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Prevalence and Diversity of Tick-Borne Pathogens in Nymphal *Ixodes scapularis* (Acari: Ixodidae) in Eastern National Parks

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Abstract

Tick-borne pathogens transmitted by *Ixodes scapularis* Say (Acari: Ixodidae), also known as the deer tick or blacklegged tick, are increasing in incidence and geographic distribution in the United States. We examined the risk of tick-borne disease exposure in 9 national parks across six Northeastern and Mid-Atlantic States and the District of Columbia in 2014 and 2015. To assess the recreational risk to park visitors, we sampled for ticks along frequently used trails and calculated the density of *I. scapularis* nymphs (DON) and the density of infected nymphs (DIN). We determined the nymphal infection prevalence of *I. scapularis* with a suite of tick-borne pathogens including *Borrelia burgdorferi*, *Borrelia miyamotoi*, *Anaplasma phagocytophilum*, and *Babesia microti*. *Ixodes scapularis* nymphs were found in all national park units; DON ranged from 0.40 to 13.73 nymphs per 100 m^2. *Borrelia burgdorferi*, the causative agent of Lyme disease, was found at all sites where *I. scapularis* was documented; DIN with *B. burgdorferi* ranged from 0.06 to 5.71 nymphs per 100 m^2. *Borrelia miyamotoi* and *A. phagocytophilum* were documented at 60% and 70% of the parks, respectively, while *Ba. microti* occurred at just 20% of the parks. *Ixodes scapularis* is well established across much of the Northeastern and Mid-Atlantic States, and our results are generally consistent with previous studies conducted near the areas we sampled. Newly established *I. scapularis* populations were documented in two locations: Washington, D.C. (Rock Creek Park) and Greene County, Virginia (Shenandoah National Park). This research demonstrates the potential risk of tick-borne pathogen exposure in national parks and can be used to educate park visitors about the importance of preventative actions to minimize tick exposure.

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Keywords

Ixodes scapularis; *Borrelia burgdorferi*; tick-borne disease; recreational exposure

In the eastern United States, the blacklegged tick, *Ixodes scapularis* Say, is the primary vector of *Borrelia burgdorferi*, the causative agent of Lyme disease, which is the most commonly reported vector-borne disease in the United States (Mead 2015). *Ixodes scapularis* also vectors other pathogens that can cause potentially serious disease, including *Borrelia miyamotoi*, *Anaplasma phagocytophilum*, and *Babesia microti* (Barbour and Fish 1993, Homer et al. 2000, Jin et al. 2012, Krause et al. 2015). Established blacklegged tick populations are nearly continuous across counties in the Northeastern and North-Central United States where the majority of *I. scapularis*-borne disease cases are reported (Mead 2015, Eisen et al. 2016). The risk of acquiring Lyme disease is influenced by spatiotemporal variation in the density of host-seeking infected nymphs (Diuk-Wasser et al. 2012). This metric often correlates with Lyme disease incidence, though to varying degrees (Mather et al. 1996, Stafford et al. 1998, Falco et al. 1999, Pepin et al. 2012). Human behavior, including time spent in tick-infested areas or engaged in behaviors that enhance or reduce the likelihood of encounters with ticks (Orloski et al. 2000, Connally et al. 2009), also influences the likelihood of acquiring Lyme disease and may explain some of the lack of concordance between measures of density of infected host-seeking nymphs and Lyme disease incidence (Pepin et al. 2012).

Understanding where people may come into contact with infected vector-competent ticks is central to mitigating tick-borne disease risk. For example, in the Mid-Atlantic and Northeastern United States, peridomestic exposure to *I. scapularis* likely occurs frequently (Falco and Fish 1988, Maupin et al. 1991, Klein et al. 1996, Connally et al. 2006, Feldman et al. 2015), whereas in the North-Central United States, recreational exposures are believed to be more common than peridomestic exposures (Kitron and Kazmierczak 1997, Paskewitz et al. 2001). Regardless of geographic region, previous studies have demonstrated a risk of human exposure to infected host-seeking *I. scapularis* nymphs in recreational settings (Falco and Fish 1989, Schulze et al. 1992, Oliver and Howard 1998, Paskewitz et al. 2001, Han et al. 2014, Prusinski et al. 2014, Ford et al. 2015). National parks are popular recreation destinations and may represent areas of elevated acarological risk, yet one cannot adequately infer the risk of tick-borne disease for park visitors or employees from the epidemiological surveillance conducted at the county spatial scale (Eisen et al. 2013). National parks often vary ecologically from surrounding areas, and thus the density of infected ticks may differ between settings; further, human behavior within the parks may differ from behavior in surrounding communities.

In this study, we sought to characterize the acarological risk, that is, the risk of human exposure to tick-borne pathogens, in national parks in the Eastern United States. We surveyed frequently used trails in national park units across six Northeastern and Mid-Atlantic States and the District of Columbia, ranging from Maine in the north to Virginia in the south. Our collection efforts focused on the nymphal stage of *I. scapularis.* This stage likely poses the greatest threat of transmission of *B. burgdorferi* and other pathogens to

humans, as peak activity of questing nymphs occurs in late spring and early summer which coincides with peak onset of human disease (Piesman 1989, Fish 1993, Falco et al. 1999, Mead 2015). Here, we describe the diversity of ticks collected by drag sampling during summer months, density of host-seeking *I. scapularis* nymphs, and diversity and prevalence of *B. burgdorferi*, *B. miyamotoi*, *A. phagocytophilum*, and *Ba. microti* infection in *I. scapularis* nymphs.

Materials and Methods

Tick Collection

Ticks were collected between 29 May and 1 July, 2014, by drag sampling in seven National Park Service (NPS) units in the Northeastern United States. In 2015, Acadia and Shenandoah National Parks were added. Shenandoah National Park in Virginia represents an area of expansion of human Lyme disease cases as well as geographic expansion of *I. scapularis.* Acadia National Park has a history of *I. scapularis* expansion on the island and also represents a site where tick phenology differs, likely due to climate. In 2015, all sites except Acadia National Park were sampled between 27 May and 2 July (Table 1, Fig. 1). At Acadia National Park, the northern-most study site, the peak activity of questing *I. scapularis* nymphs occurs later in the year (Rand et al. 2007). Therefore, we visited each transect at this site three times; sampling was initiated in June to synchronize with other parks being sampled and sampling occurred again in mid-July and early August when peak questing activity was expected (Rand et al. 2007). In each park unit, we consulted NPS staff to choose high visitor use areas with suitable habitat and a history of reports from visitors or staff about tick abundance as our sampling sites. We aimed to sample three 750-m transects in each NPS unit twice per year. However, at Monocacy National Battlefield, only a single transect was sampled in 2014 and only two transects were sampled in 2015. Further, due to poor sampling conditions, transects at some parks were only sampled on a single occasion in a given year (Table 1). To best represent the risk to park visitors of encountering host-seeking *I. scapularis* nymphs, transects were located along established trails in closed canopy deciduous forest with ample leaf litter on or near the trail. Because we sought to maximize the number of host-seeking *I. scapularis* nymphs captured, we targeted timing and site selection to this species. Although we did not conduct phenology studies at our study sites, sampling corresponded with the period of peak *I. scapularis* nymphal host-seeking activity (Wilson and Spielman 1985, Piesman et al. 1987, Sonenshine and Mather 1994, Ostfeld et al. 1996, Diuk-Wasser et al. 2006, Gatewood et al. 2009, Orr et al. 2013) and the time of year when most human cases of Lyme disease are reported (Mead 2015). We report the highest number of nymphs encountered during a single visit to represent the “peak” density of nymphs (DON). All other ticks captured were considered incidental captures.

Ticks were collected from the leaf litter directly adjacent to the trail edge by dragging a 1-m^2 rubber-bonded cotton sheet (JoAnn Fabric #1491315) with a rope attached to a 48″ dowel inside the top edge and washers were sewn into the bottom edge to enhance ground contact. Trail transects were 750 m in length except at Manassas National Battlefield Park, where one transect was split into two 375-m segments. To minimize the number of ticks missed due to falling off the drag, we checked the drags and removed ticks every 15 m. All species

of ticks encountered were collected and preserved immediately in RNAlater (Ambion, Austin, TX) or 70% ethanol, with the exception of five *I. scapularis* nymphs from Catoctin Mountain Park that were initially collected on tape and later transferred to RNAlater. For pathogen detection, we aimed to collect at least 50 *I. scapularis* nymphs from each transect. If a total of 50 *I. scapularis* nymphs had not been collected after both sampling sessions in a given year, additional sampling was done; nearby suitable habitat was selected for sampling and often the other side of the trail was sampled or suitable habitat parallel with and <5 m off the trail. Nymphs collected during extra sampling were not included in density calculations.

DNA Extraction and Pathogen Detection, *Ixodes scapularis*

We prepared 375-µl triturates from up to 155 individual *I. scapularis* nymphs per park and extracted DNA from a 150-µl aliquot of each triturate as described in Graham et al. (2016). Leftover triturate was stored at 4 °C or −80 °C. For every 18 field-collected samples, we included one tick-free extraction as a negative control. Extracts were stored at 4 °C or −80 °C or tested immediately for *B. burgdorferi*, *A. phagocytophilum*, and *Ba. microti* using a pair of previously described multiplex real-time polymerase chain reaction (PCR) assays (Hojgaard et al. 2014). Each of the two assays, hereafter M1 and M2, included one target for each pathogen. M1 also targeted the *I. scapularis* actin gene to allow us to verify the presence of amplifiable DNA in each tick extract (Hojgaard et al. 2014). Each 10-µl reaction contained 4.8 µl eluate (~2% of the original tick), 1X iQ Multiplex Powermix (Bio-Rad, Hercules, CA), 300 nM each forward and reverse primer, and 200 nM each probe. Cycling conditions comprised a 3-min denaturation at 95 °C followed by 40 cycles of 95 °C for 10 s and 60 °C for 45 s. We tested each extract for *B. miyamotoi* using a second pair of real-time PCR assays targeting the adenylosuccinate lyase (*purB*) and glycerophosphodiester phosphodiesterase (*glpQ*) genes (Graham et al. 2016). One of the paired *B. miyamotoi* real-time PCR assays also detected the *I. scapularis* actin gene, which allowed us to verify the integrity of DNA that had been held at −80 °C for up to a year or at 4 °C for up to 2 mo after extraction and testing with M1 and M2.

Each set of real-time PCRs included negative extraction controls and a no-template control. Positive controls were composed of DNA from plasmid constructs containing the pathogen target sequences (Hojgaard et al. 2014, Graham et al. 2016). We carried out sample DNA extractions, PCR set-up, and amplification in three separate rooms. All real-time reactions were run on a C1000 Touch thermal cycler with a CFX96 real-time system (Bio-Rad).

We analyzed samples using CFX Manager 3.1 software (Bio-Rad) with the quantitation cycle (Cq) determination mode set to regression. To verify the integrity of each sample, we analyzed the distribution of *I. scapularis* actin Cq values for all samples collected in a single year and identified outliers by constructing an outlier box plot using JMP 11 statistical software (v. 11.1.1 SAS Institute Inc. 2013). Samples with an *I. scapularis* actin Cq greater than the upper whisker value (3rd quartile +1.5 [interquartile range]) were repeated. We also repeated any sample if the actin amplification curve had an end relative fluorescent unit (RFU) value <400. If the quality of the DNA was still suspect upon repeat, we extracted fresh DNA from 150 µl of the remaining tick triturate. If the second isolate repeatedly failed

to yield acceptable Cq and RFU values for the *I. scapularis* actin target, that sample was not included in infection prevalence analyses. A sample that showed acceptable *I. scapularis* actin amplification and tested positive (Cq ≤40) for both pathogen targets was considered positive for that pathogen. We repeated or reextracted any sample that initially yielded inconsistent results for one or more pathogens.

In our hands, DNA extracted from *B. burgdorferi* (ss) strains typically yields similar Cq values for both *B. burgdorferi* targets employed in the Hojgaard et al. (2014) real-time PCR panels: a segment of the flagellar filament cap gene (*fliD*) and a segment of the *B. burgdorferi* chromosome containing partial tRNA coding sequences (gB31). DNA from other *B. burgdorferi* sensu lato species, however, may test positive for neither, one, or both targets, and some strains yield dramatically different Cq values for *fliD* and gB31 (data not shown). We therefore amplified and sequenced segments of the *clpA* and *pepX* genes to verify the species identification of at least one *fliD*- and gB31-positive sample from each park, including any sample that yielded *fliD* and gB31 Cq values that differed by ≥2. We employed a (semi-)nested approach based on the MLST typing protocol originally developed by Margos et al. (2008), and detailed by Wang et al. (2014), with minor modifications, to amplify both targets. Each 25-μl outer reaction contained 12.5 μl 2X HotStar Taq Master Mix (Qiagen, Valencia, CA), 500 nM each outer primer, 2.0 mM $MgCl_2$, and 5–10 μl template. Cycling conditions included a 15-min activation and denaturation at 95 °C followed by 8 cycles (*clpA*) or 9 cycles (*pepX*) of 30 s at 94 °C, 30 s at 55 °C to 48 °C (*clpA*) or 60 °C to 52 °C (*pepX*), decreasing 1 °C each cycle, and 1 min at 72 °C. This was followed by an additional 20–30 cycles of 30 s at 94 °C, 30 s at 48 °C (*clpA*) or 52 °C (*pepX*), and 1 min at 72 °C, and a final 5 min extension at 72 °C. Each 50-μl inner reaction contained 25 μl 2X HotStar Taq Master Mix (Qiagen), 500 nM each outer primer, 2.0 mM $MgCl_2$, and 5–10 μl product from the outer reaction. Cycling conditions were as indicated in the Wang et al. (2014) simplified (semi-)nested PCR protocol, with the denaturation step extended to 30 s and 50 °C and 52 °C annealing temperatures for *clpA* and *pepX*, respectively. We verified the presence of an ~850-nt *clpA* amplicon or an ~668-nt *pepX* amplicon by visualizing 10 μl of the inner product on a 1% agarose gel. The remaining product was purified using the QIAquick PCR Purification Kit (Qiagen). We sequenced ~4 ng of each amplicon in both directions using the inner amplification primers and BigDye Terminator 3.1 Ready Reaction Mix (ThermoFisher). The BigDye Xterminator Kit (ThermoFisher) was used to remove unincorporated dyes before analyzing the samples on an ABI 3130XL genetic analyzer. We used Lasergene 12 software (DNASTAR, Madison) to construct a consensus sequence for each amplicon based on at least double coverage of every nt and queried the GenBank and pubMLST (http://pubmlst.org/borrelia/; Margos et al. 2015) (accessed July 2016) databases for similar sequences. We verified the species identification of all samples that tested positive for *B. miyamotoi* by using a similar approach, described in Graham et al. (2016), to amplify, sequence, and analyze a segment of the *clpA* gene.

Molecular Verification of Tick Species Identification

It is difficult to distinguish *I. scapularis* nymphs from *Ixodes affinis* Neumann nymphs using morphologic keys, and some of our collection sites were in areas in which *I. affinis* may be expanding its range (Nadolny et al. 2011). We therefore tested a subset (378/679) of samples

from our 2014 collection using a single-tube molecular assay to distinguish *I. scapularis* from *I. affinis* (Wright et al. 2014). Each 15-µl reaction contained 1X SsoAdvanced SYBR Green Supermix (Bio-Rad), 500 nM forward and reverse *I. affinis* primers, 500 nM forward and reverse *I. scapularis* primers, and 2 µl template. Reactions were performed on a C1000 Touch thermal cycler with a CFX96 real-time system (Bio-Rad), as previously described (Wright et al. 2014). We analyzed samples using CFX Manager 3.1 (Bio-Rad) with the Cq determination mode set to single threshold. Wright et al. (2014) reported that a melting peak range of 84.0–85.5°C was characteristic of *I. scapularis*, whereas a range of 81.5–82.5°C was characteristic of *I. affinis*. In our hands, DNA from laboratory-reared *I. scapularis* (Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO) processed alongside field-collected samples occasionally yielded melting peaks as high as 86.0°C. We therefore considered a Cq ≤30 and a melting temperature between 84.0 and 86.0°C confirmation that a specimen had been correctly identified as *I. scapularis*.

Statistical Analyses

We calculated the density of *I. scapularis* nymphs for each collection period by dividing the number of nymphs collected on each transect by the total drag area. To represent the highest potential risk in a given area, we then identified the single collection period during each year that yielded the highest nymphal density. We report this observed peak nymphal density as the density of nymphal *I. scapularis* (DON). We report the percent of all *I. scapularis* nymphs collected at a site over all collection periods within a year infected with *B. burgdorferi*, *B. miyamotoi*, *A. phagocytophilum*, or *Ba. microti* as the nymphal infection prevalence (NIP). We calculated the peak density of infected nymphs (DIN) by multiplying NIP by the peak DON to estimate the number of infected *I. scapularis* nymphs per 100 m^2. We used the Wilcoxon signed rank tests to detect differences in DON and DIN between years. We used a likelihood ratio test with a chi-square approximation to test for differences in the proportion of infected ticks between years. Differences in density of infected nymphs among pathogens were analyzed using the non-parametric Kruskal–Wallis rank sums test with the Steel–Dwass correction for multiple comparisons. We assessed pathogen diversity using two diversity indices, Shannon–Wiener Index (H) and Simpson's Index (D). These two indices measure species diversity in distinct ways, each accounting for richness and evenness differently. Simpson's Diversity Index is a dominance index that accounts for number and abundance of each species and is sensitive to dominant or abundant species. The Shannon–Wiener Diversity Index is an evenness index that is equally sensitive to rare and abundant species. We used Pearson correlation (r) to detect relationships between the geographic location (latitude and longitude) of each park and pathogen diversity and the abundance of coinfected ticks. We also used Pearson correlation to examine the relationship between the numbers of coinfected ticks and peak DON. All statistical tests were carried out at a significance level of $\alpha = 0.05$ and performed using JMP statistical software (v. 11.1.1 SAS Institute, Inc. 2013).

Results

Seven national parks were sampled between late May and early August in 2014 and 2015; two additional parks were sampled only in 2015, for a total of nine parks sampled during the duration of the 2-yr study. Ticks characterized as *I. scapularis* based on morphology were found at all sites and in both years (Table 1). Given the morphological similarities, particularly among immature life stages, and coincidental timing of host-seeking activity between *I. scapularis* and *I. affinis*, we tested a subset of nymphs using molecular methods to confirm morphological identification. All nymphs tested were identified as *I. scapularis.*

We collected a total of 1,305 *I. scapularis* nymphs by drag sampling at seven national parks in 2014, and we collected 1,282 *I. scapularis* nymphs from nine national parks in 2015 (Table 1). Among the national parks sampled in 2014, we observed the lowest DON (0.67 nymphs per 100 m^2) on the single transect sampled at Monocacy National Battlefield and the highest peak DON (13.73 nymphs per 100 m^2) at Rock Creek Park. In 2015, Gettysburg National Military Park yielded the lowest DON (0.27 nymphs per 100 m^2), whereas the highest DON (20.40 nymphs per 100 m^2) occurred at Fire Island National Seashore, William Floyd Estate (Table 2). There was considerable variation among transects within national parks. The largest amount of variation in peak DON within a single national park was at Manassas in 2014, where there was an eightfold difference in DON among transects, and at Rock Creek Park in 2015, where there was a sevenfold difference in DON between the highest and lowest density transects. National parks showing the least amount of variation in DON across transects in both years were Monocacy National Battlefield and Prince William Forest Park, which had variation among transects of <1 nymphs per 100 m^2 (Table 2). The median DON over all sites and years was 2.4 *I. scapularis* nymphs per 100 m^2. Although a trend of fewer *I. scapularis* nymphs was noted in 2015 when the median peak DON was 1.6 nymphs per 100 m^2, as compared with 3.0 nymphs per 100 m^2 in 2014, there was no significant difference in the number of *I. scapularis* nymphs or DON detected between years (Wilcoxon signed ranks; W =10.00, P =0.11).

We tested 679 and 781 *I. scapularis* nymphs collected in 2014 and 2015, respectively, for a suite of zoonotic *I. scapularis*-borne pathogens (Table 2). *Borrelia burgdorferi* was the most widespread pathogen, and was detected in nymphs collected at all sites in both years of the study. We were able to amplify and sequence two *Borrelia* targets (*clpA* and *pepX*) from 31 of the nymphs that had tested positive for *B. burgdorferi* by real-time PCR, and at least one target from two additional samples. This included at least one sample from each park for each collection year and all samples that had yielded suspect real-time PCR results. BLAST analysis indicated that all target sequences were ≥99% identical to homologous sequences from *B. burgdorferi* sensu stricto (ss) isolates, and ≤96% identical to the corresponding sequences from all other species, including other *B. burgdorferi* sensu lato species, in the GenBank database. In three cases, the sequence data indicated that the sample contained a mix of more than one species or strain, so we could not conclusively identify the infecting species. The sequence data were consistent, however, with coinfection with multiple *B. burgdorferi* (ss) strains. All *pepX* amplicons contained 570-nt segments identical to *pepX* alleles associated exclusively with *B. burgdorferi* (ss) isolates in the pubMLST database (alleles 1, 5, 6, 7, 8, and 18). All but two of the *clpA* amplicons contained a 579-nt segment

identical to *clp*A alleles also associated exclusively with *B. burgdorferi* (ss) isolates (alleles 1, 4, 5, 6, 7, 9, 10, 18, 24, and 158). Two of the nymphs yielded identical *clpA* alleles that differed by a single nucleotide from *clpA* allele 21 (426A >G). Allele 21 is also associated only with *B. burgdorferi* (ss). Given that we positively identified 30 (11%) of the *B. burgdorferi*-positive samples as *B. burgdorferi* (ss), and given that we did not identify any other *Borrelia* spp. in any of the samples, we considered all *B. burgdorferi*-positive nymphs positive for *B. burgdorferi* (ss).

Borrelia burgdorferi (ss) NIP per park in *I. scapularis* ranged from 5.1% at Prince William Forest Park to 28.6% at Gettysburg National Military Park in 2014, and from 3.2% at Prince William Forest Park to 35.6% at Catoctin Mountain Park in 2015 (Table 2). There was no difference in the NIP with *B. burgdorferi* between years (likelihood ratio: χ^2 =0.20, df =1, P =0.65). Among parks that were sampled in consecutive years, there was no significant difference detected in the density of *I. scapularis* nymphs infected with *B. burgdorferi* between years (Wilcoxon signed ranks; W =2.00, P =0.81); DIN was lowest at Prince William Forest Park in both years, with 0.07 and 0.04 infected nymphs per 100 m^2 in 2014 and 2015, respectively. The highest DIN was detected at Rock Creek Park in 2014 (3.3 infected nymphs per 100 m^2) and from Fire Island National Seashore, William Floyd Estate in 2015 (2.2 infected nymphs per 100 m^2).

We also tested *Ixodes scapularis* nymphs for *B. miyamotoi*, *A. phagocytophilum*, and *Ba. microti* infection. Amplification and sequencing of the *clpA* target confirmed all *B. miyamotoi*-positives. Amplicon sequences from all *B. miyamotoi*-positive nymphs were identical to homologous sequences from *B. miyamotoi* CT14D4 (CP010308.1) and LB-2001 (CP006647.2), and all amplicons contained a 570-nt segment identical to *clpA* allele 200 in pubMSLT. As of July 26, 2016, this allele was associated with only one isolate in the pubMLST database, *B. miyamotoi* M1029. *Borrelia miyamotoi*-infected nymphs had a more sporadic distribution than nymphs infected with *B. burgdorferi* and were found at 66.7% of the sites sampled (Table 2). When present, NIP across all sites ranged from <1% at Acadia National Park to as high as 4.4% at Catoctin Mountain Park. *Anaplasma phagocytophilum*-infected nymphs were found at 77.8% of sites; NIP among sites ranged from 1% at Rock Creek Park to 10.7% of *I. scapularis* nymphs tested from Fire Island National Seashore, William Floyd Estate. *Babesia microti* was the least commonly detected pathogen and was only detected in the two most northerly national parks. At Acadia National Park, NIP for *Ba. microti* in 2015 was 4% and as many as 9% of *I. scapularis* nymphs from a given transect tested positive. NIP rates with *Ba. microti* at Fire Island National Seashore in 2014 and 2015 were 8.7% and 15.3%, respectively, with as many as 28% of nymphs infected from a single transect in 2015 (Table 2).

We detected multiple pathogens in *I. scapularis* from all sites except Monocacy National Battlefield, where we only detected *B. burgdorferi*. We calculated the Shannon–Wiener (H) and Simpson's (D) Diversity Indices for all national parks and both years. Based on these indices, pathogen diversity in *I. scapularis* was lowest at Monocacy National Battlefield, whereas Fire Island National Seashore harbored the greatest pathogen diversity. There were no significant differences in Shannon–Wiener Diversity or Simpson's Diversity among sites (Kruskal–Wallis; $|z| \leq 1.29$, $P \geq 0.96$), and neither index of diversity was related to the density

of *I. scapularis* nymphs (Pearson's correlation: $r \leq 0.48$, $P \geq 0.07$). Further, there was no association between diversity and latitude (Pearson's correlation: $r \leq 0.37$, $P \geq 0.17$); however, a significant association was found between longitude and both measures of species diversity (Pearson's correlation: $r \leq 0.53$, $P \geq 0.03$). Higher diversity sites were located closer to the coast, although geographic position only explained ~50% of the observed variation in diversity.

A small percentage (2.7%) of *I. scapularis* nymphs tested were coinfected with multiple pathogens (Table 3). The majority of coinfected nymphs, 97.5%, were simultaneously infected with two different pathogens; coinfections with *B. burgdorferi* and *Ba. microti* were the most common, occurring in 16 of 39 (41.0%) coinfected nymphs, followed by coinfections with *B. burgdorferi* and *A. phagocytophilum*, which occurred in 14 of 39 (35.9%). We only observed coinfections with *B. burgdorferi* and *B. miyamotoi* in a single nymph collected from Manassas National Battlefield Park and a single nymph from Rock Creek Park. Other pathogen combinations in coinfected nymphs are shown in Table 3. Two parks accounted for the majority of coinfected nymphs; 62% were collected at Fire Island National Seashore and 26% at Acadia National Park. Fire Island National Seashore had the highest pathogen diversity of all sites and also had the highest rate of coinfected ticks; one tick found there was infected with three pathogens: *B. burgdorferi*, *Ba. microti*, and *A. phagocytophilum*. We were significantly more likely to observe coinfected ticks as DON increased (Pearson's correlation: $r = 0.65$, $P = 0.01$), and coinfected ticks were most likely to occur at northern (Pearson's correlation: $r = 0.55$, $P = 0.03$) and coastal sites (Pearson's correlation: $r = 0.72$, 0.48, $P = 0.002$).

Although we sampled habitat conducive to *I. scapularis* survival, we encountered and collected other species of ticks at most national parks sampled. *Ixodes scapularis* was the only species collected at Acadia National Park, whereas at least one other species was encountered at each of the other parks (Table 1). *Dermacentor variabilis* (Say) adults were collected from six national parks in 2014 and from three parks in 2015; a single *D. variabilis* nymph was collected at Gettysburg National Military Park and two *D. variabilis* nymphs were collected from Shenandoah National Park in 2015 (Table 1). Three sites accounted for the majority of the *Amblyomma americanum* (L.) found, and when present, *A. americanum* often occurred in high numbers. We collected >3,000 *A. americanum* adults and nymphs and >2,500 adults and nymphs at Fire Island National Seashore in 2014 and 2015, respectively (Table 1). A single *Rhipicephalus sanguineus* (Latreille) male and one *Haemaphysalis leporispalustris* (Packard) nymph was caught in 2015 at Fire Island National Seashore and one *Amblyomma maculatum* Koch was found in 2014 at Manassas National Battlefield Park (Table 1).

Discussion

Avoiding tick bites is essential to reducing the risk of tick-borne pathogen exposure, and this is best accomplished, particularly in recreational settings, by avoiding high-risk habitats during peak nymphal tick activity (Piesman and Eisen 2008). Assessing when and where people are at highest risk for exposure to vectors and what pathogens are present in those vectors are primary steps in risk assessment and prevention (Piesman and Eisen 2008, Eisen

et al. 2013). In this study, we documented acarological risk for exposure to *I. scapularis*-borne pathogens on frequently used hiking trails in nine eastern national parks, but we observed great variability in acarological risk within and among parks. Compared with *B. burgdorferi*, ticks infected with *B. miyamotoi*, *A. phagocytophilum*, and *Ba. microti* were less widespread and less prevalent.

The results presented here for host-seeking *I. scapularis* distribution are generally consistent with previous studies conducted near the national parks we sampled. The establishment of *I. scapularis* is well documented across much of the study area (Eisen et al. 2016). However, to our knowledge, previous studies had not documented established *I. scapularis* populations in Washington, D.C., where we collected *I. scapularis* at Rock Creek Park, or in Greene County, Virginia, where we collected *I. scapularis* at Shenandoah National Park. Additionally, we confirmed recent reports of established *I. scapularis* populations in Albemarle, Warren, and Prince William Counties in Virginia, and in Adams County, Pennsylvania (Han et al. 2014, Eisen et al. 2016). National park units in Pennsylvania and Maryland are located within counties that have been considered high Lyme disease incidence counties since the mid- to late 1990s, while the District of Columbia and counties sampled in Virginia have only more recently achieved high-incidence Lyme disease status (Kugeler et al. 2015, Nelson et al. 2015).

Pathogen prevalence was also consistent with findings from previous research conducted at locations near our study sites. Han et al. (2014) reported an NIP of *B. burgdorferi* of 18% at Gettysburg National Military Park, Adams County, Pennsylvania. Similarly, investigations in Cumberland and York Counties, which border Adams County to the north and east, respectively, found a 39% to 52% infection prevalence of *I. scapularis* with *B. burgdorferi* (Diuk-Wasser et al. 2012, Hutchinson et al. 2015). In this study, *I. scapularis* nymphal infection prevalence with *B. burgdorferi* was 20–30% at Gettysburg National Military Park. At the same site, as many as 3% of nymphs collected in 2014 and 8% of nymphs collected in 2015 were found to be infected with *A. phagocytophilum*. A single *I. scapularis* nymph collected at this park was coinfected with *B. burgdorferi* and *A. phagocytophilum*, a coinfection prevalence of 1.6%. This is the first report of *A. phagocytophilum* from this park, but in Cumberland County, Pennsylvania, bordering Adams County to the north, *B. burgdorferi*, *Ba. microti*, and *A. phagocytophilum* are known to infect *I. scapularis*, and investigators have observed *B. burgdorferi* and *A. phagocytophilum* coinfections in about 1.5% of adult ticks (Hutchinson et al. 2015). We documented *B. burgdorferi* in 12–26% of *I. scapularis* nymphs from Acadia National Park, results which are consistent with other studies that reported infection prevalence of 11.8% (Ginsberg 1992) to 23% (Diuk-Wasser et al. 2012) in host-seeking nymphs, and as high as 45% in nymphs collected from field-caught rodents (Rand et al. 1993). Previous studies conducted at Fire Island National Seashore and Long Island reported higher infection prevalence (20–30%) of *B. burgdorferi* infecting *I. scapularis* nymphs (Ginsberg 1992, Diuk-Wasser et al. 2012); our results show NIP on individual transects at Fire Island National Seashore ranged from 8% to 28%. Across all transects, 14% and 19% of nymphs caught in 2014 and 2015 Fire Island National Seashore were infected with *B. burgdorferi*. Across study sites, coinfection in ticks was observed in 2.6% of nymphs tested. The most common coinfections were ticks infected with *B. burgdorferi* and *Ba. microti* (1.1% of all nymphs tested) or *B. burgdorferi* and *A.*

phagocytophilum (0.7% of all nymphs tested). Coinfections of *B. burgdorferi* and *Ba. microti* were only documented in ticks collected from Acadia National Park and Fire Island National Seashore, whereas coinfections of *B. burgdorferi* and *A. phagocytophilum* occurred at Acadia National Park, Fire Island National Seashore, Gettysburg National Military Park, and Shenandoah National Park (Table 3). The rates of coinfection documented here are in general agreement with published rates of coinfections in *I. scapularis* nymphs with *B. burgdorferi* and *Ba. microti* or *B. burgdorferi* and *A. phagocytophilum* (Diuk-Wasser et al. 2016, Feldman et al. 2015). The highest rate of coinfection with *B. burgdorferi* and *Ba. microti* at our study sites was 5.3% at Fire Island National Seashore, whereas coinfection rates as high as 7.7% have been documented at residential sites elsewhere in New York (Feldman et al. 2015).

Over the past two decades, the distribution of Virginia counties reporting Lyme disease cases has expanded to the southwest, as has the distribution of counties classified as having established *I. scapularis* populations (Brinkerhoff et al. 2014, Eisen et al. 2016). Diuk-Wasser et al. (2012) reported the highest DON and DIN in the northeastern portion of Virginia, and they reported few *I. scapularis* nymphs and did not detect *B. burgdorferi* at sites located throughout the rest of the state. They sampled from 2004 to 2006, however, when Lyme disease cases occurred less frequently in central and western Virginia than in recent years (Sonenshine et al. 1995, Casteel and Sonenshine 1996, Brinkerhoff et al. 2014). In 2013, Ford et al. (2015) did not find *I. scapularis* at any of six sampling areas along the Appalachian Trail in west, central, and north-central Virginia. We sampled Shenandoah National Park in the north-central part of the state and Manassas National Battlefield and Prince William Forest park in the northeast. We documented *I. scapularis* and *B. burgdorferi* on each transect at all three national parks sampled in Virginia, with the exception of a single transect at Prince William Forest Park from which 0/26 nymphs tested positive for any pathogen in 2015. Across the state, prevalence of *B. burgdorferi* ranged from 6 to 29%, and prevalence of *A. phagocytophilum* was as high as 8% at three transects at Shenandoah National Park. Findings were similar <100 km east at Manassas National Battlefield Park, where the number of *I. scapularis* nymphs was comparable, prevalence of *B. burgdorferi* ranged from 8 to 39%, and prevalence of *B. miyamotoi* was as high as 8%. Interestingly, Manassas National Battlefield Park and Prince William Forest Park are located <40 km apart in Prince William County in northeast Virginia, yet the DON was roughly three times greater at Manassas National Battlefield Park than at Prince William Forest Park, and NIP with *B. burgdorferi* was 10 times higher at Manassas National Battlefield Park than at Prince William Forest Park in 2014 and 20 times higher at Manassas National Battlefield Park than at Prince William Forest Park in 2015. It is not surprising to observe considerable differences in NIP among park units located in close proximity, as national park units are often quite different from surrounding areas and may be managed differently depending on the type or park unit, e.g., battlefield park versus forest park, and thus the density of infected ticks may differ between settings. Thus, it is difficult to infer risk of surrounding areas based on a single measurement and inference to the risk of tick-borne disease for park visitors or employees should be based on park-specific surveillance efforts. Generally, our findings thus suggest that the distribution *of B. burgdorferi*-infected *I. scapularis*, like the distribution of

counties reporting Lyme disease, is expanding westward from known human disease foci in the eastern United States (Nelson et al. 2015).

For this study, we aimed to sample for ticks during the period of peak *I. scapularis* nymphal host-seeking activity which occurs between May and August in the Mid-Atlantic and Northeastern United States (Wilson and Spielman 1985, Piesman et al. 1987, Sonenshine and Mather 1994, Ostfeld et al. 1996, Diuk-Wasser et al. 2006, Gatewood et al. 2009, Orr et al. 2013). Although this was our aim and we report peak observed nymphal density, we did not conduct phenology studies simultaneously to document the nymphal peak and therefore cannot infer that the numbers of ticks reported here represent the absolute peak in nymphal host-seeking density each year. Peak density of *I. scapularis* nymphs was highly variable across national park units sampled and ranged from a low of less than one nymph per 100 m^2 at Monocacy National Battlefield, to 12 nymphs per 100 m^2 at Fire Island National Seashore, William Floyd Estate, in both years of the study. Most other sites had between two and five *I. scapularis* nymphs per 100 m^2. Moreover, although we targeted *I. scapularis* habitat, primarily deciduous forests with canopy and adequate leaf litter (Ginsberg and Ewing 1989, Siegel et al. 1991), both *A. americanum* and *D. variabilis* overlap with *I. scapularis* in both questing activity timing and habitat (Bishopp and Trembley 1945, Sonenshine 1991), and thus were incidental captures.

This research demonstrates the potential risk of tick-borne disease in eastern national parks and can be used to promote awareness among park visitors of the potential for recreational tick and tick-borne pathogen exposure. This work represents a step toward understanding public health risks in these national parks, and the data will aid in improving prevention and education programs for park visitors. To reduce vector-borne disease exposures, national parks focus on encouraging visitors to use appropriate personal protective measures. Strategies include informing visitors when and where they are at highest risk, which pathogens are present and what symptoms result from infection (National Park Service 2006, Piesman and Eisen 2008, Eisen et al. 2013), and providing guidance on personal protective measures to prevent tick bite (http://www.cdc.gov/ticks/avoid/on_people.html) (accessed July 2016), the importance of prompt and safe removal of attached ticks (http://www.cdc.gov/ticks/removing_a_tick.html) (accessed July 2016) and seeking medical attention if signs and symptoms occur after being bitten by a tick (http://www.cdc.gov/ticks/symptoms.html) (accessed July 2016). Promoting prevention efforts in these areas may be particularly important for those visitors traveling from nonendemic areas that may not be as mindful to the prevention of tick bites or symptoms of tick-borne diseases. Although this work identified the diversity, density, and distribution of ticks and medically important tick-borne pathogens, further studies are needed to fully assess acarological risk, including studies that incorporate communication with visitors to evaluate how frequently people encounter ticks in national parks and to identify specific behaviors that may result in increased risk of tick encounter.

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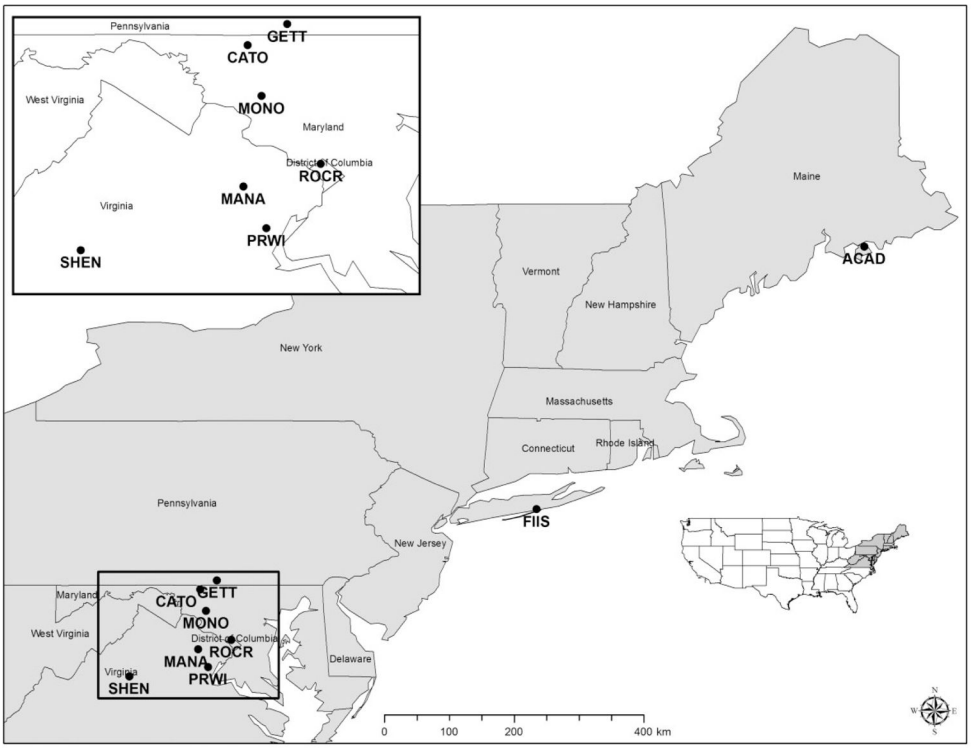

Fig. 1.

Nine national park units with established populations of *I. scapularis* in 2014 and 2015. ACAD, Acadia National Park; CATO, Catoctin Mountain Park; FIIS, Fire Island National Seashore; GETT, Gettysburg National Military Park; MANA, Manassas National Battlefield Park; MONO, Monocacy National Battlefield; PRWI, Prince William Forest Park; ROCR, Rock Creek Park; SHEN, Shenandoah National Park.

Table 1

Dates, number, and area of transects sampled and number of ticks collected from eastern United States national parks—2014–2015

Site[a]/year	Sample date range	No. transects sampled[b]	Total area (m^2)	*Ixodes scapularis*			*Dermacentor variabilis*		*Amblyomma americanum*		
				Larva	Nymph	Adult	Nymph	Adult	Larva	Nymph	Adult
ACAD											
2015	6/30–8/14	6	6,750	116	154	1	0	0	0	0	0
CATO[c]											
2014	6/17–7/1	6	4,500	0	104	2	0	2	0	0	0
2015	6/11–6/25	6	4,500	15	46	2	0	0	0	0	0
FIIS[c,d]											
2014	5/29–6/12	6	4,500	6	339	9	0	6	24	2,628	887
2105	5/29–6/19	6	4,500	1	539	10	0	6	1	1,630	1,027
GETT[c]											
2014	6/4–6/19	4	3,000	0	63	0	1	30	0	0	0
2015	6/1–7/2	6	4,500	19	51	2	0	37	0	0	0
MANA[e,f]											
2014	6/2–6/18	6	4,500	0	196	2	0	77	0	610	224
2015	5/27–6/16	6	4,500	0	106	1	1	23	0	217	139
MONO											
2014	6/3–6/13	2	1,500	0	10	0	0	1	0	0	0
2015	6/25	2	1,500	0	10	0	0	0	0	0	0
PRWI[c]											
2014	6/1–6/10	6	4,500	0	79	2	0	15	0	139	50
2015	5/28–6/11	6	4,500	1	62	6	0	0	0	29	16
ROCR											
2014	6/7–6/19	6	4,500	0	514	0	0	0	0	2	0
2015	5/26–6/5	6	4,500	0	124	3	0	0	0	0	3
SHEN											
2015	5/28–6/24	6	4,500	30	190	4	2	0	0	22	9

[a] ACAD, Acadia National Park; CATO, Catoctin Mountain Park; FIIS, Fire Island National Seashore; GETT, Gettysburg National Military Park; MANA, Manassas National Battlefield Park; MONO, Monocacy National Battlefield; PRWI, Prince William Forest Park; ROCR, Rock Creek Park; SHEN, Shenandoah National Park.

[b] This represents the total number of 750-m transects sampled at each park. Most sites had three transects sampled two times (N=6).

[c] Transects from which extra sampling in surrounding suitable habitat was conducted in effort to obtain ≥50 nymphal *I. scapularis* for *B. burgdorferi* infection prevalence estimates; extra ticks collected off transect were not included in density estimates.

[d] One *Rhipicephalus sanguineus* male and one *Haemaphysalis leporispalustris* nymph caught in 2015.

[e] Transects 1 and 2 at Monocacy National Battlefield were only sampled a single time in 2015.

[f] One *A. maculatum* male caught in 2014.

Table 2

Density and infection prevalence of nymphal *I. scapularis* collected at national parks in the eastern United States—2014–2015

Park[a]/Year		Peak DON[b]/100 m^2 median (range)	No. nymphs PCR	*Borrelia burgdorferi*		*Borrelia miyamotoi*		*Babesia microti*		*Anaplasma phagocytophilum*	
				NIP[c]	Peak DIN[d]/100 m^2 (range)	NIP	Peak DIN/100 m^2 (range)	NIP	Peak DIN/100 m^2 (range)	NIP	Peak DIN/100 m^2 (range)
ACAD	2015	3.20 (1.60–4.93)	154	0.18	0.56 (0.28–1.48)	0.01	0.02 (0.00–0.07)	0.04	0.13 (0.00–0.19)	0.03	0.10 (0.00–0.06)
CATO	2014	2.93 (1.33–3.47)	103	0.19	0.57 (0.39–0.80)	0.02	0.06 (0.00–0.14)	0.00	NA	0.03	0.09 (0.00–0.20)
	2015	1.33 (0.27–2.53)	45	0.36	0.47 (0.09–0.88)	0.04	0.06 (0.00–0.22)	0.00	NA	0.02	0.03 (0.00–0.07)
FIIS	2014	10.67 (2.40–13.07)	149	0.15	1.57 (0.20–3.14)	0.04	0.43 (0.05–1.05)	0.09	0.93 (0.20–1.60)	0.07	0.79 (0.15–1.49)
	2015	12.00 (8.13–20.40)	150	0.19	2.24 (1.20–5.71)	0.02	0.24 (0.000–0.48)	0.15	1.81 (0.72–5.71)	0.11	1.28 (0.48–1.46)
GETT	2014	2.27 (1.33–4.00)	63	0.29	0.65 (0.000–0.93)	0.00	NA	0.00	NA	0.02	0.04 (0.00–0.04)
	2015	0.67 (0.27–2.67)	51	0.16	0.11 (0.05–0.89)	0.00	NA	0.00	NA	0.04	0.03 (0.00–0.05)
MANA	2014	4.87 (1.47 – 12.53)	120	0.17	0.81 (0.29–1.33)	0.03	0.12 (0.00–0.12)	0.00	NA	0.00	NA
	2015	3.87 (1.33–8.53)	87	0.24	0.93 (0.42–2.05)	0.02	0.09 (0.00–0.34)	0.00	NA	0.00	NA
MONO[e]	2014	0.67 (NA)	10	0.10	0.07	0.00	NA	0.00	NA	0.00	NA
	2015	0.67 (0.40–0.93)	10	0.20	0.13 (0.00–0.27)	0.00	NA	0.00	NA	0.00	NA
PRWI	2014	1.47 (1.47–2.00)	79	0.05	0.07 (0.06–0.12)	0.03	0.04 (0.00–0.06)	0.00	NA	0.03	0.04 (0.00–0.06)
	2015	1.33 (1.33–2.00)	62	0.03	0.04 (0.00–0.10)	0.02	0.02 (0.00–0.10)	0.00	NA	0.00	NA
ROCR	2014	12.13 (7.73 – 13.73)	155	0.27	3.29 (1.69–3.88)	0.01	0.16 (0.00–0.28)	0.00	NA	0.00	NA
	2015	3.60 (1.33–9.47)	98	0.20	0.73 (0.61–0.97)	0.00	NA	0.00	NA	0.01	0.04 (0.000–0.19)
SHEN	2015	1.33 (1.33–2.93)	124	0.12	0.16 (0.08–0.39)	0.00	NA	0.00	NA	0.02	0.03 (0.00–0.11)

[a]ACAD, Acadia National Park; CATO, Catoctin Mountain Park; FIIS, Fire Island National Seashore; GETT, Gettysburg National Military Park; MANA, Manassas National Battlefield Park; MONO, Monocacy National Battlefield; PRWI, Prince William Forest Park; ROCR, Rock Creek Park; SHEN, Shenandoah National Park.

[b]DON, density of nymphal *I. scapularis* (range across transects).

[c]NIP, nymphal infection prevalence.

[d]DIN, density of infected nymphs (NIP × DON; range across transects).

[e]Only one transect sampled at MONO in 2014.

Table 3

Coinfection of *I. scapularis* nymphs and density per 100 m^2 from eastern United States national parks—2014–2015

Site[a]	*Borrelia burgdorferi*	*Babesia microti*	*Anaplasma phagocytophilum*	*Borrelia miyamotoi*	No. of ticks/total tested	Coinfection prevalence	Density of coinfected nymphs per 100 m^2
ACAD							
2015	+	+			5/154	0.032	0.10
	+		+		5/154	0.032	0.10
FIIS							
2014	+	+			3/149	0.020	0.17
	+		+		5/149	0.034	0.30
			+	+	1/149	0.007	0.06
2015	+	+			8/150	0.053	0.72
	+		+		3/150	0.020	0.27
		+	+		2/150	0.013	0.18
		+		+	1/150	0.007	0.09
	+	+	+		1/150	0.007	0.09
GETT							
2014	+		+		1/63	0.016	0.04
MANA							
2015	+			+	1/87	0.011	0.05
PRWI							
2014			+	+	1/79	0.013	0.02
ROCR							
2014	+			+	1/155	0.006	0.07
SHEN							
2015	+		+		1/124	0.008	0.01

[a]ACAD, Acadia National Park; FIIS, Fire Island National Seashore; GETT, Gettysburg National Military Park; MANA, Manassas National Battlefield Park; MONO, Monocacy National Battlefield; PRWI, Prince William Forest Park; ROCR, Rock Creek Park; SHEN, Shenandoah National Park.