133), and *M. rutilus* (*n*=89: adult=49, sub-adult=40).

Detection of borrelial DNA in rodent specimens

The urinary bladder of each of the 879 rodents was processed through to sequencing. Blood samples were processed and analyzed for 549 rodents, including 143 adults and 406 sub-adults (Table 1). A total of 139 urinary bladder samples (15.8%) and 40 blood samples (7.3%) were positive for one or more of the three borreliae of interest to this study (Table 1). *B. garinii* and *B. afzelii* were detected among 89 (10.1%) and 49 (5.6%) urinary bladder samples, respectively, whereas *B. miyamotoi* was rarely detected (1.5%). However, 38 of the 40 positive blood samples were identified as *B. miyamotoi* infections. Blood samples were available for 9 of the rodents positive for *B. miyamotoi* using the urinary bladder. All of these were also positive for *B. miyamotoi* (see supplementary material online at www.liebertpub.com/vbz).

B. garinii and *B. afzelii* infection rates were both significantly higher for adults compared to sub-adults, whereas there was no significant difference between the two groups in the case of *B. miyamotoi* (Table 2). When calculated separately by species, *A. speciosus* was the only species showing a significantly higher infection rate of *B. afzelii* in adults when compared to sub-adults (adult=23/121, sub-adult=16/325, $p<0.01$, Fisher exact test). However, *A. speciosus* (adult=37/121, sub-adult=32/325) and *M. rufocanus* (adult=7/62, sub-adult=0/133) both had significantly higher ($p<0.01$, Fisher exact test) infection rates of *B. garinii* among adults. Although the overall infection rates and sample sizes were too small to determine significant difference for the other species, the general tendency was for infection rates of adults to be higher than sub-adults for both *B. garinii* and *B. afzelii* (data not shown). The exception was *M. rex*, for which no infections with either borrelia were detected. Given the above, only the adult and sub-adult totals including all species are reported (Table 2).

In contrast to *B. garinii* and *B. afzelii*, *B. miyamotoi* infection rates were not significantly different between adults and sub-adults for any of the rodent species for either sample type (i.e., for blood samples, *A. speciosus*: adult=3/62 [4.8%], sub-adult=21/229 [9.2%]; *M. rufocanus*: adult=2/22 [9.1%], sub-adult=8/84 [9.5%] [remaining data not shown]). Consequently, only the totals were reported for the adults and sub-adults of all species collectively for *B. miyamotoi* as well (Table 2). Furthermore, *B. miyamotoi* infection rates remained statistically stable for both adults and sub-adults and in total across all months, whereas both *B. garinii* and *B. afzelii* showed an increase in infection rates across months for adult and sub-adult groups with significantly higher values in later months (Fig. 1). Using the Kruskal–Wallis test with the Dunn multiple comparisons test, *B. garinii* infection rates among adults in June and July ($p<0.05$) and August ($p<0.01$) were all higher than in May. Among sub-adults, September had significantly higher infection rates than June ($p<0.01$) or August ($p<0.05$). *B. afzelii* infection rates were higher among adults in July and August than in May ($p<0.01$), and higher in July than in June ($p<0.01$). Sub-adults also showed an increase in *B. afzelii* with infection rates higher in September than in June, July, or August ($p<0.01$).

Feeding ticks collected from rodents

A total of 597 rodents trapped in 2011 and included in this study were examined for the attachment of feeding ticks. A total of 1604 ticks were found, and of those, 955 (59.5%) were identified as *I. persulcatus* larvae, and 177 (11.0%) were identified as *I. persulcatus* nymphs. In all, 275 rodents had *I. persulcatus* larvae attached and 80 had *I. persulcatus* nymphs attached (see supplementary material online at www.liebertpub.com/vbz).

Using the Mann–Whitney test, there was no significant difference between the mean number of *I. persulcatus* larvae attached to adults and sub-adults of all rodent species

TABLE 2. PREVALENCE OF *BORRELIA* SPP. IN URINARY BLADDER AND BLOOD SAMPLES BY AGE GROUP

Age group	Urinary bladder				Blood			
	No. tested	<i>B. afzelii</i>	<i>B. garinii</i>	<i>B. miyamotoi</i>	Blood tested	<i>B. afzelii</i>	<i>B. garinii</i>	<i>B. miyamotoi</i>
Adult	293	29 (9.9%) ^a	54 (18.4%) ^b	3 (1.0%)	143	1 (0.7%)	1 (0.7%)	6 (4.2%)
Sub-adult	586	20 (3.4%) ^a	35 (6.0%) ^b	10 (1.7%)	406	0 (0%)	0 (0%)	32 (7.9%)

^{a,b}Significant difference in rates between age groups determined using Fisher's Exact test ($P<0.01$).

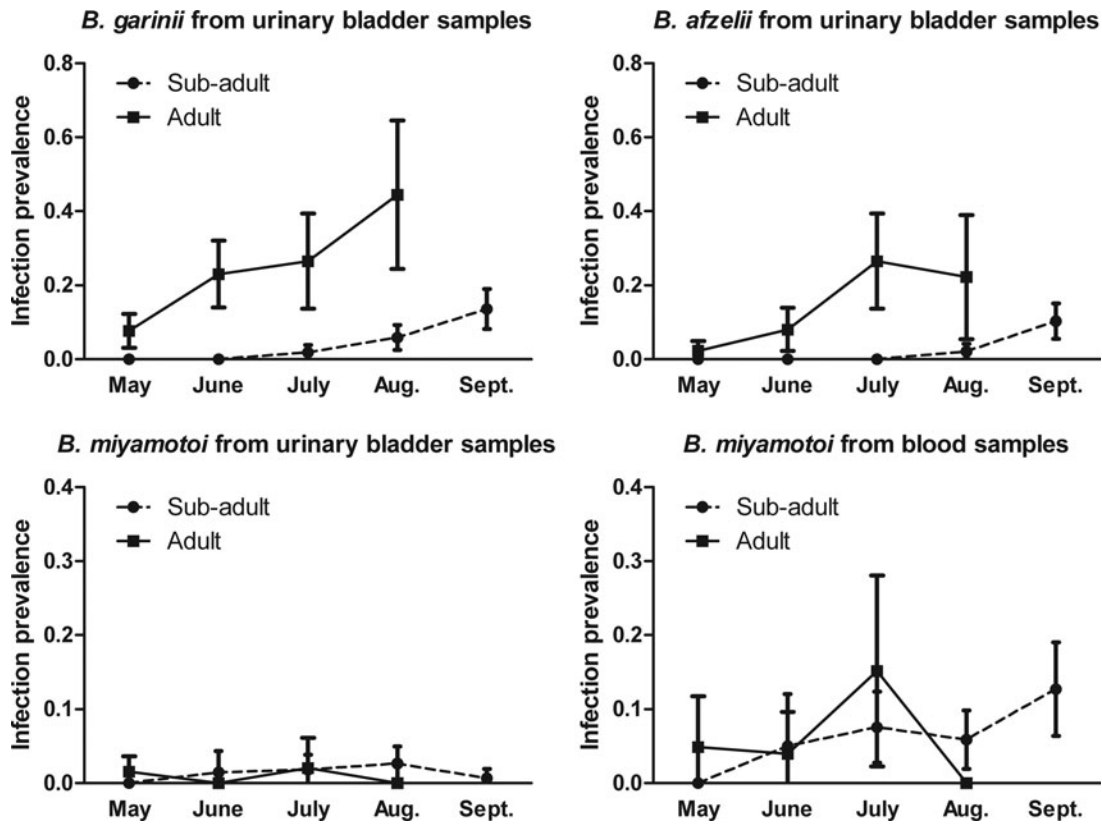


FIG. 1. *Borrelia*-positive means by month with 95% confidence interval (CI) for urinary bladder and blood samples.

collectively (adults, mean=1.5, standard deviation [SD]=2.7, $n=199$; sub-adults, mean=1.6, SD=3.2, $n=398$). Upon analysis of total adults and sub-adults for the mean number of nymphs attached, however, the difference was significant ($p<0.01$), with adults accounting for a larger portion of the nymphs (adults, mean=0.6, SD=1.5, $n=199$; sub-adults, mean=0.1, SD=0.6, $n=398$).

Tick burdens across months were charted for both *I. persulcatus* larvae and nymphs (Fig. 2). Rodents trapped in August had a significantly lower mean burden of larvae than all other months ($p<0.01$, Kruskal–Wallis with the Dunn multiple comparisons test). Also, the mean larval burden of rodents trapped in September was significantly higher than that of rodents trapped in July ($p<0.01$, Kruskal–Wallis with Dunn). As for the mean burden of nymphs, rodents trapped in June had a significantly higher burden than those trapped in August or September ($p<0.01$ and $p<0.05$, respectively, Kruskal–Wallis with Dunn).

Cross-evaluation, using the Fisher exact test, of positive results for borreliae and the number of *I. persulcatus* larvae or nymphs attached to rodents showed that *B. garinii* positive, adult rodents were more likely to have both nymphs and larvae attached ($p<0.01$ and $p<0.05$, respectively) than negative adults. Sub-adult rodents showed no such association for *B. garinii*. Sub-adult rodents that were positive for *B. miyamotoi*, on the other hand, were more likely to carry a larval burden than negative rodents when considering results from either urinary bladder or blood samples ($p<0.05$ and $p<0.01$, respectively). Adult rodents positive for *B. miyamotoi* showed no correlation with either nymph or larval numbers. *B. afzelii*-positive rodents showed no significant association with larval or nymph numbers.

Questing ticks

Of a total 240 *I. persulcatus* ticks collected by flagging and included in this study, 163 were adults, and 77 were nymphs. PCR and sequencing analysis indicated that 49 (30.1%) adults and 6 (7.8%) nymphs were positive for the borrelial *flaB* gene. Among the adult samples, 44 (27.0%) were positive for *B. garinii*, 5 (3.1%) were positive for *B. afzelii*, and 3 (1.8%) were positive for *B. miyamotoi*. Of the nymphs, 6 (7.8%) were positive for *B. garinii*, and 1 (1.3%) sample was positive for *B. afzelii*. None of the nymphs we tested carried *B. miyamotoi*.

Discussion

In the present study, we attempted to elucidate the ecology of the borrelial spirochete *B. miyamotoi*, a potential human pathogen in Japan. Although it is well documented that *B. miyamotoi* is carried by ixodid ticks, the natural reservoirs have not yet been determined. We provide relative infection rates of multiple rodent species found in Hokkaido, Japan, and also show that the ecology of *B. miyamotoi* within wild rodents differs from that of *B. garinii* and *B. afzelii*, which are also transmitted by *I. persulcatus* ticks.

Our results, showing the highest *B. garinii* and *B. afzelii* infection rates among *A. speciosus*, are consistent with a previous study in Hokkaido (Nakao et al. 1993), suggesting that sampling and testing methods were appropriate. For *B. miyamotoi*, however, significant differences could not be determined among species with the current sample sizes, although trends suggest that *A. speciosus* and *M. rufocanus* may harbor higher infection rates than the other species. This may be important, given that these are prominent rodent species in

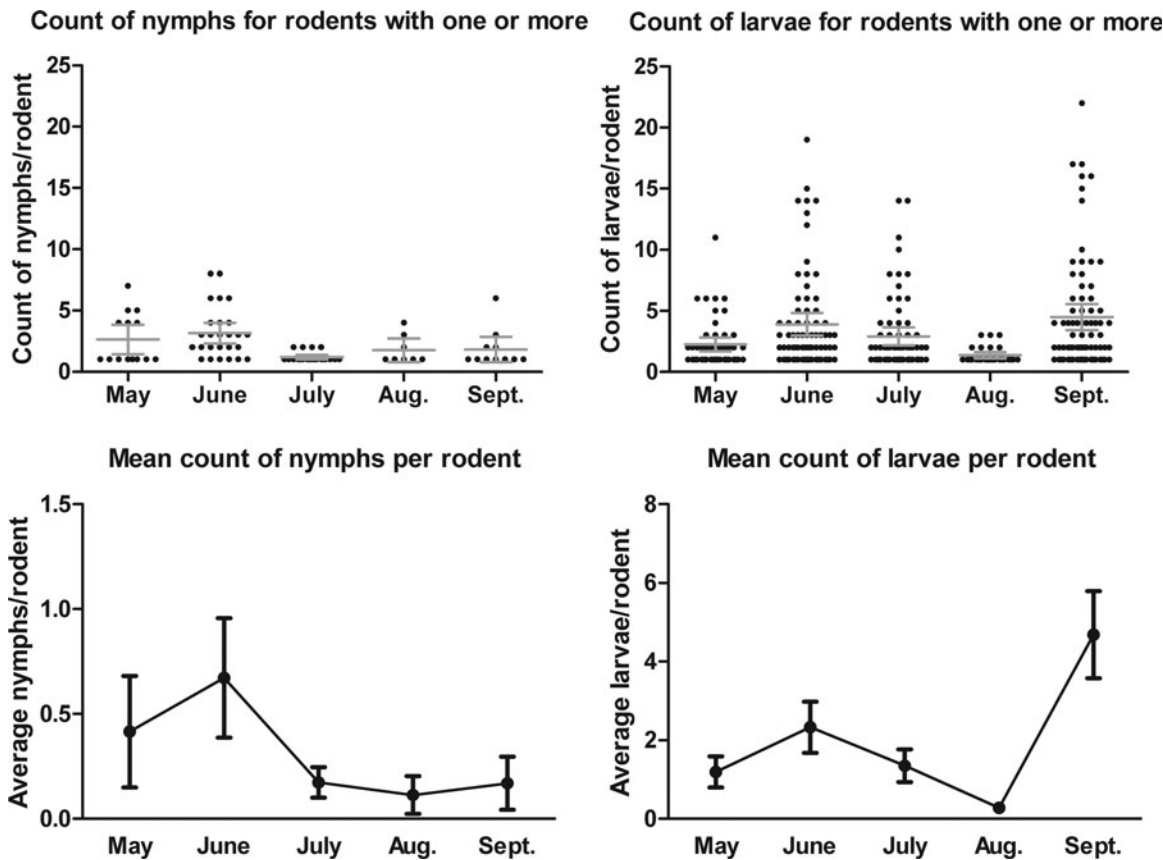


FIG. 2. (Top graphs) Counts (with means and 95% confidence interval [CI]) of nymphs and larvae for rodents which had one or more nymphs attached. (Bottom graphs) Mean number of nymphs and larvae attached per rodent in total (with 95% CI).

Hokkaido (Forestry Research Institute 2011). Regardless, because no statistical difference was found, we grouped all species together to compare the difference in infection rates of the borreliae between adult and sub-adult rodents.

We interpret the significantly higher infection rates of *B. garinii* and *B. afzelii* among adults compared to sub-adults, as consistent with persistent infection, which has been shown to occur for *Borrelia burgdorferi*, another Lyme disease pathogen, in rodents (Schwan et al. 1991). Whereas *B. garinii* and *B. afzelii* showed this age dependence, *B. miyamotoi* did not. The lack of age dependence for *B. miyamotoi* infection, along with the absence of significant difference in infection rates of the bladder across months, suggests that, unlike *B. garinii* and *B. afzelii*, it does not result in persistent infections of the urinary bladder. It has also been shown that skin samples from mice had lower infection rates than blood (Barbour et al. 2009), and evaluation of spleen samples suggested that this organ is also not a focus of persistent infection (data not shown). However, we cannot yet exclude the possibility that *B. miyamotoi* persists in other organs. *B. duttonii*, another tick-borne relapsing fever *Borrelia* sp., for instance, is reported to remain in the brain of mice in the absence of bacteremia, and recurrence of bacteremia can be induced by stress (Larsson et al. 2006). Furthermore, we have not evaluated the possibility that rodents infected with *B. miyamotoi* have reduced life spans.

Given the data that age is not positively correlated with *B. miyamotoi* infection, we propose that *B. miyamotoi* infection rates are largely due to *I. persulcatus* larval burden. Using both

urinary bladder and blood samples, sub-adult rodents positive for *B. miyamotoi* were more likely to carry larvae. Given this, the fact that larvae are responsible for a far larger portion of the overall tick burden than are nymphs, and the fact that larvae are known to harbor *B. miyamotoi* infections in the United States (Richter et al. 2012), it seems reasonable that the majority of transmission events from tick to rodent be due to larvae (whether vertical transmission of *B. miyamotoi* occurs among ticks in Japan still needs to be determined). This is in direct contrast to *B. garinii* and *B. afzelii*, for which vertical transmission is thought to seldom occur, and therefore cannot be transmitted to rodents by larvae. The lack of correlation between *B. miyamotoi*-positive adults and larval burden may simply be due to the small number of samples.

Although statistically insignificant, *B. miyamotoi* infection rates in blood show a mild incline suggestive of month dependence. However, because *B. miyamotoi* was deemed not age dependent, we suspect this incline reflects trends in *I. persulcatus* larval burden. The two graphs are suggestive of parallel change with a mild lag of infection compared to larval burden, as we would expect.

The 1.8% prevalence of *B. miyamotoi* among questing adult *I. persulcatus* ticks from our study areas was similar to the infection rates found among ticks of the same species in sampled areas of Russia and among *I. scapularis* ticks in parts of the northeastern United States (Scoles et al. 2001, Fomenko et al. 2010). The consistently low prevalence of *B. miyamotoi* among ticks and rodents compared to prevalences of *B. garinii*

and *B. afzelii* may be, in part, associated with the unique ecology of the spirochete (e.g., the possible absence of persistent infections).

In conclusion, we suspect that, unlike *B. garinii* and *B. afzelii*, which cause persistent infections in the urinary bladder, *B. miyamotoi* may not. Furthermore, we predict that the prevalence of *B. miyamotoi* among wild rodents is largely a consequence of transmission from *I. persulcatus* larvae, and that infection rates among rodents may reflect seasonal changes in the population of questing larvae. However, further research, including infection trials, is necessary to fully substantiate these hypotheses.

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Author Disclosure Statement

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