Prevalence of *Borrelia burgdorferi* and *Anaplasma phagocytophilum* in *Ixodes scapularis* (Acari: Ixodidae) Nymphs Collected in Managed Red Pine Forests in Wisconsin

XIA LEE,[1] DAVID R. COYLE,[1,2] DIEP K. HOANG JOHNSON,[3] MATTHEW W. MURPHY,[4] MICHAEL A. MCGEEHIN,[4] ROBERT J. MURPHY,[5] KENNETH F. RAFFA,[1] AND SUSAN M. PASKEWITZ[1,6]

J. Med. Entomol. 51(3): 694–701 (2014); DOI: http://dx.doi.org/10.1603/ME13140

ABSTRACT Changes in the structure of managed red pine forests in Wisconsin caused by interacting root- and stem-colonizing insects are associated with increased abundance of the blacklegged tick, *Ixodes scapularis* Say, in comparison with nonimpacted stands. However, the frequency and variability of the occurrence of tick-borne pathogens in this coniferous forest type across Wisconsin is unknown. Red pine forests were surveyed from 2009 to 2013 to determine the prevalence of *Borrelia burgdorferi* and *Anaplasma phagocytophilum* in questing *I. scapularis* nymphs. Polymerase chain reaction analysis revealed geographical differences in the nymphal infection prevalence (NIP) of these pathogens in red pine forests. In the Kettle Moraine State Forest (KMSF) in southeastern Wisconsin, NIP of *B. burgdorferi* across all years was 35% (range of 14.5–53.0%). At the Black River State Forest (BRSF) in western Wisconsin, NIP of *B. burgdorferi* across all years was 26% (range of 10.9–35.5%). Differences in NIP of *B. burgdorferi* between KMSF and BRSF were statistically significant for 2010 and 2011 and for all years combined ($P < 0.05$). NIP of *A. phagocytophilum* (human agent) averaged 9% (range of 4.6–15.8%) at KMSF and 3% (range of 0–6.4%) at BRSF, and was significantly different between the sites for all years combined ($P < 0.05$). Differences in coinfection of *B. burgdorferi* and *A. phagocytophilum* were not statistically significant between KMSF and BRSF, with an average of 3.4% (range of 1.7–10.5%) and 2.5% (range of 0–5.5%), respectively. In 2013, the density of infected nymphs in KMSF and BRSF was 14 and 30 per 1000m^2, respectively, among the highest ever recorded for the state. Differences in the density of nymphs and NIP among sites were neither correlated with environmental factors nor time since tick colonization. These results document significant unexplained variation in tick-borne pathogens between coniferous forests in Wisconsin that warrants further study.

KEY WORDS blacklegged tick, coinfection, Lyme disease, anaplasmosis, *Anaplasma phagocytophilum* variant 1

Lyme disease (LD), caused by a pathogen transmitted by the blacklegged tick *Ixodes scapularis* Say, is the most commonly reported vector-borne disease in the United States. Wisconsin is one of the 13 northeastern and upper Midwestern states that consistently reports high incidence of LD. The average annual incidence of confirmed LD in Wisconsin during 2006–2010 was 32.9 cases per 100,000 persons, compared with 9.2 during 1995–1999, a 3.6-fold increase. In 2012, there was a change in the surveillance guidelines for reporting LD cases to the Wisconsin Division of Public Health. Even with the lowered surveillance levels, 1,766 confirmed and probable cases were reported (Wisconsin Division of Public Health 2013), and this may be underestimated by as much as 10-fold (Kuehn 2013). The incidence of human anaplasmosis, caused by *Anaplasma phagocytophilum* transmitted by *I. scapularis*, is also increasing. Minnesota and Wisconsin account for >70% of all reported cases of human anaplasmosis in the United States, with Wisconsin reporting 516 total cases (incidence of 9.03 cases per 100,000 persons) in 2012 (Centers for Disease Control and Prevention [CDC] 2012, Wisconsin Division of Public Health 2013, Minnesota Department of Health 2013). Both enhanced reporting and changes in disease ecology may be contributing to the increased incidence (Lee et al. 2013).

Although *I. scapularis* and tick-borne diseases are common in the upper Midwest, there have been few studies examining the relationship between habitat and acarological risk in the region. One exception was

[1] Department of Entomology, University of Wisconsin, Madison, WI 53706.
[2] Current address: D.B. Warnell School of Forestry and Natural Resources, University of Georgia, Athens, GA 30602.
[3] Wisconsin Department of Health Services, Division of Public Health, Madison, WI 53701.
[4] National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA 30341.
[5] Wisconsin Department of Natural Resources, Madison, WI 53707.
[6] Corresponding author, e-mail: paskewit@entomology.wisc.edu.

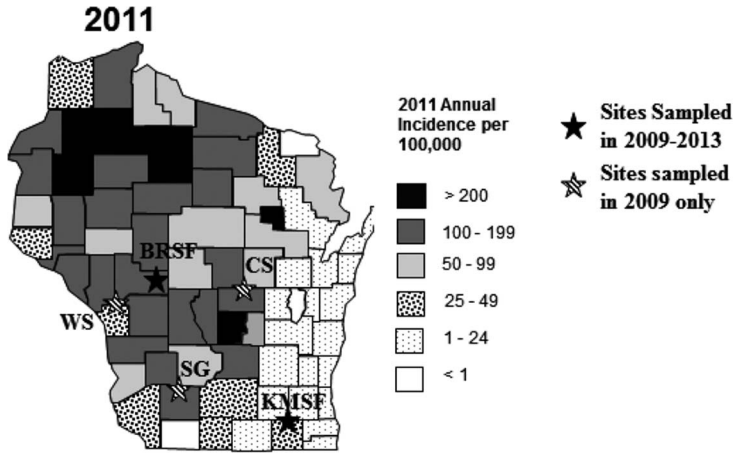

Fig. 1. Map of Wisconsin with LD incidence by county in 2011 (last year with available county-level incidence data) and sampling sites from 2009 to 2013. Incidence data provided by the Wisconsin Division of Public Health. BRSF, Black River State Forest; WS, West Salem; SG, Spring Green; CS, Central Sands; and KMSF, Kettle Moraine State Forest.

the work by Guerra et al. (2002), who sampled 138 locations in Wisconsin, Illinois, and Michigan during the 1990s and determined that tick presence was associated with certain soil types, grasslands, and specific forest types. Specifically, tick densities were highest in oak forests and lowest in conifer-dominated forests. Approximately 22% of Wisconsin forests are dominated by conifers, including spruce–fir (9%), pine (9%), and mixed oak–pine (4%). Recently, we described the surprising finding that managed red pine (*Pinus resinosa* Solander ex Aiton) stands in Wisconsin support *I. scapularis* populations at levels similar to many deciduous forests (range of 8–28 nymphs per $1000m^2$; Diuk-Wasser et al. 2012), partly because of insect-caused disturbance (Coyle et al. 2013). The study sites included both healthy stands and stands that have undergone structural changes in their plant communities caused by native insect–fungal complexes. Cascading effects due to root-feeding insects and their fungal symbionts lead to the death of red pine trees, causing gaps in the stands (Klepzig et al. 1991, Aukema et al. 2010). These openings are colonized by early successional plants (Aukema et al. 2010), and seem likely to provide favorable habitat for white-tailed deer (*Odocoileus virginianus*) and white-footed mice (*Peromyscus* spp.), which may contribute to the modest increases in *I. scapularis* densities that were measured in those stands (Coyle et al. 2013). These stands are currently managed for multiple uses depending on their location, and visitors to them include foresters, government employees, hunters, and recreational users.

Nymphal infection prevalence (NIP) for *Borrelia burgdorferi* and *A. phagocytophilum* did not differ between diseased and disease-free stands (Coyle et al. 2013). However, there were striking variations in NIP among locations in 2009, with higher prevalence of both pathogens in red pine stands located in southeastern than in central and western Wisconsin. To further explore the patterns of acarological risk in red pine forests, we report the results of multiyear surveys for *B. burgdorferi* and *A. phagocytophilum* in questing nymphs collected from these forests from 2009 to 2013.

Materials and Methods

Study Sites. Ticks were collected from 31 managed mature red pine stands in five regions of Wisconsin in 2009. These stands are located in West Salem, Black River State Forest (BRSF), Central Sands, Kettle Moraine State Forest (KMSF), and Spring Green (Fig. 1). During 2010–2013, additional samples were collected from KMSF in Walworth County and BRSF in Jackson County (Fig. 1) to explore spatial and temporal differences in infection prevalence. KMSF (Southern Unit) is a landscape that encompasses 8,903 ha of hills, kettles, and lakes carved out by the last ice age, located near the southern edge of the *I. scapularis* range expansion in Wisconsin (Fig. 1; Lee et al. 2013). *I. scapularis* populations have been described in this forest since the middle 1990s (Guerra et al. 2002, Caporale et al. 2005). BRSF and West Salem are situated within west–central Wisconsin, where *I. scapularis* has been detected since 1981 (Davis et al. 1984). These sites lie at the edge of the glaciated central plains, encompassing 27,518 ha of mixed hardwood forest. The Central Sands region is located between the boundaries of Portage, Waupaca, and Waushara Counties in Central Wisconsin, and Spring Green is located within Iowa County in southwestern Wisconsin (Fig. 1). The presence of *I. scapularis* in these latter two regions was detected in 1981 (Davis et al. 1984). The incidence of LD in humans varies considerably across the areas where the sites are located (Fig. 1).

Weather Data. Weather data were acquired from the National Oceanic and Atmospheric Administration (http://www.noaa.gov/). The closest site to KMSF was a station located 16 miles away in the city of Whitewater, WI. For BRSF, precipitation data were available for the nearby city of Black River Falls, WI.

Table 1. PCR oligonucleotide primers used in this study, from Caporale and Kocher (1994) and Steiner et al. (2006)

Primer name	Species	Gene	Nucleotide sequence (5′–3′)
FOspB-1	*B. burgdorferi*	OspB operon	GGTGCTGAGTCAATTGGTTCT
ROspB-2			TTCTAGGCTGGTTCCAGCTGT
Nested			
OspB-3			TTTTCCGACTACAAGACTTCC
OspB-4			TTAGAAGCATTTGATGCCAGC
ge3a	*A. phagocytophilum*	16S rRNA	CACATGCAAGTCGAACGGATTATTC
ge10r			TTCCGTTAAGAAGGATCTAATCTCC
Nested			
ge9f			AACGGATTATTCTTTATAGCTTGCT
ge2			GGCAGTATTAAAAGCAGCTCCAGG

The closest weather station was located 40 miles away in Dodge, WI.

Tick Collections. Questing *I. scapularis* ticks were collected by dragging a 1-m^2 white flannel cloth along the forest floor. In 2009, drags were checked every 50 m, while in 2012 and 2013, drags were checked every 10 m to reduce the number of ticks that were lost during the dragging process. In 2010 and 2011, drags were checked after 1 min rather than by distance because of limited staffing. Sampling was continued until at least 100 nymphal *I. scapularis* were collected. Thus, the density of nymphs (DON) and the density of infected nymphs (DIN) were not calculated for these two years, but the NIP is still obtainable. During 2009, sampling was conducted in June, July, and August. During 2010, 2011, and 2013, ticks were collected during the peak of the nymphal activity period in June. Few ticks were collected in 2012, when dragging did not occur until early July.

In 2009, ticks were collected along the edge of a diseased tree pocket (inside ring) and 10 m away from the edge (outside ring; Coyle et al. 2013). In disease-free stands, a permanently marked central point was established and tick drags were performed in rings established 25 m and 35 m away from the point. Because pockets varied in size, the number of 50-m^2 drags varied between 2 and 13. There were 21 symptomatic sites and 10 asymptomatic sites (Coyle et al. 2013).

Because there were no differences in NIP between symptomatic and asymptomatic forests (Coyle et al. 2013), we chose one symptomatic stand in KMSF and an area adjacent to an asymptomatic stand in BRSF for repeated sampling. These stands were selected because of similar understory and vegetation density. Drags were carried out along random transects or trails to maximize nymphal collections and were checked after each minute of dragging in 2010–2011. At least 1000 m^2 was sampled at each stand.

In 2013, we established 1-ha grids at BRSF and KMSF to facilitate small mammal trapping for future studies. Ticks were sampled along 100-m transects spaced every 10 m. Grids were established within 200 m of the sampling locations for 2010 and 2011 in areas that were matched for density of shrubs, saplings, and other vegetation.

Any ticks found on the cloth drag were removed by forceps and placed alive into 8-ml tubes. All ticks were transported to the University of Wisconsin–Madison Medical Entomology laboratory for immediate identification and storage at −20°C before DNA extraction.

DNA Extraction. DNA extraction, polymerase chain reaction (PCR) amplification, gel electrophoresis, and gel visualization were each done in separate rooms and under strict conditions with dedicated pipettes and aerosol-resistant filter pipette tips to reduce the risk of contamination. Nymphal ticks were sterilized in 100% ethanol for 5 min, placed into 1.7-ml centrifuge tubes, and bisected using a sterile 18-gauge hypodermic needle. Extraction was done using a DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) following the Animal Tissue (Spin-Column) protocol provided by the manufacturer. The resulting DNA sample was aliquoted with one part of the sample placed at −80°C for long-term storage and the remainder stored at −20°C for immediate use.

PCR Analysis. PCR was performed using EconoTaq Plus Green 2× Master Mix (Lucigen Corporation, Middleton, WI). Each PCR reaction contained a total volume of 25 μl using 1.25 U of EconoTaq DNA polymerase, reaction buffer, 400 μM dATP, 400 μM dGTP, 400 μM dCTP, 400 μM dTTP, 3 mM $MgCl_2$, 1 pmol/μl of each primer pair, and 1 μl of DNA template. Primer sequences used for detection of *B. burgdorferi* and *A. phagocytophilum* are listed in Table 1. At least two negative controls containing nuclease-free water were included in each PCR set to detect contamination. Positive controls were also included. Reaction products were separated by gel electrophoresis in 1% agarose gels in 1× TAE buffer and gels were stained with ethidium bromide before visualization with ultraviolet transillumination.

Detection of *B. burgdorferi*. A nested PCR assay was performed on nymphs using the primer pair FOspB-1–ROspB-2 for the primary reaction and OspB-3–OspB-4 for the nested reaction targeting a 200-bp fragment of the OspB operon gene segment. One microliter of the primary reaction was used as a template for the nested PCR reaction. The cycling conditions were initial denaturation at 94°C for 30s and 30 cycles each of denaturation at 93°C for 30s, annealing for 1 min at 52°C, and extension at 72°C for 2 min. An additional final extension at 72°C for 10 min was performed (Caporale and Kocher 1994). The same cycling conditions were used for both the primary and nested reactions. All positive samples from 2010 and 2011 were sequenced in both directions to confirm that amplicons were from *B. burgdorferi* rather than a

congeneric species (e.g., *Borrelia miyamotoi* or *Borrelia bissettii*).

Detection of *A. phagocytophilum*. A nested PCR assay using the primer pair ge3a–ge10r for the primary reaction and the primer pair ge9f–ge2 for the nested reaction targeting a 546-bp fragment of the 16S rRNA gene was performed for nymphs (Steiner et al. 2006). The nested reaction used 1 μl of the primary reaction as a template. The cycling conditions were performed according to Steiner et al. (2006). All positive PCR products were extracted and sequenced to determine the *A. phagocytophilum* genotype variants. At least two variants of *A. phagocytophilum* exist in Wisconsin, the human agent (AP-ha) and the variant 1 (AP-variant 1). The human agent, as the name implies, is the cause of human anaplasmosis. AP-variant 1 uses white-tailed deer as its host and does not infect white-footed mice or humans (Belongia et al. 1997, Massung et al. 2003).

DNA Sequencing. DNA from positive PCR products was extracted and purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA). Sequencing reactions were run using BigDye v.3.1, and sent to the University of Wisconsin–Madison Biotechnology Center for sequencing.

Data Analyses. DON is reported as the mean number of nymphs per 1,000 m^2. NIP is reported as the percentage of nymphs infected with a pathogen, and DIN is reported as the mean number of infected nymphs per 1,000 m^2. DON and DIN are reported for 2009 and 2013 only, whereas NIP is reported for all 5 yr. The expected probability of coinfection was calculated as the product of the prevalence of each pathogen individually. For each year, differences in NIP of *B. burgdorferi* and *A. phagocytophilum* from KMSF and BRSF as well as the actual and expected coinfection numbers for each stand were compared using Fisher's exact test, which is preferred over chi-square tests when some cells in the data table have values <10. Chi-square goodness-of-fit tests with Yates' correction were used for comparisons based on summarizing data from all years. All results were considered significant when $P < 0.05$.

Results

DON, NIP, and DIN for *B. burgdorferi*. In total, 1,190 *I. scapularis* nymphs were collected from 2009 to 2013. Of these, 411 were collected from KMSF and 672 were collected from West Salem and BRSF; the remaining 107 nymphs were collected from Spring Green and Central Sands in 2009.

All 31 stands at the five locations were sampled in 2009. The number of stands and total distance sampled at each location are summarized in Table 2. For this year, the DON was highest at Spring Green with 28 nymphs per 1,000 m^2 and was ≈3.5 times higher than that at Central Sands and KMSF. Conversely, NIP for *B. burgdorferi* was highest at KMSF (47.4%) and was 2–3 times higher than the NIPs at the other locations (Table 2). DIN varied from 2 to 5 infected nymphs per 1,000 m^2 across the five locations.

Table 2. Estimates of *I. scapularis* nymphal density and pathogen infection prevalence for 31 mature red pine plantations at five locations in Wisconsin in 2009

	West Salem	Black River	Kettle Moraine	Spring Green	Central Sands
Total nymphs collected	282	30	47	58	49
Total distance sampled (m^2)	10,042	2,524	5,592	2,052	6,070
Number of stands	11	3	6	3	8
BB NIP (Infected Nymphs/Total Nymphs) (%, 95% confidence interval)	58/282 (20.6, 16.1–25.9)	4/30 (13.3, 4.4–31.6)	20/47 (42.6, 28.6–57.7)	8/58 (13.8, 6.6–25.9)	13/49 (26.5,15.4–41.1)
AP NIP (Infected Nymphs/Total Nymphs) (%, 95% confidence interval)	7/282 (2.5, 1.1–5.3)	0/30 (0, 0–14.1)	5/47 (10.6, 4–23.9)	0/58 (0, 0–7.7)	3/49 (6.1, 1.6–17.9)
DON (Nymphs/1,000 m^2)[a]	28	12	8	28	8
BB DIN (Infected nymphs/1,000 m^2)	6	2	4	4	2

NIP, nymphal infection prevalence; DON, density of nymphs; DIN, density of infected nymphs; BB, *B. burgdorferi*; AP, *A. phagocytophilum*–human agent.

[a] Data from Coyle et al. (2013).

While NIP was strikingly high at KMSF in 2009, the number of nymphs collected was low at this and other locations. To improve confidence in NIP estimates in subsequent years, collections were focused on one stand each in BRSF and KMSF, and attempts were made to collect at least 100 nymphs each year during the peak of the season. In Table 3, these results as well as the data for these single stands for 2009 are reported. During 2010 and 2011, NIP was significantly higher at the KMSF stand (Table 3). In 2012, few nymphs were collected (one from BRSF [uninfected] and none from KMSF). In 2013, NIP was not significantly different between these stands (14.5% at KMSF and 23% at BRSF [$P = 0.48$]).

In 2013, a team of students established and sampled 1-ha grids at both locations. The number of nymphs collected from 1-ha grids was high (DON = 130/1,000 m^2 for BRSF and 90/1,000 m^2 for KMSF; DIN = 14 at KMSF and 30 at BRSF).

Sequencing of all positives samples from KMSF and BRSF in 2010 and 2011 identified *B. burgdorferi* sensu stricto. None of the congeneric genotypes were present.

Detection of *A. phagocytophilum* and Coinfection Prevalence. The prevalence of AP-ha was compared among all five locations in 2009. Nearly 10% of nymphs sampled at KMSF were infected with this pathogen, while prevalence at Central Sands (6%) and West Salem (2.5%) was much lower (Table 2). There were no positive nymphs from stands located at Spring Green or BRSF.

For the single stand comparisons from 2009 to 2011 and 2013, infection prevalence for AP-ha was always higher at the KMSF stand than for the BRSF stand (Table 3). This difference was significant when all years were combined ($P = 0.006$), but not for any individual year. No ticks were infected with this pathogen in 2012.

Sequencing of all positive samples from KMSF and BRSF from 2010 to 2013 confirmed the presence of both AP-ha and AP-variant 1. Of the 47 ticks that tested positive for *A. phagocytophilum*, 6 (12.8%) were identical to AP-variant 1. These included one sample from KMSF in 2011, one sample from BRSF, and four samples from KMSF in 2013. All others from 2010 to 2013 were identical to AP-ha.

There were significant differences in coinfection prevalence between sites in 2009, with coinfection prevalence for AP-ha and *B. burgdorferi* at KMSF ≈16 times higher than West Salem (data not shown). However, coinfection prevalence in 2010, 2011, and 2013 was similar at both KMSF and BRSF ($P = 1$, Table 3). Coinfection prevalence did not deviate significantly within a site from the expected prevalence based on the prevalence of the individual pathogens.

Table 3. Infection and coinfection prevalence in *I. scapularis* nymphs collected from a single stand repeatedly sampled at KMSF and BRSF from 2009 to 2013

Sampling year	KMSF			BRSF		
	B. burgdorferi (Infected nymphs by total nymphs) (%, 95% CI)	*A. phagocytophilum*[a] (Infected nymphs bytotal nymphs) (%, 95% CI)	*B. burgdorferi*/*A. phagocytophilum*[a] Coinfection prevalence (infected nymphis bytotal nymphs) (%, 95% CI)	*B. burgdorferi* (Infected nymphs by total nymphs) (%, 95% CI)	*A. phagocytophilum*[a] (Infected nymphs by total nymphs) (%, 95% CI)	*B. burgdorferi*/*A. phagocytophilum*[a] Coinfection prevalence (infected nymphs by total nymphs) (%, 95% CI)
2009	9/19 (47.4, 25.2–70.5)	3/19 (15.8, 4.2–40.5)	2/19 (10.5, 1.8–34.5)	2/6 (33.3, 6–75.9)	0/6 (0, 0–39)	0/6 (0, 0–39)
2010[b]	62/117 (53.0, 43.6–62.2)	13/117 (11.1, 6.3–18.6)	7/117 (6, 2.6–12.3)	39/110 (35.5, 26.7–45.2)	7/110 (6.4, 2.8–13.1)	6/110 (5.5, 2.2–12)
2011[b]	43/130 (33.1, 25.2–41.9)	5/130 (3.8, 1.4–9.2)	2/130 (1.5, 0.2–6)	14/128 (10.9, 6.3–18)	1/128 (0.8, 0.04–4.9)	1/128 (0.8, 0.04–4.9)
2013	17/117 (14.5, 8.9–22.5)	9/117 (7.7, 3.8–14.5)	2/117 (1.7, 0.2–6.7)	28/121 (23.1, 16.2–31.9)	3/121 (2.5, 0.6–7.6)	2/121 (1.7, 0.3–6.4)

At least 1,000 m^2 was sampled at each location.

[a] *A. phagocytophilum*–human agent.

[b] Years where *B. burgdorferi* infection prevalence were statistically significant, $P < 0.05$.

Discussion

Although coniferous forests and managed pine plantations are not usually considered highly suitable habitat for *I. scapularis* ticks (Ginsberg and Zhioua 1996, Guerra et al. 2002, Bunnell et al. 2003, Carroll

2003), the results documented tick populations and infection prevalence for tick-borne pathogens in Wisconsin red pine forests that are similar to or higher than those reported for other Midwestern state parks and forests (Shukla et al. 2003; Diuk-Wasser et al. 2010, 2012; Hamer et al. 2012). For example, Hamer et al. (2012) reported peak June densities of 34 nymphs per 1,000 m^2 and DIN of 4 infected nymphs per 1,000 m^2 in an oak-dominated state park in southwestern Michigan. In the current study, DONs ranged from 8 to 130 nymphs per 1,000 m^2 and DIN from 1.6 to 30 infected nymphs per 1,000 m^2 depending on the year and site. When red pine plantations are established, trees are planted in parallel rows for ease of maintenance and harvest. Undisturbed plantations are characterized by a litter layer of pine needles and little understory vegetation and do not support many *I. scapularis.* However, pine plantations often experience disturbance when trees die due to weather or insect damage, and these losses create more diverse environments where *I. scapularis* can thrive. Studies in natural red cedar, pitch pine, white pine, and mixed hardwood–pine forests from New Jersey and Maryland also documented instances where conifer forests supported tick populations that exceeded those in deciduous forest (Lord 1995, Schulze et al. 1998, Carroll 2003).

There have been few studies of the prevalence of AP-ha in Wisconsin, although 30% of human anaplasmosis cases in the United States occurred in the state in 2010 (the last year for which national statistics are available). The NIP of *A. phagocytophilum* in ticks from BRSF was similar to nymphal prevalences reported by Shukla et al. (2003) for four state parks in Wisconsin (3–4%). However, AP-ha NIP at KMSF was consistently higher, averaging 9.5% across the 4 yr. Similarly, *B. burgdorferi* NIP tended to be higher in KMSF (average of 35.5%) in comparison with BRSF (average of 25.8%). Recent colonization by ticks might be associated with lower infection prevalence. However, neither DON nor NIP was low at KMSF, the most recently colonized of the sites. This suggests that ticks and pathogen cycles can both become well-established in an area within a decade of tick colonization. The most likely explanation for consistently higher pathogen prevalence at KMSF is that the reservoir community differs between KMSF and BRSF. Mice and other small mammals play key roles as reservoirs for both *A. phagocytophilum* and *B. burgdorferi.* Track plate assessments in 2010 and small mammal trapping in 2013 verified the presence of *Peromyscus* sp. mice and *Myodes gapperi* (red-backed voles) at both sites and of *Tamias striatus* (eastern chipmunks) at KMSF (S.M.P., unpublished data). Future work will focus on examining the impact of these potential reservoirs and hosts on pathogen dynamics.

The occurrence of AP-variant 1 in questing nymphs is an indicator that larvae might be feeding on white-tailed deer. AP-variant 1 has been isolated from white-tailed deer and is not thought to infect white-footed mice or other small mammals (Belongia et al. 1997, Massung et al. 2003). This variant is not associated with human or canine disease. We found AP-variant 1 in nymphs collected at both KMSF and BRSF. In 2013, 31% of the positive samples from KMSF were AP-variant 1 and this was 3.4% of all nymphs from that site. By inference, a minimum of 3–4% of the larvae that survived to nymphal stages may have fed on deer as larvae in 2012 at the KMSF stand. This raises several interesting questions. First, while deer are known to host immature stages of *I. scapularis* (Watson and Anderson 1976, Main et al. 1981), they are generally regarded as unimportant in this regard (Ostfeld 2011). Our results document 3–4% as a lower bound for 2012 at KMSF, but use of deer could be much higher because larvae would also feed on uninfected animals. Second, the processes leading to the elevated levels of AP-variant 1 NIP observed in 2013 are unknown. We are unable to determine whether infected ticks derived from larvae feeding on a single animal that was active in the stand in 2012 or from multiple animals, which might indicate a deer population-level phenomenon. It is also possible that ecological conditions during 2012–2013 affected tick behavior or survivorship of infected ticks. The year 2012 was extreme in two regards. First, spring warming occurred earlier in the year than had been documented before, resulting in an early spring and a prolonged period that was likely suitable for *I. scapularis* host-seeking. Temperatures averaged 5.8–6.8°C (10–12°F) warmer than the 30-yr average during January through March of 2012 in southern and central Wisconsin, and warmer weather continued during April through June. Larvae are most abundant during the spring and early summer in Wisconsin, and it is conceivable that this unusual season impacted host availability for this stage. For example, the absence of an insulating snow cover may have increased the mortality of important small mammal hosts due to predation or heat loss. In addition, moderate to severe drought occurred at both locations during the spring and summer of 2012. The precipitation for each month from June through August ranged from 5.1 to 8.8 cm below the 30-yr average for the weather station nearest to KMSF. Drought was less pronounced at BRSF, but July precipitation was only 1.4 cm compared with normal precipitation between 7.6 and 12.7 cm. Studies have shown that infection with pathogens can alter tick physiology in ways that affect survival under laboratory conditions of low humidity or unfavorable temperature (Hermann and Gern 2010, Neelakanta et al. 2010). Although the studies are intriguing, the significance of these observations for AP-variant 1 or other pathogens effects on nymphal survivorship under field conditions is unclear. However, it is possible that fed larvae or the resulting nymphal ticks infected with the AP deer variant strain were more likely to survive the dry conditions of 2012.

The unusual weather in 2012 may also have impacted DON in 2013. Although we were unable to perform density estimates for all years, data are available for 2009 and 2013. Distributed sampling across 31 stands at five locations in 2009 yielded DON estimates of 8–28 nymphs per 1000 m^2, a range that is similar to estimates achieved by other studies (Diuk-Wasser et al. 2012). More focused sampling in 2013 resulted in

much higher estimates of 90–130 nymphs per 1,000 m^2 at KMSF and BRSF. Although this could be the result of the altered sampling protocol, nymph density was much higher across multiple locations in Wisconsin during 2013 (S.M.P. and X. L. unpublished data). In 2013, DIN at KMSF and BRSF was 14 and 30, respectively. These estimates are among the highest ever recorded for Wisconsin forests (M. A. Diuk-Wasser, personal communication) and were driven by the extremely high DON. The warmer, prolonged spring of 2012 may have increased successful host-seeking by larval ticks, resulting in elevated populations of nymphs in 2013. Considerable interannual variation in DON and DIN was also documented for deciduous forests by Diuk-Wasser et al. (2012). Additional work is needed to understand the underlying causes of interannual and interforest variation in DON in both coniferous and deciduous forests.

The presence of infected ticks in an area does not necessarily imply that human exposure is occurring. Risk is a result of DIN as well as patterns of use at a location. At BRSF, the area under study is primarily used for recreational all-terrain vehicles and by foresters. The primary risk in this area may be to homeowners who live within similar forests nearby and to forest managers who are exposed as a result of their occupation. In contrast, the KMSF stand is located in an area that is heavily used by the public for horseback riding, hiking, dog walking, and mountain biking. The Wisconsin Department of Natural Resources estimated ≈1.3 million visitors to the Kettle Moraine Southern Unit (the Unit under study) during 2012, with peak attendance during June and July. Sampling along random transects and trails at this location in 2010 and 2011 demonstrated the potential for exposure, as sampling was frequently interrupted by passersby. Expanded education and outreach in these areas may help to reduce exposure and the incidence of tick-borne disease in these forests.

Acknowledgments

We thank Scott Larson and University of Wisconsin–Madison undergraduate students Justin Berg, Amanda Maegli, Colin Sinnot, Karli Reifschneider, Charles Opitz, Oliver MacDonald, and Elizabeth Hemming for their assistance with tick collecting and PCR analysis. We also thank Mike Sieger and Peter Bakken, site managers for KMSF and BRSF, respectively, for their cooperation in this project. Funding for this project was provided by the CDC, National Center for Environmental Health.

References Cited

Aukema, B. H., J. Zhu, J. Møller, J. G. Rasmussen, and K. F. Raffa. 2010. Predisposition to bark beetle attack by root herbivores and associated pathogens: roles in forest decline, gap formation, and persistence of endemic bark beetle populations. For. Ecol. Manage. 259: 374–382.

Belongia, E. A., K. D. Reed, P. D. Mitchell, C. P. Kolbert, D. H. Persing, J. S. Gill, and J. J. Kazmierczak. 1997. Prevalence of granulocytic *Ehrlichia* infection among white-tailed deer in Wisconsin. J. Clin. Microbiol. 35: 1465–1468.

Bunnell, J. E., S. D. Price, A. Das, T. M. Shields, and G. E. Glass. 2003. Geographic information systems and spatial analysis of adult *Ixodes scapularis* (Acari: Ixodidae) in the middle Atlantic region of the U.S.A. J. Med. Entomol. 40: 570–576.

Caporale, D. A., and T. D. Kocher. 1994. Sequence variation in the outer-surface-protein genes of *Borrelia burgdorferi.* Mol. Biol. Evol. 11: 51–64.

Caporale, D. A., C. M. Johnson, and B. J. Millard. 2005. Presence of *Borrelia burgdorferi* (Spirochaetales: Spirochaetaceae) in southern Kettle Moraine State Forest, Wisconsin, and characterization of strain W97F51. J. Med. Entomol. 42: 457–472.

Carroll, J. F. 2003. Survival of larvae and nymphs of *Ixodes scapularis* Say (Acari: Ixodidae) in four habitats in Maryland. Proc. Entomol. Soc. Wash. 105: 120–126.

(CDC) Centers for Disease Control and Prevention. 2012. Lyme Disease. (http://www.cdc.gov/lyme/).

Coyle D. R., M. W. Murphy, S. M. Paskewitz, J. L. Orrock, X. Lee, R. J. Murphy, M. A. McGeehin, and K. F. Raffa. 2013. Belowground herbivory initiates a cascade that increases abundance of Lyme disease vectors. For. Ecol. Manage. 302: 354–362.

Davis, J. P., W. L. Schell, T. E. Amundson, M. S. Godsey, A. Spielman, W. Burgdorfer, A. G. Barbour, M. LaVenture, and R. A. Kaslow. 1984. Lyme disease in Wisconsin: epidemiologic, clinical, serological, and entomological findings. Yale J. Biol. Med. 57: 685–696.

Diuk-Wasser, M. A., G. Vourc'h, P. Cislo, A. G. Hoen, F. Melton, S. A. Hamer, M. Rowland, R. Cortinas, G. J. Hickling, J. I. Tsao, et al. 2010. Field and climate-based model for predicting the density of host-seeking nymphal *Ixodes scapularis,* an important vector of tick-borne disease agents in the eastern United States. Glob. Ecol. Biogeogr. 19: 504–514.

Diuk-Wasser, M. A., A. G. Hoen, P. Cislo, R. Brinkerhoff, S. A. Hamer, M. Rowland, R. Cortinas, G. Vourc'h, F. Melton, G. J. Hickling, et al. 2012. Human risk of infection with *Borrelia burgdorferi,* the Lyme disease agent, in eastern United States. Am. J. Trop. Med. Hyg. 86: 320–327.

Ginsberg, H. S., and E. Zhioua. 1996. Nymphal survival and habitat distribution of *Ixodes scapularis* and *Amblyomma americanum* ticks (Acari: Ixodidae) on Fire Island, New York, USA. Exp. Appl. Acarol. 20: 533–544.

Guerra, M., E. Walker, C. Jones, S. M. Paskewitz, R. Cortinas, A. Stancil, L. Beck, M. Bobo, and U. Kitron. 2002. Predicting the risk of Lyme disease: habitat suitability for *Ixodes scapularis* in the North Central United States. Emerg. Infect. Dis. 8: 289–297.

Hamer, S., G. J. Hickling, J. L. Sidge, E. D. Walker, and J. Tsao. 2012. Synchronous phenology of juvenile *Ixodes scapularis,* veterbrate host relationships, and associated patterns of *Borrelia burgdorferi* ribotypes in the Midwestern United States. Ticks Tick-borne Dis. 3: 65–74.

Hermann, C., and L. Gern. 2010. Survival of *Ixodes ricinus* (Acari: Ixodidae) under challenging conditions of temperature and humidity is influenced by *Borrelia burgdorferi* sensu lato infection. J. Med. Entomol. 47: 1196–2004.

Klepzig, K. D., K. F. Raffa, and E. B. Smalley. 1991. Association of an insect-fungal complex with red pine decline in Wisconsin. For. Sci. 37: 1119–1139.

Kuehn, B. M. 2013. CDC estimates 300,000 US cases of Lyme disease annually. JAMA 310: 1110.

Lee, X., K. Hardy, D. H. Johnson, and S. M. Paskewitz. 2013. Hunter-killed deer surveillance to assess changes in the

prevalence and distribution of *Ixodes scapularis* (Acari: Ixodidae) in Wisconsin. J. Med. Entomol. 50: 632–639.

Lord, C. C. 1995. Seasonal activity of nymphal *Ixodes scapularis* (Acari: Ixodidae) in different habitats in New Jersey. J. Med. Entomol. 32: 66–70.

Main, A. J., H. E. Sprance, K. O. Kloter, and S. E. Brown. 1981. *Ixodes dammini* (Acari: Ixodidae) on white-tailed deer (*Odocoileus virginianus*) in Connecticut. J. Med. Entomol. 18: 487–492.

Massung, R. F., R. A. Priestley, N. J. Miller, T. N. Mather, and M. L. Levin. 2003. Inability of a variant strain of *Anaplasma phagocytophilum* to infect mice. J. Infect. Dis. 188: 1757–1763.

Neelakanta, G., H. Sultana, D. Fish, J. F. Anderson, and E. Fikrig. 2010. *Anaplasma phagocytophilum* induces *Ixodes scapularis* ticks to express an antifreeze glycoprotein gene that enhances their survival in the cold. J. Clin. Invest. 120: 3179–3190.

Ostfeld, R. S. 2011. Lyme disease: The ecology of a complex system. Oxford University Press, Oxford, United Kingdom.

Schulze, T. L., R. A. Jordan, and R. W. Hung. 1998. Comparison of *Ixodes scapularis* (Acari: Ixodidae) populations and their habitats in established and emerging Lyme disease areas in New Jersey. J. Med. Entomol. 35: 64–70.

Shukla, S. K., M. F. Vandermause, E. A. Belongia, K. D. Reed, S. M. Paskewitz, and J. J. Kazmierczak. 2003. Importance of primer specificity for PCR detection of *Anaplasma phagocytophila* among *Ixodes scapularis* ticks from Wisconsin. J. Clin. Microbiol. 41: 4006.

Steiner, F. E., R. R. Pinger, C. N. Vann, M. J. Abley, B. Sullivan, N. Grindle, K. Clay, and C. Fuqua. 2006. Detection of *Anaplasma phagocytophilum* and *Babesia odocoilei* DNA in *Ixodes scapularis* (Acari; Ixodidae) collected in Indiana. J. Med. Entomol. 43: 437–442.

Watson, T. G., and R. C. Anderson. 1976. *Ixodes scapularis* Say on white-tailed deer (*Odocoileus virginianus*) from Long Point, Ontario. J. Wildlife Dis. 12: 66–71.

Received 12 July 2013; accepted 4 March 2014.