HOW IS *BORRELIA MIYAMOTOI* MAINTAINED AMONG ITS VECTOR*, IXODES SCAPULARIS*, AND VERTEBRATE HOST POPULATION?

By

Seungeun Han

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ABSTRACT

HOW IS *BORRELIA MIYAMOTOI* MAINTAINED AMONG ITS VECTOR*, IXODES SCAPULARIS*, AND VERTEBRATE HOST POPULATION?

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Borrelia miyamotoi is a relapsing fever spirochete transmitted by ticks in the *Ixodes ricinus* complex, which are known to vector many pathogens, including *Borrelia burgdorferi* sensu lato, the agents of Lyme borreliosis. *Borrelia miyamotoi* was first discovered in Japan in 1995, and then in 1999 it was detected in *I. scapularis* in the eastern United States. After being recognized as a human pathogen in Russia in 2011, human case of *B. miyamotoi* infection has been continuously reported in the United States and Eurasia.

Despite being discovered more than 20 years ago, very little is known about how *B. miyamotoi* is maintained in nature. Most knowledge about the ecology of *B. miyamotoi* has been acquired incidentally from studies focused on *B. burgdorferi* sensu lato. My dissertation research, therefore, aimed to improve understanding about how *B. miaymotoi* is maintained among the vector and wildlife hosts. In each chapter of my dissertation, I investigated fundamental components of *B. miyamotoi* maintenance in the enzootic cycle and discussed potential implication of my finding for public health.

In chapter 1, I estimated acarological risk of *B. miyamotoi* infection by investigating *B. miyamotoi* infection prevalence and density of infected ticks (DIT) from field collected larval, nymphal and adult *I. scapularis* from Wisconsin (WI) and Massachusetts (MA), where *I. scapularis* and associated pathogens are highly abundant, and where human cases of *B. miyamotoi* have been reported. I found that larvae and nymphs pose broadly similar *B.*

miyamotoi acarological risk, and the two juvenile stages may pose greater risk than do the adults. Therefore, I suggested that estimates of acarological risk as well as the seasonality for *I. scapularis*-borne diseases should be expanded to incorporate larvae.

Reservoir hosts of juvenile and adult ticks, small mammals and white-tailed deer (*Odocoileus virginianus*), respectively, were investigated for their roles in *B. miyamotoi* maintenance. *Borrelia miyamotoi* infection prevalence of small mammal hosts captured in Wisconsin was investigated to identify potential reservoir host species in chapter 2, and I compared the infection prevalence of *B. miyamotoi* in adult ticks removed from white-tailed deer with that in questing adults in chapter 3. The results showed potentially low or limited reservoir competence of white-footed mice for *B. miyamotoi* and highest infection prevalence from eastern chipmunks. Significantly higher infection prevalence of deer blood-fed females than that of questing females was observed, suggesting deer may be amplifying hosts for *B. miyamotoi*.

Transovarial transmission (TOT) rate and filial infection prevalence (FIP) of B. miyamotoi were investigated from engorged females and their larval clutches, which were collected from hunter-harvested white-tailed deer in chapter 4. The result showed that TOT and FIP are high, but < 100%, which suggests that vertical transmission alone will be insufficient to support the enzootic maintenance of *B. miyamotoi* infection.

In chapter 5, I used the next-generation matrix model to investigate the effects of systemic, non-systemic (co-feeding), and vertical transmission on *B. miyamotoi* maintenance by R_0. Model results indicated potential significant contributions of non-systemic and vertical transmission on *B. miyamotoi* maintenance, whereas systemic transmission showed minimal effects on R_0 of *B. miyamotoi*.

To my beloved wife, Wonhee,
and precious daughter, Peet.

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KEY TO ABBREVIATIONS

LD	Lyme Disease
s.l.	sensu lato
US	United States
WI	Wisconsin
MA	Massachusetts
IGS	Intergenic spacer region
DOT	Density of ticks
DIT	Density of infected ticks
MLE	Maximum Likelihood Estimates
BMD	*Borrelia miyamotoi* disease
TOT	Transovarial transmission
FIP	Filial infection prevalence
s.s.	sensu stricto
EI	Engorgement index
ORs	Odds ratios
RFG	Relapsing fever group
HTBRF	Hard tick-borne relapsing fever
ME	Maine
NH	New Hampshire
TN	Tennessee
RH	Relative humidity

CI Confidence interval

SE Standard error

CHAPTER 1

Estimating the acarological risk of *Borrelia miyamotoi* among all questing life stages of *Ixodes scapularis* in the north central and northeastern United States

ABSTRACT

Borrelia miyamotoi has been detected in multiple *Ixodes ricinus* complex species, including *I. scapularis* in the eastern US. Human cases of *B. miyamotoi* infection have been reported in all Lyme disease endemic areas. *Borrelia miyamotoi* can be transmitted by larvae, nymphs and adult ticks; transmission can occur within 24 hours of feeding. To clarify the contribution of all questing life stages to the acarological risk of *B. miyamotoi*, we assayed questing larval, nymphal and adult *I. scapularis* at one field site in each of the northeastern and north central United States over 3 years. Average densities of questing larvae, nymphs, females and males (DOT) were 36.4, 5.2, 1.1, and 1.5 (ticks per 1,000 m^2) from Wisconsin (WI), and 10.2, 2.0, 0.5, and 0.5 from Massachusetts (MA). Infection prevalence among life stages ranged from 0.3 to 2.6%. Densities of infected ticks (DIT; the product of DOT and infection prevalence) of larval, nymphal, female, and male ticks were estimated as 0.32, 0.11, 0.007, and 0.018 ticks per 1,000 m^2 from WI and 0.034, 0.042, 0.013, and 0.010 ticks per 1,000 m^2 from MA, respectively. We suggest that larvae and nymphs pose broadly similar *B. miyamotoi* acarological risk, and the two juvenile stages may pose greater risk than do the adults. Estimates of acarological risk as well as the seasonality for *I. scapularis*-borne diseases should be expanded to incorporate larvae.

INTRODUCTION

Borrelia miyamotoi, a relapsing fever group spirochete, has been detected throughout the range of *Ixodes ricinus* complex ticks, which also vector the Lyme disease (LD) pathogen, *B. burgdorferi* sensu lato (s.l.) (Scoles et al., 2001; Fraenkel et al., 2002; Fomenko et al., 2010). *Borrelia miyamotoi* was first discovered in questing *Ixodes persulcatus* ticks in Japan in 1995 (Fukunaga et al., 1995). Human cases of *B. miyamotoi* infection were first reported in 2011 from Russia (Platonov et al., 2011) and subsequently from Lyme disease endemic areas in North America, Europe and Asia (Gugliotta et al., 2013; ,Krause et al., 2013; Hovius et al., 2013; Krause et al., 2014; Sato et al., 2014; Molloy et al., 2015; Jobe et al., 2016; Krause et al., 2016; Fiorito et al., 2017; Jiang et al., 2018).

In the U.S. *B. miyamotoi* has been detected in the western blacklegged tick (*I. pacificus*) (Mun et al., 2006) and the blacklegged (=deer) tick, *I. scapularis*, both of which vector several other pathogens. Four of the disease reportable to the CDC, LD, human anaplasmosis, human babesiosis, and a tick-borne encephalitis (caused respectfully by *B. burgdorferi sensu stricto* and *B. maynonii, Anaplasma phagocytophilum, Babesia microti, and* Powassan/deer tick virus) (Holman et al., 2004; Biggs et al., 2016; Pritt et al., 2016; Hermance and Thangamani, 2017) have been increasing in annual incidence and geographic spatial distribution (Eisen et al., 2017), due to in large part to the range expansion of *I. scapularis* (Dennis et al., 1998; Eisen et al., 2016; Eisen et al., 2017). Thus, although not reportable to the CDC at this time, *B. miyamotoi* disease is clearly an emerging disease and its risk to public health should be investigated, especially as its transmission biology has additional implications for epidemiology and public health messaging compared to the other bacterial zoonoses transmitted by *I. scapularis*.

There is no vertical transmission among ticks of the agents of Lyme disease, human anaplasmosis, and human babesiosis; larvae emerge uninfected, with these agents being subsequently acquired by horizontal transmission between ticks and reservoir hosts, followed by transstadial transmission within the tick population.(Patrican, 1997; Dumler et al., 2001; Piesman and Happ, 2001; Rollend et al., 2013). In contrast, like POWv/deer tick virus (Costero and Grayson, 1996), *B. miyamotoi* can be transmitted transovarially from infected females to their progeny (Scoles et al., 2001; Breuner et al., 2017). Transmission of *B. miyamotoi* to laboratory mice by vertically infected *I. ricinus* larvae and *I. scapularis* nymphs has been demonstrated (van Duijvendijk et al., 2016; Scoles et al., 2001), and transmission via a single transovarially infected larval *I. scapularis* to laboratory mice has been reported recently (Breuner et al., 2018). Therefore, vertically infected larval *I. scapularis*, in addition to nymphs (and presumably adults) may vector *B. miyamotoi* to humans.

In northern states, the nymphal stage is responsible for the majority of cases of Lyme disease, human anaplasmosis, and babesiosis, with the seasonality of human cases matching that of the nymphal activity period (Mather et al., 1996b; Biggs et al., 2016). Although adult ticks may be infected, the density of host-seeking infected nymphs is much larger compared to that of adults (due to mortality and the lack of true feeding by adult males), leading to a greater exposure risk. Similarly, there is greater use of the outdoors by people during the nymphal activity period (late spring/summer) compared to that of the adults (spring and fall) (Schwartz et al., 2017). Finally, as the probability of pathogen transmission increases the longer the infected tick feeds, adult females pose a lower risk because their larger size and conspicuous color allow them to be detected sooner. Thus,

the metric used of assessing the acarological risk of LD, human anaplasmosis and human babesiosis is the density of infected nymphal ticks (Mather et al., 1996a; Diuk-Wasser et al., 2012; Hahn et al., 2017).

Although nymphs likewise pose epidemiological risk for *B. miyamotoi* disease, evidence highly suggestive of the epidemiological importance of infected larvae has emerged from two recent case series reports of human *B. miyamotoi* infection in the northeastern United States (Molloy et al. 2015 and Fiorito et al. 2017). Researchers observed the peak incidence of *B. miyamotoi* infection in the patients to occur in August continuing into September, which corresponds to the peak activity period of questing larval *I. scapularis* in northeastern United States (Molloy et al., 2015; Krause and Barbour, 2015; Fiorito et al., 2017). Although prevalence of *B. miyamotoi* infection, and less commonly the density of infected ticks, has been reported for nymphal and adult ticks in North America, Europe and Asia (Barbour et al., 2009; Hamer et al., 2014; Ruyts et al., 2017; Iwabu-Itoh et al., 2017; Jiang et al., 2018), to our knowledge, *B. miyamotoi* prevalence or density of questing larvae has been reported in two studies in questing larval *I. ricinus* in Europe (van Duijvendijk et al., 2016; Szekeres et al., 2017) but not yet in *I. scapularis*.

Thus the primary aim of the present study was to estimate and compare acarological risk of *B. miyamotoi* infection (Mather et al., 1996a; Diuk-Wasser et al., 2012) from questing larval, nymphal and adult *I. scapularis*. We sampled ticks from two sites -- one in the north-central and one in the northeastern US -- where *I. scapularis* and associated pathogens are abundant (Barbour et al., 2009) and where human cases of *B. miyamotoi* have been reported (Molloy et al., 2015)Jobe et al., 2016).

MATERIALS AND METHODS

Field sites

Descriptions of the Wisconsin (WI) and Massachusetts (MA) field sites and tick collection methods have been provided elsewhere (Rulison et al., 2013; Ogden et al., 2018). Briefly, tick sampling was conducted as part of a larger study at field sites in selected LD endemic states. The WI site was established at Fort McCoy (Monroe County), where the forest is dominated by various oaks (*Quercus* spp.), red maples (*Acer rubrum*) interspersed with pines (*Pinus* spp.) and with a shrub layer of tree saplings. The MA field site was at Cape Cod National Seashore (Barnstable County), where the forest consisted of pitch pine (*Pinus rigida*) and mixed oak woodland with shrub layers of mostly bear oaks (*Quercus ilicifolia*). At each field site, we set up 3 replicate 1-ha sampling arrays. Each array comprised a 7 x 7 grid with 15 m spacing between grid points.

Tick collections

From 2010 - 2012, tick sampling was conducted at 2- 3 week intervals from April to November in WI and May to December in MA. Sampling was undertaken on rain-free days, avoiding the driest periods of the day. Ticks were dragged or flagged (Ginsberg and Ewing, 1989; Falco and Fish, 1992; Rulison et al., 2013) on 8 transects (90 m) parallel with grid lines. Researchers inspected the 1 - m^2 drag cloths for ticks every 15 m. Ticks were stored in 95% ethanol and later identified morphologically to species, life stage and sex using published dichotomous keys (Keirans and Clifford, 1978)Sonenshine, 1979; Durden and Keirans, 1996).

DNA extraction and quantitative PCR

Subsamples of each life stage of *I. scapularis* ticks were selected to investigate *B. miyamotoi* infection prevalence by molecular assay. Ticks were selected randomly from all three grids for all three years. Total DNA was extracted from individual nymphal and adult ticks by using the Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA) as previously described (Tsao et al., 2004). Larvae were pooled in groups of five for DNA extraction. Extracted DNA was stored at -20 °C until assays were performed. DNA extracts were screened by real-time PCR with specified primers and probe for a portion of the 16S rDNA of *B. miyamotoi* (Tsao et al., 2004). The identity of *B. miyamotoi* positive samples by real-time PCR was confirmed by direct sequencing of a fragment of the 16S-23S intergenic spacer region (IGS) (Tsao et al., 2004; Hamer et al., 2010; Bunikis et al., 2004). Molecular assays were conducted at Michigan State University.

Statistical analysis

The number of collected ticks were divided by the dragged areas to calculate the density of each life stage and sex of *I. scapularis*, referred to as the density of ticks (DOT). We present the data for male and female adult ticks separately because females are more likely to feed on hosts and so pose a greater risk of transmission of tick-borne pathogens. Overall DOT (per 1000 m^2) by life stage and sex for a given field site was calculated by summing the total number of ticks collected divided by the total area sampled among the three arrays and three years.

Statistical analysis for DOT was confined to ticks collected during the main host-seeking season for each life stage (i.e., May-October in WI and MA for larva; May-September

in WI and MA for nymph; April-June and October-November in WI, May-June and October-December in MA for adult) these periods encompassed > 90% of the ticks collected. Seasonal variation (by month) in DOT for each life stage was analyzed for each field site by General Linear Model in STATISTIX 8 (Analytical Software, Tallahassee, Florida, USA), while accounting for variation in DOT among arrays and years. Tick count data were log(x+1) transformed to normalize the data structure. Mean monthly DOT (averaged among 3 arrays over 3 years) was estimated from the log-transformed data, and then back-transformed for graphical presentation of the seasonal phenology curve for each life stage.

The back-transformed DOT estimates were multiplied by the infection prevalence of ticks to estimate density of infected ticks (DIT). Because molecular assays had been conducted on pooled larvae and individual nymphs and adults, point estimates of *B. miyamotoi* infection prevalence were bias-corrected Maximum Likelihood Estimates (MLE) obtained using the Excel add-in program PooledInfRate (Version 4.0; Biggerstaff, 2009) that estimates infection rates from pooled samples. Overall infection prevalence per life stage and sex per field site was estimated from ticks sampled among the three arrays and three years. We estimated seasonal (monthly) acarological risk of *B. miyamotoi* infection by adding the DIT of larvae, nymphs and adult females.

Two-tailed Fisher Exact Tests were used for pairwise comparison of *B. miyamotoi* infection prevalence among life stages and sexes of *I. scapularis* between field sites. A significance level of $\alpha = 0.05$ was used for all statistical analyses.

RESULTS

Density of ticks (DOT)

At the research area in WI, we flagged or dragged 528,570 m^2 total area and collected 19,249 larval, 2,761 nymphal, 582 female, and 775 male *I. scapularis* from April to November 2010 - 2012 (Table 1.1A). Overall DOT per 1,000 m^2 of each life stage and sex of ticks collected from 3 arrays through 3 years was estimated as 36.4 for larvae, 5.2 for nymphs, 1.1 for females and 1.5 for males (2.6 for adults). Larval *I. scapularis* were collected only from May to October (represented as main host-seeking season). Therefore, larval DOT of active season (6 months) from 3 arrays over 3 years were used for statistical analysis and seasonal graph. Seasonal larval DOT showed a bimodal distribution with peaks in June (43.9/1000 m^2) and August (41.7/1000 m^2). The majority of nymphs (99.6%) were collected from May to September. Peak nymphal DOT was observed in June (12.9/1000 m^2), which overlapped with the June larval peak. Over 97% of female and male ticks were collected from April – June and October-November. Female and male *I. scapularis* showed a trend of high DOT in spring, April and May (female, 3.2 and 3.7/1000 m^2, respectively; male 3.4 and 4.9/1000 m^2, respectively).

In MA, we sampled 316,530 m^2 and collected 3,231 larval, 634 nymphal, 162 female and 163 male adult *I. scapularis* (Table 1.1B). Tick sampling was conducted from May to December 2010 - 2012. Overall DOT (per 1000 m^2) was 10.2 for larvae, 2.0 for nymphs, 0.5 for females and 0.5 for males. Larvae were collected from May to October. Seasonal phenology of larvae showed a single peak in August (13.28/1000 m^2). Over 99% of nymphs were active from May to September. Peak nymphal DOT was observed in June (3.0/1000 m^2). Over 99% of female and male ticks were collected from May, June, October –

December. There was a trend that higher DOT of females and males were observed in late fall season than in spring.

There were some significant spatial and temporal (annual) differences in the DOT within life stages at each site. In WI, array A and sampling year 2010 showed significantly higher mean DOT of larvae than other arrays and years (ANOVA, array's $p=0.0015$; year's $p<0.0001$; Tukey's HSD, array A $p<0.05$; year 2010 $p<0.05$). Nymphal DOT was significantly higher at array A (ANOVA $p=0.0005$; Tukey's HSD, $p<0.05$) than other arrays, and significantly lower in year 2012 (ANOVA, $p<0.0001$; Tukey's HSD, $p<0.05$). There was no significant difference among 3 years for both female and male DOT (ANOVA, $p>0.97$). Array C showed significantly lower DOT of female and male than other arrays (ANOVA, $p=0.005$; Tukey's HDS, $p<0.05$). Similarly, in MA, from all life stages and sexes, array C showed significantly lower DOT through tick sampling period (ANOVA, $p=0.0003$; Tukey's HSD, $p<0.05$). There was no DOT variation among larvae among sampling years (ANOVA, $p=0.12$). Similarly, there was no DOT variation in either female and male ticks among 3 years (ANOVA, $p>0.122$). Significantly higher DOT of nymphs was observed in year 2012 (ANOVA, $p=0.0065$; Tukey's HSD, $p<0.05$).

Distinct seasonal activity patterns among life stages were apparent within each site (Fig. 1.1A – 1.2D). Similar activity patterns between WI and MA were observed for nymphs (highest peak in June) and adults (early spring and late fall peaks), however nymphal peak density in WI ($12.9/m^2$) was significantly higher than that of MA ($3.0/m^2$; ANOVA, $p=0.0002$). Seasonal patterns of larval DOT were apparently different between WI (bimodal peaks in June and August) and MA (single peak in August). Larval DOT in August was significantly higher in WI ($41.7/m^2$) than MA ($13.3/m^2$; ANOVA, $p=0.037$).

Infection prevalence and density of infected ticks (DIT)

From WI, 2555 larval, 782 nymphal, 319 female and 411 male *I. scapularis* were assayed for infection with *B. miyamotoi*. The infection prevalence of *B. miyamotoi* in questing larval, nymphal, female and male *I. scapularis* were 0.9, 2.1, 0.6, and 1.2%, respectively (Table 1.2A). There was significant difference in infection prevalence between larvae and nymphs (Fisher's Exact Test, $p=0.011$), but no statistical difference in comparisons of other life stages ($p> 0.095$) or between sexes ($p=0.48$).

From MA, 915 larval, 351 nymphal, 116 female and 104 male *I. scapularis* were assayed for infection with *B. miyamotoi*. The infection prevalence of larval, nymphal, female and male ticks were observed as 0.3, 2.3, 2.6, and 1.9%, respectively (Table 1.2B). Larval infection prevalence was significantly lower than nymphal and adult prevalence ($p<0.0088$). There was no significant difference in infection prevalence among nymphs, female adults and male adults ($p=0.74$).

Infection prevalence of each life stage and sex was not different between the two field sites ($p=0.12$). Overall density of *B. miyamotoi* infected ticks (DIT) of larval, nymphal, female, and male *I. scapularis* were estimated as 0.32, 0.11, 0.007, and 0.018 from WI (Table 1.2A) and 0.034, 0.042, 0.013, and 0.010 tick per 1,000 m^2 from MA (Table 1.2B), respectively. DIT of larvae, nymphs and adult females were added for each month to present seasonal pattern of overall DIT in WI (Fig. 1.3) and MA (Fig. 1.4).

We sequenced a fragment of the 16S-23S rRNA intergenic spacer region in a subset of *B. miyamotoi*-positive samples, and sequences showed 99% identity with published sequences for *B. miyamotoi* in GenBank. Sequences of representative positive samples have

been deposited in Genbank (accession nos. KT321367, KT321368, MH931391~MH931398).

DISCUSSION

Borrelia miyamotoi was detected from all life stages of *I. scapularis* throughout the research period at field sites in MA and WI. The infection prevalence of *B. miyamotoi* was consistent between questing nymphs and adults, which is similar with previous reports for *I. scapularis* (Barbour et al., 2009; Hamer et al., 2014). We observed, however, significantly lower infection prevalence of questing larval *I. scapularis* than other life stages.

We observed spatial and temporal variation in DOT among sampling arrays and between field sites over the 3-year research period, which is similar with previously described spatiotemporal variations of tick abundance in North America and Europe ;Ruyts et al., 2017). Overall DOT of each life stage and sex in MA was significantly lower than that of each life stage and sex in WI. It is unclear whether the observed lower DOT in MA were within the range of expected spatiotemporal variation. The seasonal activity pattern of each life stage in MA, however, was consistent with previous reports (Daniels et al., 1989; Diuk-Wasser et al., 2006; Gatewood et al., 2009; Ogden et al., 2018).

Larval DOT was consistently 5∼7 times and > 20 times higher than nymphal and adult DOT, respectively, over 3 years, which might indicate highest abundance of larvae than other life stages of ticks. Although the nymphal activity periods were similar between field sites, distinct activity patterns of larvae were observed between WI and MA, resulting in different levels of overlap between the two stages. We observed bimodal activity of larvae in June and August from WI (Fig. 1.1A), where the first larval peak coincided with the nymphal peak (Fig. 1.1B). In MA, however, the nymphal peak (Fig. 1.2B) preceded and did not overlap with the larval peak (Fig. 1.2A), which occurred in August. These

phenological patterns were consistent with previous reports (Gatewood et al., 2009; Ogden et al., 2018).

The infection prevalence of *B. miyamotoi* ranged from 0.3 % to 2.3 % among the life stages. The infection prevalence of questing larvae was significantly lower than that of nymphs in WI and lower than all other life stages and sexes in MA. The observed low infection prevalence of questing larvae, however, might be related with our assay method. From each sampling effort (dragging/flagging), we pooled larvae in groups of five for DNA extraction to screen *B. miyamotoi* infection, whereas nymphs and adults were assayed individually. Infection prevalence was estimated by a bias-corrected Maximum Likelihood Estimate (MLE), which provides point estimation of infection prevalence from pooled data. This method does not require a single-individual-positive assumption (i.e., when a pool tests positive then assumes that only individual in that pool is positive), which is the compulsory assumption of the traditional analysis for estimating infection rates from pooled samples using the minimum infection rate (MIR) estimation (Biggerstaff, 2009). However, a female *I. scapularis* can lay several thousands of eggs in one spot, resulting in dense aggregations of larvae (Daniels and Fish, 1990; Stafford, 1992). Vertically infected larvae, therefore, could be highly clumped, and result in multiple infected larvae being collected simultaneously by a single sampling effort. The aggregated distribution of questing larvae thus may result in underestimating questing larval infection prevalence by screening pooled larvae. Alternatively, *B. miyamotoi* may be maintained at low prevalence in questing larvae by vertical transmission as we observed in this study, and then may increase in nymphs via horizontal transmission involving wildlife reservoir hosts and/or non-systemic, co-feeding transmission. Increased nymphal prevalence may be maintained

through transstadial and horizontal (systemic and/or non-systemic) transmission to questing adults. Further research on how vertical, transstadial and horizontal transmission influences *B. miyamotoi* infection prevalence is needed.

Transmission efficiency from reservoir hosts to ticks and duration of host animal infectivity, along with the capacity of co-feeding transmission, have been considered crucial measures of fitness determinants for *I. scapularis*-borne pathogens, and thus are hypothesized to be affected by the phenologies of the larval and nymphal host-seeking periods (Randolph et al., 2000; Ogden et al., 2006). Specifically, seasonal synchronous or asynchronous activities between larvae and nymphs may affect the reproductive number (R_0) and infection prevalence of pathogens. For instance, it has been suggested that asynchronous pattern, where nymphs host-seek prior to larval *I. scapularis* in northeastern US, results in the high transmission efficiency and infection prevalence of *B. burgdorferi* (Wilson and Spielman, 1985). *Borrelia miyamotoi* infection prevalence of each life stage and sex, however, was not significantly different between WI and MA even though different seasonal activity patterns of larvae and nymphs were observed between the two sites. This observation might indicate that transmission and maintenance of *B. miyamotoi* infection is not much affected by synchronous (WI) or asynchronous (MA) phenologies of larval and nymphal *I. scapularis*. Further studies on the duration of infectivity and transmission efficiency of hosts may help elucidate the importance of larval and nymphal phenologies on *B. miyamotoi* maintenance.

Acarological risk for Lyme disease is measured by the density of *B. burgdorferi* infected host-seeking *I. scapularis* nymphs (DIN) (Mather et al., 1996a; Diuk-Wasser et al., 2012) because nymphs cause the majority of Lyme disease (Schwartz et al. 2017). Here,

however, we report the density of *B. miyamotoi*-infected ticks (DIT) for all life stages to estimate the acarological risk of *B. miyamotoi* disease. Transmission of *B. miyamotoi* by vertically infected larval *I. ricinus* (van Duijvendijk et al., 2016) and single larval *I. scapularis* (Breuner et al., 2018) have been demonstrated in laboratory animal experiments, which highly suggests vector competency of larval ticks for *B. miyamotoi* transmission. *Borrelia miyamotoi* DNA has been detected in the salivary glands of unfed nymphal *I. scapularis* and transmission from infected nymphs to laboratory mice could occur < 24 hrs (Breuner et al., 2017). Thus, because transmission and infection of *B. miyamotoi* may be initiated immediately after infected ticks start blood feeding, infected adult females should also be considered as possible vectors despite the potential shorter attachment duration due to their noticeable appearance. Because males do not readily feed, we did not consider their contribution to DIT.

In both WI and MA, there was a higher DIT of larvae and nymphs than adult ticks, which reflects the higher abundances of the juvenile life stages, given the lower observed infection prevalence in larvae. In general, there was a trend of DIT in larvae being 3x that of nymphs and 10x that of adults, and with DIT being greater in WI versus MA. We estimated seasonal (monthly) acarological risk of *B. miyamotoi* infection by adding the DIT of larvae, nymphs and adult females. DIT of *B. miyamotoi* infected larvae, nymphs and adult females was maintained at similar magnitude through most of sampling period, mainly from May to September in WI (Fig. 1.3) and, at lower magnitude but more extended, from May to November in MA (Fig. 1.4). This observation might indicate that risk period of *I. scapularis*-borne disease could be extended beyond the known high risk period of Lyme disease (June and July).

Recent epidemiological reports of human *B. miyamotoi* infection in northeastern US showed the highest peak of human *B. miyamotoi* incidence to occur in August (Molloy et al., 2015; Fiorito et al., 2017), corresponding with the peak activity of questing larval *I. scapularis*. Consequently vertically infected larvae have been suggested as the major vector for human *B. miyamotoi* disease (Krause and Barbour, 2015). Our result in MA also showed that the highest acarological risk of *B. miyamotoi* infection posed by vertically infected questing larvae was observed in August. However, we observed that similar or even higher magnitude of overall DIT in spring and early summer, which was mostly contributed by nymphal and adult female DIT. Observed consistent seasonal pattern of cumulative DIT resulted from distinct seasonal activity of larvae, nymphs and females is not consistent with reported epidemiological pattern of *B. miyamotoi* disease. The discrepancy between cumulative DIT pattern and seasonal pattern of human *B. miyamotoi* incidence could be explained if vertically infected larvae have higher *B. miyamotoi* transmission efficiency (tick to host) than transstadially or horizontally infected nymphs or adult females. However, Breuner et al. (2017) reported that the probability of *B. miyamotoi* transmission to laboratory mice exposed to feeding by a single nymphal *I. scapularis* reached at 73% by complete blood feeding. Alternatively, *B. miyamotoi* transmission by infected nymphs might be reduced by co-infection with other pathogens. If we consider that extremely high infection prevalence of *B. burgdorferi* in ticks has been observed and co-infection of *B. miyamotoi* and *B. burgdorferi* in questing nymphs and also in humans have been reported in the northeastern US (Barbour et al., 2009; Hamer et al., 2014; Molloy et al., 2015), there could be high possibility of co-infection in ticks with *B. miyamotoi* and *B. burgdorferi*. Transmission efficiency of *B. miyamotoi* in co-infected *I. scapularis* is unknown. Hamer et al.

(2014), however, reported significantly lower co-infection prevalence (0.05%) of *B. miyamotoi* and *B. burgdorferi* in adult *I. scapularis*, which was 2.1 times lower than expected co-infection prevalence given individual prevalence of each *Borrelia* species. Further research on transmission dynamics of *B. miyamotoi* in coinfected ticks may help resolve this question.

From an epidemiological perspective, human cases of *B. miyamotoi* disease might be underestimated during June and July, when incidence of LD cases is high. Patients suspected to have LD might receive antibiotics immediately to resolve their symptoms, with no laboratory tests run that could detect *B. miyamotoi* disease (or other tick-borne pathogens). In contrast, for patients who present with flu-like symptoms outside of the expected LD seasons, patients and health professionals may be more likely to run laboratory tests for other tick-borne diseases. Prospective epidemiological studies of *B. miyamotoi* disease throughout the activity periods of *I. scapularis* with effective laboratory assays in both north central and northeast LD endemic areas would help to elucidate the relative importance of larval, nymphal and adult life stages as *B. miyamotoi* vectors as well as the seasonality of risk.

We can estimate *B. burgdorferi* DIN in WI by multiplying observed nymphal DOT (5.2/1,000 m^2) with reported nymphal infection prevalence of *B. burgdorferi* (16.7%) in WI (Barbour et al., 2009). The estimated *B. burgdorferi* DIN of 0.87 tick per 1,000 m^2 is just 2 times higher than the overall DIT of *B. miyamotoi* infected larvae, nymphs and adult females (0.43/1,000 m^2) despite that infection prevalence of *B. burgdorferi* is generally 10 times higher than that of *B. miyamotoi* in *I. scapularis* in the Northeast US (Barbour et al., 2009).

One of the most distinct biologically different characters between *B. miyamotoi* and several other pathogens vectored by *I. scapularis,* such as *B. burgdorferi* s.l., *Anaplasma phagocytophilum, Babesia microti* (Nelder et al., 2016), is that transovarial transmission (TOT) has been observed in *B. miyamotoi* (Scoles et al., 2001; Breuner et al., 2017), but is considered as non-existent or rare for other pathogens except Powassan/deer tick virus (Costero and Grayson, 1996; Patrican, 1997; Dumler et al., 2001). Therefore, public health concern has been focused on nymphal *I. scapularis* as the major vector stage for tick-borne human disease, which resulted in a lack of published data regarding larval density and prevalence of vertically infected ticks. If we consider the minute size of larvae which may make larval bites easily missed, the number of people who are bitten by larvae and the acarological risks posed by larvae could be underestimated. In The Netherlands, however, it has been reported that larval *I. ricinus* are responsible for 1.3 to 4.3% of human tick bites, which results in ~ 30,000 people were bitten by larvae annually (Hofhuis et al., 2013; Lindblom et al., 2014).

In this article, we provided density of questing larval, nymphal and adult *I. scapularis* and their infection prevalence of *B. miyamotoi*, which allowed us to estimate acarological risk of *B. miyamotoi* for all life stage of *I. scapularis* from Wisconsin and Massachusetts where Lyme disease is highly endemic. Based on our data, it is suggested that estimates of acarological risk for *I. scapularis*-borne disease should be re-considered and incorporate larval *I. scapularis* as an important vector stage for tick-borne human disease.

APPENDIX

Table 1.1A. Tick numbers and density of *Ixodes scapularis* by life stages and sexes in Wisconsin († density of ticks).

Year	No. of ticks						Ticks per 1000 m² (DOT)†				
	Larvae	Nymphs	Females	Males	Adults	Dragged area (m²)	Larvae	Nymphs	Females	Males	Adults
2010	13,826	1,743	162	204	366	248,940	55.5	7	0.7	0.8	1.5
2011	3,295	600	187	253	440	128,430	25.7	4.7	1.5	2	3.4
2012	2,128	418	233	318	551	151,200	14.1	2.8	1.5	2.1	3.6
Total	**19,249**	**2,761**	**582**	**775**	**1,357**	**528,570**	**36.4**	**5.2**	**1.1**	**1.5**	**2.6**

Table 1.1B. Tick numbers and density of *Ixodes scapularis* by life stages and sexes in Massachusetts († density of ticks).

Year	No. of ticks						Ticks per 1000 m^2 (DOT)†				
	Larvae	Nymphs	Females	Males	Adults	Dragged area (m^2)	Larvae	Nymphs	Females	Males	Adults
2013	1,989	208	55	38	93	122,040	16.3	1.7	0.5	0.3	0.8
2014	675	187	79	84	163	116,640	5.8	1.6	0.7	0.7	1.4
2015	567	239	29	40	69	77,760	7.3	3.1	0.4	0.5	0.9
Total	**3,231**	**634**	**163**	**162**	**325**	**316,440**	**10.2**	**2**	**0.5**	**0.5**	**1**

Table 1.2A. *Borrelia miyamotoi* infection prevalence and density of infected *Ixodes scapularis* by life stages and sexes in Wisconsin (§ density of infected ticks).

Year	Infection prevalence (%)					Infected ticks per 1000 m^2 (DIT)§				
	Larvae	Nymphs	Females	Males	Adults	Larvae	Nymphs	Females	Males	Adults
2010	0.4	2.6	0	1.5	0.9	0.222	0.183	0	0.013	0.013
2011	1.3	0.4	0.7	0.4	1.6	0.336	0.016	0.01	0.007	0.056
2012	1.1	3.5	0.8	0	0.3	0.156	0.096	0.012	0	0.012
Total	**0.9**	**2.1**	**0.6**	**1.2**	**1**	**0.32**	**0.107**	**0.007**	**0.018**	**0.025**

Table 1.2B. *Borrelia miyamotoi* infection prevalence and density of infected *Ixodes scapularis* by life stages and sexes in Massachusetts (§ density of infected ticks).

Year	Infection prevalence (%)					Infected ticks per 1000 m^2 (DIT)§				
	Larvae	Nymphs	Females	Males	Adults	Larvae	Nymphs	Females	Males	Adults
2013	0.5	0	8.3	0	4.8	0.075	0	0.038	0	0.036
2014	0.4	3.2	1.4	1.4	1.4	0.021	0.051	0.01	0.01	0.02
2015	0	1.4	0	10	3.4	0	0.043	0	0.051	0.031
Total	**0.3**	**2.1**	**2.6**	**2**	**2.3**	**0.034**	**0.042**	**0.014**	**0.01**	**0.024**

Figure 1.1A. Monthly density of questing larval *Ixodes scapularis* (DOT, mean tick per 1,000 m² ± standard error) in Wisconsin (April - Nov 2010 - 2012).

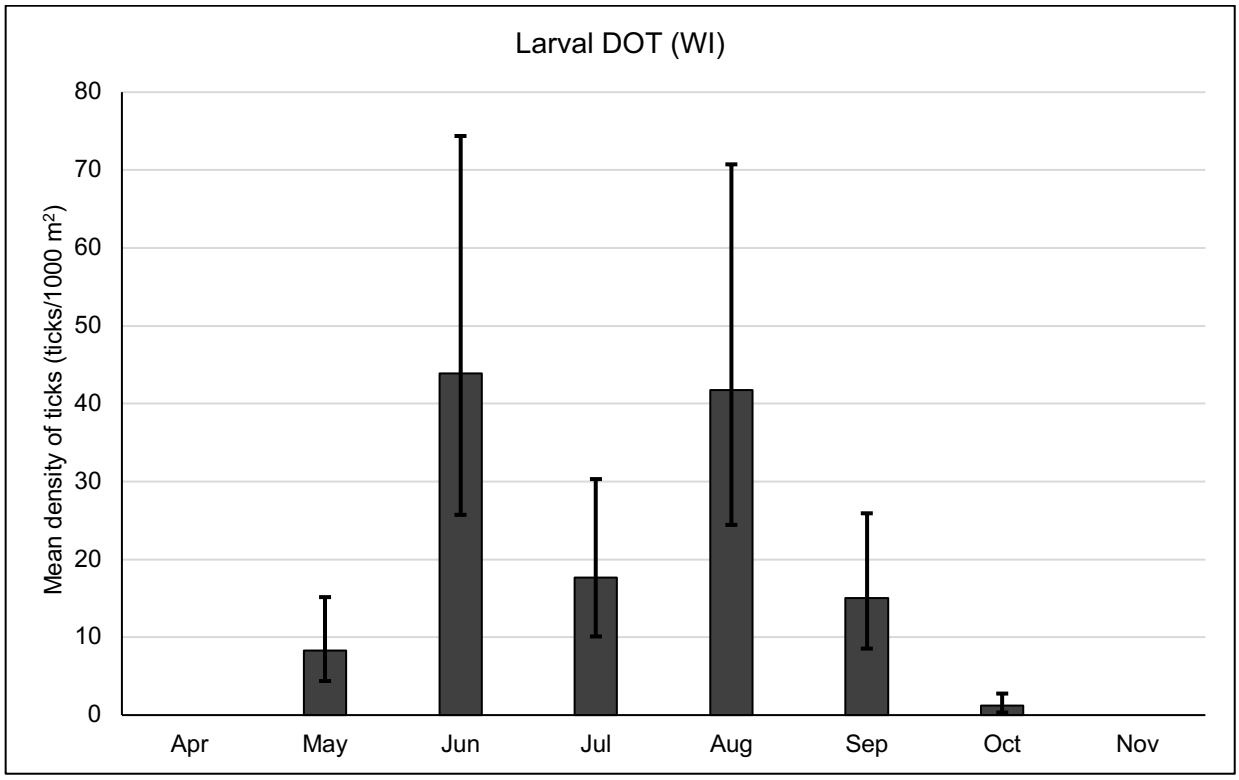


Figure 1.1B. Monthly density of questing nymphal *Ixodes scapularis* (DOT, mean tick per 1,000 m² ± standard error) in Wisconsin (April - Nov 2010 - 2012).

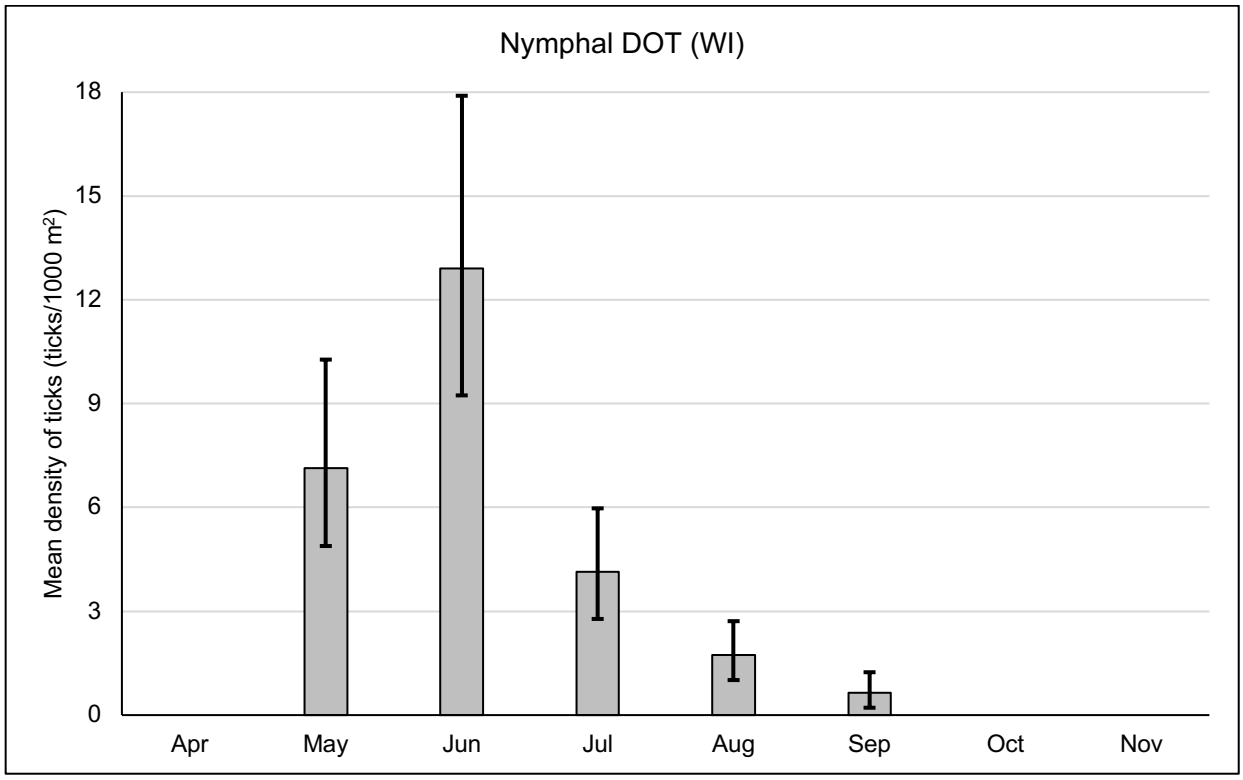


Figure 1.1C. Monthly density of questing female *Ixodes scapularis* (DOT, mean tick per 1,000 m² ± standard error) in Wisconsin (April - Nov 2010 - 2012).

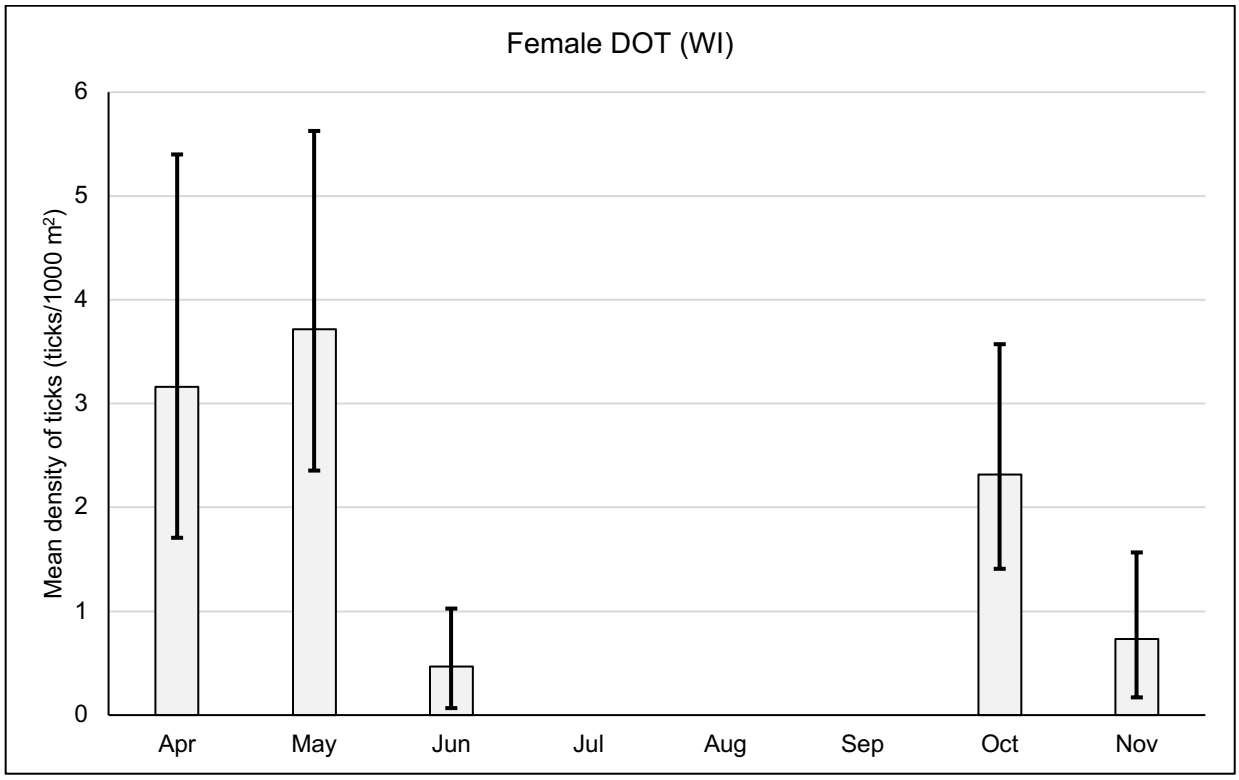


Figure 1.1D. Monthly density of questing male *Ixodes scapularis* (DOT, mean tick per 1,000 m² ± standard error) in Wisconsin (April - Nov 2010 - 2012).

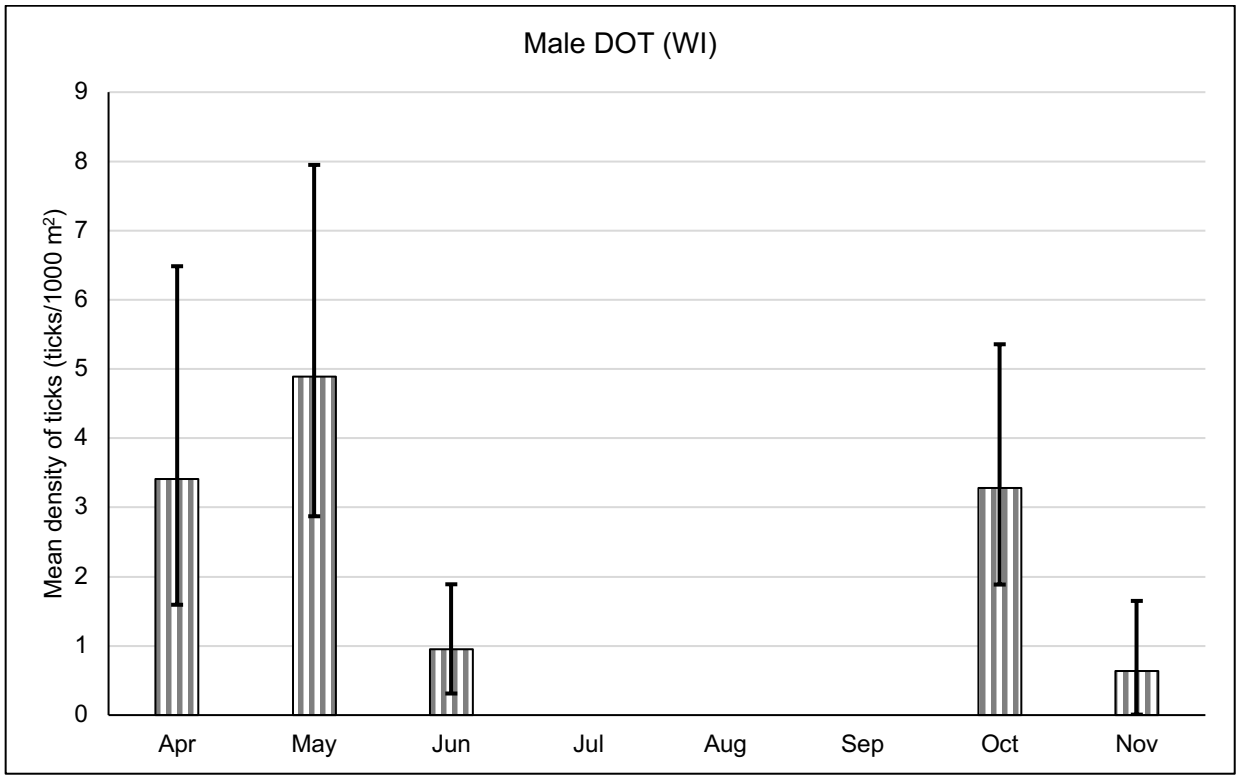


Figure 1.2A. Monthly density of questing larval *Ixodes scapularis* (DOT, mean tick per 1,000 m² ± standard error) in Massachusetts (May - December 2010-2012).

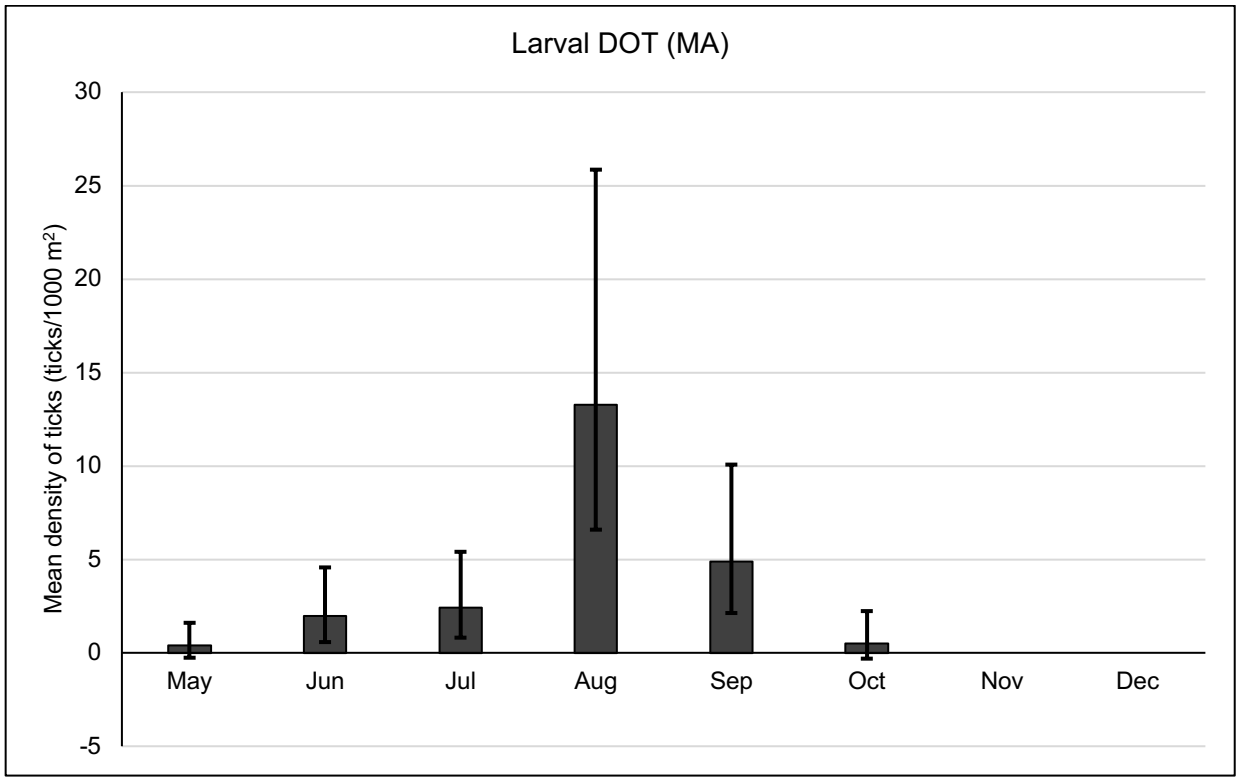


Figure 1.2B. Monthly density of questing nymphal *Ixodes scapularis* (DOT, mean tick per 1,000 m² ± standard error) in Massachusetts (May - December 2010-2012).

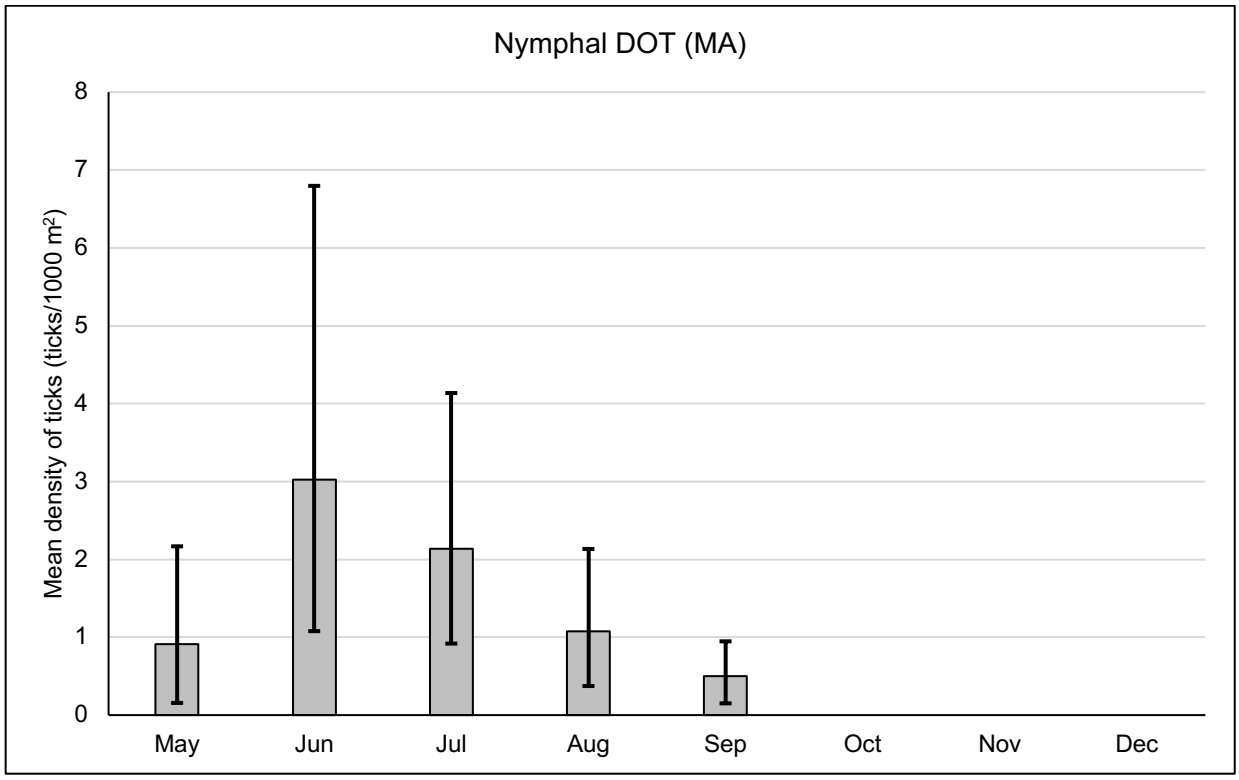


Figure 1.2C. Monthly density of questing female *Ixodes scapularis* (DOT, mean tick per 1,000 m² ± standard error) in Massachusetts (May - December 2010-2012).

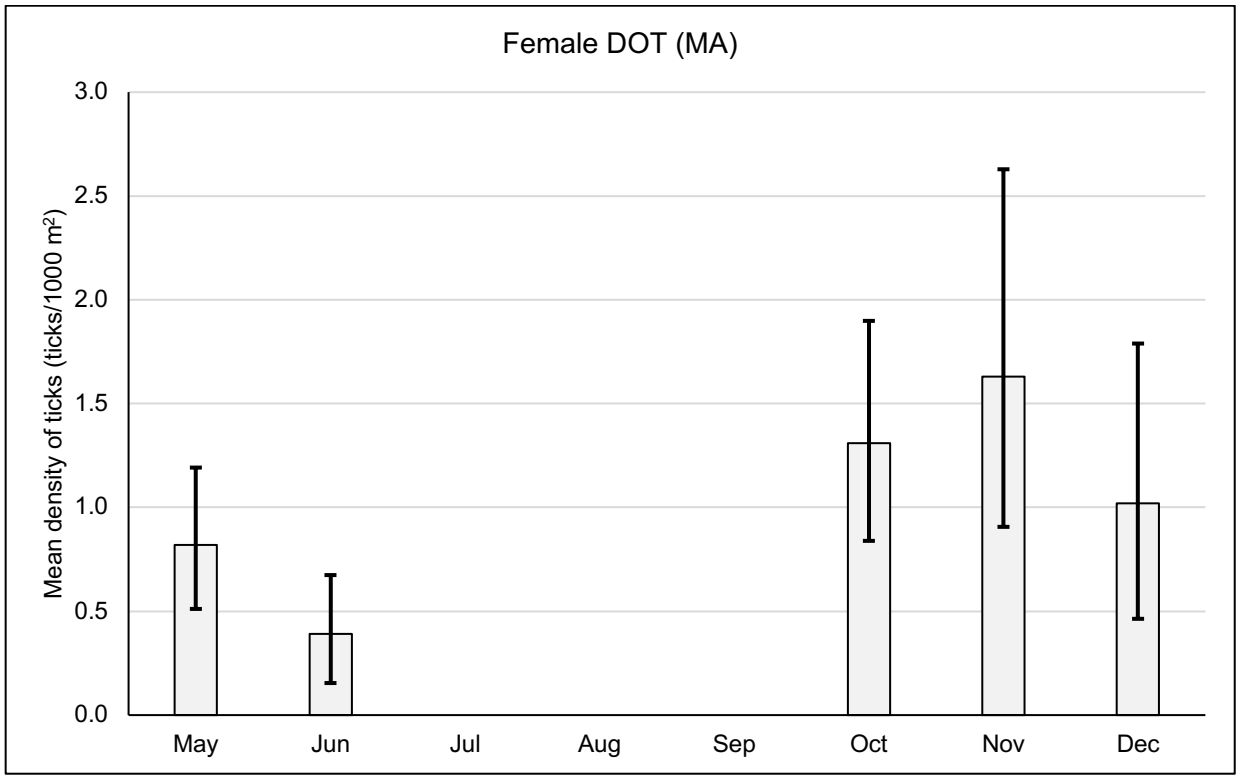


Figure 1.2D. Monthly density of questing male *Ixodes scapularis* (DOT, mean tick per 1,000 m² ± standard error) in Massachusetts (May - December 2010-2012).

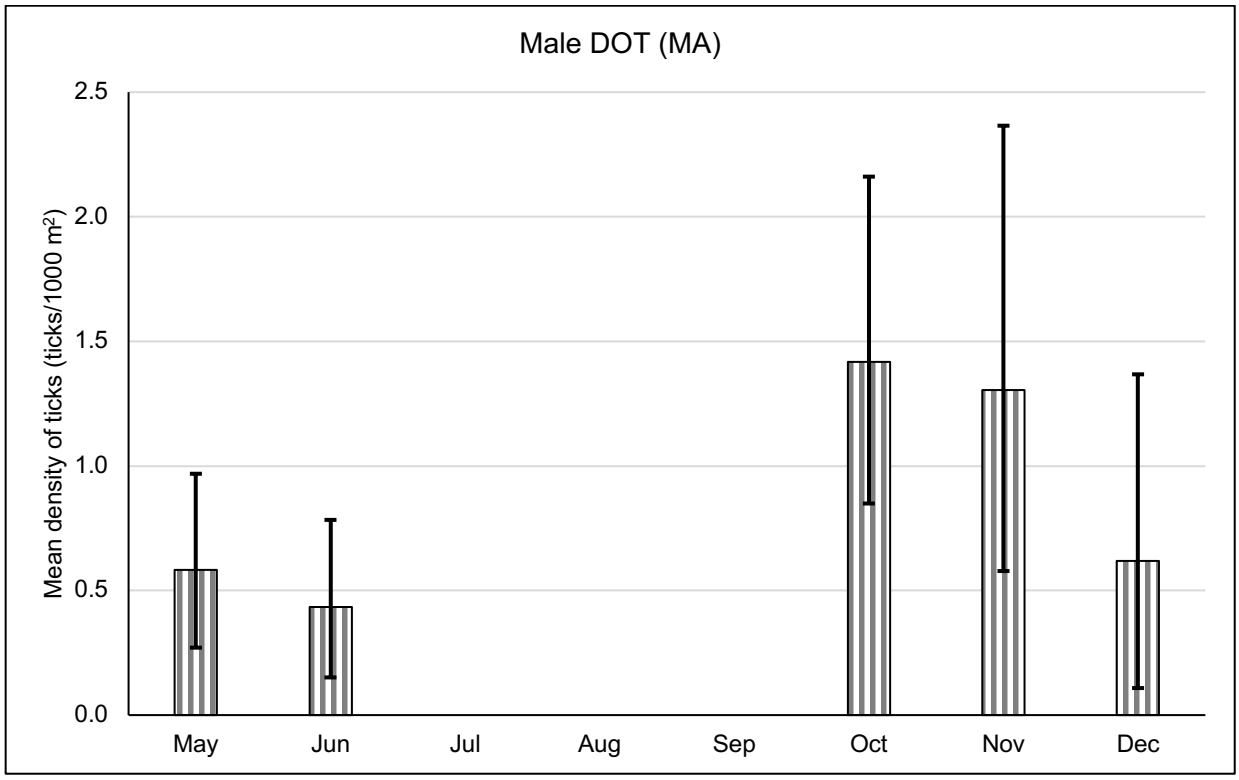


Figure 1.3. Cumulative density of *Borrelia miaymotoi* infected larval, nymphal, and adult female *Ixodes scapularis* (DIT; ticks per 1,000 m^2) by sampling month in Wisconsin.

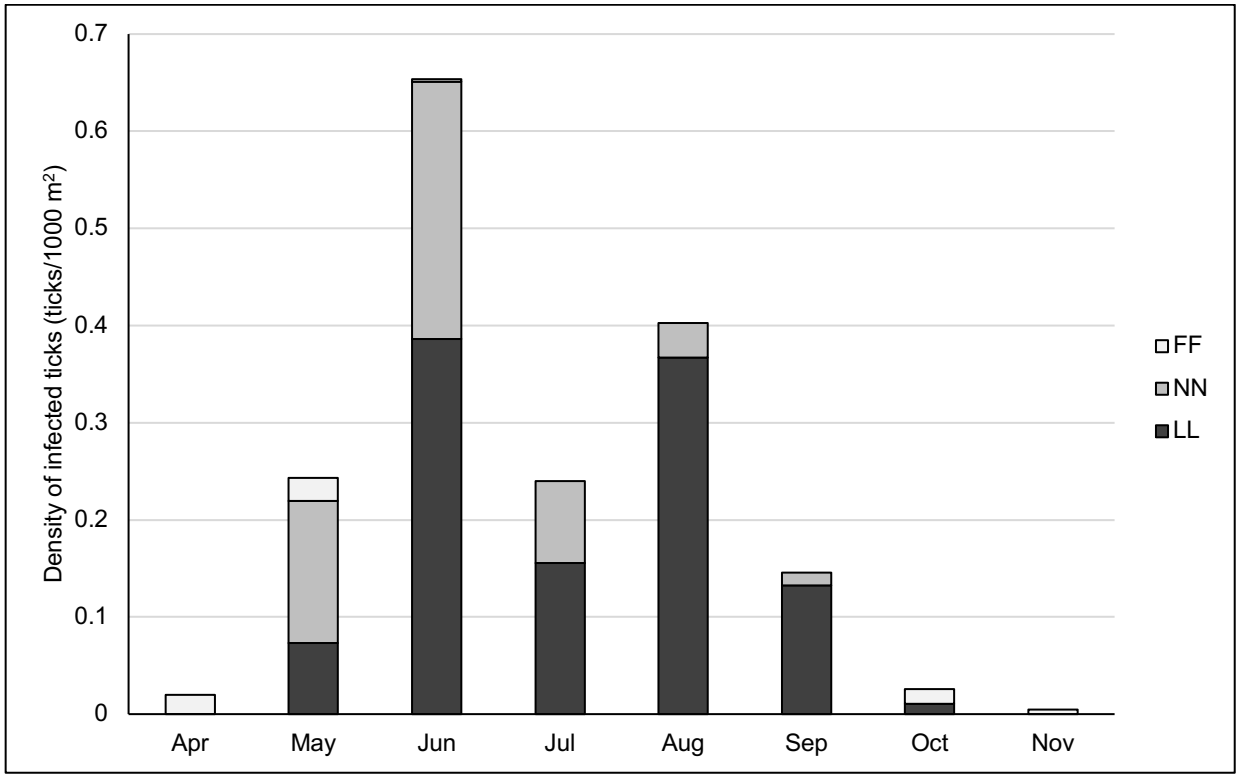
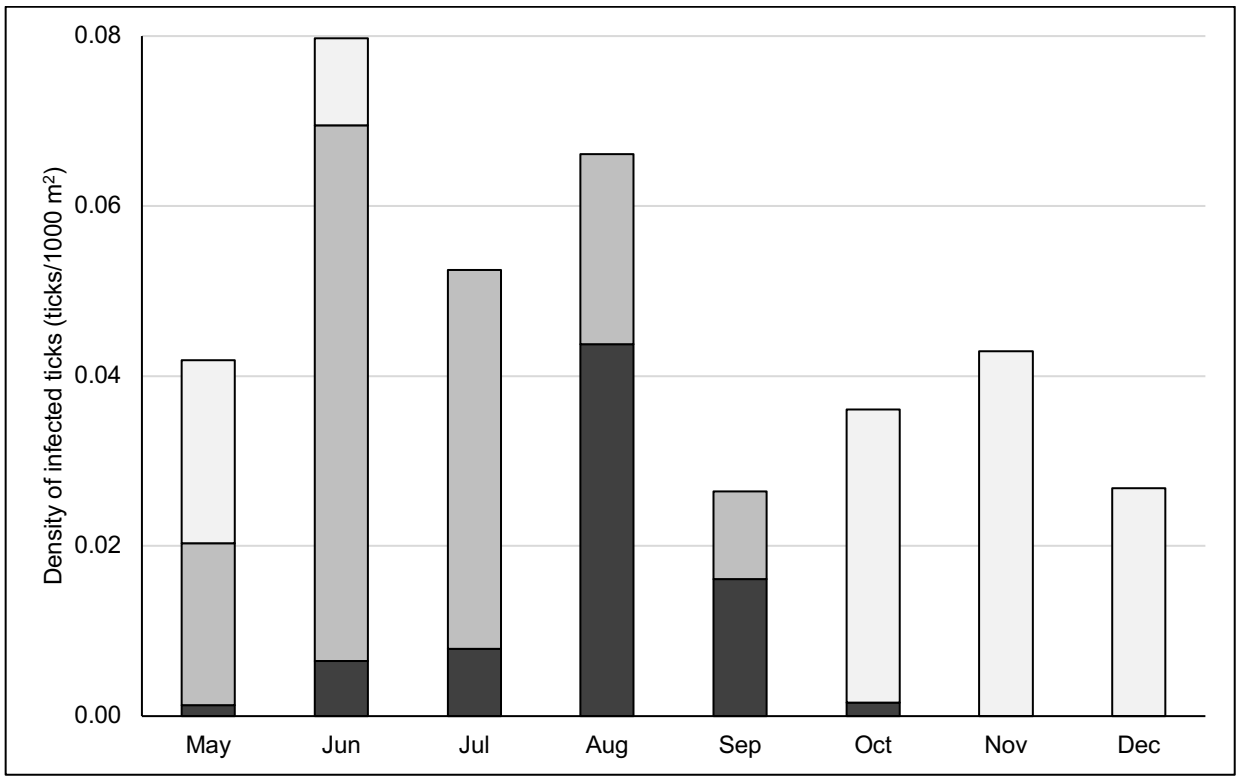


Figure 1.4. Cumulative density of *Borrelia miyamotoi* infected larval, nymphal, and adult female *Ixodes scapularis* (DIT; ticks per 1,000 m^2) by sampling month in Massachusetts.



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CHAPTER 2

Investigating small and medium-sized mammals to identify potential reservoirs of *Borrelia miyamotoi* in the North Central U.S.A.

ABSTRACT

Borrelia miyamotoi, a tick-borne zoonotic bacterium, was first discovered over two decades ago, but the enzootic cycle is still undefined. In the eastern United States, *Borrelia miyamotoi* is vectored by the tick, *Ixodes scapularis*. Several rodent species have been suggested to be reservoir hosts for *B. miyamotoi*. Therefore, as a first step, in an area where *I. scapularis* is highly abundant and which is endemic for multiple *I. scapularis*-borne diseases, we surveyed small and medium-sized mammals to find evidence suggestive of reservoir potential. We trapped mammals on three 1-ha grids from May - October 2010-2012, and screened blood and ear tissue biopsies for *B. miyamotoi* infection by polymerase chain reaction. On-host ticks were also collected from trapped animals, allowing us to analyze tick loads and the seasonal activity of *I. scapularis*. From a total of 2152 capture events consisting of 14 mammal species, six species (northern short-tailed shrew, southern red-backed vole, white-footed mouse, raccoon, *Sorex* spp. shrew, eastern chipmunk) comprised 98.5% of captures. The white-footed mouse (*Peromyscus leucopus*) was the most frequently trapped host species. Likewise, the majority of larval (72.2%) and nymphal (50.7%) *I. scapularis* were recovered from white-footed mice. The highest mean larval loads were observed from northern short-tailed shrews (5.5 ticks/capture) and white-footed mice (5.1 ticks/capture), whereas raccoons (2.2 ticks/capture) and eastern chipmunks (2.6 ticks/capture) harbored the highest mean nymphal loads. *Borrelia miyamotoi* infection was most frequently observed from white-footed mice, with 1/705 tissue (0.1%) and 12/444 blood (2.7%) samples infected, which may support the potential significant role of white-footed mice for *B. miyamotoi* maintenance. None of the attached ticks (n=26), however, recovered from 9 spirochetemic white-footed mice were infected,

and none of 3 spirochetemic white-footed mice that were recaptured 1 - 56 days later were spirochetemic. These results suggest low or limited reservoir competence of white-footed mice for *B. miyamotoi*. These results confirm those from the Northeast that blood appears to be more sensitive compared with ear tissue for detecting *B. miyamotoi* (Barbour et al. 2009); that the white-footed mouse exhibits low infection prevalence (Barbour et al. 2009); and that infection is short-lived in white-footed mice (Scoles et al. 2001). Further research on reservoir competence of hosts is required to better understand *B. miyamotoi* maintenance in nature and one species to further investigate is the eastern chipmunks as it showed the highest infection prevalence (18.8% blood samples, n=16) and typically feeds many juvenile ticks. Host infection with *B. miyamotoi* peaked in June, which is several months earlier than that published in the Northeast. Peaks in host infection track the respective peak larval activity periods in both regions, providing evidence for the importance of larvae for *B. miyamotoi* enzootic maintenance, and by extension, for human disease as well.

INTRODUCTION

Borrelia miyamotoi was first discovered from *Ixodes persulcatus* in 1994 in Japan (Fukunaga et al., 1995), and soon after, it was detected throughout the range of *I. ricinus* complex ticks in all Lyme disease endemic areas, such as from *I. ricinus* in Europe (Fraenkel et al., 2002; Richter et al., 2003), *I. persulcatus* in Russia (Fomenko et al., 2010), *I. pacificus* in the western United States (Mun et al., 2006), and *I. scapularis* in the eastern United States (Scoles et al., 2001). Since the first human cases of *Borrelia miyamotoi* disease (BMD) were reported from Russia in 2011(Platonov et al., 2011), cases subsequently have been reported from the United States (Gugliotta et al., 2013; Krause et al., 2013; Krause et al., 2014; Molloy et al., 2015; Jobe et al., 2016; Krause et al., 2016; Fiorito et al., 2017) and Eurasia (Hovius et al., 2013; Sato et al., 2014; Boden et al., 2016; Platonov et al., 2017; Hoornstra et al., 2018; Jiang et al., 2018).

In contrast with Lyme disease spirochetes (*B. burgdorferi*), *B. miyamotoi* can be vertically transmitted by transovarial transmission from infected females to their offspring (Scoles et al., 2001; Breuner et al., 2017). From feeding female *I. scapularis* collected from white-tailed deer in nature, we observed a transovarial transmission (TOT) rate of 90.9% and a filial infection prevalence (FIP) of 84.4% (Han et al. in review). Although high, the TOT rate and FIP of *B. miyamotoi* were <100%, suggesting that the vertical transmission value may not be sufficient for *B. miyamotoi* maintenance in the enzootic cycle (Fine, 1975). Horizontal transmission through reservoir host animals and/or non-systemic, co-feeding transmission thus might be required for enzootic maintenance of *B. miyamotoi*. In that case, identifying reservoir host species and estimating their contribution to enzootic maintenance would increase our understanding of the disease ecology of *B. miyamotoi*.

Several approaches to characterize reservoir host animals have been suggested and applied to vector-borne disease research. The concept of 'reservoir potential' was used to compare the contribution of different rodent hosts to the transmission of Lyme disease spirochetes, *B. burgdorferi*, which is described as a function of (1) their relative abundance, (2) their degree of contact with vector ticks, and (3) their infection prevalence (Mather et al., 1989). The term 'reservoir competence' could be characterized by three parameters: (1) susceptibility of the host to infection by vector, (2) persistence of infection in the host, (3) transmission efficiency of the host to on-host ticks (Kahl et al., 2002) . Each of those parameters defining 'reservoir potential' and 'reservoir competence' has been investigated to understand Lyme disease cycle and it has been suggested that white-footed mice are major reservoir hosts for *B. burgdorferi* maintenance, with many other small mammals contributing at apparently lower levels (Godsey et al., 1987; Anderson, 1991; Bunikis et al., 2004b). Persistent infectivity and high transmission efficiency of white-footed mice for *B. burgdorferi* (Donahue et al., 1987) along with seasonal activity pattern of juvenile *I. scapularis* (Wilson and Spielman, 1985; Spielman et al., 1985; Thompson et al., 2001) have been considered as the main factors contributing on the efficiency of *B. burgdorferi* transmission cycle and observed high prevalence in questing ticks (Mather et al., 1990).

Required information to identify reservoir competent hosts of *B. miyamotoi,* such as susceptibility, duration of infection, and transmission efficiency, could be obtained from well designed and controlled laboratory experiments by using xenodiagnostic ticks as conducted previously (Scoles et al., 2001). Field evidence, however, of *B. miyamotoi* infection from wild animal hosts may help target potential host species for xenodiagnosis studies.

Borrelia miyamotoi has been detected in white-footed mice and other various wild rodents or birds globally (Table 1) (Fukunaga et al., 1995; Barbour et al., 2009; Hamer et al., 2010; Scott et al., 2010; Taylor et al., 2013; Cosson et al., 2014; Burri et al., 2014; Cerar et al., 2015; Szekeres et al., 2015; Tokarz et al., 2016; Wagemakers et al., 2017; Hamšíková et al., 2017; Ruyts et al., 2017; Shannon et al., 2017; Yang et al., 2018; Salkeld et al., 2018). Several rodent species have been suggested as reservoir hosts for *B. miyamotoi* infection/transmission (Scoles et al., 2001; Burri et al., 2014); however, we still have limited knowledge of their contribution on *B. miyamotoi* enzootic cycle in nature and in different geographic regions. Thus, we investigated wild small and medium mammal hosts to identify potential reservoir hosts for *B. miyamotoi* maintenance by providing field information required to describe 'reservoir potential' and 'reservoir competence'. We trapped mammals and screened *B. miyamotoi* infection in an area with abundant *I. scapularis* populations, where *B. miyamotoi* has been previously detected from ticks (Hamer et al., 2010; Han et al., 2016; Han et al. in review) and where Lyme disease and other diseases vectored by *I. scapularis* are highly endemic. Density and phenology of on-host larval and nymphal *I. scapularis* collected from captured animals were estimated. We also screened on-host *I. scapularis* for *B. miyamotoi* infection to better understand *B. miyamotoi* transmission between animal hosts and ticks.

MATERIALS AND METHODS

Field sites

The field site and study design have been described elsewhere (Rulison et al., 2013; Ogden et al., 2018). Briefly, we sampled three replicate 1.1 hectare arrays at Fort McCoy military installation in central Wisconsin (WI), where *I. scapularis* is well-established (Jackson and DeFoliart, 1970; Lee et al., 2013), and where several *I. scapularis*-borne pathogens have been detected, including *B. burgdorferi* sensu stricto (s.s.), *B. mayonii*, *Anaplasma phagocytophilum*, *Babesia microti*, and *B. miyamotoi* (Steiner et al., 2008; Hamer et al., 2014; Pritt et al., 2016). The arrays were located > 3 km from one another to minimize captures of the same individual animal at multiple arrays. At all three arrays, there was mostly sandy soils, with oak (*Quercus* spp.) and maple (*Acer rubrum*)-dominated forest interspersed with pine (*Pinus* spp.), with a shrub layer of mostly tree saplings.

Mammal trapping

Mammal trapping method was described elsewhere (Ogden et al., 2018). Briefly, mammal trapping was conducted from May to October from 2010 to 2012. At each array, we placed forty-nine aluminum Sherman live trap (H. B. Sherman Traps, Tallahassee, FL) in a 7 x 7 grid with 15 m of inter-trap distance and four Tomahawk-live traps (Tomahawk Live Trap Co., Tomahawk, WI) at the midpoint of each of the four edges. Sherman traps were baited with crimped oatmeal for capturing small mammals and Tomahawk traps were baited with sardines intended to trap medium size mammal, raccoons or opossums. Traps were opened at each evening (1700 to 2000 hours) and checked the following morning (0630 to 0800 hours). Animal trapping was conducted for 2-3 consecutive nights

on each trapping session with approximately every 3 weeks interval through trapping period. Each captured mammal was examined for basic data (species, sex and mass) and ticks for a maximum of 5 min. Animals were received a metal ear tag (National Band and Tag, New-port, KY) upon initial capture. Attached on-host ticks and 2 mm (small mammals) or 4 mm (medium mammals) ear biopsies were collected and placed in plastic vials with 95% ethanol. Blood samples were collected from tail tips or femoral vein and stored in serum separation tubes. Blood tubes were centrifuged for serum separation and kept at –80 °C until DNA extraction. All animals were released at the point of capture. Any individuals that were recaptured within the same trapping session were inspected for ticks, but no additional blood nor biopsies were taken. In addition to on-host ticks, another ear biopsy and blood sample were taken for any individual recaptured in a subsequent trapping session. Collected on-host ticks were later identified to species and life stages based on morphological criteria (Sonenshine, 1979; Durden and Keirans, 1996). The frequency of captures for each animal species was interpreted as an index of relative abundance. Animal trapping and processing protocols were approved through Michigan State University's Institutional Animal Care and Use Committee (AUF # 06-09-094-00).

DNA extraction and quantitative PCR

Total DNA was extracted from individual ear tissue biopsies, 30 µl volume of blood clot, and individual engorged juvenile *I. scapularis* by using DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) following the manufacturer's animal tissue protocol as described previously (Tsao et al., 2004). We used one 2 mm ear biopsy for DNA extraction from each small mammal, but we only used a quarter piece (cut with a sterile scalpel blade) of a 4mm

ear tissue biopsy from medium sized mammals. A subset of collected on-host juvenile *I. scapularis* were examined with a dissecting microscope using an ocular micrometer to calculate an engorgement index (EI; ratio of body length/scutal width) (Yeh et al., 1995; Couret et al., 2017). Larvae and nymphs with > EI 2 were selected from individuals screened previously by tissue and/or blood assay. DNA was eluted with 100 µl AE buffer previously incubated at 70°C and stored at -20 °C. Each extraction batch included extraction negative controls consisting of extraction reagent without sample materials.

We assayed for infection with *B. miyamotoi* using a quantitative real-time PCR (qPCR) that targeted a fragment of the 16S rDNA as previously described (Tsao et al., 2004). The specificity of the qPCR used in this study to discriminate *B. miyamotoi* from *B. burgdorferi* was verified previously (Tsao et al., 2004; Barbour et al., 2009). Extracted DNA of previously-identified *B. miyamotoi*-infected field collected *I. scapularis* nymphs and water served as positive and negative PCR controls, respectively. To confirm the identity of *B. miyamotoi* positive samples detected by the qPCR, we sequenced a fragment of the 16S-23S intergenic spacer (IGS) region from a subset of positive DNA samples as previously described (Hamer et al., 2010)(Bunikis et al., 2004a). Quantitative PCR assay and DNA sequencing were conducted by using the ABI Prism 7900HT Sequence Detection System and ABI 3730 xl DNA Analyzer (Applied Biosystems, Foster City, CA), respectively, at the Michigan State University Research Technology Support Facility.

Statistical analysis

For each host species, the mean number of on-host (= attached or feeding) *I. scapularis* per capture was calculated by dividing the total number of on-host juvenile ticks

collected from that species by the total number of individuals of that species captured (including individuals from which no ticks had been detected). The Wilcoxon rank sum test (unpaired) and Wilcoxon signed rank test (paired) were used for pairwise comparisons of overall mean number of on-host ticks by host species and tick life stages.

The seasonal patterns of on-host larval and nymphal parasitism collected from all 6 abundant host species and from white-footed mice only were estimated by General Linear Models (STATISTIX 8 (Analytical Software, Tallahassee, Florida, USA)), while accounting for array and year. Mean numbers of on-host ticks were log(x+1) transformed to normalize the data structure. Monthly means of on-host ticks collected from 3 arrays over 3 years were estimated from the log-transformed values. Back transformed means were used for on-host tick phenology graphs.

Two-tailed Fisher's exact tests were used for pairwise comparisons of infection prevalence. A significance level of α=0.05 was used for all statistical analysis. Odds ratios (ORs) were accompanied by 95% CI in brackets and the respective p-value.

RESULTS

Mammal capture and on-host tick collection

Over three years there were 2152 mammal captures comprising 14 different species. Six mammal species contributed to 98.5% of total captures, which consisted of northern short-tailed shrews (*Blarina brevicauda*, n=119), southern red-backed voles (*Clethrionomys gapperi*, n=436), white-footed mice (*Peromyscus leucopus*, n=1315), raccoons (*Procyon lotor*, n=53), *Sorex* spp. shrews (n=164), and eastern chipmunks (*Tamias striatus*, n=32). The white-footed mouse was the most frequently captured species (61.1%) followed by southern red-backed voles (20.3%) and *Sorex* spp. shrews (7.6%). We used capture data for these 6 mammal species for all further analyses.

For on-host tick density and phenology analyses, if the same individual was captured multiple times within a single trapping session, we used only the data from the first time it was captured. We collected a total of 7278 larval, 540 nymphal, 5 female and 2 male *I. scapularis* from 1766 captures (Table 2). Larval *I. scapularis* were most frequently collected from white-footed mice (72.2%), northern short-tailed shrews (8.9%) and *Sorex* spp. shrews (9.2%). The highest mean numbers of on-host larvae per capture was observed from northern short-tailed shrews (mean=5.5) and the white-footed mice (mean=5.1), whereas southern red-backed voles showed the lowest mean number of on-host larvae (mean=1.1). The majority of on-host nymphs were recovered from white-footed mice (50.7%) and raccoons (21.5%). Eastern chipmunks and raccoons had the highest mean number of on-host nymphs per capture as 2.6 and 2.2, respectively, which were significantly higher than that of white-footed mice (mean=0.3, Wilcoxon rank sum test, $p<0.0001$ for both comparisons). The overall mean number of larvae infested on each

animal (mean=4.1) was significantly higher than that of nymphs (mean=0.3, Wilcoxon signed rank test, p<0.0001). All adult *I. scapularis* (5 females and 2 males) were recovered from raccoons. Monthly mean numbers of on-host larvae and nymphs per capture were estimated from 3 arrays over 3 years for all 6 species together and for white-footed mice alone. The seasonal patterns of on-host larvae from both all 6 species (Fig. 2.1A) and white-footed mice alone (Fig. 2.2A) appeared to have a bimodal distribution with the first peak in June and a second peak of similar magnitude in August, but mean July larval burdens were not statistically lower. Similarly, while mean on-host nymphs per capture for all 6 species trended higher in May and July, mean on-host nymphs per capture in June was not significantly lower (Fig. 2.1B). The mean on-host nymph per capture for white-footed mice peaked in May and gradually decreased through the rest of sampling period (Fig. 2.2B).

Assaying ear tissues for *B. miyamotoi*

We assayed a total of 1218 ear tissues collected from 12 species (Table 3). The majority of ear tissues were collected from white-footed mice (n=705; 57.9%). We detected *B. miyamotoi* DNA in only 5 tissues (0.4%) comprising 4 species: 1 northern short-tailed shrew (2.0%, n=52), 1 southern red-backed vole (0.3%, n=292), 2 *Sorex* spp. shrews (2.6%, n=76) and 1 white-footed mouse (0.1%, n=705). There was no significant difference between the proportion of total captured animals that were white-footed mice (61.1%) and the proportion of ear tissues that were from white-footed mice (57.9%) (OR, 1.01 [0.90-1.13], p=0.85). Blood samples were not collected from any of the 5 individual animals identified as *B. miyamotoi* positive by the tissue assay, whereas from three of these

individuals (1 southern red-backed vole and 2 *Sorex* spp. Shrew), on-host ticks were available and assayed for *B. miaymotoi* (see below).

Assaying blood samples for *B. miyamotoi*

A total of 516 blood samples collected from 10 species were screened for *B. miyamotoi* infection, of which 444 blood samples (86.1%) were collected from white-footed mice (Table 3). Blood samples were provided from 381 individual animals. Among them, 91 animals (86 white-footed mice, 3 raccoons, and 2 eastern chipmunks) were captured multiple times and provided at least two blood samples. The proportion of white-footed mice in blood samples was significantly higher relative to their proportion among captured animals (OR, 0.68 [0.59-0.78], p<0.0001). We detected *B. miyamotoi* DNA from a total of 16 blood samples (3.1% [1.8~5.1%]), which were collected from 1 northern short-tailed shrew, 12 white-footed mice, and 3 eastern chipmunks. All 16 *B. miyamotoi* positive blood samples were collected from 16 different individuals. The eastern chipmunk showed a significantly higher infection prevalence (18.8% [5.0 - 46.3%]) than the overall or white-footed mice (2.7% [1.5 - 4.8%]) infection prevalence (p < 0.016).

Among 516 tested blood samples, 409 samples were collected from individual animals which also provided ear biopsies at the same time when the blood was sampled. For seven white-footed mice and three eastern chipmunks that were identified as *B. miyamotoi* positive by blood assay, *B. miyamotoi* was detected from none (0/10) of their biopsies. Three of the *B. miyamotoi* spirochetemic white-footed mice were re-captured 1, 34, and 56 days later, but *B. miyamotoi* was not detected again. Nine of twelve white-footed

mice and all three eastern chipmunks from which *B. miyamotoi* was detected in the blood also had attached ticks that were assayed for *B. miyamotoi* (see below).

Assaying attached ticks for *B. miyamotoi*

Briefly, larvae or nymphs with > EI 2 (> 48 hours of attachment) were selected for pathogen assay to increase the chance of detecting *B. miyamotoi* due to acquisition from the host. On-host ticks were screened individually for *B. miyamotoi* infection. A total of 287 larvae and 13 nymphs with > EI 2 were selected from 156 captures. Of the 156 captures, 24 were screened previously only by blood, 58 were screened previously only by tissue, and 74 were screened by both assays, which include 12 of 16 infected individuals screened by blood assay and 3 of 5 infected individuals screened by tissue assay. Up to 17 engorged juvenile ticks were selected from animals tested by tissue or/and blood assay and screened for *B. miyamotoi* infection to compare the result with tissue/blood assays. There could be 4 possible scenarios to interpret the result of the attached tick assay by comparing it with the result of tissue/blood assay: (1) on-host tick positive and host tissue/blood positive, (2) on-host tick positive and host tissue/blood negative, (3) on-host tick negative and host tissue/blood positive, (4) on-host tick negative and host tissue/blood negative. From scenario 1, we may not be able to identify the origin of infection between ticks and hosts. Larvae may have been infected vertically or by feeding on the host, which was infected previously. Nymphs may have been infected vertically or during their larval blood meal or nymphal blood meal. The host may have been infected previously or from the attached ticks. Detection of simultaneous *B. miyamotoi* infection of a host and the attached ticks will be reported. In scenario 2, larvae may have been infected vertically and nymphs may have

been infected vertically or during their larval blood meal. The host may not be infected yet, or are infected with a spirochete load that is below the threshold of detection by our tissue/blood assays. From scenario 3, the host may have been infected previously, and *B. miyamotoi* has not been transmitted from hosts to attached ticks. This could be interpreted as low transmission efficiency of the hosts or low detection efficiency of the attached tick assay. Scenario 4 is not a subject of interpretation in this study.

At least one *B. miyamotoi* positive attached tick was recovered from 5 individuals (3.2%), consisting of 1 southern red-backed vole, 1 *Sorex* spp. shrew, and 3 eastern chipmunks (Table 3). All 5 animals were ones from which *B. miyamotoi* also had been detected either from blood (3 eastern chipmunks) or from tissue (1 southern red-backed vole and 1 *Sorex* spp. shrew) (scenario 1; we did not observe any examples of scenario 2). *Borrelia miyamotoi* was detected from none of the 26 on-host larvae that were recovered from 9 *B. miyamotoi*-spirochetemic white-footed mice (scenario 3) (Table 4).

Borrelia miyamotoi positive animals

A total of 21 individual animals were identified as *B. miyamotoi* positive by at least one of three assays: 2 northern short-tailed shrews, 1 southern red-backed vole, 2 *Sorex* spp. shrews, 13 white-footed mice, and 3 eastern chipmunks. There were no positive animals captured during the first (May) and the last (October) trapping months. Positive animals were captured most frequently in June (n=9) and similar numbers in July (n=3), August (n=5), and September (n=4) (Fig. 2.3).

We sequenced a fragment (478bp) of the ribosomal RNA intergenic spacer region from 5 representative *B. miyamotoi* positive samples. All 5 sequences were 100% identical

to the LB-2001 strain listed in GenBank (Accession no. CP006647) and have been deposited in GenBank (Accession numbers KY293396-KY293400).

DISCUSSION

A reservoir host for tick-borne disease could be characterized by at least three components: (1) accessibility to the vector ticks; (2) susceptibility to and persistence of infection when bitten by infectious ticks; and (3) capacity of pathogen transmission to feeding ticks (Kahl et al., 2002). Different host species may have various degrees of efficiency for each component. As a consequence, each host species may vary in their role or contribution to pathogen transmission and infection dynamics in the enzootic cycle (Woolhouse et al., 1997). The most direct evidence of competence of a host (one component of being a reservoir) for a tick-borne pathogen would come from conducting xenodiagnoses experiments. Xenodiagnosis, however, requires many resources (e.g., infectious challenge ticks; naive pathogen-free ticks; animals of the desired species, and all the infrastructure that is needed to conduct such experiments), and thus before conducting such experiments, field data may provide guidance on identifying host species with greater potential to be reservoirs. For pathogens that cannot be transmitted vertically, one can assess the competence of wildlife hosts by assaying naturally feeding larvae for infection. For *B. miyamotoi*, which can be transmitted vertically, 'natural xenodiagnosis' cannot be used to assess host competence status because it cannot be certain whether an infected attached larva acquired *B. miyamotoi* from the host or transovarially. Thus, identification of potential reservoir hosts still requires detection of *B. miyamotoi* from the host.

Although still relatively few, the number and geographic distribution of wildlife surveys for *B. miyamotoi* has been growing. From surveillance studies in Europe, Asia, and North America, *B. miyamotoi* has been detected in several small mammal and bird species (Table 1); however, their roles as reservoir hosts are uncertain. Here we captured mammal

hosts of juvenile *I. scapularis* to find potential reservoir hosts of *B. miyamotoi* based on the parameters required to define 'reservoir potential' or 'reservoir competence' as described previously (Mather et al., 1989; Kahl et al., 2002). Relative abundance, tick loads, and infection prevalence was measured for several small and medium-sized mammal species captured at a field site in the north central US where *I. scapularis* and several associated pathogens are well-established.

Relative abundance of mammal species and their juvenile *I. scapularis* loads

Over a three year period, we captured 14 different mammal species, and 6 species accounted for over 98% of the captures. Moreover, 97.9% and 90% of the larvae and nymphs respectively were recovered from these 6 species, which may indicate their importance for *I. scapularis* ecology and *B. miyamotoi* enzootic maintenance. Therefore, we focused our data analysis and interpretation on these 6 species. The white-footed mouse was the most frequently trapped species (58.4%), and the majority of larvae (72.2%) and half the nymphs (50.7%) collected from captured hosts were collected from white-footed mice. These findings are consistent with previous publications (Levine et al., 1985; Godsey et al., 1987; Lane et al., 1991), and might suggest high 'reservoir potential' of white-footed mice and relative importance for *B. miyamotoi* maintenance. Despite over half of the feeding nymphs were collected from white-footed mice, on average, the mean number of nymphal loads on raccoons and eastern chipmunks were over 7 and 8 times greater than on white-footed mice, respectively, whereas lowest larval burden was on the southern red-backed vole. Observed variation in larval and nymphal tick infestation among host species were consistent with published studies from the northeastern US (Levine et al., 1985;

Davidar et al., 1989; Schmidt et al., 1999). Data on questing larval and nymphal *I. scapularis* density collected contemporaneously with the wildlife trapping reported here were 36.4 and 5.2 tick per 1000 m^2, respectively (See Chapter 1). If we compare the ratio of overall mean larval and nymphal load per each animal (4.1L:0.31N) with that of questing larval and nymphal densities (36.4L:5.2N), a significantly smaller proportion of nymphs were recovered from animals we captured than that of questing nymphs (OR 1.89 [1.19-3.01]; p=0.0075). Taken together, these observations suggest that relative to other host species, these 6 species may feed relatively more larval versus nymphal *I. scapularis*.

Transmission of *B. miyamotoi* by vertically infected larval *I. ricinus* (van Duijvendijk et al., 2016) and larval *I. scapularis* (Breuner et al., 2018) to laboratory mice has been demonstrated. Hatched flat larvae, therefore, may also vector *B. miyamotoi* to wild animals or humans. In a study located in the Northeast, Barbour et al. (2009) reported that the frequency of *B. miyamotoi* spirochetemia in white-footed mice captured from late May through early September increased in prevalence and peaked in early September, coinciding with peak larval questing activity. These data implicate a greater role for larvae, rather than nymphs, which peak in activity in June and July (Gatewood et al., 2009; Ogden et al., 2018), as vectors of *B. miyamotoi* for infecting white-footed mice. The epidemiological importance of larval ticks for *B. miyamotoi* transmission was also evidenced by two recently published epidemiological studies carried out in the northeastern US, which also showed a peak incidence of human disease in late summer (August) (Molloy et al., 2015; Fiorito et al., 2017).

In contrast to Barbour et al. (2009), we observed that *B. miyamotoi*-positive animals were captured most frequently in June, with similar, but lower numbers in months

throughout the rest of summer months and early fall (Fig. 2.3). In the north central US where we conducted this study, the seasonal activities of questing larvae and nymphs overlap substantially, and peak in July (Gatewood et al., 2009; Ogden et al., 2018). Because of the overlapping phenologies of nymphs and larvae, we cannot say unequivocally that larvae are the main enzootic vectors in the north central US. The shift in the seasonality of infection in hosts from later summer to mid-summer, however, tracks the same relative shift in larval questing activity between the two regions and thus still provides data supporting the prediction of the importance of larvae as vectors. Further research on *B. miyamotoi* prevalence from a greater diversity of host species and seasonal activity pattern of on-host *I. scapularis* in other areas may help clarify the relative roles of larvae and nymphs as vectors.

***Borrelia miyamotoi* infection of mammal hosts**

Significantly higher *B. miyamotoi* infection prevalence was observed by mammal blood assays (3.1%) than ear biopsy assays (0.4%), which is consistent with that observed in white-footed mice in the northeastern US (Barbour et al., 2009). In the current study, *B. miyamotoi* was not detected from any ear tissues collected from individual animals identified as *B. miyamoto*i spirochetemic when both samples were obtained during the same capture event. Our results, therefore, support the previous observation that blood appears to be more sensitive compared with tissue for detecting *B. miyamotoi*.

Combining data from both tissue and blood assays, *B. miyamotoi* was detected from a total of 21 individual animals representing 5 mammal species, of which the white-footed mouse (n=13) was the most frequently identified species. Overall, however, only 2.7% of

white-footed mouse were infected, whereas *B. miyamotoi* was detected from 18.8% of eastern chipmunks. These results may indicate significant roles for both the white-footed mouse, which is very abundant, and the eastern chipmunk in *B. miyamotoi* enzootic maintenance.

Reservoir competence of *B. miyamotoi*

Transmission of *B. miyamotoi* from host animals to larvae was demonstrated from wild-caught bank voles and yellow-necked mice to xenodiagnostic larval *I. ricinus* (Burri et al., 2014). However, there was no published data regarding on transmission efficiency (defined as the efficiency of the host at transmitting the spirochete to feeding vectors) of *B. miyamotoi* from host to ticks. The probability that a vector becomes infected by feeding on a host species has been defined as 'realized reservoir competence' and used for investigating the role of host animals in vector-borne disease system (Schauber and Ostfeld, 2002; LoGiudice et al., 2003; Brunner et al., 2008). The 'realized reservoir competence' of a host species can be characterized as the combined probabilities that: (1) an individual of that host species is infected, and (2) a feeding tick becomes infected, given an infected host. Our results provide limited information regarding on realized reservoir competence of white-footed mice for *B. miyamotoi* transmission. None of on-host ticks (n=26) recovered from 9 *B. miyamotoi* spirochetemic white-footed mice were infected (Table 4). White-footed mice, therefore, may have a low realized reservoir competence. We could not distinguish whether infected on-host larvae collected from other spirochetemic hosts were infected from host animals by horizontal transmission or from transovarial transmission. Therefore, realized reservoir competence of other host species could not be

able to be determined with our data. However, we may be able to provide a pattern of association between infected hosts and infection of attached ticks. Infection of attached ticks was observed from one red-backed vole (1/1), one of two *Sorex* spp. shrews (1/2), and all 3 eastern chipmunks (3/3). Among 16 *B. miyamotoi* infected animals identified by blood assay, three white-footed mice were re-captured after 1 day and 1-2 months from the captures identified as *B. miyamotoi* infection. None of the three white-footed mice showed persistent or recurrent spirochetemia, which may indicate short duration of *B. miyamotoi* spirochetemia in white-footed mice. It is possible, however, that number of *B. miyamotoi* spirochetes in mouse blood may have decreased below the detection limit of our qPCR. Alternatively, it may be that *B. miyamotoi* colonized other organs rather than remaining in blood stream.

Controlled laboratory experiments measuring over time *B. miyamotoi* infection in various tissues and transmission efficiency and dynamics to xenodiagnostic larvae are required to better interpret our field observations and to identify potential reservoir host species and their relative roles in *B. miyamotoi* enzootic maintenance.

We observed that the white-footed mouse was the dominant species among the most frequently trapped six mammal species and served as hosts for the majority of attached juvenile (especially larval) *I. scapularis*. Given larvae may be infected transovarially, white-footed mice may play an important role for *B. miyamotoi* maintenance as we also observed the high proportion of white-footed mice from *B. miyamotoi* infected animals. However, white-footed mice may not have strong reservoir potential or reservoir competence for *B. miyamotoi* as indicated by potentially short spirochetemia duration and low transmission efficiency to larval *I. scapularis*. This limited reservoir potential or

reservoir competence of white-footed mice as reservoir hosts could be one explanation for low *B. miyamotoi* infection prevalence generally observed in questing *I. scapularis*. Eastern chipmunks had a significantly higher infection prevalence than white-footed mice. Despite their lower abundance and relatively lower contribution to feeding larvae, eastern chipmunks may help facilitate long term maintenance of *B. miyamotoi*.

Limitations and suggestions for future research

In this study, the relative abundance of each host species was estimated based on the frequency of capture for the animal species, therefore, the observed high abundance of white-footed mice and relatively low abundance of other host species may not exactly represent their abundance in the given host community due to our study design. In addition, only 516 blood samples were screened from total 1766 captures while 1218 ear tissues were screened. Moreover, the proportion of white-footed mice in the blood tested individuals was significantly higher than their proportion of total captures, which resulted from the difficulties in sampling a large enough blood volume from small mammals due to their small size.

We only targeted small and medium size mammals in this study; however, the vector, *I. scapularis*, is known to be a generalist and feed on a wide range of vertebrate hosts especially as larvae and nymphs (Eisen and Lane, 2002). Infection of *B. miyamotoi* has been observed from several bird species (Scott et al., 2010; Wagemakers et al., 2017), and higher prevalence of infection in females engorged with white-tailed deer blood compared with that of questing females was previously reported (Han et al., 2016). If we consider that females can transmit *B. miyamotoi* vertically to their offspring, white-tailed deer, which are

the major hosts of adult *I. scapularis*, may play a significant role in the *B. miyamotoi* enzootic cycle (Lane et al., 1991).

Determining the contributions of reservoir hosts in the *B. miyamotoi* enzootic cycle can be complex because of the need to take into the account the qualitative effects of reservoir competence for *B. miyamotoi* transmission and quantitative roles as blood feeding hosts of *I. scapularis*. Our results along with the identified limitations highlight the necessity of further research with additional trapping methods which might allow one to sample additional host species, including birds and white-tailed deer, to infer their contributions to *B. miyamotoi* infection/transmission dynamics.

APPENDIX

Table 2.1. Reported *Borrelia miyamotoi* surveillance of wildlife.

Species		Origin	Total	Positive		Sample type	Assay type	Reference
Scientific name	Common name		(n)	(n)	(%)			
Apodemus argenteus	Small Japanese field mouse	Japan (Wild)				Blood	Culture	(Fukunaga et al., 1995)
Peromyscus leucopus	White-footed mouse	U.S.A (Wild)	556	36	6.5%	Blood	PCR	(Barbour et al., 2009)
			86	2	2.3%	Ear tissue	PCR	
Peromyscus leucopus	White-footed mouse	U.S.A (Lab.)				Xeno.	PCR	(Scoles et al., 2001)[ϕ]
Peromyscus leucopus	White-footed mouse	U.S.A (Wild)	1249	12	1.0%	Ear tissue	PCR	(Hamer et al., 2010)
Meleagris gallopavo	Wild turkey	U.S.A (Wild)	42	11	26.2%	Blood	PCR	(Scott et al., 2010)
			59	30	50.8%	Skin tissue	PCR	
Apodemus argenteus	Small Japanese field mouse	Japan (Wild)	137	1	0.7%	Urinary bladder	PCR	(Taylor et al., 2013)
			87	1	1.1%	Blood	PCR	
Apodemus speciosus	Large Japanese field mouse		446	10	2.2%	Urinary bladder	PCR	
			291	24	8.2%	Blood	PCR	
Myodes rufocanus	Gray red-backed vole		195	2	1.0%	Urinary bladder	PCR	
			106	10	9.4%	Blood	PCR	
Myodes rutilus	Northern red-backed vole		89	0	0.0%	Urinary bladder	PCR	
			56	3	5.4%	Blood	PCR	
Myodes rex	Hokkaido red-backed vole		12	0	0.0%	Urinary bladder	PCR	

Table 2.1 (cont'd)

			9	0	0.0%	Blood	PCR	
Myodes glareolus	Bank vole	France (Wild)	72	4	5.6%	Spleen	PCR	(Cosson et al., 2014)
Apodemus flavicollis	Yellow-necked mouse	Switzerland (Wild)	45	0	0.0%	Blood	PCR	(Burri et al., 2014)[f]
Myodes glareolus	Bank vole		19	0	0.0%	Blood	PCR	
Apodemus flavicollis	Yellow-necked mouse	Slovenia (Wild)	297	2	0.7%	Heart tissue	PCR	(Cerar et al., 2015)[λ]
Apodemus flavicollis	Yellow-necked mouse	Hungary (Wild)	102	1	1.0%	Skin tissue	PCR	(Szekeres et al., 2015)
			67	1	1.5%	Spleen	PCR	
Apodemus agrarius	Striped field mouse		202	0	0.0%	Skin tissue	PCR	
			92	0	0.0%	Spleen	PCR	
Myodes glareolus	Bank vole		29	0	0.0%	Skin tissue	PCR	
			11	0	0.0%	Spleen	PCR	
Microtus arvalis	Common vole		7	0	0.0%	Skin tissue	PCR	
			4	0	0.0%	Spleen	PCR	
Micromys minutus	Eurasian harvest mouse		3	0	0.0%	Skin tissue	PCR	
Mus musculus	House mouse		5	0	0.0%	Skin tissue	PCR	
			3	0	0.0%	Spleen	PCR	
Apodemus agrarius	Striped field mouse	Croatia (Wild)	53	7	13.2%	Kidney, Lung	PCR	(Tokarz et al., 2016)
Apodemus flavicollis	Yellow-necked mouse		131	1	0.8%	Kidney, Lung	PCR	
Apodemus sylvaticus	Wood mouse		3	0	0.0%	Kidney, Lung	PCR	

Table 2.1 (cont'd)

Myodes glareolus	Bank vole		43	0	0.0%	Kidney, Lung	PCR	
Sorex araneus	Common shrew		6	1	16.7%	Kidney, Lung	PCR	
Microtus arvalis	Common vole		4	0	0.0%	Kidney, Lung	PCR	
Microtus agrestis	Field vole		1	0	0.0%	Kidney, Lung	PCR	
Muscardinus avellanarius	Hazel dormouse		1	0	0.0%	Kidney, Lung	PCR	
Apodemus sylvaticus	Wood mouse	Netherlands (Wild)	21	3	14.3%	Spleen	PCR	(Wagemakers et al., 2017)
Apodemus flavicollis	Yellow-necked mouse		2	0	0.0%	Spleen	PCR	
Cocidura russula	Greater white-toothed shrew		4	0	0.0%	Spleen	PCR	
Microtus arvalis	Common vole		8	1	12.5%	Spleen	PCR	
Myodes glareolus	Bank vole		34	3	8.8%	Spleen	PCR	
Sorex araneus	Common shrew		5	0	0.0%	Spleen	PCR	
Capreolus capreolus	Roe deer		10	0	0.0%	Spleen	PCR	
Caruelis chloris	European greenfinch		4	1	25.0%	Spleen	PCR	
Coccothraustes coccothraustes	Hawfinch		2	0	0.0%	Spleen	PCR	
Fringilla coelebs	Common chaffinch		3	0	0.0%	Spleen	PCR	
Parus major	Great tit		2	1	50.0%	Spleen	PCR	
Phylloscopus trochilus	Willow warbler		1	0	0.0%	Spleen	PCR	
Pyrrhula pyrrhula	Eurasian bullfinch		1	0	0.0%	Spleen	PCR	

Table 2.1 (cont'd)

Turdus iliacus	Redwing		5	0	0.0%	Spleen	PCR	
Turdus merula	Common blackbird		2	0	0.0%	Spleen	PCR	
Turdus philomelos	Song thrush		6	0	0.0%	Spleen	PCR	
Apodemus flavicollis	Yellow-necked mouse	Slovakia (Wild)	356	33	9.3%	Skin tissue	PCR	(Hamšíková et al., 2017)
Myodes glareolus	Bank vole		226	10	4.4%	Skin tissue	PCR	
Microtus arvalis	Common vole		19	0	0.0%	Skin tissue	PCR	
Apodemus sylvaticus	Wood mouse		2	0	0.0%	Skin tissue	PCR	
Micromys minutus	Eurasian harvest mouse		1	0	0.0%	Skin tissue	PCR	
Microtus subterraneus	Eurasian pine vole		1	0	0.0%	Skin tissue	PCR	
Sciurus vulgaris	Eurasian red squirrel	Belgium (Wild)	45	3	6.7%	Spleen	PCR	(Ruyts et al., 2017)$^{\sigma}$
Felis catus	Domestic cat	U.S.A (Domestic)	49	2	4.1%	Blood	PCR	(Shannon et al., 2017)
Elahurus davidianus	Pere David deer	China (Natural reserve)	43	1	2.3%	Blood	PCR	(Yang et al., 2018)
Neotoma fuscipes	Dusky-footed woodrat	California, U.S.A. (Wild)	6	1	17.0%	Blood	PCR	(Salkeld et al., 2018)
Peromyscus boylii	Brush mouse		71	2	3.0%	Ear tissue	PCR	
Peromyscus californicus	California mouse		24	4	17.0%	Ear/Blood$^{\theta}$	PCR	

ϕ Animals screened by xenodiagnostic larvae in laboratory

λ Animal number n=251 in retrospective and n=46 in prospective study

Table 2.1 (cont'd)

σ Road killed animals were screened

θ 3 ear tissues and 1 blood

Table 2.2. On-host larval and nymphal *Ixodes scapularis* collected from the first capture of each trapping session for the 6 most frequently captured species.

Host species		Capture	On-host Larva		On-host Nymph	
Scientific name	**Common name**	**No.**	**No.**	**Mean**	**No.**	**Mean**
Blarina brevicauda	Northern short-tailed shrew	119	650	5.5	14	0.12
Clethrionomys gappei	Southern red-backed vole	377	401	1.1	54	0.14
Peromyscus leucopus	White-footed mouse	1026	5252	5.1	274	0.27
Procyon lotor	Raccoon	52	221	4.3	116	2.23
Sorex spp.	Long-tailed shrew	164	668	4.1	8	0.05
Tamias striatus	Eastern chipmunk	28	86	3.1	74	2.64
Total		1766	7278	4.1	540	0.31

Table 2.3. Detection of *Borrelia miyamotoi* in tissue, blood and/or on-host ticks collected from captured mammals.

Host species		Tissue		Blood		On-Host tick	
Scientific name	**Common name**	**Tested No.**	**Positive No.**	**Tested No.**	**Positive No.**	**Tested No.**	**Positive No.**
Blarina brevicauda	Short-tailed shrew	52	1	1	1	5	0
Clethrionomys gapperi	Red-backed vole	292	1	5	0	11	1
Peromyscus leucopus	White-footed mouse	705	1	444	12	128	0
Procyon lotor	Raccoon	47	0	40	0	2	0
Sorex spp.	Long-tailed shrew	76	2	0	0	5	1
Tamias striatus	Eastern chipmunk	15	0	16	3	4	3
Others$^{\phi}$		31	0	10	0	1	0
Total		**1218**	**5**	**516**	**16**	**156**	**5**

ϕ Others include star nosed mole, opossum, southern flying squirrel, meadow vole, gray fox, jumping mouse

Table 2.4. Comparison of *Borrelia miyamotoi* detection among tissue, blood, and on-host tick assays for 21 individuals.

Host species		Ear	Capture	Tissue	Blood	On-Host Tick assay	
Scientific name	Common name	Tag	date	assay	assay	Pos.	Tested
Blarina brevicauda	Northern short-tailed shrew	-	6/5/10	-	Pos.	-	-
		-	8/17/11	Pos.	-	-	-
Clethrionomys gapperi	Southern red-backed vole	2943	6/3/10	Pos.	-	3	5
Peromyscus leucopus	White-footed mouse	3022	7/10/10	Neg.	Pos.	-	-
		3033	8/14/10	Neg.	Pos.	0	4
		3047	6/22/10	Neg.	Pos.	0	3
		3748	7/6/11	Neg.	Pos.	0	2
		K006	6/13/12	Neg.	Pos.	0	1
		K007	6/13/12	Neg.	Pos.	-	-
		K013	6/14/12	Neg.	Pos.	0	6
		3231	8/18/11	-	Pos.	0	3
		3264	6/14/11	-	Pos.	0	4
		3760	7/28/11	-	Pos.	0	2
		3875	9/11/11	-	Pos.	-	-
		3877	8/18/11	-	Pos.	0	1
		3119	10/9/11	Pos.	-	-	-
Sorex spp.	Long-tailed shrew	-	10/9/12	Pos.	-	0	2
		-	8/17/11	Pos.	-	1	2
Tamias striatus	Eastern Chipmunk	K064	6/14/12	Neg.	Pos.	4	16
		K061	6/14/12	Neg.	Pos.	17	17
		K395	9/15/12	Neg.	Pos.	2	4

Figure 2.1A. Observed seasonal activity of on-host larval *Ixodes scapularis* collected from 6 species that contributed to 94% of mammal captures from 3 1-ha arrays from May – October 2010 – 2012 at Ft. McCoy, Wisconsin (Error bar indicates 95% confidence interval).

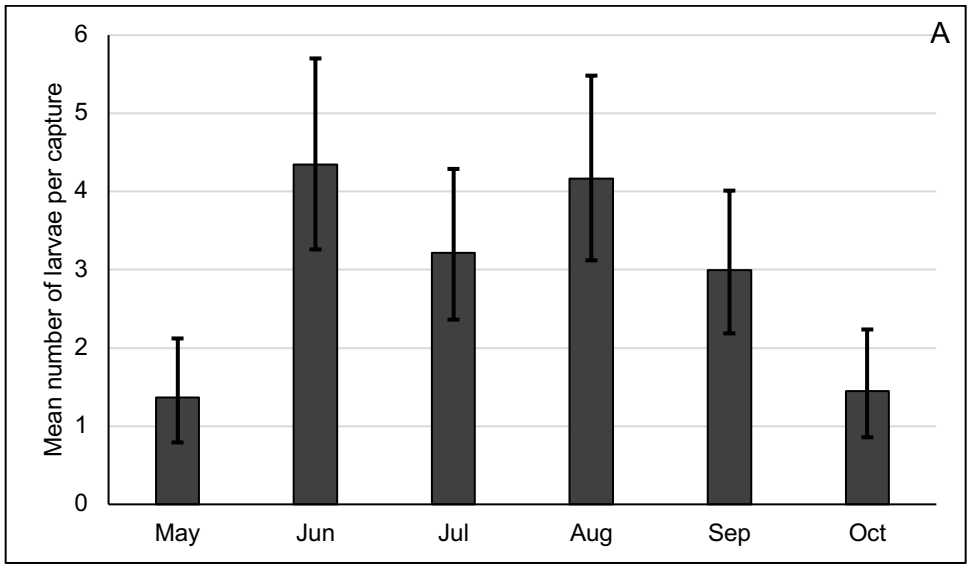


Figure 2.1B. Observed seasonal activity of on-host nymphal *Ixodes scapularis* collected from 6 species that contributed to 94% of mammal captures from 3 1-ha arrays from May - October 2010-2012 at Ft. McCoy, Wisconsin (Error bar indicates 95% confidence interval).

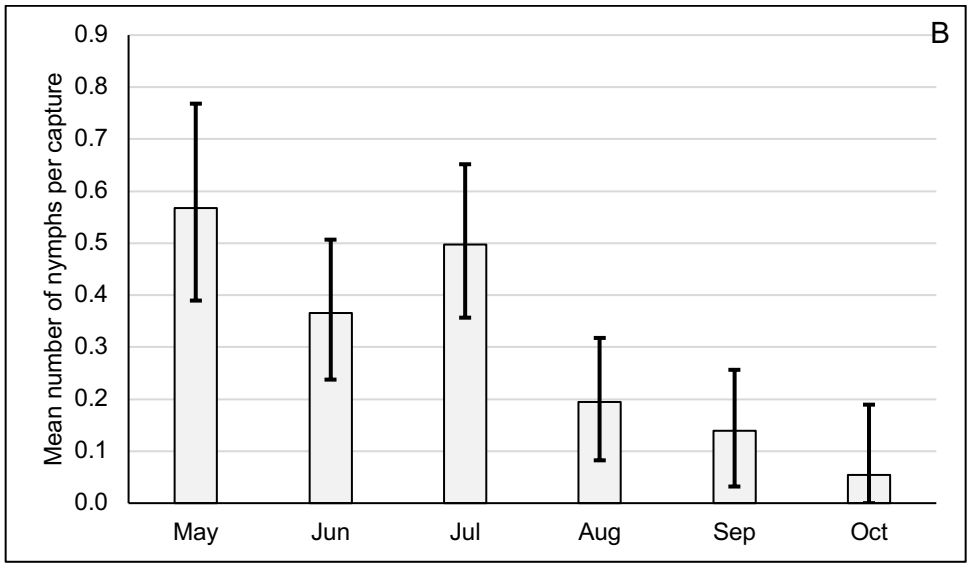


Figure 2.2A. Observed seasonal activity of on-host larval *Ixodes scapularis* collected from white-footed mice from 3 1-ha arrays from May - October 2010-2012 at Ft. McCoy, Wisconsin (Error bar indicates 95% confidence interval).

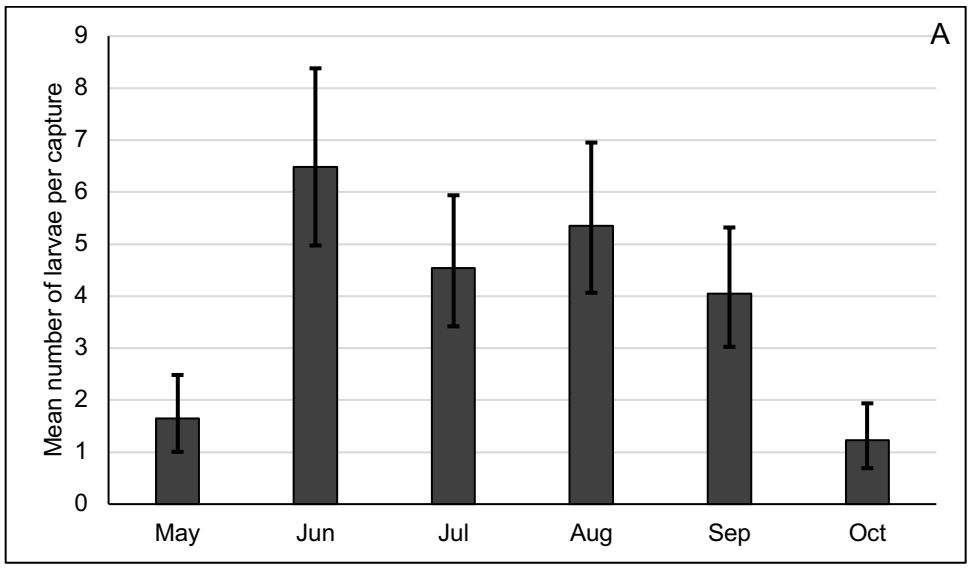


Figure 2.2B. Observed seasonal activity of on-host nymphal *Ixodes scapularis* collected from white-footed mice from 3 1-ha arrays from May - October 2010-2012 at Ft. McCoy, Wisconsin (Error bar indicates 95% confidence interval).

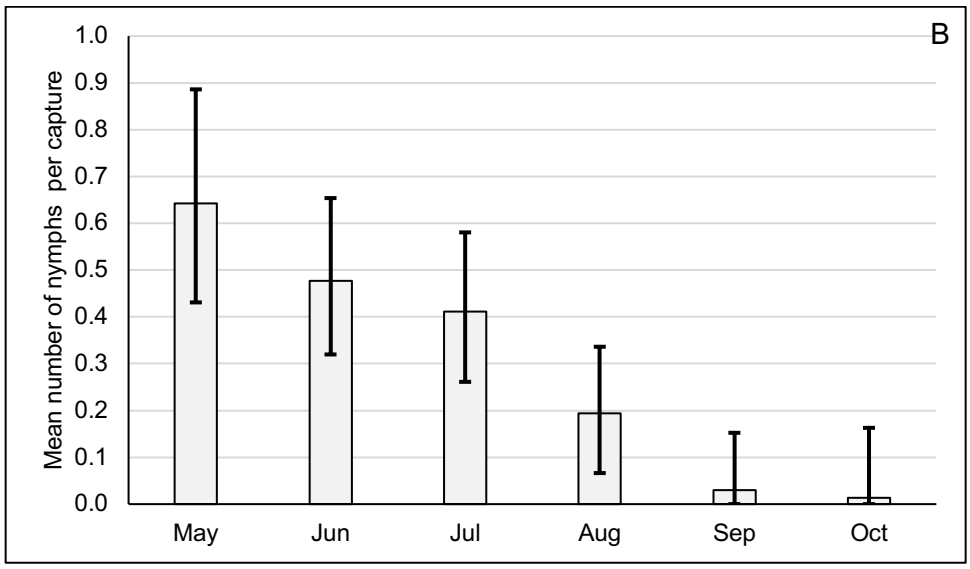
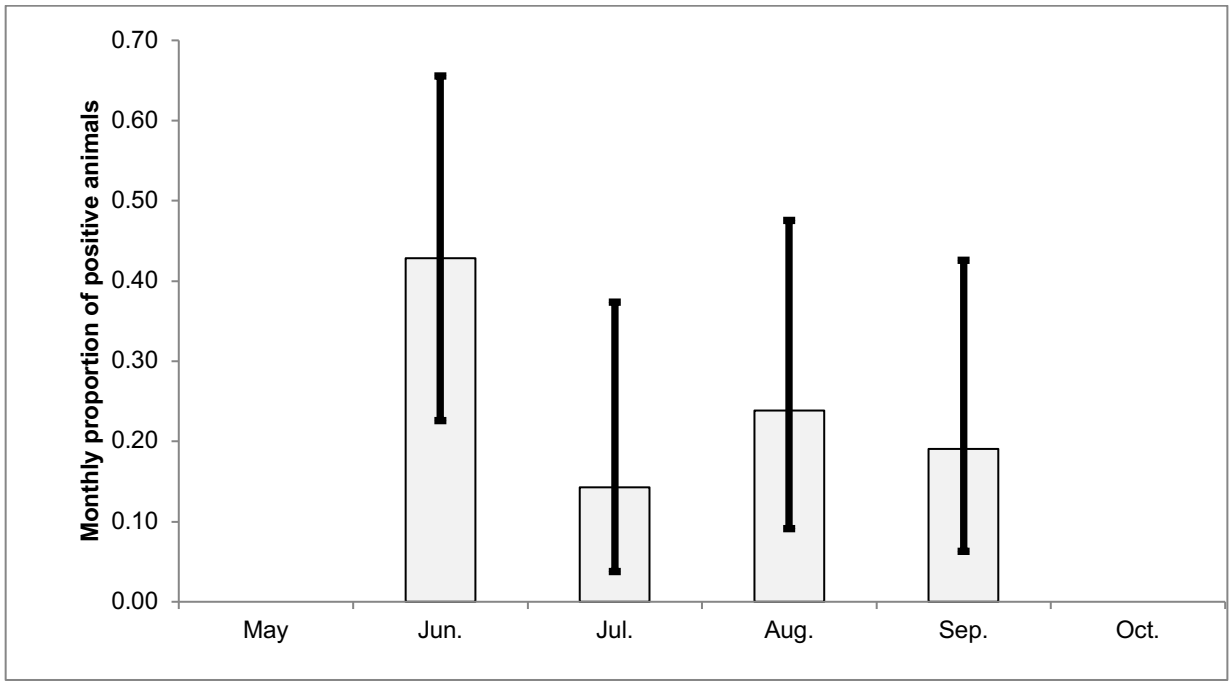


Figure 2.3. Monthly proportion of *Borrelia miyamotoi* positive animals (n= 21); animals were considered positive if they were infected by any one of the assays.



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CHAPTER 3

High prevalence of *Borrelia miyamotoi* in adult blacklegged ticks (*Ixodes scapularis*) sampled from white-tailed deer (*Odocoileus virginianus*)

ABSTRACT

Borrelia miyamotoi is the bacterial agent of a newly recognized zoonosis, with a global distribution but no clearly identified reservoir host. In eastern North America, it is vectored by the blacklegged tick, *Ixodes scapularis.* White-tailed deer (*Odocoileus virginianus*) are important for the ecology and disease risk of several other *I. scapularis*-borne pathogens, so we investigated the role of deer in the enzootic cycle of *B. miyamotoi.* When 730 questing adult and 355 deer-associated adult *I. scapularis* in Wisconsin were compared, *B. miyamotoi* infection prevalence of deer-associated ticks (4.5%) was significantly higher than that of the questing ticks (1.0%). Furthermore, the infection prevalence of blood-fed females (7.1%) was 11.8 times higher than that of questing females (0.6%), suggesting deer are amplifying hosts for *B. miyamotoi*. *Borrelia miyamotoi* can be transmitted both vertically and horizontally, so deer management to reduce tick populations offers the possibility of mitigating both routes of transmission.

INTRODUCTION

Borrelia miyamotoi, a relapsing fever group (RFG) spirochete detected throughout the range of *Ixodes persulcatus-ricinus* ticks, has been implicated recently in human disease in Russia, the United States, Europe and Japan (Platonov et al., 2011; Gugliotta et al., 2013) (Krause et al., 2013; Hovius et al., 2013; Sato et al., 2014). Assessing and mitigating the health risk posed by this zoonotic pathogen requires a better understanding of its eco-epidemiology. Enzootic maintenance of *B. miyamotoi* has remained largely enigmatic since its discovery in Japan in 1995 (Fukunaga et al., 1995), in part because features of its biology – including low prevalence in the vector population and difficulties with spirochete cultivation – have made it challenging to study in the field and laboratory. A lack of distinct symptoms and obvious epidemiological impact compared to (and potentially obscured by) its sympatric relatives, *B. burgdorferi* sensu lato, the agents of Lyme disease, have meant that most knowledge about the ecology of *B. miyamotoi* has been acquired incidentally from studies focused on the former species.

In the eastern U.S., western U.S., Europe, and Asia, *B. miyamotoi* is transmitted by *I. scapularis* (Scoles et al., 2001), *I. pacificus* (Mun et al., 2006), *I. ricinus* (Fraenkel et al., 2002), and *I. persulcatus* (Fukunaga et al., 1995; Fomenko et al., 2010) respectively. These ticks commonly vector *B. burgdorferi* sensu lato, with *B. miyamotoi* detected at much lower prevalence. For instance, in a survey across 46 sites in the eastern U.S., *B. miyamotoi* was 10 - 20 times less prevalent in questing nymphal blacklegged ticks than was *B. burgdorferi* (Barbour et al., 2009). This is despite the fact that unlike *B. burgdorferi*, which only can be transmitted horizontally via a reservoir host, *B. miyamotoi* also can be transmitted vertically (Scoles et al., 2001). Observed vertical transmission rates, however, may be too

low on their own to ensure enzootic maintenance (Scoles et al., 2001; Barbour et al., 2009). If so, horizontal transmission facilitated by feeding on reservoir hosts and/or cofeeding on permissive hosts must occur for *B. miyamotoi* enzootic maintenance.

Borrelia miyamotoi has been detected from several vertebrate species and their attached ticks, but it remains unknown which hosts are the most ecologically important reservoirs. Certain rodent species are competent for *B. miyamotoi*, but the magnitude of their contributions to its maintenance in North America (Scoles et al., 2001; Barbour et al., 2009) and Eurasia (Taylor et al., 2013; Burri et al., 2014) is not yet clear.

Borrelia miyamotoi also has been detected in ticks feeding on European red deer (*Cervus elaphus*)(Wodecka, 2007*)*, domestic ruminants (Richter and Matuschka, 2010) and, recently, by our group, in blacklegged ticks removed from white-tailed deer (*Odocoileus viginianus*) in the eastern U.S. (Rosen, 2009). White-tailed deer are important hosts for adult *I. scapularis* and also feed juveniles (Lane et al., 1991). Thus, if deer are reservoirs for *B. miyamotoi*, they could play an important role in the epizootiology of this transovarially transmitted pathogen in addition to maintaining the tick population.

As a test of this 'competent reservoir' hypothesis, we predicted that ticks attached to deer should exhibit a greater infection prevalence than that seen in sympatric, questing ticks of the same life stage. We tested this prediction – in an area of Wisconsin endemic for both *I. scapularis* and *B. miyamotoi* – by comparing the infection prevalence of *B. miyamotoi* in adults ticks removed from white-tailed deer with that in questing ticks. Because on-host male *I. scapularis* imbibe very small volumes of blood, we further predicted that engorged females would have the highest infection prevalence compared with deer-associated males and questing females. Finally, we compared *B. miyamotoi* infection prevalence between

questing nymphs and questing adults to infer the amplifying effects of different hosts of each life stage.

MATERIALS AND METHODS

Field site

We sampled three 1.1 ha deciduous forest sites at Fort McCoy in central Wisconsin, USA, where *I. scapularis* is well-established (Jackson and DeFoliart, 1970; Lee et al., 2013), and where several *I. scapularis*-borne pathogens have been detected, including *B. burgdorferi, Anaplasma phagocytophilum, Babesia microti*, and *B. miyamotoi* (Steiner et al., 2008; Hamer et al., 2014). At all three sites, oaks and maples dominated the upper story and tree saplings dominated the shrub layer.

Tick collection

Our methods for collecting questing ticks are described in detail in Rulison et al. (2013). Briefly, on a sampling array at each site, we collected questing nymphs and adults from vegetation by dragging or flagging a square piece of white flannel cloth (1 m^2) along eight 90 m^2 transects. Dragcloths were checked every 15 m and ticks were collected, stored in 95% ethanol, and brought back to Michigan State University for species identification using dichotomous keys (Keirans and Clifford, 1978; Durden and Keirans, 1996). We sampled from April to November either weekly (2010) or every three weeks (2011-2012). Additionally, in November 2010, adult ticks were collected from hunter-harvested deer on opening day of the gun season at a mandatory check station at Fort McCoy. Each deer was inspected for a maximum of 10 min; all ticks were stored in 95% ethanol. Deer age and sex were recorded.

***Borrelia miyamotoi* detection**

We selected a subset of questing nymphal and adult *I. scapularis* for pathogen detection. Ticks within a life stage, year, and array were randomly chosen; within a given year, we assayed a similar number of ticks from each of the three arrays. Total DNA was extracted from cryo-pulverized individual ticks by using a DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) as described previously (Tsao et al., 2004; Hamer et al., 2010). Briefly, individual ticks were submerged in liquid nitrogen, immediately pulverized, and then incubated overnight in lysis buffer at 56 °C. DNA was eluted with 100 µl AE buffer previously incubated at 70°C and stored at -20 °C. Each extraction batch included extraction negative controls consisting of extraction reagent without ticks.

We assayed for infection with *B. miyamotoi* using a quantitative PCR that targeted a fragment of the 16S rDNA as previously described (Tsao et al., 2004). The specificity of the real-time PCR used in this study to discriminate *B. miyamotoi* from *B. burgdorferi* was verified previously (Tsao et al., 2004; Barbour et al., 2009). The qPCR was conducted in a 15 µl reaction volume consisting of 7.5 µl of TaqMan® Universal PCR Mater Mix (Applied Biosystem, Foster City, CA), 1 µl of each 10µM primer, 1 µl of 3µM probe and 3 µl DNA template. We used sterile water as a negative PCR control. For positive controls we used extracted DNA from previous field samples (two white-footed mouse (*Peromyscus leucopus*) ear biopsies) that had tested positive for *B. miyamotoi* (and confirmed by sequence analysis) (Hamer et al., 2010). Quantitative PCR assays were conducted by using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) at the Michigan State University Genomics facility.

To confirm the identity of all positive samples identified by the qPCR reaction, we sequenced a fragment of the 16S-23S intergenic spacer (IGS) region (Bunikis et al., 2004) as previously described (Hamer et al., 2010). Briefly, we conducted a nested PCR in a 25 µl reaction volume consisting of 12.5 µl of FailSafe PCR 2X PreMix (Epicentre, Madison, WI), 1.5 µl of total forward and reverse 10 µM primers for each outer and inner PCR cycle, 0.25 µl of FailSafe PCR Enzyme Mix (0.625 units, Epicentre), and 2.5 µl of DNA template, visualized the PCR product by gel electrophoresis. PCR products were purified using ExoSAP-IT (USB Corporation, Cleveland, OH) as per manufacturer's instructions. The forward sequence of the ~ 500 bp fragment was determined using an ABI 3730xl platform (Applied Biosystems, Foster City, CA) at the Michigan State University Genomics facility. Sequence chromatographs were visually inspected and cleaned. Sequences were identified as *B. miyamotoi* based on alignments with sequences published in GenBank.

Statistical analysis

We compared *B. miyamotoi* infection prevalence between groups using Fisher's exact test with $\alpha = 0.05$.

RESULTS

Ticks

We tested 782 questing nymphal and 730 questing adult *I. scapularis* collected at Ft. McCoy from 2010-2012, plus 355 adult *I. scapularis* removed from hunter-harvested deer in November 2010 (Table 3.1). A total of 49 deer were checked (35 male, 7 female and 7 undetermined sex), with *I. scapularis* collected from 44 deer (89.8%; comprising 33 male, 7 female and 4 undetermined sex).

Infection in questing ticks

Borrelia miyamotoi infection prevalence for a given questing tick life stage did not vary significantly among years (Table; $P > 0.05$ for each life stage); so we pooled data across years to increase statistical power. Numbers of positive ticks were nevertheless low and our nymphal and adult infection prevalence estimates (2.1% vs. 1.0%) did not differ statistically ($P = 0.095$). Similarly, the estimated infection prevalence of male adults (1.2%) was not significantly different from that of female adults (0.6%; $P = 0.476$).

Infection in deer-associated ticks

Borrelia miyamotoi infected adult ticks were collected from 9 (20.5%) of 44 tick-infested deer (all 9 were male). Five of the deer carrying infected ticks were 1 year of age, and the other four were 2, 3, > 4 years, and undetermined age. Young deer were less likely to carry ticks, however infestation with *B. miyamotoi* infected ticks was not correlated with deer age or sex (both $P > 0.1$).

The infection prevalence of deer-associated adult *I. scapularis* (4.5%) was significantly higher than that of questing adults (1.0%; P = 0.0004). As predicted *a priori*, the infection prevalence of male ticks was significantly lower than that of female ticks (2.5% vs. 7.1%; P = 0.035, 1-tailed test). Similarly, as predicted, the infection prevalence of attached female ticks (7.1%) was 11.8 times greater than that of questing female ticks (0.6%; P < 0.0001, 1-tailed test).

We successfully sequenced the fragment of the IGS region from 34 of 39 samples identified as positive by qPCR. All sequences showed 99% similarity with published sequences for *B. miyamotoi* (for example, accession number AY363706) in GenBank. Four representative sequences have been deposited in GenBank (Accession numbers KT321365 - KT321368).

DISCUSSION

The recent findings that *B. miyamotoi* can cause human disease emphasizes the need to understand its natural maintenance cycle and more specifically to identify key reservoir host species. *Borrelia miyamotoi* DNA has been detected in various vertebrate species and in ticks feeding on various mammal and avian species (Hamer et al., 2010; Burri et al., 2014; Lommano et al., 2014), but assigning reservoir status based on infected, individual attached ticks is problematic, given that *B. miyamotoi* can be transmitted transovarially. Xenodiagnosis experiments have identified several rodent species that are competent reservoirs, but transmission rates are low (Scoles et al., 2001) or uncertain because they are based on small sample sizes (Burri et al., 2014).

In the U.S., an early investigation of *B. burgdorferi* ecology reported 32% of white-tailed deer (*Odocoileus virginianus*) sampled on Long Island, NY, a Lyme endemic area, to be spirochetemic (Bosler et al., 1984). When Telford et al. (1988) assayed nymphs in Massachusetts that had fed upon deer as larvae, however, only 1% were found to be infected. Telford et al. (1988) attributed these positive ticks to occasional transovarial transmission and concluded that deer were not competent reservoirs for *B. burgdorferi*. Later, Kurtenbach et al. (1998) showed that deer complement efficiently kills *B. burgdorferi* sensu lato spirochetes. Given these findings, we speculate that the spirochetes previously observed in deer blood and in deer-fed larvae may have been *B. miyamotoi* rather than *B. burgdorferi.* These historical data, along with recent detections in ticks fed on European red deer (Wodecka, 2007), domestic ruminants (Richter and Matuschka, 2010) and eastern U.S. white-tailed deer (Rosen, 2009) prompted us to investigate the hypothesis that white-tailed deer are permissive to infection with *B. miyamotoi* and may serve as reservoirs. We

compared the infection prevalence in questing adult *I. scapularis* with that of adults attached to white-tailed deer, assuming that infection due to transovarial transmission would be accounted for in the questing tick infection prevalence. Infection prevalence of adult ticks removed from deer was 4.5 times greater than that of sympatric, host-seeking adults. Furthermore, engorging females had the highest infection prevalence, which was 11 times greater than that of questing females. And, even though they do not imbibe much blood, there was also a trend for increased infection prevalence in deer-associated males compared to questing males. These results show that unlike for *B. burgdorferi*, the blood of white-tailed deer is at least permissive for infection with *B. miyamotoi*; moreover, these data support the predictions of the hypothesis that white-tailed deer are reservoir hosts.

We have considered alternative explanations for the observed increase in *B. miyamotoi* infection prevalence between questing adult *I. scapularis* and adults removed from deer. First, the spirochete burden in infected questing adults could have been below the detection threshold of our qPCR so that ticks appeared to be negative when they actually were positive (a Type II error), whereas upon acquiring a bloodmeal, *B. miyamotoi* spirochetes may have reproduced sufficiently to rise above the threshold of detection. We note, however, that the initial cycle values at which *B. miyamotoi* was detected in questing ticks fell within the range detected for ticks collected from deer and indicated high burdens in some of the questing ticks. Nevertheless, until more is known about the growth kinetics of *B. miyamotoi* in ticks engorging on a competent host, we cannot rule out the possibility that blood meal amplification could explain some of the observed increase in infection prevalence in deer associated ticks.

Second, the increase in infection prevalence of ticks attached to deer may have resulted from venereal transmission between male and female ticks during copulation. No literature exists regarding the sexual transmission of *B. miyamotoi;* however, sexual transmission of relapsing fever borrelia species *B. duttonni* (*Ornithodorus moubata*)(Geigy and Aeschlimann, 1964; Wagner-Jevseenko, 1958) and *B. crocidurae (O. erraticus)*(Gaber et al., 1982) has been recorded in soft tick vectors. Further work is needed to test the possibility of sexual transmission of *B. miyamotoi* in *I. scapularis* ticks.

Our data indicate that white-tailed deer at least are permissive for infection with *B. miyamotoi* and furthermore, with the two caveats above, are probably reservoir competent (and/or may support non-systemic or cofeeding transmission)(Tsao, 2009). Inference based on genetic relatedness to several other RFG borreliae supports this suggestion. Three other RFG borreliae transmitted by hard ticks are known to infect ungulates, including cervids. *Borrelia theileri* infects cattle, is transmitted by *Rhipicephalus microplus* ticks, and is the etiologic agent of bovine theileriosis (Laveran, 1903). *Borrelia lonestari* is associated with *A. americanum* and infects white-tailed deer in the southern U.S. (Varela-Stokes, 2007). A *B. lonestari*-like spirochete recently discovered in Japan infects sika deer (*Cervus nippon yesoensis*)(Lee et al., 2014) and has been detected in *Haemaphysalis* spp. ticks. Thus, while detections of *B. miyamotoi* in ticks attached to wild and domestic ungulates (Wodecka, 2007; Richter and Matuschka, 2010) could be the result of transovarial infection, they may also be the result of horizontal transmission between deer and tick..

These infection prevalences reported here are slightly lower than, but broadly consistent with, levels reported for previous surveys undertaken at Fort McCoy (Hamer et al., 2014) and elsewhere in the eastern U.S. (Barbour et al., 2009). There was an indication

in our data that infection prevalence of questing nymphs (2.1%) may be higher than in adult ticks (1.0%), however sample sizes were low and the difference was not statistically significant. A recent study in Norway (Kjelland et al., 2015) reported a similar, non-significant decrease in *B. miyamotoi* infection prevalence from nymphs (0.89% (10/1130) to adults 0.22% (1/449). Lack of amplification in infection prevalence between the nymphal and adult stages differs markedly from the pattern seen with *B. burgdorferi*, which typically doubles in infection prevalence between these two life stages (Tsao, 2009) because larvae and nymphs both typically feed on host species that are *B. burgdorferi* competent. Lack of amplification, which needs to be confirmed by further sampling, may indicate that nymphal hosts are typically less competent for *B. miyamotoi* than are larval hosts.

Borrelia miyamotoi was discovered 20 years ago (Fukunaga et al., 1995), but incidental glimpses about its ecology have until now only been gleaned during investigations of other pathogens, primarily *B. burgdorferi* sensu lato. Our results strongly support the hypothesis that in the eastern U.S., white-tailed deer are amplifying hosts for *B. miyamotoi* and suggest that deer may contribute to the transmission ecology of *B. miyamotoi* beyond serving as the main host of adult *I. scapularis*. Future studies should be performed to confirm the generality of these findings both throughout the range of *I. scapularis* and for the cervid host species of *I. pacificus, I. ricninus, and I. persulcatus*. Although logistically challenging, controlled transmission studies that test for reservoir competence and characterize the course of infection in deer would be very valuable.

Lyme borreliosis is the most common vector-borne disease in the United States, with approximately 30,000 new cases reported to the Centers for Disease Control and

Prevention each year (Mead, 2015). Recently it has been shown that *B. miyamotoi* can and has caused human disease (Platonov et al., 2011; Krause et al., 2013; Gugliotta et al., 2013). Given that *B. miyamotoi* uses the same vector ticks, exists in the same ecosystems with *B. burgdorferi*, and given the continuous range expansion of *I. scapularis*, it seems inevitable that the human population will be increasingly exposed to *B. miyamotoi* infection (Mead, 2015). If deer are an important reservoir for this pathogen, deer management to reduce tick populations and Lyme disease risk (Piesman and Eisen, 2008) may provide additional health benefits by also weakening *B. miyamotoi* transmission dynamics. Further research is needed therefore to better understand the ecoepidemiology of *B. miyamotoi* – and how it differs from that of *B. burgdorferi* – to help inform public health management of diagnosis, treatment, and prevention of disease.

APPENDIX

Table 3.1. Prevalence of *Borrelia miyamotoi* in *Ixodes scapularis* collected at Ft. McCoy, Wisconsin, by origin, sex, and year.

Origin of ticks	Year	No. of *Borrelia miyamotoi* positive ticks / No. of ticks tested (% positive)		
		Males	Females	Total
Questing on vegetation	2010	1/65 (1.5)	0/49 (0)	1/114 (0.9)
	2011	4/169 (2.4)	1/140 (0.7)	5/309 (1.6)
	2012	0/177 (0.0)	1/130 (0.8)	1/307 (0.3)
	Total	5/411 (1.2)	2/319 (0.6)	7/730 (1.0)
Removed from deer	2010	5/199 (2.5)	11/156 (7.1)	16/355 (4.5)

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CHAPTER 4

Vertical transmission rates of *Borrelia miyamotoi* in *Ixodes scapularis* collected from white-tailed deer

ABSTRACT

Borrelia miyamotoi is a relapsing fever spirochete transmitted by ticks in the *Ixodes ricinus* complex. In the eastern United States, *B. miyamotoi* is transmitted by *I. scapularis,* which also vectors several other pathogens including *B. burgdorferi* sensu stricto. In contrast to Lyme borreliae, *B. miyamotoi* can be transmitted vertically from infected females to their progeny. Therefore, in addition to nymphs and adults, larvae can vector *B. miyamotoi* to wildlife and human hosts. Two widely varying filial infection prevalence (FIP) estimates - 6% and 73% - have been reported previously from two vertically infected larval clutches; to our knowledge, no other estimates of FIP or transovarial transmission (TOT) rates for *B. miyamotoi* have been described in the literature. Thus, we investigated TOT and FIP of larval clutches derived from engorged females collected from hunter-harvested white-tailed deer in 2015 (n=664) and 2016 (n=599) from Maine, New Hampshire, Tennessee, and Wisconsin. After engorged females oviposited in the lab, they (n=492) were tested for *B. miyamotoi* infection by PCR. Subsequently, from each clutch produced by an infected female, larval pools, as well as 100 individual eggs or larvae, were tested. The TOT rate of the 11 infected females were 90.9% (95% CI; 57.1-99.5%) and the mean FIP of the resulting larval clutches was 84.4% (95% CI; 68.1 - 100%). Given that the overall observed vertical transmission rate (the product of TOT and FIP; 76.7%, 95% CI; 44.6-93.3%) may be close to but < 100%, additional horizontal transmission may be required for enzootic maintenance of *B. miyamotoi* based on the results of a previously published deterministic model. Further investigation of TOT and FIP variability and the underlying mechanisms, both in nature and the laboratory, will be needed to resolve this question. Meanwhile,

studies quantifying the acarological risk of *Borrelia miyamotoi* disease need to consider not only nymphs and adults, but larval *I. scapularis* as well.

INTRODUCTION

Borrelia miyamotoi is a relapsing fever group spirochete transmitted by ticks in the *Ixodes ricinus* complex (Scoles et al., 2001; van Duijvendijk et al., 2016; Breuner et al., 2017), which also transmit *B. burgdorferi* sensu lato spirochetes that may cause Lyme borreliosis, the most common vector-borne disease in Europe and North America (Mead, 2015). *Borrelia miyamotoi* has been detected in Lyme borreliosis endemic areas in Eurasia and North America and now known to cause human disease (Platonov et al., 2011; Gugliotta et al., 2013; Krause et al., 2013; Chowdri et al., 2013; Hovius et al., 2013; Krause et al., 2014; Sato et al., 2014; Molloy et al., 2015; Sudhindra et al., 2016; Jobe et al., 2016; Boden et al., 2016; Krause et al., 2016; Fiorito et al., 2017; Kadkhoda et al., 2017; Jiang et al., 2018; Sato et al., 2018) that has been referred to *Borrelia miyamotoi* disease (BMD) and as hard tick-borne relapsing fever (HTBRF) (Krause and Barbour, 2015).

In the eastern United States, *B. miyamotoi* and *B. burgdorferi* sensu stricto (s.s.) are transmitted by the same vector species, *I. scapularis*, and so they coexist in the same geographical areas and interact with the same vertebrate host communities (Barbour et al., 2009). The two spirochetes, however, differ in how they are maintained enzootically. *Borrelia burgdorferi* s.s. spirochetes rely entirely on horizontal and transstadial transmission for their maintenance in the vector population. In other words, *I. scapularis* larvae hatch uninfected from the eggs (Rollend et al., 2013) and only can acquire *B. burgdorferi* s.s. by feeding on an infected host or through non-systemic, co-feeding transmission (Gern and Rais, 1996; Piesman and Happ, 2001). An infected engorged larva will maintain *B. burgdorferi* transstadially and molt into an infected nymph, which subsequently can transmit the spirochetes to susceptible hosts, including humans. In

contrast, in addition to horizontal transmission, *B. miyamotoi* can be transmitted vertically from infected females to their progeny (Scoles et al., 2001; Breuner et al., 2017). Consequently, larvae - as well as nymphs and adults - can vector *B. miyamotoi*. Transmission of *B. miyamotoi* from vertically infected larvae to laboratory mice has been demonstrated in *I. ricinus* (van Duijvendijk et al., 2016) and *I. scapularis* (Breuner et al., 2018). While the majority of Lyme borreliosis cases occur in mid-summer coinciding with the peak nymphal *I. scapularis* activity period (Schwartz et al., 2017), recent epidemiological studies have revealed that the majority of BMD cases occur later (July to September) during the larval questing period (Molloy et al., 2015; Fiorito et al., 2017).

To understand better how *B. miyamotoi* is maintained in nature and to help predict disease risk from *B. miyamotoi*-infected larvae, we set out to obtain field estimates of the magnitude of vertical transmission. We measured both the transovarial transmission rate (TOT= the proportion of infected females that produce infected egg masses) and filial infection prevalence (FIP = the proportion of larvae in a clutch that are infected) (Burgdorfer and Varma, 1967). In the first description of *B. miyamotoi* in *I. scapularis*, Scoles et al. (2001) observed transovarial transmission (but did not quantify TOT) and reported FIP estimates of 6% and 73% for two vertically infected larval clutches. To our knowledge, no other previous data regarding TOT rates and FIP of *B. miyamotoi* from *I. scapularis* exist in the literature. We therefore investigated TOT and FIP from engorged female *I. scapularis* collected from hunter-harvested white-tailed deer (*Odocoileus viginianus*) in 2015 and 2016 from Maine, New Hampshire, Tennessee, and Wisconsin. White-tailed deer (hereafter referred to as deer) serve as an important host for adult *I. scapularis* (Main et al., 1981) and we previously detected amplification of *B. miyamotoi* in

deer-fed adult female *I. scapularis* compared with questing adult ticks collected at the same time (Han et al. 2016).

MATERIALS AND METHODS

Sample sites and collection

Ticks were collected at hunter-harvested deer check stations in Maine (ME), New Hampshire (NH), Tennessee (TN), and Wisconsin (WI) during the firearm season (November) in 2015 and 2016. Sampling sites spanned Lyme borreliosis endemic areas in the United States, where *B. miyamotoi* infected ticks (Barbour et al., 2009; Hamer et al., 2014) and BMD cases (Molloy et al., 2015; Jobe et al., 2016) were previously reported. TN was included as a representative Lyme borreliosis non-endemic area, where *B. miyamotoi* had previously been detected (Rosen et al., 2012). Check stations were located in the following counties: Kennebec and York (ME); Rockingham, Hillsborough, and Carroll (NH); Anderson (TN); and Monroe (WI).

Hunters brought freshly-killed deer carcasses to check stations, and each carcass was inspected for ticks for an average of 10 min, prioritizing the head, neck, and ventral regions. Feeding female ticks were collected using forceps and stored alive in ventilated polystyrene tubes plugged with pieces of mesh and caps modified to allow airflow. Collected ticks were labelled by deer ID and held at ambient temperature and 99% relative humidity (RH) during transport to the lab.

Oviposition conditions

Upon arrival at Michigan State University, the species, life stage, and sex of the collected ticks were confirmed morphologically using published keys (Keirans and Clifford, 1978; Sonenshine, 1979). In 2015, the body weights of all female ticks were measured (nearest 1 mg) to determine engorgement levels, and female *I. scapularis* > 10 mg were

selected for the TOT experiment. In 2016, female ticks > 20 mg were selected for monitoring, as the 2015 data showed that females weighing > 20 mg had markedly higher oviposition success. Selected females were sterilized with 10% bleach for 30 seconds and then rinsed twice with water for 30 seconds to prevent fungal infection. Sterilized female ticks were placed individually in clear polystyrene tubes with ventilated caps and maintained at 22 ˚C, 99% RH, and 16:8 L:D (hrs) photoperiod (Fig. 4.1). The date that oviposition commenced was recorded for each female. Female ticks were removed from tubes after oviposition ceased, typically 15 days after the first day of oviposition, and stored in 95% ethanol. Egg batches were monitored and allowed to hatch into larvae. In 2016, for logistical reasons, females were first stored for 42 - 87 days at 10˚C, 99% RH in darkness to delay oviposition, before switching to the conditions described above to allow for oviposition (Fig. 4.1).

Screening for TOT and FIP

Total DNA from females was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's protocol for animal tissue, with minor modifications as previously described (Hamer et al., 2010). Each extraction batch included extraction negative controls consisting of extraction reagents without eggs or ticks. Presence of *B. miyamotoi* DNA was screened with a quantitative PCR (qPCR) that targeted 16S rRNA of *B. miyamotoi* as described previously (Tsao et al., 2004). We used sterile water as a negative PCR control and extracted DNA of *B. miyamotoi* M1029 strain as a positive PCR control (kindly provided by Dr. G. Margos; Margos et al., 2014). To determine the TOT rate of infected females, a pool of either 5 or 10 larvae from the larval clutch produced by

each infected female was screened. If *B. miyamotoi* was not detected, at least 10 more pools of 5 larvae were screened for each female; if *B. miyamotoi* was detected, we then tested 100 individual eggs or larvae from the infected egg/larval batch to estimate its FIP.

As part of a separate study to obtain field isolates of *B. miyamotoi* for cultivation and to confirm TOT, in 2015 larvae produced by infected females (one of two Maine clutches, a portion of one TN larval clutch, and a portion of one WI larval clutch), were sent to Yale University in an attempt to infect immunodeficient laboratory mice susceptible to infection with *B. miyamotoi* (L. Bockenstedt, pers. comm.). One of the larval clutches from Maine was very small and thus no pools or individual larvae were screened for TOT or FIP; instead, TOT was deduced by assaying blood of the challenged mouse (and FIP could not be measured). TOT and FIP were estimated of TN and WI clutches from the remaining sibling ticks that were not used for transmission experiments.

To confirm the identity of *B. miyamotoi* DNA detected in these field-derived females (n=10), larvae (n=10) and eggs (n=1), we sequenced a fragment of the 16S-23S intergenic spacer region of *Borrelia* species (Bunikis et al., 2004).

Statistical analysis

We report prevalence estimates with binomial 95% confidence intervals (CI), and we use Fisher's exact test to evaluate differences among proportions (i.e., infection prevalence, oviposition success, hatching rate, TOT rate and FIP). To estimate the overall expected prevalence of infection in the next generation of larvae due to vertical transmission, we calculated the product of female infection prevalence, TOT rate and FIP.

RESULTS

Borrelia miyamotoi infection prevalence and oviposition rates of deer-blood engorged females collected from hunter-harvested deer

In 2015, we collected 664 female *I. scapularis* feeding on hunter-harvested white-tailed deer, of which 431 females (11 – 370 mg) were selected for further monitoring (Table 1). Of these, 230 (53.4%; CI 48.5-58.1%) oviposited, and the mean weight was 116 mg (Standard Error (SE) 6.0 mg) with the smallest weighing 20 mg. In 2016, we collected 1030 females, and monitored 599, of which ranged from 20 – 423 mg (Table 1). Of these, 262 (43.7%; CI 39.71-47.8%) oviposited, and the mean weight was 140 mg (SE 5.7 mg) with the smallest weighing 23 mg. The oviposition rate was significantly lower in 2016 than in 2015 (p=0.0024).

The overall infection prevalence of *B. miyamotoi* in deer-blood engorged *I. scapularis* females was 2.6% (n=1263; CI 1.8-3.7%). In 2015 we tested all collected female *I. scapularis* (n=664) and detected a total 16 infected females (2.4%), comprising 2 ME (2.9%), 1 TN (0.8%) and 13 WI (3.0%) females. In 2016 we tested only females weighing > 20mg (n=599) and detected 17 infected females (2.8%), comprising 1 ME (8.0%), 2 NH (2.5%), and 14 WI (2.8%) females. There was no significant difference in engorged female infection prevalence between 2015 and 2016 at any site (all p>0.4), and no significant difference in prevalence among the four sites was apparent (2015 and 2016 data pooled; Chi-sq=2.84, 3df, p=0.42).

Overall, eleven females (4 in 2015 and 7 in 2016) infected with *B. miyamotoi* produced egg batches (Table 2). Oviposition rates of infected females were 40.0% (n=10; CI 13.7-72.6%) in 2015 and 41.2% (n=17; CI 19.4-66.6%) in 2016, whereas oviposition rates

of uninfected females were 53.7% (n=421; CI 48.8-58.5%) in 2015 and 43.8% (n=582; CI 39.8-48.0%) in 2016. There was no statistical difference in the mean weight of oviposited females between infected (174.6 mg, SE 35.9 mg) and uninfected (127.7 mg, SE 4.2 mg; t=1.6662, 491 df, p=0.0963). There was no significant difference between the oviposition rates of infected and uninfected females in either year (p=0.53 in 2015 and p=1.00 in 2016). Combining data from both years, larvae hatched from 10/11 egg batches (90.9%, CI 57.1-99.5%) produced by infected females, although one batch produced only 4 larvae (i.e., most eggs did not hatch). Considering only egg batches in which the majority of eggs hatched, larvae hatched from 9/11 infected females (81.8%, CI 47.8-96.8%). Overall 384/481 (79.8%; CI 75.9-83.3%) egg batches produced by uninfected females hatched into larvae. There was no significant difference between the hatching rates of infected and uninfected egg batches (p = 0.827).

TOT and FIP rates

TOT of *B. miyamotoi* was observed in 10 of 11 clutches (90.9%, CI 57.1-99.5%; Table 2) produced by *B. miyamotoi* infected females collected from white-tailed deer. Considering only vertically infected clutches from which larvae hatched (n= 7), average FIP was estimated as 84.4% (CI 68.1-100%; Table 2).

The following section provides details on the data obtained from each of the 11 *B. miyamotoi*-infected females, given the different approaches used. For the one infected clutch from TN, *B. miyamotoi* was not detected from a pool of 10 larvae, 13 pools of 5 larvae, nor from blood of two mice infested with 80 larvae. One clutch from ME was small, comprising -28 larvae; these larvae were screened individually by qPCR for *B. miyamotoi*

infection, demonstrating transovarial transmission and resulting in an estimate of 92.9% FIP. Transovarial transmission of *B. miyamotoi* in the other ME clutch was deduced by at Yale University by observing spirochetemia in a mouse infested with 60 of its larvae, with confirmation by PCR and sequencing. Transovarial transmission was detected in pools of 10 larvae from 2/2 NH clutches, and FIP was subsequently estimated as 36.0% and 92.0%, respectively. Transovarial transmission was detected in pools of 5 -10 larvae from 4/4 WI larval clutches from which all larvae hatched, and FIP ranged from 83.0% to 100.0%. From one WI egg batch that did not produce any larvae; *B. miyamotoi* was detected in a pool of 30 eggs indicating transovarial transmission, with FIP estimated as 73.0% from 100 individual eggs. From the remaining WI egg batch, 60 eggs and 4 hatched larvae were screened individually, demonstrating transovarial transmission with FIP estimated as 54.7%. The FIP values of these latter 2 egg batches were not included in the calculation of overall FIP due to potential differences in DNA extraction and qPCR detection efficiencies between larvae and eggs.

Prediction of larval infection prevalence

Larval infection prevalence of the next generation was predicted by multiplying the observed engorged female infection prevalence of each state (3.7% ME, 1.7% NH, 2.9% WI, 0.8% TN), and average TOT rate (90.9%) and FIP (84.4%). Predicted larval infection prevalence in the next generation ranged from 0.6% (TN) to 2.8% (ME), with 2.0% as the mean expected infection prevalence (Fig. 4.2).

All DNA extraction and PCR negative controls produced negative results and our positive controls produced positive results (for all assays, including those for ticks of all life

stages). All 16S-23S rRNA intergenic spacer region sequences (460 or 478 bp length; n = 21) showed 100% identity with published sequences for *B. miyamotoi* in GenBank. Sequences of females and representative larval clutches have been deposited in GenBank (accession nos. MF444832-MF444845, MF460836-MF460842).

DISCUSSION

Although *B. miyamotoi* was first detected in the US nearly twenty years ago (Scoles et al., 2001), its enzootic cycle remains poorly understood. While the role of horizontal transmission still needs to be clarified, here we begin to evaluate the role of vertical transmission. As a first step, we sought to estimate transmission rates in field-infected and fed females. We investigated engorged female *I. scapularis* collected from hunter-harvested white-tailed deer, one of the most important hosts for adult *I. scapularis* (Main et al., 1981; Bosler et al., 1984; Telford et al., 1988), from four states in the eastern U.S., including three (Maine, New Hampshire, and Wisconsin) that are designated as high risk for Lyme borreliosis and one (Tennessee) that is currently considered to be of very low risk (Schwartz et al., 2017).

The overall *B. miyamotoi* infection prevalence of engorged females collected in 2015-2016 (n= 1263) was 2.6% (95% CI 1.8-3.7%). There was no apparent geographic difference in infection prevalence, but small sample sizes at some sites resulted in low power to discern differences at such low prevalence. There also was no significant difference in overall infection prevalence between years. Considering the overall infection prevalence of engorged females as well as just those data from Wisconsin from either/both years, the infection prevalence values observed in this study are significantly lower than previously observed at the Wisconsin site in 2010 (Han et al., 2016, 7.1%, Fisher's exact test, p=0.0057). Published studies exist regarding the infection prevalence of other tick-borne pathogens in deer-fed *I. scapularis* ticks, but few have looked explicitly at *B. miyamatoi* infection. A recent examination of male *I. scapularis* collected in 2002 from hunter-harvested deer check stations located throughout New Jersey found 2.7% (95% CI

1.6-4.3%) to be infected (Egizi et al., 2018). The female *I. scapularis* attached to these deer were not assayed for pathogens, but based on Han et al. (2016), *B. miyamotoi* infection prevalence of engorging females on white-tailed deer tends to be higher than that of attached males. Thus if we take 2.7% infection as a conservative estimate for infection prevalence of engorged females, the level is similar to or greater than that estimated in our current study. We can also try to glean comparable information from laboratory studies. Rollend et al. (2013) reviewed laboratory records for 1214 female *I. scapularis* collected from three sites in Connecticut and fed on laboratory rabbits or sheep and reported the infection prevalence of resultant egg batches for *B. miyamotoi* as 1.6% (95% CI 0.9-2.4%). This result is similar to the estimates for infection prevalence of engorged females reported above (and note, females fed on different host species), but because vertical transmission may have been <100%, this value should be considered a minimum infection prevalence of engorged females.

We observed high TOT of *B. miyamotoi* from the infected *I. scapularis* engorged with deer blood; infected progeny were detected from 10 of 11 females (90.9%, CI 57.1-99.5%; Table 2). FIP, however, varied from 36.0% to 100.0% for 7 larval clutches; this wide variation is consistent with previously reported estimates of 6% and 73% (Scoles et al., 2001).

In an attempt to view our results in a larger context, here we cautiously compare our data with historical studies published prior to the detection of *B. miyamotoi* in *I. scapularis* (Scoles et al., 2001), on the apparent vertical transmission of "*B. burgdorferi*" that were later refuted by laboratory transmission experiments (Patrican, 1997). Because direct observation of spirochetes and the use of cross-reactive immunoassays would not

have differentiated between the two *Borrelia* species, these researchers most likely detected *B. miyamotoi* (or another yet-to-be discovered relapsing fever *Borrelia*) rather than *B. burgdorferi* in infected larvae (Rollend et al., 2013).

Magnarelli et al. (1987) tested 34 moribund female *I. scapularis* collected from hunter-harvested deer or domestic dogs in Connecticut after their oviposition and found 7 clutches of larvae produced by *Borrelia* infected females to be infected with spirochetes. If these spirochetes were *B. miyamotoi*, this infection prevalence of engorged females (20.6%; 95% CI 8.7 - 37.9%) is much higher than the values observed in our current study, and is trending higher than the 7.1% (95% CI 3.6-12.3%) we previously observed in deer-fed females collected from hunter-harvested deer in 2010 in Wisconsin (Han et al., 2016). The researchers reported 3.3% to 27.3% FIP, but these values may be underestimates, as it is unclear whether the denominator included larvae of *B. burgdorferi* positive/*B. miyamotoi*-negative females.

Investigations of vertical transmission of *B. burgdorferi* by *I. pacificus* have also been published. Lane and Burgdorfer (1987) fed field-collected *I. pacificus* females (n=100) on laboratory rabbits and assayed the females and their clutches for *Borrelia* infection. Because the immunoassay they used did not differentiate between *B. burgdorferi* and *B. miyamotoi* (Rollend et al. 2013) (or other uncharacterized *Borrelia* spp.), the minimum *B. miyamotoi* infection prevalence of the field-collected females fed on rabbits may have ranged from 1% - 3%, which is similar with values seen in our current study and that of Rollend et al. (2013). To quantify TOT and FIP, the researchers assayed 50 eggs and up to 100 larvae from each of the 3 *Borrelia*-infected *I. pacificus* females. Only one of the infected females transmitted spirochetes to offspring; depending on whether females actually were

infected with *B. burgdorferi, B. miyamotoi,* or both, the TOT estimate would be 33% - 100%. From the one female that transmitted spirochetes to offspring, FIP was 100% and transstadial survivorship of *Borrelia* spirochetes was also 100% for subsequent nymphs and adults. In tissue smears from this infected female, a disseminated heavy infection was observed, with spirochetes visible in multiple tissues including the central ganglion, Malpighian tubules, midgut, ovaries, and salivary glands. In contrast, spirochetal infection was not as generally disseminated in the two females that did not transmit spirochetes to offspring, despite moderate to heavy infection of spirochetes in their ovaries. These data are suggestive of a positive relationship between spirochete load and the probability of transovarial transmission, but are highly speculative because we do not definitively know spirochete identity (i.e., *B. miyamotoi* v. *B. burgdorferi*) of the infected ticks.

In our current study, there is additional, albeit sparse, support for a positive relationship between spirochete load and the probability of transovarial transmission. Only one of 11 infected females in our study did not transmit *B. miyamotoi* to her progeny. The quantitative PCR threshold cycle (C_t) value of the female that did not transmit spirochetes to offspring (C_t 39.7) was above the upper 95% confidence limit of the mean C_t value for the infected females that did transmit spirochetes to offspring (mean C_t = 21.5, 95% CI 20.6-22.4). This suggests that the relative spirochete burden of the 'non-TOT' female was significantly lower than that of TOT females. To confirm *B. miyamotoi* infection of the 'non-TOT' female, we sequenced DNA of the female and observed 100% identity with published *B. miyamotoi* sequences in GenBank.

Overall *B. miyamotoi* spirochete load in engorged females and/or heavy disseminated infections as seen Lane and Burgdorferi (1987) may be indicative of ovarian

tissue that has been successfully colonized by *B. miyamotoi*. It is unknown whether the high spirochete load may result from an initially high spirochete number in flat females and/or efficient spirochete amplification during feeding or through horizontal transmission from the adult tick's host (Han et al. 2016). Thus, future experiments that study spirochete dynamics within the engorged female, and mechanisms (e.g., timing and route) by which eggs are colonized by *B. miyamotoi,* may also reveal the relative importance of transstadial (through either vertical or horizontal transmission) and horizontal transmission during the adult bloodmeal. Ecologically, given the infection prevalence of engorged females is an important parameter influencing the overall contribution of vertical transmission, future research should document how infection prevalence of engorged females varies temporally, geographically, and among potential reservoir hosts used by blacklegged ticks (as well as for other *I. ricinus* complex ticks to understand how *B. miyamotoi* maintained in those enzootic cycles).

How important is vertical transmission for *B. miyamotoi* maintenance?

Overall, it appears that the prevalence of infection of engorged infected *I. scapularis* females is usually low (< 3%), that TOT is high (e.g. > 90%), and that FIP also can be high, but appears to be quite variable. By multiplying the observed engorged female infection prevalence, TOT rate and FIP together, we estimate that larval *I. scapularis* infection prevalence of the next generation would be ~2.0% (Fig. 4.2). This value is not significantly different from what Piesman et al. (1986) estimated for the transovarial transmission of *B. burgdorferi* in questing larval *I. scapularis*, which given their method of detection, probably was *B. miyamotoi*. They collected larval *I. scapularis* from vegetation in Massachusetts

(northeastern U.S.), fed them in the laboratory on naive hamsters. They found 2 of 274 subsequently molted nymphs to be infected, which represents a minimum of 0.7% (95% CI 0.1 - 2.9%) of transovarially infected *B. miyamotoi* prevalence in questing larval *I. scapularis*. Our predicted value of 2.0% in questing larvae based on observed TOT and FIP in this study also is consistent with reported prevalence of questing nymphal and adult *I. scapularis* (Barbour et al., 2009; Hamer et al., 2014).

This consistent prevalence of infection among larvae, nymphs and adults may seem to support the importance and adequacy of transovarial transmission for the maintenance of *B. miyamotoi* in the enzootic cycle. Our observed values of TOT and FIP, however, may not be high enough to allow *B. miyamotoi* infection to persist in the host population without input via horizontal transmission (Fine, 1975). Fine (1975) developed a deterministic mathematical model to investigate the sufficiency of vertical transmission for the maintenance of a vector-borne pathogen. According to that model, the maintenance of infection by vertical transmission alone can occur when the symbiote fitness criterion is met. This criterion is expressed as $\alpha\beta(r + v) > 1$, where r and v are defined respectively as the maternal and paternal vertical transmission rates, and α and β respectively are the relative fertility and survival rates of infected to uninfected hosts (in this case, the tick) (Fine, 1975). Fine interpreted the product of $\alpha\beta$ as a quantitative definition of symbiotic relationships between host (in this case, the tick) and agent as mutualistic, commensal, or parasitic if the product $\alpha\beta$ were >1, =1 and <1, respectively. Hosts receive selective advantage, no selective effect and selective disadvantage from the infection with 'mutual', 'commensal' and 'parasite' agent, respectively, when compared with uninfected individuals (Fine, 1975).

Here we use Fine's model as an initial quantitative exploration of the relative importance of vertical transmission. When we substitute our vertical transmission value of 76.7% (product of 90.9% TOT and 84.4% FIP) (or 93.3%, the upper 95% confidence limit) for the sum ($r + v$) and set the symbiote fitness criterion > 1, then the value of $\alpha\beta$ > 1.30 (or 1.07), which suggests that *B. miyamotoi* infection provides a > 30% (or 7%) increase in the fertility or survival of infected versus uninfected *I. scapularis*. Currently there have been no studies to indicate either positive or negative effects of *B. miyamotoi* infection on the fertility or survival of *I. scapularis.*

Our study was not designed to compare fitness effects of *B. miyamotoi* infection, but our data allow us to compare the oviposition and hatching rates of infected and uninfected females. The overall oviposition rate was significantly lower in 2016 than in 2015, which could be related to a change in our laboratory practices between the years (in 2016, to accommodate workflow logistics, we delayed oviposition by keeping females at a lower temperature and darkness for up to 87 days). Within each year, however, there was no statistical difference between the oviposition rates of *B. miyamotoi* infected and uninfected females. In addition, we did not observe any significant difference in hatching rates between infected and uninfected egg batches. Therefore, our results did not show any measurable positive or negative effect of *B. miyamotoi* infection on oviposition rates of *I. scapularis* females or of hatching success of their egg batches. Future research should compare fitness parameters of infected v. uninfected ticks throughout the entire life cycle.

For now, if we assume no fitness effects of *B. miyamotoi* infection on *I. scapularis*, then the value of $\alpha\beta$ is set to 1.0 and the sum of the maternal and paternal vertical transmission rates ($r + v$) would need to be > 1.0 for vertical transmission to maintain *B.*

miyamotoi. Thus, under this assumption, our observed vertical transmission rate of 76.7% appears to be insufficient (but closer to being sufficient if we take the upper 95% CI value of 93.3%) to support the maintenance of *B. miyamotoi* infection in the tick population and suggests that horizontal transmission involving reservoir hosts and/or co-feeding, non-systemic transmission likely is required for *B. miyamotoi* enzootic maintenance. If so, identifying reservoir hosts and quantifying horizontal transmission parameters would increase our understanding regarding the biological processes underlying *B. miyamotoi* maintenance. Additional mathematical modeling (e.g., Hartemink et al., 2008) that considers the vertebrate host population may be helpful to further evaluate the contributions of vertical and horizontal transmission for *B. miyamotoi* maintenance and extend our knowledge about the disease ecology of *B. miyamotoi*.

Implications for public health

First recognized as a human pathogen in Russia in 2011(Platonov et al., 2011), the number of human case reports of *B. miyamotoi* infection since then has been increasing in the United States and Eurasia (Gugliotta et al., 2013; Krause et al., 2013; Chowdri et al., 2013; Hovius et al., 2013; Krause et al., 2014; Sato et al., 2014; Molloy et al., 2015; Sudhindra et al., 2016; Jobe et al., 2016; Boden et al., 2016; Krause et al., 2016; Fiorito et al., 2017; Kadkhoda et al., 2017; Jiang et al., 2018; Sato et al., 2018). This increase undoubtedly is due in part to increased surveillance effort and improved diagnostics to detect BMD. Nevertheless, the range of *I. scapularis* continues to expand (Dennis et al., 1998; Eisen et al., 2016), so the incidence of BMD is likely to increase over time, as has already been seen

with Lyme borreliosis, human anaplasmosis, and human babesiosis (Mead et al., 2015; Schwartz et al., 2017).

One of the epidemiologically important biological differences between *B. miyamotoi* and several other *I. scapularis*-borne pathogens is that transovarial transmission occurs in *B. miyamotoi* (Scoles et al., 2001; Barbour et al., 2009), but is non-existent for most other *I. scapularis*-borne pathogens (Macleod and Gordon, 1933; Patrican, 1997; Dumler et al., 2001; Rollend et al., 2013) (Powassan/deer tick virus is an exception; Ebel and Kramer, 2004) (see van Duijvendijk et al., 2016, for differences in the *I. ricinus* system). Thus, while nymphs pose the greatest epidemiological risk for several *I. scapularis*-borne pathogens, larvae may pose an important, and possibly the greatest, risk for BMD due to their high abundance and tiny size, as is already suggested by the seasonality of BMD incidence seen in two studies from the Northeast (Molloy et al., 2015; Fiorito et al., 2017). Future studies should quantify the infectious dose of *B. miyamotoi* in infected larvae; as well as measure the acarological risk for *B. miyamotoi* (i.e., the density of infected ticks; Szekeres et al., 2017; Ruyts et al., 2017) as a function of all questing life stages.

APPENDIX

Table 4.1. Infection prevalence of *Borrelia miyamotoi* positive (pos) female *Ixodes scapularis* ticks collected from hunter-harvested white-tailed deer.

State	2015			2016		
	No. of females	No. of pos.	% Infection prevalence (95% CI†)	No. of females	No. of pos.	% Infection prevalence (95% CI†)
Maine	69	2	2.9 (0.5-11.0)	13	1	7.7 (0.4-37.9)
New Hampshire	32	0	0.0 (0.0-13.3)	85	2	2.4 (0.4-9.0)
Tennessee	133	1	0.8 (0.0-4.7)	--	--	--
Wisconsin	430	13	3.0 (1.7-5.2)	501	14	2.8 (1.6-4.8)
Total	664	16	2.4 (1.4-4.0)	599	17	2.8 (1.7-4.6)

† 95% confidence interval for a proportion including continuity correction

Table 4.2. Transovarial transmission (TOT) and filial infection prevalence (FIP) of *Borrelia miyamotoi* from 11 infected female *Ixodes scapularis* ticks collected from hunter-harvested white-tailed deer in Tennessee, Maine, New Hampshire, and Wisconsin.

State	Egg Batch ID	Weight of female (g)	C_t Value of female DNA	TOT	FIP		
					No. of larvae tested	No. of positive larvae	% Infection prevalence (95% CI¥)
Tennessee	F15-011	0.052	39.7	No	75	0	0 (0-6.1)
Maine	F15-042	0.046	18.7	Yes	28	26	92.9 (75.1-98.8)
	F15-044†	0.062	20.7	Yes	NA	NA	NA
New Hampshire	F16-573	0.221	21.6	Yes	100	36	36.0 (26.8-46.3)
	F16-596	0.354	23.4	Yes	100	92	92.0 (84.4-96.2)
Wisconsin	F15-279	0.191	21.8	Yes	100	100	100.0 (95.4-100.0)
	F16-099	0.262	21.6	Yes	100	98	98.0 (92.3-99.7)
	F16-276	0.306	22.3	Yes	200	178	89.0 (83.6-92.8)
	F16-311	0.293	22.5	Yes	100	83	83.0 (73.9-89.5)
	F16-108	0.043	20.8	Yes	64	35	54.7 (41.8-67.0)‡
	F16-288	0.091	21.6	Yes	100	73	73.0 (63.0-81.2)‡
Mean % (95% CI)¥				**90.9% (57.1-99.5%)**			**84.4 (68.1-100%)€**

Table 4.2 (cont'd)

† All larvae were infested on a RAG-deficient mouse

‡ FIP was estimated from unhatched eggs (F16-288) and unhatched eggs and 4 hatched larvae (F16-108)

¥ 95% confidence interval for a proportion including continuity correction or for mean

€ The estimate of the average FIP of larval ticks did not include F16-108 and F16-288

Figure 4.1. Workflow for estimating transovarial transmission (TOT) and filial infection prevalence (FIP) of *Borrelia miyamotoi*-infected deer-fed *Ixodes scapularis* females in 2015 (RH – Relative Humidity, L:D - Light : Dark lighting schedule). See text for modifications made in 2016 to improve workflow logistics.

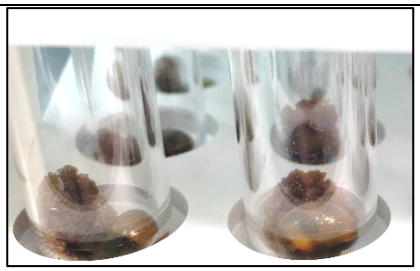
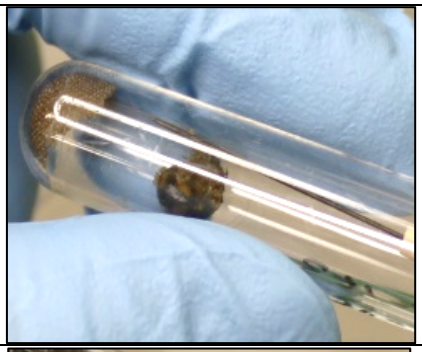
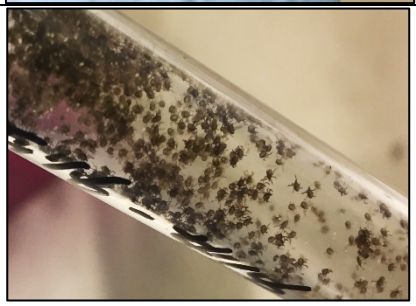
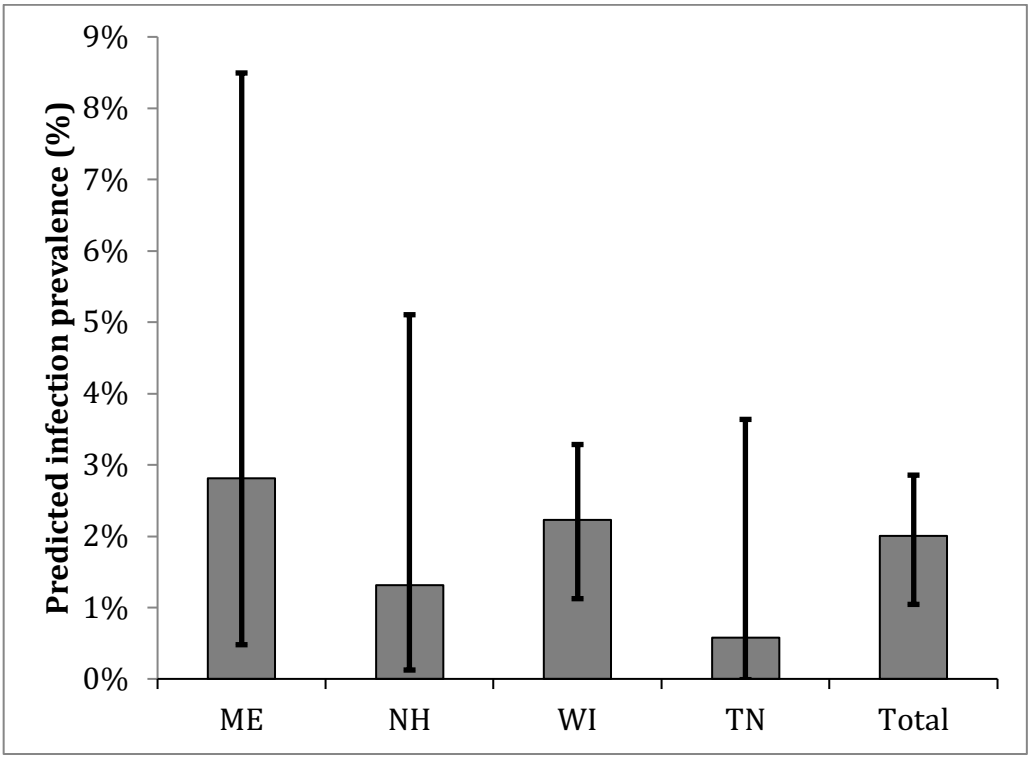
A. Field collections 1) Collect engorged *I. scapularis* females from hunter-harvested white-tailed deer.	
B. Laboratory: Oviposition 1) Sterilize and weigh females; 2) Store females individually at 22C, 99% RH with 16:8 L:D (hrs) photoperiod schedule.	
C. Screen females for *B. miyamotoi* 1) Remove females 15 days post onset of oviposition; 2) Assay each female for infection.	
D. Quantify TOT/FIP 1) TOT - Screen pools of larvae from each infected female (minimum of 10 pools, if no infections found); 2) FIP - Screen 100 individual larvae from each infected clutch.	

Figure 4.2. Predicted *Borrelia miyamotoi* prevalence (95% CI) in the next generation of larvae; calculations based on observed female infection prevalence, TOT rate, and FIP from ticks at each sampling site. ME = Maine, NH = New Hampshire, WI = Wisconsin, TN = Tennessee. The overall predicted infection prevalence value for these sites combined (= Total) is also shown.



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CHAPTER 5

Investigating the contribution of systemic, non-systemic and vertical transmission on *Borrelia miyamotoi* maintenance by analysis of R_0 using the next-generation matrix model

ABSTRACT

The basic reproduction number (R_0) has been used for infectious disease research as an indicator of whether a pathogen can persist or go extinct in a given population, and tick-borne disease systems are not an exception for its application. We used the next-generation matrix model to investigate the effects of systemic, non-systemic (co-feeding), and vertical transmission on *Borrelia miyamotoi* maintenance by R_0. Pathogen related values in the model were parameterized in the context of the white-footed mouse (*Peromyscus leucopus*) and blacklegged tick (*Ixodes scapularis*) system found in eastern North America. Model results indicated potential significant contributions of non-systemic and vertical transmission on *B. miyamotoi* maintenance, whereas systemic transmission showed minimal effects on R_0 of *B. miyamotoi*. The importance of aggregation of larval ticks and related co-feeding transmission efficiency between larvae with larvae or with nymphs were also highlighted. Our results suggested that white-footed mice may have limited direct effects on *B. miyamotoi* enzootic cycle as reservoir competent hosts, but rather they may provide important quantitative contributions as hosts of juvenile ticks, not just by increasing survivorship of ticks but through facilitating co-feeding transmission. Further field and laboratory studies to provide precise parameter values are required to better understand *B. miyamotoi* enzootic cycle by using the next-generation matrix model.

INTRODUCTION

Even in the simplest infectious disease systems (single pathogen – single host system), the dynamics can be highly complex due to heterogeneity among the pathogen and host populations spatiotemporal variation of environments in which pathogen-host interactions occur. However, most infectious diseases involve multi-host species and/or multiple transmission routes, which makes difficult to study infectious disease system. Mathematical modeling approaches, therefore, have been applied to understand better epidemiological dynamics of infectious disease, and one of the most central organizing concepts is the basic reproduction number (R_0).

The basic reproduction number (R_0) is most commonly defined as the expected number of secondary cases produced by an infected individual in a completely susceptible population during its entire infectious period (Anderson and May, 1990)(Diekmann et al., 1990). R_0, therefore, provides the threshold value of pathogen persistence or extinction such as if $R_0 > 1$, a pathogen can invade or persist, whereas $R_0 < 1$ indicates it will go extinct. Several applications of R_0 have been used for studying infectious disease epidemiology. The risk whether a disease outbreak will actually occur can be predicted by R_0. For example, if a disease outbreak occurs, the rate of the initial increase of infected individuals can be predicted by R_0. Also, the required proportion of a population to vaccinate to prevent a disease outbreak can be determined using R_0 (Anderson and May, 1990)(Hartemink et al., 2008).

As with many other infectious disease systems, the concept of R_0 has been applied to tick-borne disease systems to investigate ecological and epidemiological complexities (Randolph, 1998)(Norman et al., 1999)(Caraco et al., 2002)(Rosà et al., 2003)(Rosà and

Pugliese, 2007)(Ogden et al., 2014). Various approaches to define R_0 for tick-borne disease systems have been attempted due to the extreme complexities created by multiple host interactions in infection and/or transmission of pathogens and multiple transmission routes connected with multiple life stages of the vectors.

Hartemink et al. (2008) introduced a 'next-generation matrix' approach to characterize R_0 for tick-borne infections, which projects the growth in the number of infected individuals on a generational basis. Furthermore, the elements and properties of the matrix have clear biological interpretations. One of the features that makes the next-generation matrix model appropriate for modeling tick-borne disease systems is that the model is constructed based on a so-called 'type-at-birth' matrix, which categorizes individuals by the stage when they become infected by one of the multiple transmission routes. The next-generation matrix model, therefore, allows to compare the multi-transmission routes for their contributions on R_0.

Borrelia miyamotoi, a relapsing fever group spirochete, is a recently discovered zoonotic pathogen transmitted by *Ixodes ricinus* complex ticks, which also vector Lyme disease (LD) spirochetes in North America, Europe and Asia (Fukunaga et al., 1995)(Scoles et al., 2001)(Fraenkel et al., 2002)(Fomenko et al., 2010). In the United States, *B. miyamotoi* is transmitted by *I. scapularis* (Scoles et al., 2001) in the northeastern and upper midwestern US and *I. pacificus* in the Pacific coast areas (Mun et al., 2006). *Borrelia miyamotoi* has been detected from various wildlife species (Barbour et al., 2009)(Hamer et al., 2010)(Wagemakers et al., 2017) Several rodent species have been considered as potential reservoir hosts (Scoles et al., 2001)(Burri et al., 2014); however their roles in and contributions to the *B. miyamotoi* enzootic cycle are still unclear. In contrast to LD

spirochetes, *B. miyamotoi* can be transmitted vertically from infected females to their offspring (Scoles et al., 2001)(Breuner et al., 2017), and horizontal (systemic or non-systemic) and transstadial transmission have been demonstrated in the laboratory between *I. scapularis* and white-footed mice (*Peromyscus leucopus*) (Scoles et al., 2001) and immunocompromised mice (SCID *Mus musculus*) (Lynn et al., 2018). Most biological or ecological features of *B. miyamotoi*, however, have still remained enigmatic especially its maintenance in enzootic cycle. No equivocal reservoir host species have been identified, and specifically, aside from Scole's et al. (2001) initial xenodiagnostic experiment which showed limited transmission, no further laboratory experiments have been conducted on other potential reservoir host species to characterize infection/transmission of *B. miyamotoi* from ticks to hosts, and vice versa.

Thus, we applied the next-generation matrix model to explore how *B. miyamotoi* might be maintained in nature with specific focus on characterizing the potential contributions of three transmission routes observed in *B. miyamotoi-I.scapularis*-hosts cycle. Tick-related parameter values in the model were defined by previously published literature described in Hartemink et al. (2008). As there is limited published information regarding much of the biology and ecology of *B. miyamotoi*, we used field data derived from our previous research to provide estimates of initial values of model parameters related with hosts and *B. miyamotoi*.

MATERIALS AND METHODS

Calculating R_0 using the next-generation matrix model

The next-generation matrix model has the ability to estimate R_0 from highly complex disease systems that include multi-hosts and multi-transmission routes as tick-borne diseases. Hartemink et al. (2008) distinguished five 'types-at-birth' of infection comprising acquisition of the pathogen by different tick life stages as well as infection in the host. The identified five types of infection consist of (1) infected as an egg (by vertical transmission); (2) infected as a larva (from first blood meal); (3) infected as a nymph (from second blood meal); (4) infected as an adult female (from third blood meal); (5) systemically infectious vertebrate host (see Scheme 5.1-5.3 for the details of model matrix). The next generation matrix was constructed as a 5x5 matrix of elements. Each element (k_{ij}) in the matrix represents the number of new infections of tick stage (i) infected by another infected individual which was infected at life stage (j). Note that type-at-birth refers to the tick life stage during which the tick became infected, not the actual life stage. For example, the element k_{31} indicates the number of nymphs (=stage 3) which were infected at type-at birth 1 (i.e., infected as an egg (=stage 1) by vertical transmission). Consequently, a tick infected at type-at-birth 1 can have 3 chances to transmit infection to nymphs by co-feeding transmission: during at its first (=i.e., larval), second (= nymphal) and third (= adult) blood meals. The corresponded equation is below:

$$k_{31} = (s_{\mathrm{L}}\theta_{\mathrm{LN}}C_{\mathrm{NL}} + s_{\mathrm{L}}\, s_{\mathrm{N}}\, \theta_{\mathrm{NN}}C_{\mathrm{NN}} + s_{\mathrm{L}}\, s_{\mathrm{N}}\, s_{\mathrm{A}}\, \theta_{\mathrm{AN}}C_{\mathrm{NA}})h_{\mathrm{c}}$$

The formula incorporates survival probability of each stage (s), the transmission efficiency from tick to tick (θ) (i.e. co-feeding transmission), the tick burden on a host (C), and the proportion of competent hosts (h_c) (Table 1, 2).

We used the next generation matrix model to explore how assumptions about different transmission routes (systemic, non-systemic (co-feeding), and vertical transmission) might affect R_0. Series of R_0 values were calculated as a function of the proportion of competent hosts (h_c) for low, medium, high efficiency of each transmission parameter. Parameterized results of the next-generation matrix were spectrally decomposed by using the eigen (matrix) function in R, which computes eigenvalues and eigenvectors of complex matrices. The dominant (largest) eigenvalue of a matrix, which characterizes the growth of the elements of the matrix after they have reached a stable stage distribution, is considered as R_0.

A single value of R_0 calculated from initial values of all parameters was referred as 'Standard R_0,'and a series of R_0 values generated by iterating the model over the range of proportion of competent hosts from 0 to 1 (by 0.01 increments) was referred to as 'Standard R_0 series'. The Standard R_0 and Standard R_0 series were used to estimate sensitivity and elasticity values of each parameter.

Parameters in the next-generation matrix model

Initial values of tick related parameters were determined from previously published data described in Hartemink et al. (2008) (Table 1). Many biological features of *B. miyamotoi* infection and transmission among ticks and hosts are unknown. Therefore, *B. miyamotoi* specific parameters were determined based on limited information from the

literature or expert knowledge (see Table 2 for a full list and description of *B. miyamotoi* related parameters). From a previous study in which we trapped mammal hosts of juvenile *I. scapularis* in the north central US (Chapter 2), we observed that the majority of juvenile *I. scapularis* were recovered from white-footed mice, which also were identified as the most frequently trapped small mammal species. Therefore, the proportion of white-footed mice (58.1%) from total captured small and medium-sized animals was used as the initial parameter value of the proportion of competent host. Our field data suggested a potentially short infection duration in white-footed mice as none of the recaptured mice showed persistent infection with *B. miyamotoi*. Lack of persistent infection of *B. miyamotoi* was reported from wild *Apodemus* spp. and *Myodes* spp. in Japan where *B. miyamotoi* is vectored by *I. persulcatus* (Taylor et al., 2013). Thus we set the initial value of *B. miyamotoi* infection duration of competent hosts as 3 days to represent a potentially short infection duration of rodent hosts.

There are two types of systemic transmission parameters in the model, host-to-tick transmission (p_L, p_N, p_A) and tick-to-host transmission (q_L, q_N, q_A). Scoles et al. (2001) reported a combined systemic (horizontal) and transstadial transmission rate from infected white-footed mice to xenodiagnostic larval *I. scapularis* that then molted into nymphs as 2.8-7.1%. Therefore, the initial value of host-to-tick transmission efficiency was set as 0.1 to generate the standard R_0. The probability of *B. miyamotoi* transmission to laboratory mice (*Mus musculus*) exposed to feeding by a single infected nymphal *I. scapularis* was reported as 73% by the completion of blood feeding (Breuner et al., 2017). In addition, serological evidence of *B. miyamotoi* exposure was observed from 88% of laboratory mice exposed to a complete blood meal by a single vertically infected larval *I.*

scapularis (Breuner et al., 2018). Based on these data, the tick-to-host transmission efficiency of *B. miyamotoi* was set as 0.8 for the initial value. Non-systemic (co-feeding) transmission of *B. miyamotoi* was observed from xenodiagnostic nymphal *I. scapularis* which fed as xenodiagnostic larvae simultaneously with infected nymphs on uninfected laboratory white-footed mice (Scoles et al., 2001). There was, however, no other literature regarding non-systemic transmission efficiency of *B. miyamotoi* in *I. scapularis*. Therefore, the initial value of non-systemic transmission efficiency for *B. miyamotoi* was borrowed from previously reported transmission efficiency of *B. burgdorferi* (0.56) between cofeeding *I. ricinus* nymphs and larvae (Gern and Rais, 1996).

Vertical transmission can contribute to R_0 when infected eggs (E) are produced from an infected female, which had either hatched herself from a vertically infected egg or become infected by feeding on an infected host as larva, nymph or adult. The expected number of infected eggs per individual was estimated from the product of the average number of eggs per female and the transmission efficiency from adult female to egg, while taking into account the survival probability of each type-at-birth. The probability that an egg in an egg batch produced by an infected female is infected could be estimated as a product of the infection prevalence of adult females, the transovarial transmission rate from an infected female to an egg batch, and the filial infection prevalence of the egg batch. Using data from a prior study we conducted (Chapter 4), we calculated 0.02 as the initial value of vertical transmission efficiency.

Next-generation matrix model simulation

The next-generation matrix model was simulated to investigate the effects of changes in the efficiency of three different transmission routes (systemic, non-systemic (co-feeding) and vertical) on R_0 of *B. miyatmotoi*. Changes in R_0 were visualized also as a function of the proportion of competent hosts. For *systemic transmission*, we focused on varying the host-to-tick transmission efficiency due to expected large variation of the parameter values among host species, which may provide us a better understanding regarding the role of reservoir hosts in *B. miyamotoi* enzootic cycle. The initial value of tick-to-host transmission efficiency were fixed as 0.8 (as described above) while low (0.1), medium (0.5) and high (0.9) transmission efficiency of host-to-tick transmission parameters were used for the model simulation.

Non-systemic (co-feeding) transmission can be subdivided by tick life stages into donors and recipients for pathogen transmission. The model has nine non-systemic transmission parameters representing combinations of three infectious life stages (larva, nymph and adult female) for each donor and recipient. Non-systemic transmission parameters pertaining to juvenile ticks (θ_{LL}, θ_{NL}, θ_{LN}, and θ_{NN}) were varied for model simulation, while non-systemic transmission parameters pertaining to adult ticks were fixed at the initial value of 0.56. The efficiency of non-systemic transmission was also tested as low (0.1), medium (0.5) and high (0.9) values to calculate R_0 by the model simulation.

As a proportional value, *vertical transmission efficiency* could range between 0 and 1. The effects of changes in vertical transmission efficiency, however, were tested using low, medium and high values of transmission efficiency from adult to egg as 0.001, 0.05, and 0.1, respectively, by considering realistic possible ranges of the values (the infection prevalence

of questing larvae that we previously observed at the same field site where mammal data were obtained was used as the vertical transmission efficiency value in the model; see Chapter 1).

Elasticity of R_0

Elasticity is defined as the proportional change in R_0 due to a proportional change in a parameter, whereas sensitivity refers to an absolute change in R_0 as a result of a change in a parameter (H. Caswell, 2001)(Matser et al., 2009). Elasticity analysis, however, may be considered as a fairer means of comparison when parameters that are measured on different scales are compared for their contributions to R_0. Therefore, we conducted elasticity analyses for model parameters by measuring proportional changes in R_0 from the standard R_0 resulting from proportional changes of initial values of model parameters. Changes in elasticities of three transmission parameters were plotted as a function of the proportion of competent hosts to elucidate the relative contribution of each transmission route to R_0.

RESULTS

Model simulations for three transmission routes: systemic, non-systemic, and vertical transmission

R_0 was calculated with three hypothetical values (low, medium and high) of each transmission route parameter as a function of the competent host proportion by simulating the next-generation matrix model, while the other two transmission parameters were fixed as initial values.

First, the persistence threshold value of R_0 (=1) was easily reached at ~ 40-60% proportion of competent hosts from all 3 values of systemic host-to-tick transmission (Fig. 5.1). Variation in the required competent host proportion to reach the threshold R_0 was small regardless of the extreme variation of systemic transmission parameter values. The threshold of R_0 =1 was reached at 58.6% of competent host proportion even without systemic transmission.

Second, the threshold value for R_0 =1 was never reached with low (10%) non-systemic transmission efficiency regardless of the proportion of competent hosts (Fig. 5.2). The required competent host proportion to reach the threshold value of R_0 =1 with medium (50%) and high (90%) non-systemic transmission efficiency were 62.6% and 36.4%, respectively. If we fix the competent host proportion value at the initial value of 0.581 represented by proportion of white-footed mice from our field data as a hypothetical true competent host proportion, then a minimum of 53.9% of non-systemic transmission efficiency between larvae and nymphs was required to reach the threshold value of R_0.

The required competent host proportions to reach the threshold value of R_0 with low (0.001), medium (0.05) and high (0.1) vertical transmission efficiency were 82.8%,

36.4% and 22.2%, respectively (Fig. 5.3). The required minimum infection prevalence of questing larvae represented as vertical transmission efficiency in the model to reach the threshold value of R_0 was 1.79% at 58.1% proportion of competent hosts.

Elasticity of R_0

Elasticity values of model parameters associated with juvenile ticks, competent hosts and three transmission routes were plotted in Figure 5.4. Tick-associated parameters such as average number of eggs per adult (E), survival provability of ticks (s_L, s_N, s_A), and attachment days of ticks (D_L, D_N, D_A) were excluded from elasticity analyses. Proportion of competent hosts (h_c) showed highest elasticity value, whereas infection duration of the competent host (i) showed the lowest elasticity value. Parameters which showed moderate elasticity values were mean number of larvae cofeeding with a larva (C_{LL}), mean number of larvae cofeeding with a nymph (C_{LN}), efficiency of cofeeding transmission from larva to larva (θ_{LL}) and from nymph to larva (θ_{NL}), and transmission efficiency from adult to egg (r_A). All six systemic transmission parameters (transmission efficiency from host to larva/nymph/adult ($p_{L,N,A}$) and from larva/nymph/adult to host ($q_{L,N,A}$)) showed low elasticity values.

Apparent differences in relative contributions to R_0 by the three transmission routes were observed from simulation results for the elasticity of R_0 (Fig. 5.5). Non-systemic and vertical transmission efficiency contributed greatly to R_0 of *B. miyamotoi*, whereas systemic transmission efficiency showed minimal effects on R_0 regardless of competent host proportion. There was, however, an obvious contrast in elasticity values responding to competent host proportion between non-systemic and vertical transmission. Elasticity

values of non-systemic transmission increased logarthmically with increasing proportion of competent hosts, whereas elasticity values of vertical transmission efficiency decreased exponentially with increasing the proportion of competent hosts.

DISCUSSION

Systemic transmission can be defined as transmission of pathogens via infected vertebrate hosts to naïve ticks by blood feeding on the hosts. Non-systemic (co-feeding) transmission can be defined as transmission from infected to non-infected ticks during blood feeding in close spatiotemporal proximity on a host that is not systemically infected. Pathogens also can be transmitted vertically (transovarially) from infected females to their progeny. All three modes of transmission have been observed for *B. miyamotoi*, however, relative contribution of each transmission route on the enzootic maintenance of *B. miyamotoi* is still uncertain. We investigated the effects of various components of the enzootic cycle of *B. miyamotoi* on the basic-reproduction number (R_0) by using the next-generation matrix model, with particular focus on the potential contributions of systemic, non-systemic, and vertical transmission. We used the next-generation matrix model presented by Hartemink et al. (2008) to estimate R_0 of *B. miyamotoi* in the white-footed mouse and *I. scapularis* system found in the eastern U.S. The next-generation matrix model can take into account multiple types-at-birth infections and all three transmission routes, which exist in tick-borne disease systems and create complexities in the enzootic cycle of tick-borne pathogens. In the model matrix, each type-at-birth represents reproduction numbers of infected individuals that acquired the infection by only one transmission route, which made it possible to compare the effects of three transmission routes on the basic reproduction number. Our simulation results showed that R_0 of *B. miyamotoi* responded sensitively to the changes of non-systemic and vertical transmission parameters while the threshold of R0 was constantly reached regardless of the extreme variation of systemic transmission parameter values, suggesting that survival or extinction of *B. miyamotoi* could

be determined by non-systemic and vertical transmission rather than systemic transmission. Even though systemic transmission (specifically host-to-tick transmission which may vary by host species and could be interpreted as the part of host contribution) showed little effect on R_0, proportion of competent hosts provided most significant effects on R_0 of *B. miyamotoi*.

There are several steps involved that may influence the efficiency of systemic transmission. In the systemic transmission route, *B. miyamotoi* is transmitted from infected ticks to a host by blood feeding (a, transmission efficiency from tick to host); then the host develops spirochetemia (systemic infection) for a certain period (b, host competency and infection duration); and during the infectious period, *B. miyamotoi* is transmitted from the infected host back to other feeding ticks (c, transmission efficiency from host to tick). These factors affecting systemic transmission efficiency could vary among diverse combinations of vertebrate and tick species. In our model simulations, initial parameter values for the proportion of competent hosts (h_c), infection duration of hosts (i) and systemic transmission efficiency (q_L, q_N, q_A and p_L, p_N, p_A) were based on data collected from our prior field studies conducted in the north central US, where *I. scapularis* frequently feeds on white-footed mice in an ecosystem that supports several *I. scapularis*-borne pathogens. There was not much variation on the proportion of competent hosts (white-footed mice in our model) required for *B. miyamotoi* persistence (i.e., $R_0 = 1$) despite varying host-to-tick transmission values over a wide range. Our model simulations showed that even *B. miyamotoi* can be maintained without systemic transmission from infected host to ticks when the proportion of competent hosts reached at 58.6%.

Thus, the results of the model indicated that systemic transmission efficiency of *B. miyamotoi* from infected white-footed mice to ticks did not contribute much to *B. miyamotoi* maintenance. The proportion, or abundance, of white-footed mice, however, had large impacts on *B. miyamotoi* persistence in our model simulations, which may provide support for a positive, but limited reservoir potential or reservoir competence of white-footed mice. Reservoir potential has been described as the proportion of infected vectors produced by an individual of a given host species (Mather et al., 1989)(Brunner et al., 2008), which can be estimated as a product of the proportion of vectors fed by an individual of a given species and the probability that a vector becomes infected by feeding on the host species (Schauber and Ostfeld, 2002)(LoGiudice et al., 2003). Reservoir competence can be described by the susceptibility of the host to infection by vector, the persistence of infection in the host, and the transmission efficiency of pathogen from the host to ticks (Kahl et al., 2002)(LoGiudice et al., 2003)(Brunner et al., 2008). If a host species feeds few vectors or has low transmission efficiency of pathogen to them, the host species could be considered as having low reservoir potential and/or reservoir competence. Therefore, our model simulation result provided evidence of low reservoir potential/reservoir competence of white-footed mice for *B. miyamotoi* transmission. Further research on white-footed mice and other mammal species for susceptibility and persistence of *B. miyamotoi* infection, and transmission efficiency to ticks along with the density of juvenile *I. scapularis* on host animals are required to better explain this observation.

A positive relationship between the non-systemic (co-feeding) transmission rate and the probability of *B. miyamotoi* persistence was observed from model simulations. The

persistence threshold value of R_0 was reached rapidly by increasing the non-systemic transmission rate. This result may indicate that *B. miyamotoi* largely relies on non-systemic transmission for its maintenance in nature. If this were the case, the systemic transmission efficiency of *B. miyamotoi* from vertebrate hosts to ticks may not be important for *B. miyamotoi* maintenance, but rather accessibility or aggregation of ticks on hosts would have important implications for the *B. miyamotoi* enzootic cycle. Therefore, even if white-footed mice had low reservoir competence or even if they were completely non-competent hosts for *B. miyamotoi* infection, they still may play a significant role for *B. miyamotoi* maintenance by serving as the major hosts for juvenile *I. scapularis* (Levine et al., 1985)(Godsey et al., 1987)(Lane et al., 1991).

Simulation results of vertical transmission efficiency showed a similar pattern with that of non-systemic transmission on R_0. The persistence threshold value of R_0 was quickly reached with increasing vertical transmission efficiency, which also corresponded with a decreasing required proportion of competent hosts. Only 22.2-36.4% of the host community needed to be competent hosts to exceed the persistence threshold value of R_0 when the vertical transmission value was 0.05-0.1. Our simulation results indicated that vertical transmission facilitated *B. miyamotoi* maintenance over large range of the proportion of competent hosts: all 3 scenarios of vertical transmission efficiency values reached the threshold values of R_0, and relatively lower competent host proportions were required for $R_0 > 1$ than the other two transmission routes.

If we consider spatiotemporal variation in density of rodent species (Ostfeld et al., 1996)(Ostfeld et al., 2006), the potential independence of *B. miyamotoi* maintenance from the changes of the relative abundance of competent hosts provided by vertical

transmission could have important implications on the persistence of *B. miyamotoi* in the enzootic cycle. The initial value of vertical transmission efficiency used in the model was estimated as the probability that an egg becomes infected from infected female, which was the product of female infection prevalence, transovarial transmission rate (the proportion of infected females that produce infected egg masses), and filial infection prevalence (the proportion of larvae in a clutch that are infected) (Burgdorfer and Varma, 1967). This can also be thought of as the expected (next generation) questing larval infection prevalence, assuming uninfected and infected eggs have the same survivorship. Therefore, the infection prevalence of adult females when they oviposit may be very important for the contribution of vertical transmission to R_0. Reservoir hosts fed on by adult female ticks may have large implications on *B. miyamotoi* maintenance due to their potential effects on the infection prevalence of adult female ticks. White-tailed deer (*Odocoileus virginianus*) are known as the major hosts of adult *I. scapularis* (Lane et al., 1991). Significantly higher infection prevalence of engorged females collected from white-tailed deer than that of questing females was previously reported (Han et al., 2016); however, the role of white-tailed deer on *B. miyamotoi* maintenance still remains uncertain. Further research on reservoir hosts of adult *I. scapularis*, along with infection dynamics of *B. miyamotoi* in engorged adult females, may help us better understand *B. miyamotoi* maintenance in the nature.

As we observed from the simulation result of systemic transmission efficiency, parameters associated with systemic transmission showed minimum elasticity values, whereas moderate elasticity values were observed from parameters related with non-systemic and vertical transmission. Due to large contributions of non-systemic and vertical transmission to R_0 of *B. miyamotoi* in our model simulations, co-feeding transmission

efficiency from larva or nymph to larva and mean number of larvae co-feeding with a larva or a nymph also showed moderate elasticity values. Therefore, the result of elasticity analysis also showed that density and/or aggregation of on-host larvae and nymphs on vertebrate hosts may have large implications for *B. miyamotoi* maintenance.

Apparent differences in the respective contributions to R_0 among the three transmission routes can be observed more clearly when results from model simulation of the elasticity analyses are graphed together (Fig. 5.5). The contribution of systemic transmission on R_0 of *B. miyamotoi* varies little, whereas *B. miyamotoi* maintenance is greatly affected by non-systemic and vertical transmission. Non-systemic and vertical transmission had an inverse relationship with regard to the maintenance of *B. miyamotoi*. Vertical transmission had the greatest effect on R_0 of *B. miyamotoi* when the proportion of competent hosts in the community is low. The contribution of vertical transmission to R_0 rapidly decreased as the proportion of competent hosts increased. Non-systemic transmission started providing stable larger contribution of R_0 than vertical transmission from around 8% of competent host proportion. The contribution of non-systemic transmission for R_0 of *B. miyamotoi* increased by as the proportion of competent hosts increased.

Overall our model results showed that non-systemic (co-feeding) transmission provided the greatest contribution to *B. miyamotoi* maintenance along with large inputs from vertical transmission. In tick-borne disease systems, co-feeding transmission was first described for Thogoto virus (Jones et al., 1987) and tick-borne encephalitis virus (TBE) (Alekseev and Chunikhin, 1990)(Labuda et al., 1993b)(Labuda et al., 1993a). The importance of co-feeding transmission for TBE virus transmission and/or enzootic

maintenance has been well documented (Randolph, 2011), and the contribution of co-feeding transmission on ecological maintenance of Lyme disease has been discussed (Gern and Rais, 1996)(Piesman and Happ, 2001)(Kiffner et al., 2011)(Kjelland et al., 2011)(Voordouw, 2015). *Borrelia miyamotoi,* like other tick-borne relapsing fever spirochetes, mainly resides in the blood (Barbour and Hayes, 1986), rather than skin tissues (Barbour et al., 2009). If we consider that co-feeding transmission occurs in the spatiotemporal proximity between donor and recipient ticks on the same reservoir host (Randolph et al., 1996)(Voordouw, 2015), *B. miyamotoi* may have a lower efficiency of co-feeding transmission than TBE virus or Lyme disease spirochetes, which can persist longer at the biting site of donor ticks after blood feeding. Co-feeding transmission of *B. miyamotoi*, therefore, may occur only when aggregated and/or synchronous blood feeding of ticks on hosts is present. This potential limitation of co-feeding transmission may prevent non-systemic transmission from being sufficient for *B. miyamotoi* enzootic maintenance and thus require some level of vertical (and systemic) transmission.

The current study explored the potential relationship of systemic, non-systemic, and vertical transmission for *B. miyamotoi* maintenance by modeling effects on R_0 using the next-generation matrix model (Harteminke et al. 2008). The simulation results suggested potential significant contributions of non-systemic and vertical transmission but limited contributions of systemic transmission on *B. miyamotoi* maintenance. Consequently, the importance of aggregation of larval ticks co-feeding with larvae or nymphs and co-feeding transmission efficiency were also highlighted. Our results suggested that rodent hosts may have limited qualitative effects on *B. miyamotoi* enzootic cycle as reservoir competent hosts, but rather, they may provide important quantitative measures as blood feeding hosts

of juvenile ticks. Similarly, any hosts, not just rodents, that may feed aggregations of larvae and nymphs (e.g., deer)(Huang et al., 2018) may likewise contribute to *B. miyamotoi* maintenance. Our model relied on many parameter values derived from the literature. Further empirical field and laboratory studies to provide more precise and reliable values of parameters in the next-generation matrix model (especially that showed large elasticity values) would improve model analyses and our understanding of *B. miyamotoi* enzootic cycles.

APPENDIX

Table 5.1. Tick-related parameters as defined in Hartemink et al. (2008).

Parameter	Description	Estimate
E	Average no. eggs per adult	2000[1,2]
s_{L}	Survival probability from egg to feeding larva	0.05[1]
s_{N}	Survival probability from feeding larva to feeding nymph	0.1[1]
s_{A}	Survival probability from feeding nymph to feeding adult	0.1[1,ϕ]
C_{LL}	Mean no. larvae cofeeding with a larva	30[3]
C_{NL}	Mean no. nymphs cofeeding with a larva	2[4]
C_{AL}	Mean no. adults cofeeding with a larva	0.01[4]
C_{LN}	Mean no. larvae cofeeding with a nymph	20[2,3]
C_{NN}	Mean no. nymphs cofeeding with a nymph	1[4]
C_{AN}	Mean no. adults cofeeding with a nymph	0.01[4]
C_{LA}	Mean no. larvae cofeeding with an adult	0.01[4]
C_{NA}	Mean no. nymphs cofeeding with an adult	0.01[4]
C_{AA}	Mean no. adults cofeeding with an adult	0.01[4]
N_{LH}	Average no. larvae on competent host	6[3,5,6]
N_{NH}	Average no. nymphs on competent host	0.2[5,6]
N_{AH}	Average no. adults on competent host	0.001[4]
D_{L}	Days of attachment of larva	2.5[5]
D_{N}	Days of attachment of nymph	3.5[4]
D_{A}	Days of attachment of adult	12[4]

* Numbers in superscript refer to the following references; 1, (Randolph and Craine, 1995); 2, (Randolph, 2004); 3, (Randolph et al., 1999); 4, S. E. Randolph, unpublished; 5, (J. Gray, O. Kahl, R. S. Lane, 2002); 6, (Humair et al., 1999)

ϕ Assuming half of the 20% survivors mentioned by Randolph and Craine (1995) will be female

Table 5.2. Pathogen-specific parameters derived from previous literature and our field data for the best estimate (adapted from Hartemink et al. (2008)).

Parameter	Description	Estimate
i	Systemic infection duration	3 days[1]
θ^{λ}	Efficiency from tick to tick	0.56[2]
p_{L}	Efficiency from competent host to larvae	0.1[3]
p_{N}	Efficiency from competent host to nymph	0.1[3]
p_{A}	Efficiency from competent host to adult	0.1[3]
q_{L}	Efficiency from larvae to competent host	0.8[4]
q_{N}	Efficiency from nymph to competent host	0.8[4]
q_{A}	Efficiency from adult to competent host	0.8[4]
r_{A}	Efficiency from adult to egg	0.02[5]

* Numbers in superscript refer to the following references; 1, field evidence and (Taylor et al., 2013); 2, transmission efficiency of *Borrelia burgdorferi* between Ixodes ricinus nymph and larva was borrowed from (Gern and Rais, 1996); 3, (Scoles et al., 2001); 4, (Breuner et al., 2017) and (Breuner et al., 2018); 5, Han et al. (2018) in review

λ. All transmission efficiencies between co-feeding ticks were assumed to be the same due to the lack of data

Figure 5.1. R_0 plotted as a function of the fraction of blood meals taken on competent hosts (h_c) for *Borrelia miyamotoi*. R_0 was calculated with systemic transmission (ST; p_L, p_N, p_A) values 0.1, 0.5, and 0.9, while non-systemic and vertical transmission parameters were fixed at the initial values of 0.56 and 0.02, respectively. The threshold value of *B. miyamotoi* persistence (i.e., R_0 = 1) is indicated with a dotted line.

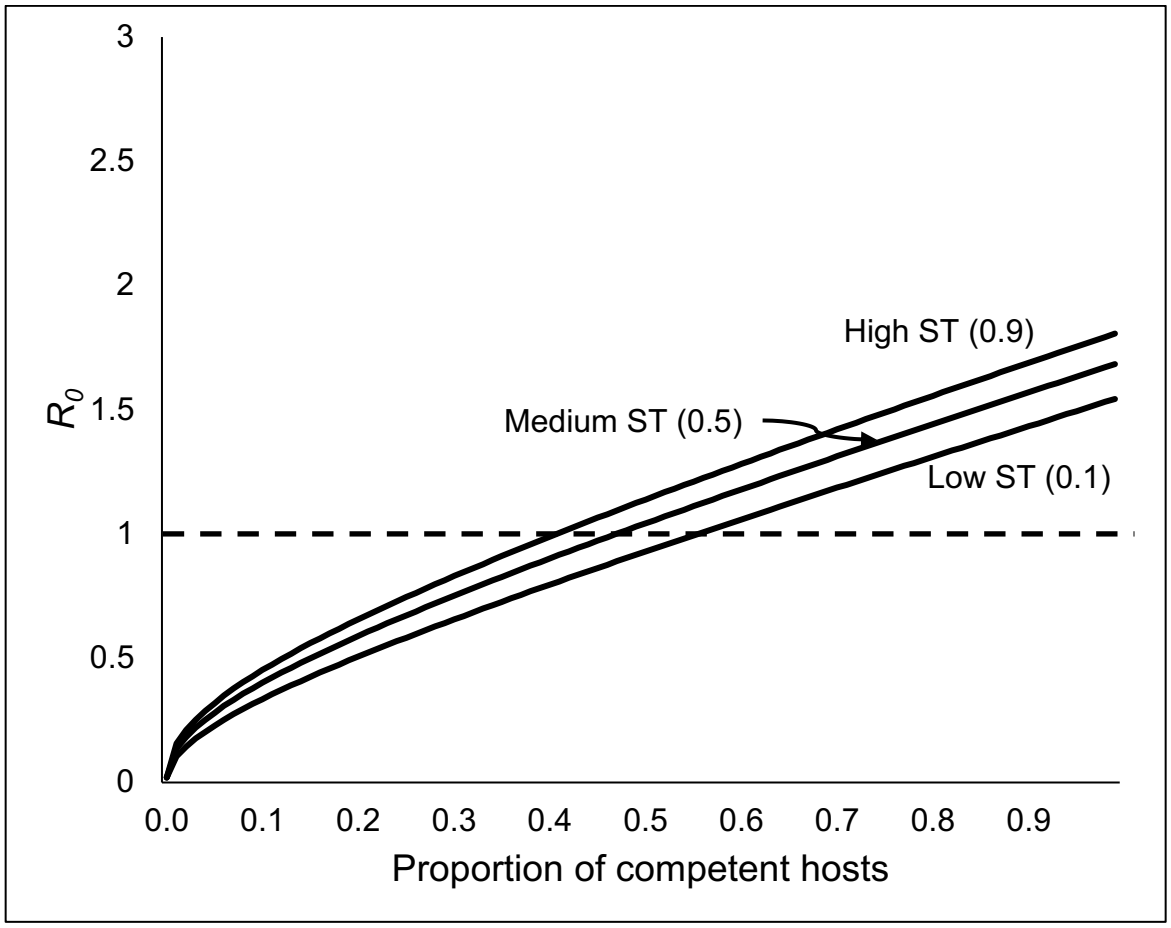


Figure 5.2. R_0 plotted as a function of the fraction of blood meals taken on competent hosts (h_c) for *Borrelia miyamotoi*. R_0 was calculated with non-systemic (co-feeding) transmission (NST; θ) value 0.1, 0.5, and 0.9, while systemic (p_L, p_N, p_A) and vertical transmission parameters were fixed at the initial values of 0.1 and 0.02, respectively. The threshold value of *B. miyamotoi* persistence (i.e., R_0 = 1) is indicated with a dotted line.

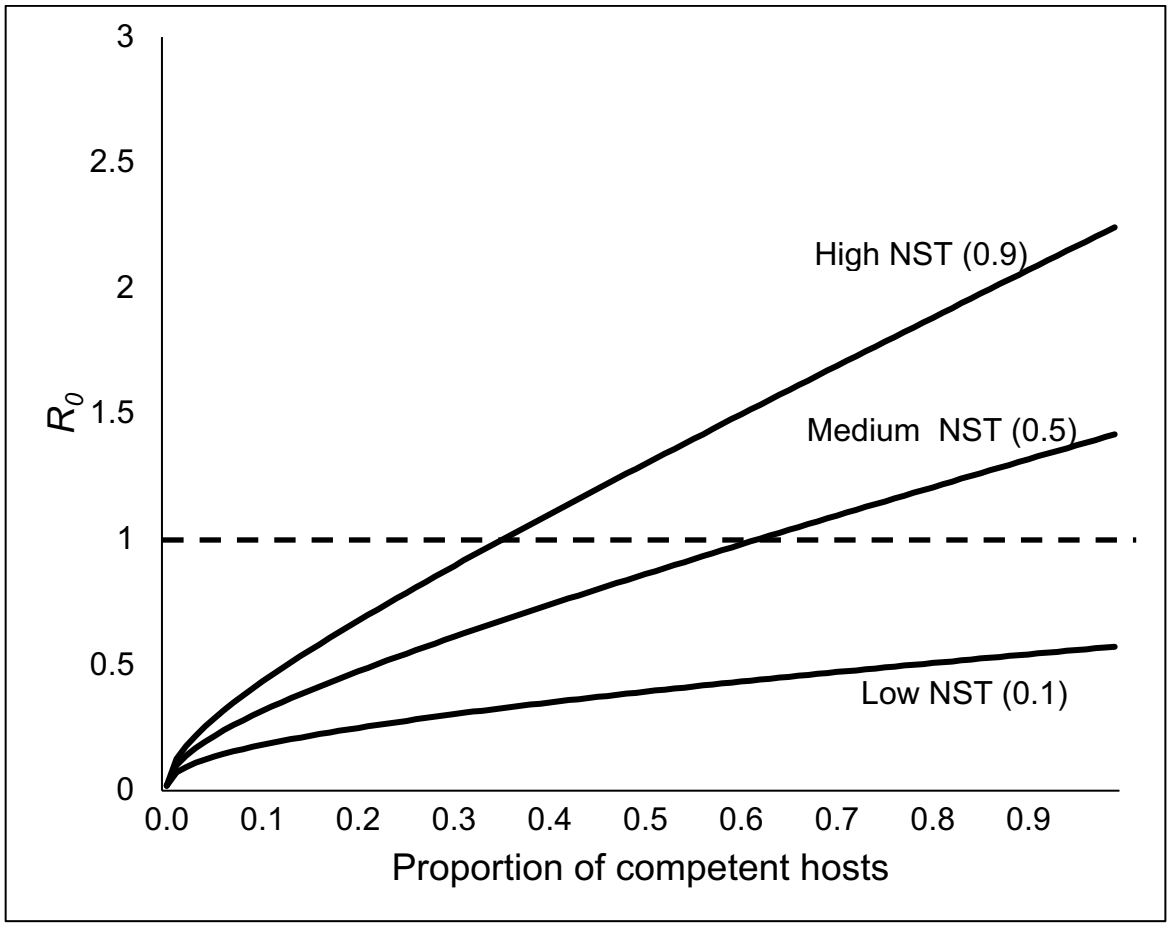


Figure 5.3. R_0 plotted as a function of the fraction of blood meals taken on competent hosts (h_c) for *Borrelia miyamotoi*. R_0 was calculated with vertical transmission (Vert) values 0.001, 0.05, and 0.1, while systemic (p_L, p_N, p_A) and non-systemic transmission parameters were fixed at the initial values of 0.1 and 0.56, respectively. The threshold value of *B. miyamotoi* persistence (i.e., $R_0 = 1$) is indicated with a dotted line.

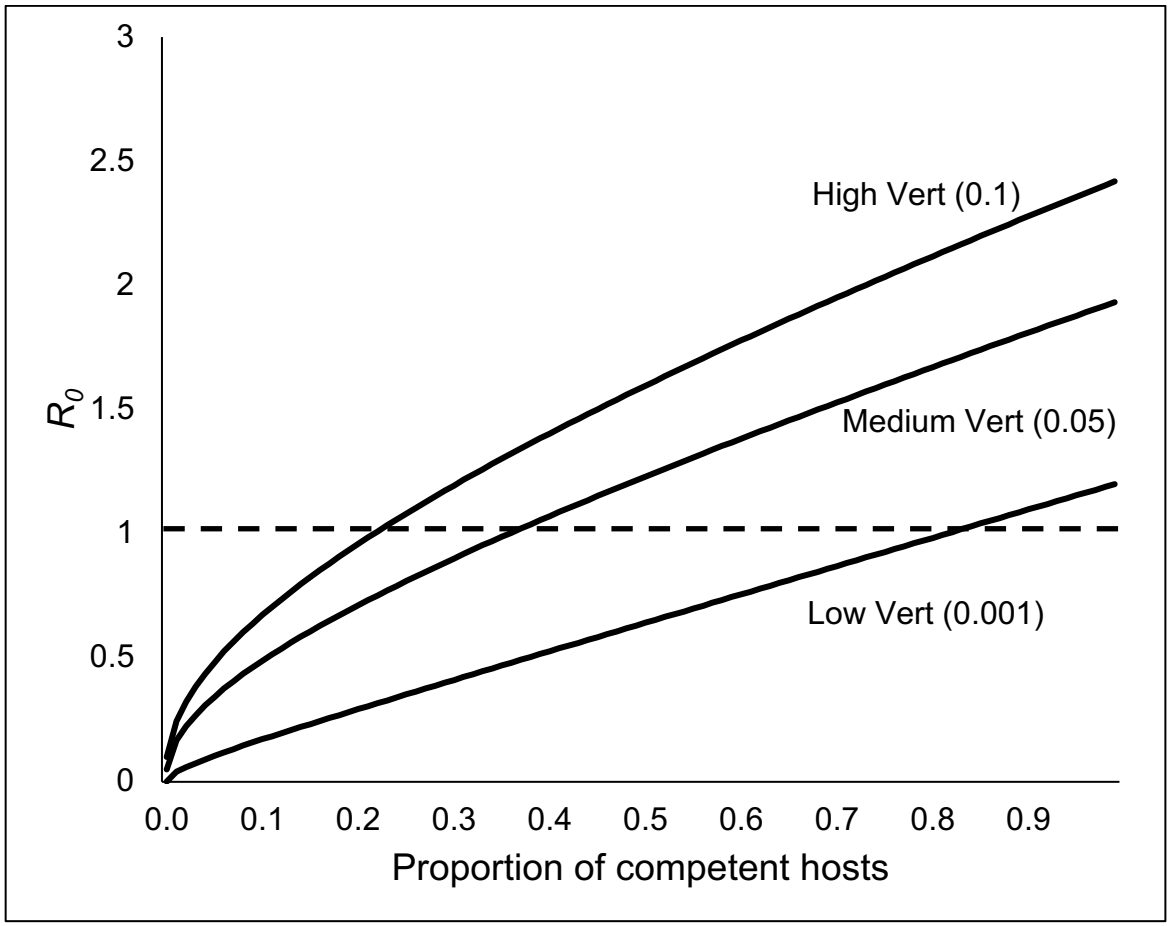


Figure 5.4. Elasticity values of parameters of interest for R_0 of *Borrelia miyamotoi* (see Table 2 for parameter definitions).

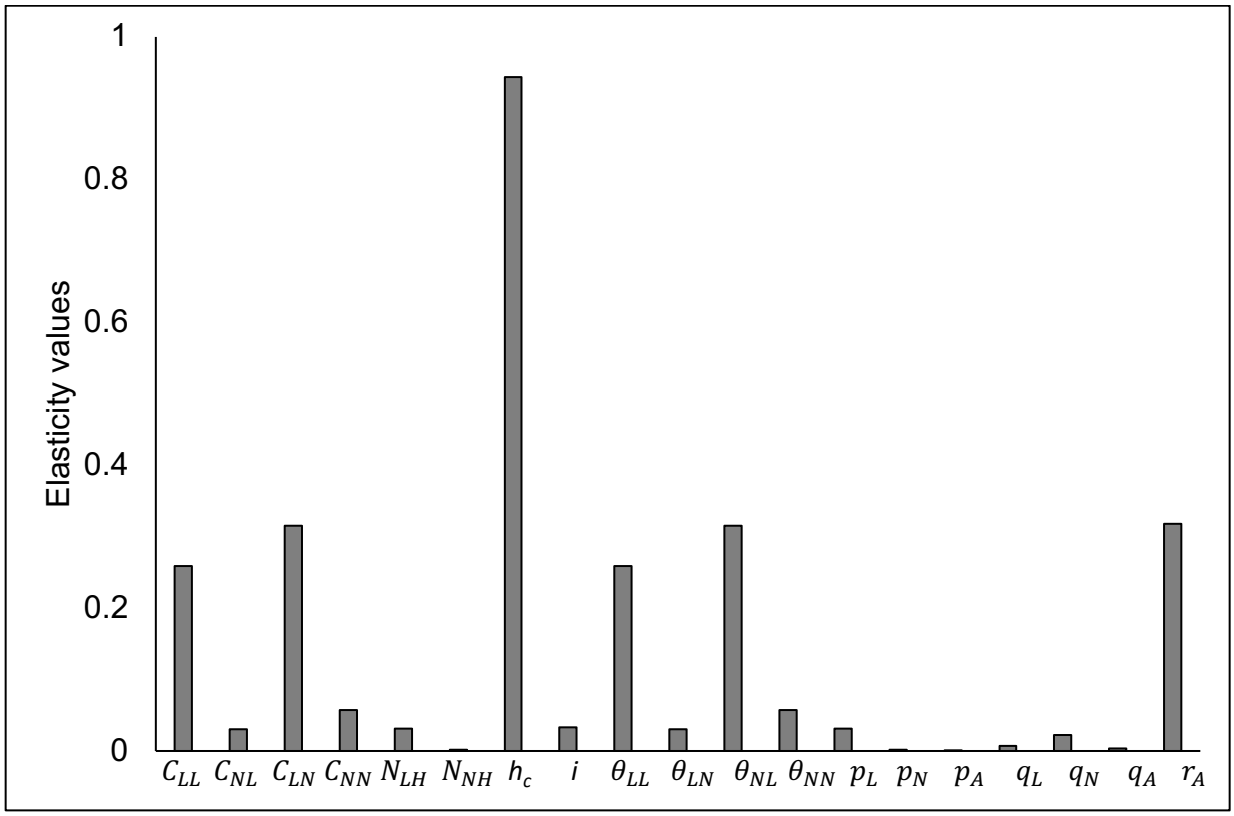
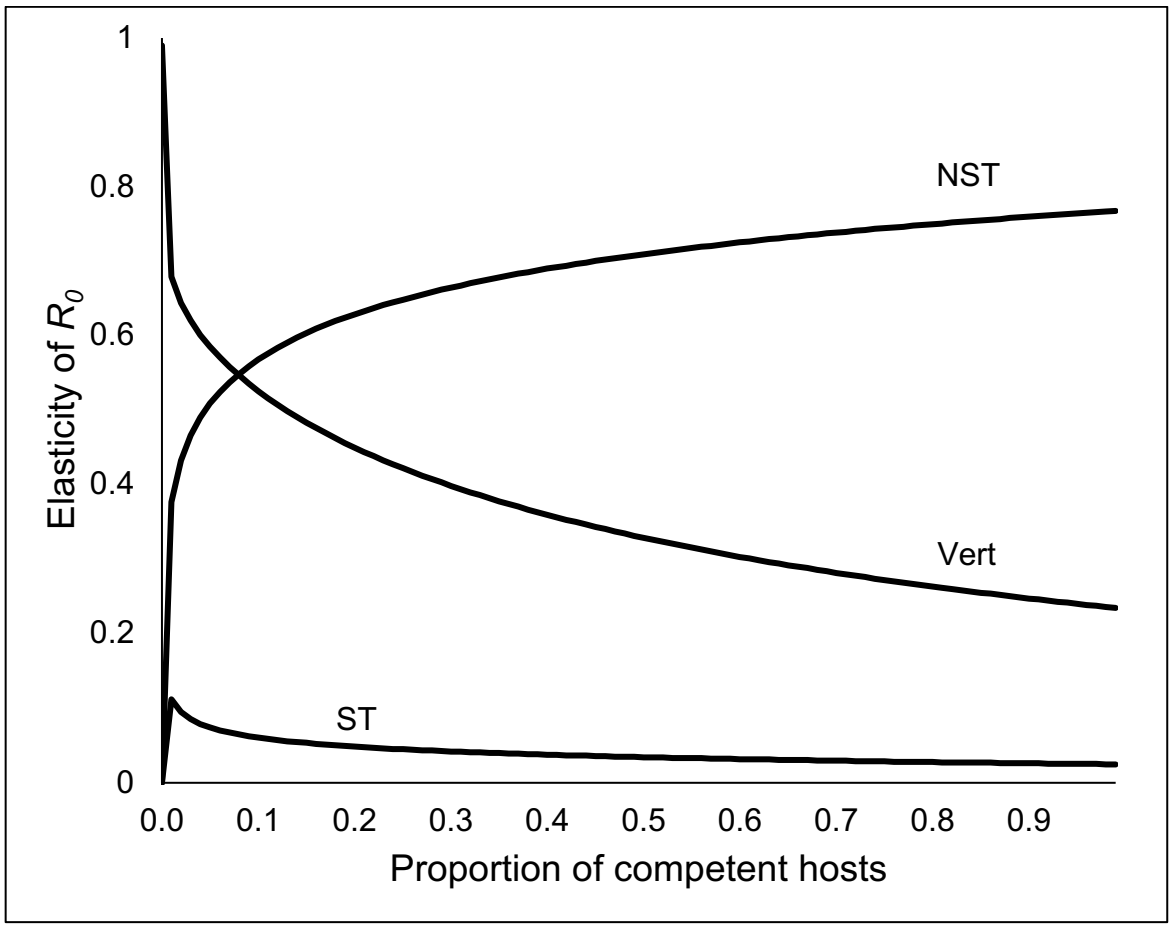


Figure 5.5. Relative contribution to R_0 of *Borrelia miyamotoi* from systemic (ST), non-systemic (NST; co-feeding), and vertical (Vert) transmission. Elasticity of R_0 for systemic (ST), non-systemic (NST; co-feeding), and vertical (Vert) transmission plotted as a function of the fraction of blood meals taken on competent hosts (h_c) for *Borrelia miyamotoi*.



Scheme 5.1. Structure of the next generation model.

$$K = \begin{Bmatrix} k_{11} & k_{12} & k_{13} & k_{14} & 0 \\ k_{21} & k_{22} & k_{23} & 0 & k_{25} \\ k_{31} & k_{32} & k_{33} & 0 & k_{35} \\ k_{41} & k_{42} & k_{43} & 0 & k_{45} \\ k_{51} & k_{52} & k_{53} & 0 & 0 \end{Bmatrix}$$

Scheme 5.2. Schematic version of the matrix indicating related transmission routes at the type-at-birth

$$\begin{pmatrix} vertical & vertical & vertical & vertical & 0 \\ cofeeding & cofeeding & cofeeding & 0 & host \rightarrow L \\ cofeeding & cofeeding & cofeeding & 0 & host \rightarrow N \\ cofeeding & cofeeding & cofeeding & 0 & host \rightarrow A \\ tick \rightarrow host & tick \rightarrow host & tick \rightarrow host & 0 & 0 \end{pmatrix}$$

Scheme 5.3. List of equations used to calculate the expected number of new cases at the type-at-birth

$$k_{11} = s_L s_N s_A Er_A$$

$$k_{12} = s_N s_A Er_A$$

$$k_{13} = s_A Er_A$$

$$k_{14} = Er_A$$

$$k_{15} = 0$$

$$k_{21} = (s_L \theta_{LL} C_{LL} + s_L s_N \theta_{NL} C_{LN} + s_L s_N s_A \theta_{AL} C_{LA}) h_c$$

$$k_{22} = (s_N \theta_{NL} C_{LN} + s_N s_A \theta_{AL} C_{LA}) h_c$$

$$k_{23} = (s_A \theta_{AL} C_{LA}) h_c$$

$$k_{24} = 0$$

$$k_{25} = \frac{p_L i N_{LH}}{D_L}$$

$$k_{31} = (s_L \theta_{LN} C_{NL} + s_L s_N \theta_{NN} C_{NN} + s_L s_N s_A \theta_{AN} C_{NA}) h_c$$

$$k_{32} = (s_N \theta_{NN} C_{NN} + s_N s_A \theta_{AN} C_{NA}) h_c$$

$$k_{33} = (s_A \theta_{AN} C_{NA}) h_c$$

$$k_{34} = 0$$

$$k_{35} = \frac{p_N i N_{NH}}{D_N}$$

$$k_{41} = (s_L \theta_{LA} C_{AL} + s_L s_N \theta_{NA} C_{AN} + s_L s_N s_A \theta_{AA} C_{AA}) h_c$$

$$k_{42} = (s_N \theta_{NA} C_{AN} + s_N s_A \theta_{AA} C_{AA}) h_c$$

$$k_{43} = (s_A \theta_{AA} C_{AA}) h_c$$

Scheme 5.3 (cont'd)

$k_{44} = 0$

$k_{45} = \frac{p_A i N_{AH}}{D_A}$

$k_{51} = (s_L q_L + s_L s_N q_N + s_L s_N s_A q_A) h_c$

$k_{52} = (s_N q_N + s_N s_A q_A) h_c$

$k_{53} = s_A q_A h_c$

$k_{54} = 0$

$k_{55} = 0$

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