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Surveillance of Ticks and Tick-Borne Pathogens in Suburban Natural Habitats of Central Maryland

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Abstract

Lyme and other tick-borne diseases are increasing in the eastern United States and there is a lack of research on integrated strategies to control tick vectors. Here we present results of a study on tick-borne pathogens detected from tick vectors and rodent reservoirs from an ongoing 5-yr tick suppression study in the Lyme disease-endemic state of Maryland, where human-biting tick species, including *Ixodes scapularis* Say (Acari: Ixodidae) (the primary vector of Lyme disease spirochetes), are abundant. During the 2017 tick season, we collected 207 questing ticks and 602 ticks recovered from 327 mice (*Peromyscus* spp. (Rodentia: Cricetidae)), together with blood and ear tissue from the mice, at seven suburban parks in Howard County. Ticks were selectively tested for the presence of the causative agents of Lyme disease (*Borrelia burgdorferi* sensu lato [s.l.]), anaplasmosis (*Anaplasma phagocytophilum*), babesiosis (*Babesia microti*), ehrlichiosis (*Ehrlichia ewingii, Ehrlichia chaffeensis*, and 'Panola Mountain' *Ehrlichia*) and spotted fever group rickettsiosis (*Rickettsia* spp.). *Peromyscus* ear tissue and blood samples were tested for *Bo. burgdorferi* sensu stricto (s.s), *A. phagocytophilum*, *Ba. microti*, and *Borrelia miyamotoi.* We found 13.6% (15/110) of questing *I. scapularis* nymphs to be *Bo. burgdorferi* s.l. positive and 1.8% (2/110) were *A. phagocytophilum* positive among all sites. *Borrelia burgdorferi* s.s. was found in 71.1% (54/76) of *I. scapularis* nymphs removed from mice and 58.8% (194/330) of captured mice. Results from study on tick abundance and pathogen infection status in questing ticks, rodent reservoirs, and ticks feeding on *Peromyscus* spp. will aid efficacy evaluation of the integrated tick management measures being implemented.

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Keywords

Lyme disease; blacklegged tick; tick-borne pathogen; *Borrelia* ; *Peromyscus*

Tick-borne diseases are increasing in the United States, and Lyme disease accounts for the majority of reported cases (Rosenberg et al. 2018). In Maryland, where the present research was carried out, Lyme disease was first reported in 1979 and became a state reportable zoonosis in 1989 (Strickland et al. 1994, Glass et al. 1995). Currently, Maryland ranks in the top 10 states in terms of annual reported Lyme disease cases (CDC 2020). In the Mid-Atlantic region, including Maryland, and the Northeast, Lyme disease is caused by *Borrelia burgdorferi* sensu stricto (s.s.) (Spirochaetales: Spirochaetaceae), a spirochete maintained in an enzootic cycle involving the blacklegged tick, *Ixodes scapularis* Say (Acari: Ixodidae), and several vertebrate reservoir species including the white-footed mouse, *Peromyscus leucopus* (Rafinesque) (Rodentia: Cricetidae), which is an important host for the immature tick life stages (LoGiudice et al. 2003). The white-tailed deer, *Odocoileus virginianus* (Zimmermann) Artiodactyla: Cervidae, does not serve as a reservoir for *B. burgdorferi* s.s. but is an important reproductive host for *I. scapularis* adults, driving tick population increases that lead to intensified enzootic spirochete transmission and increased risk of human bites by infected ticks (Telford et al. 1988, Amerasinghe et al. 1993, Halsey et al. 2018, Telford 2018).

Other factors influencing enzootic pathogen transmission and acarological risk for human bites by infected ticks include genetic variability of *B. burgdorferi* s.s., variable host species assemblages, landscape features, and climate conditions (Glass et al. 1995, Alghaferi et al. 2005, Anderson and Norris 2006, Jackson et al. 2006, Brisson et al. 2008, Simon et al. 2014, Kilpatrick et al. 2017). As a human vaccine for Lyme disease is still lacking and use of other personal protection measures has not proven adequate to prevent the increase of Lyme and other tick-borne diseases, there is a continued need to develop and evaluate environmentally based strategies to suppress populations of human-biting ticks and reduce the intensity of enzootic pathogen transmission (Carroll et al. 2009; Stafford et al. 2017; Williams et al. 2018a, b; Eisen and Stafford 2020). Surveillance of tick-borne pathogens can help shape management strategies aimed at reducing the density of infected ticks, particularly the nymphal stage, which can go undetected when biting humans (Stafford et al. 2017).

Transmission potentials are thought to be higher in edge habitat when compared to deeper forest (Williams et al. 2018a, b), though much of that risk has been attributed to increased human activity associated with forest edges rather than true acarological risk (Horobik et al. 2006). Moreover, some tick-borne pathogens, like *Anaplasma phagocytophilum*, *Babesia microti, B. burgdorferi* sensu lato (s.l.) and *Borrelia miyamotoi*, have been shown to co-occur frequently (Diuk-Wasser et al. 2016) and can demonstrate mutualistic associations when found as coinfections in *I. scapularis* (Swanson et al. 2006) or *Peromyscus* mice (Dunn et al. 2014, Kilpatrick et al. 2017). Larval and nymphal *I. scapularis* may acquire coinfections when feeding on rodents and the resulting molted nymphs and adults could transmit the co-infecting pathogens during the subsequent bloodmeal (Walter et al. 2016).

In the current study, we present baseline data on density of host-seeking ticks and pathogen infection in ticks and rodent reservoirs prior to the implementation of a 5-yr integrated pest management (IPM) project conducted in urban parks in central Maryland and including different combinations of three control technologies: topical application of acaricides to deer, topical application of acaricides to rodents, and broadcast application of entomopathogenic fungus. In addition to *I. scapularis* and its associated human pathogens (including *Bo. burgdorferi* s.l., *Bo. miyamotoi*, *A. phagocytophilum*, and *Ba. microti*), the study areas harbor two other species of humanbiting ticks—*Amblyomma americanum* (L.) and *Dermacentor variabilis* (Say) (Acari: Ixodidae)—that serve as vectors for *Ehrlichia* spp. and *Rickettsia* spp. pathogens.

Materials and Methods

Study Sites

Seven suburban parks within Howard County, Maryland were included in the study: Blandair (BL; 39°13′17.18″N, 76°49′44.50″W), Cedar Lane (CL; 39°13′52.34″N, 76°53′5.18″W), Centennial (CT; 39°14′47.65″N, 76°51′29.06″W), David Force (DF; 39°17′25.08″N, 76°52′28.25″W), Middle Patuxent Environmental Area (MPEA; 39°12′57.50″N, 76°55′2.52″W), Rockburn (RB; 39°13′8.70″N, 76°46′25.96″W), and Wincopin Trails (hereafter, Wincopin; 39°8′55.98″N, 76°50′9.73″W). Because single-family homes were adjacent to each drag transect and trapping grid, we define our parks as peridomestic forests.

Tick Sampling

The relative density of host-seeking adult and nymphal ticks of different species was determined in each park by drag-sampling along two concurrent parallel transects (Blandair = 680 m; Cedar Lane = 400 m; Centennial = 600 m; David Force = 475 m; MPEA = 450 m, RB = 500 m; and Wincopin = 630 m). The parallel transects were located within 50 m of forest edge under the assumption this forest depth had a high likelihood of potential host use (Simon et al. 2014) and would be representative of the tick assemblage composition at and near the forest edge (Gallo et al. 2017). Cloths of 1 m^2 white corduroy were dragged along vegetation following the two transects at a slow pace and checked for ticks every 10 m. Collected ticks were placed into microcentrifuge tubes with ≥70% ethanol. Sites were drag-sampled for ticks twice per month from May to November 2017. Additionally, ticks were removed from captured rodents (described below) and stored in ≥70% ethanol. Ticks were identified to species and life stage following taxonomic keys (Cooley 1946, Keirans and Clifford 1978, Keirans and Litwak 1989) and subjected to pathogen detection as described below.

PCR Assays for Pathogen Detection in Ticks

Nucleic acids were isolated from ticks using the Qiagen DNeasy Blood and Tissue Kit and the Tissues and Rodent Tails protocol on the QIAcube instrument (Qiagen, Valencia, CA). For questing ticks, all *I. scapularis* specimens were processed individually because of high expected pathogen prevalence. Questing *A. americanum* and *D. variabilis* ticks were pooled in groups of five, so that aliquots of five individual tick homogenates were combined,

isolated, and tested together. Individual samples were archived at −20°C to be used later if the pool tested positive for a pathogen. For ticks removed from mice, all ticks of the same species and life stage removed from a single mouse were combined in one tube for isolation.

Ticks were tested for human pathogens for which their species are known to serve as vectors. Therefore, *I. scapularis* were tested for presence of *A. phagocytophilum*, *Ba. microti*, and *Bo. burgdorferi* s.l.; *A. americanum* were tested *for Ehrlichia ewingii, Ehrlichia chaffeensis*, and "Panola Mountain" *Ehrlichia*; and *D. variabilis* were screened for bacteria in the genus *Rickettsia*. Nucleic acid of *Rickettsia rickettsii*, kindly provided by Abdu Azad (University of Maryland School of Medicine), was used as positive control for all reactions above. Detailed method has been reported previously by Stromdahl et al. (2011). Positive controls were used in all PCR reactions. All initial positive results were confirmed by testing the DNA extract with a second PCR for a different genetic region if available, and positive specimens were defined as samples that produced at least two separate PCR positive results. PCR cycling conditions are as described in the original articles mentioned below.

Ixodes scapularis-associated pathogens were detected as follows. Real time PCR to detect *Bo. burgdorferi* s.l. was performed using primers and a probe designed to anneal to the *OspA* gene of *Bo. burgdorferi* s.l. (Straubinger 2000). Any samples positive in this assay were tested again in a real-time PCR targeting the inner part of the *fla* gene of *Bo. burgdorferi* s.l. (Leutenegger et al. 1999). For *Ba. microti*, the PCR was performed using a real-time assay targeting the 18S rRNA gene of *Ba. microti* (Tonnetti et al. 2009) or a real-time assay targeting a different section of the 18S rRNA gene of *Ba. microti* (Rollend et al. 2013). For *A. phagocytophilum*, the primary PCR screen was performed using a melting curve analysis of amplification of the groESL gene which differentiates *A. phagocytophilum* from *Ehrlichia* spp. as described previously (Bell and Patel 2005). Any samples that were positive for *A. phagocytophilum* were confirmed using a nested PCR targeting the 16S r RNA gene of *A. phagocytophilum* (Massung et al. 1998).

Amblyomma americanum-associated *pathogens were detected as follows.* PCR was performed using a melting curve analysis of amplification of the groESL gene which differentiates *A. phagocytophilum, E. chaffeensis, E. ewingii, Ehrlichia muris eauclairensis*, and 'Panola Mountain' *Ehrlichia* (Bell and Patel 2005, Stromdahl et al. 2012, Stromdahl et al. 2014). Any samples positive for *E. chaffeensis* were reconfirmed by a PCR using primers for the 16S rRNA gene of *E. chaffeensis* (Loftis et al. 2003). Any samples positive for *E. ewingii* were reconfirmed by PCR using primers for the p28 gene of *E. ewingii* (Gusa et al. 2001). Any samples positive for *Ehrlichia* sp. 'Panola Mountain' were reconfirmed by a separate PCR using primers for the *gltA* gene of *Ehrlichia* sp. 'Panola Mountain' (Loftis et al. 2008).

Dermacentor-associated *pathogens were detected as follows. PCR was* performed for *Rickettsia* spp. using primers for the *ompB* gene (Jiang et al. 2012) and confirmed and speciated using amplification of the *ompA* gene (Rr190.70p and Rr190.602n) followed by a Pst1 restriction fragment RFLP (Regnery et al. 1991).

Rodent Capture and Sampling

All rodent sampling occurred with United States Department of Agriculture (USDA) authorization under permit 15–030, IACUC16–023. Rodents were captured with Sherman live traps (LFAHD folding trap, H. B. Sherman Traps, Inc., Tallahassee, FL). Each site was sampled twice per month from 25 May through 24 November 2017. Traps were placed in two, 36-trap grids per park and operated for two consecutive nights. The two 6 × 6 grids at each site were placed spatially independent to act as subsets per site. Grid transects began 10 m into the forest from the property-lines on the forest edge with rows continuing deeper into forest habitat at ~10 m intervals. Traps were stationed in preferred rodent microhabitat (Drickamer 1990) within a 5 m radius of grid points.

Peromyscus leucopus and *P. maniculatus* (Wagner) (Rodentia: Cricetidae) co-occur in Maryland (Hall 1981). Molecular methods, such as PCR, are a more reliable method used to differentiate the two *Permomyscus* species compared to using phenotypic traits alone, particularly when identifying juveniles (Fiset et al. 2015, Long et al. 2019). Due to logistical constraints, sequencing of rodent genotypes from samples did not occur. Thus, to avoid erroneous conclusions on tick ecology, we refer to trapped rodents as *Peromyscus* spp. (Machtinger and Williams 2020). Captured rodents were temporarily sedated with isoflurane and examined for presence of ticks. Recovered ticks were placed in ≥80% ethanol. Blood (approximately 100 µl) was collected via subocular puncture on Whatman #4 filter paper (GE Healthcare, Chicago, IL) and allowed to dry before cold storage. An ear biopsy was performed using an ear punch (Integra Miltex, York, PA) and ear samples were placed in RNA Later (Qiagen) and stored at 4°C until processing. Each rodent also was given a unique ear tag identifier (Stoelting Inc., Wood Dale, IL). Marked rodents were then released at the location of their capture.

Rodent Infection With Tick-Borne Pathogens

Nucleic acid was isolated from rodent blood samples as previously described (Fedele et al. 2020). Briefly, 400 µl of lysis buffer (376 µl ATL; 20 µl proteinase K; 2 µl Reagent DX; and 2 µl Carrier RNA, 1 µg/µl; Qiagen) was added to each tube containing the blood sample and samples were incubated for 20 min at 56°C. Nucleic acid was isolated from rodent ear tissue samples as follows. First, the ear tissue sample was placed in a tube containing 100 ml PBS/collagenase A (100 mg collagenase A/ml; Roche, Indianapolis, IN) and incubated for 4 h at 37°C. Second, 300 µl of lysis buffer (276 µl ATL; 20 µl proteinase K; 2 µl Reagent DX; and 2 µl Carrier RNA, 1 µg/µl) was added to each tube containing the ear tissue sample and the sample was incubated overnight at 56°C. Following the final incubation step for each of the sample types, 300 µl lysate of either the blood sample or the ear tissue sample was processed using the KingFisher DNA extraction system and the MagMAX Pathogen RNA/DNA Kit (ThermoFisher Scientific, Houston, TX).

For blood samples, the subsequent multiplex TaqMan PCR reactions included previously described (Fedele et al. 2020) in-house primer and probe master mixes (M73 and M74) targeting *A. phagocytophilum* (*msp2* and *msp4* genes), *Ba. microti* (*sa1* gene and *18S* rDNA), *Bo. miyamotoi* (*purB* and g*lpQ* genes), and rodent GAPDH (Applied Biosystems TaqMan Rodent GAPDH ControlReagents kit; Thermo Fisher Scientific). For ear tissue

samples, we used two different in-house primer and probe master mixes (M76 and M78; Table 1) targeting *Bo. burgdorferi* s.l. (chromosomal DNA), *Bo. burgdorferi* s.s. (*oppA2* gene), *Borrelia mayonii* (*oppA2* gene), *Bo. miyamotoi* (*purB* and g*lpQ* genes), and rodent GAPDH. The rodent GAPDH target was included as a PCR and DNA purification control. PCR reactions for M73, M74 and M78 were performed in 15 μl solutions with 7.5 μl iQ Multiplex Powermix (Bio-Rad, Hercules, CA), 5 μl DNA extract, primers/probes, and water. PCR for M76 was performed in 25 μl with 12.5 μl iQ Multiplex Powermix, 5 μl DNA extract, primers/probes, and water.

The TaqMan PCR cycling conditions for M73 consisted of: denature DNA at 95°C for 3 min followed by 40 cycles of 95°C for 10 s and 60°C for 30 s on a C1000 Touch thermal cycler with a CFX96 real-time system (Bio-Rad). The TaqMan PCR cycling conditions for M74 and M78 consisted of: denature DNA at 95°C for 3 min followed by 40 cycles of 95°C for 10 s and 65°C for 30 s. The TaqMan PCR cycling conditions for M76 consisted of: denature DNA at 95°C for 3 min followed by 40 cycles of 95°C for 10 s and 58°C for 60 s. All PCR samples were analyzed using CFX Manager 3.1 software (Bio-Rad) with the quantitation cycle (Cq) determination mode set to regression. Based on Graham et al. (2016), only Cq values <40 were considered indicative of a pathogen target being present in the tested sample.

Tick Density

Tick density was calculated as the number of ticks collected by drag-sampling divided by twice the transect distance (m). Doubling the transect length accounts for two collectors dragging for ticks side-by-side. Due to site variation in transect length, all tick densities were standardized to 100 m^2. Tukey's honestly significant difference (HSD) multiple comparisons of means was used to make parameter comparisons across temporal and geographic scales. Spatially explicit factors were mapped with ArcMap 10.6.1. (Esri, Redlands, CA) using the North American Datum of 1983 (NAD 1983) latitude/longitude projection.

Generalized Linear Mixed-Effects Model

A generalized linear mixed-effects model (glmm) was produced using the glmmML package (Broström 2020) to estimate the likelihood of abiotic and biotic factors contributing to *Bo. burgdorferi* s.l. infection in *Peromyscus*-fed *I. scapularis*. We modeled *Peromyscus*-fed *I. scapularis* infection status (binary as infected with *Bo. Burgdorferi* s.l. or not infected) as a function of trap depth into forest (spatial gradient) and at different times of the year (seasonal gradient) to estimate the likelihood of encountering rodents with *Bo. burgdorferi* s.l.-infected ticks. Three seasonal categories were assigned to capture data occurring within the spring (4 May to 20 June 2017), summer (21 June to 20 September 2017), and fall (21 September to 15 November 2017). Simulating edge use, the spatial categories were used to determine the likelihood of encountering *Bo. burgdorferi* s.l.-infected *I. scapularis* larvae or nymphs on rodents relative to forest edge or if infection probabilities were related to the season of capture. Three gradient categories indicated forest depth from the peridomestic edge-forest interface. Site was treated as a random effect with fixed effects, including time of year (season), distance from edge, and tick life stage (larva or nymph). Infection status of *I. scapularis* removed from rodents was used as the binary response. Statistical

analyses were done using R (R version 4.0.1 'See Things Now', The R Foundation for Statistical Computing, www.R-project.org). Infection frequency (prevalence) was calculated as the proportion of infected individuals within a constrained sample size. We also used the 'prevalence' package (Devleesschauwer et al. 2015) and Jeffreys Priors to calculate the 95% Bayesian Credible intervals (CI) for prevalence estimations from infection frequencies (Modarelli et al. 2020).

Results

Questing Ticks

As shown in Table 2, the total of 207 ticks collected from drag-sampling included 65 *A. americanum* (55 nymphs and 10 adults), 3 *Dermacentor albipictus* (Packard) adults, 1 *D. variabilis* adult, and 138 *I. scapularis* (110 nymphs and 28 adults; May-November). *Ixodes scapularis* nymphs were most active in the early summer (May-July) with 15/110 (13.6%) of collected nymphs positive for *B. burgdorferi* s.l. The Rockburn site yielded the highest number of *I. scapularis* ($n = 41$) with 7 (17%) *Bo. burgdorferi* s.l. infections and Blandair the lowest ($n = 8$ ticks) and no *Bo. burgdorferi* s.l. infections. *Amblyomma americanum* was the dominant tick species ($n = 44$) at Wincopin and this site accounted for the only two *E. chaffeensis* (2%) infections. However, no *A. americanum* were collected from Centennial or David Force and no questing ticks were found to carry coinfections.

Host-seeking activity of *A. americanum* and *I. scapularis* nymphs was highest from May to July and peaked in June (Fig. 1). *Ixodes scapularis* nymphs were detected throughout the study period (May–November). The mean number of *I. scapularis* nymphs collected from May to July was 0.8 (SE ± 0.4) at Blandair, 1.3 (SE ± 0.6) at Cedar Lane, 2.2 (SE ± 1.1) at Centennial, 1.7 (SE ± 0.4) at David Force, 3.0 (SE ± 1.3) at MPEA, 5.6 (SE ± 2.3) at Rockburn, and 2.5 (SE ± 1.0) at Wincopin. Nymphal tick activity, determined by drag-sampling success, was reduced between August-November compared to May–July (Fig. 1). Because sample sizes were low, sites were grouped for a more robust estimate of prevalence. We found 14% (15/110; CI = 8.5–21.4%) of *I. scapularis* nymphs to be *Bo. burgdorferi* s.l. positive among sites. Prevalence of *A. phagocytophilum* was lower, with 1.8% (2/110; CI = 0.6–6.2%) of *I. scapularis* nymphs infected.

Adult *Dermacentor* spp. were collected from August to October at four sites, including Blandair ($n = 1$, male *D. albipictus*), Cedar Lane ($n = 1$, male *D. variabilis*), Centennial ($n = 1$, female *D. albipictus*), and David Force ($n = 1$, male *D. albipictus*) (Table 2). Neither species were collected from the environment at MPEA, Rockburn, or Wincopin.

Ticks Removed From Rodents

A total of 601 rodents, including 330 uniquely identifiable individuals, were captured over 5,040 trap nights with a trap success of 12% (601/5,040), yielding a total of 602 ticks representing two species (588 *I. scapularis*, larvae = 516, nymphs = 72; 19 *D. variabilis*, larvae = 15, nymphs = 4) that were removed from rodents from May to September (Fig. 2; Table 5). Average tick burden on *Peromyscus* was found to be 3 (SE ± 0.5) *I. scapularis* larvae or nymphs per rodent, ranging from two ticks/rodent host at Blandair to five ticks/

rodent at Cedar Lane. *Ixodes scapularis* nymphs were detected on rodents at all sites from May to September, although in greatest numbers at David Force ($n = 39$). Few ($n = 9$) larvae were removed prior to July sampling. Overall, larval-burdens on *Peromyscus* were greatest from July to September and nymphal-burdens highest in May and June (Fig. 2). We also found *D. variabilis* infesting *Peromyscus* in the months of May and June, though not as frequently as *I. scapularis* overall (Fig. 2).

Prevalence of *Bo. burgdorferi* s.l. infection in *I. scapularis* from rodents was overall very high (mean = 64%; SE ± 12), although site variation was wide-ranging from 0 to 100% (Table 3). *Anaplasma phagocytophilum* was found to occur in 2/15 (13%) nymphs from rodents at Blandair and in 4/39 (10%) of nymphs at David Force. *Anaplasma phagocytophilum* occurred as a coinfection with *Bo. burgdorferi* s.l. in 100% (4/4) of nymphs removed from *Peromyscus* at David Force, and 50% (1/2) at Blandair. The number of *Bo. burgdorferi* s.l.-infected nymphs feeding on *Peromyscus* differed significantly ($T = 3.9$; df = 109; $P < 0.001$) from *Bo. burgdorferi* s.l.-infected larvae (13.0%; 64/492; CI = 10–16%) removed from captured rodents. Although *D. variabilis* ($n = 14$) were removed from rodents, none were found to carry *Rickettsia* spp.

We found no differences in the number of ticks found on *Peromyscus* spp. hosts across edge gradients ($F = 1.05$; df = 2, 213; $P = 0.35$). The relative abundance of infected *I. scapularis* larvae and nymphs was highest in the spring and summer months though there were differences in *Bo. burgdorferi* s.l. prevalence across seasons ($F = 4.68$, df = 2, 18; $P = 0.02$), particularly between summer and fall (Tukey HSD = 34.3, CI = 3–66; $P = 0.03$). Results of our glmm analysis suggest a greater likelihood of nymphs ($P < 0.01$) carrying a tick-borne pathogen when compared to larvae. Also, our model suggests the greater the burdens of *I. scapularis* nymphs on individual *Peromyscus* hosts the more likely they are to harbor *Bo. Burgdorferi* s.l. ($P = 0.02$) compared to random chance alone (Table 4). Similar capture frequencies of infected *Peromyscus* occurred across edge gradients ($F = 0.075$; df = 2, 18; $P = 0.92$) suggesting spatial heterogeneity measured by mark and recapture sampling (Fig. 3).

Rodent Pathogen Infection and Coinfection

Pathogen-infected rodents were found at each site (Table 5). *Borrelia burgdorferi* s.s. was the most frequently detected pathogen with over half of the rodents (53%; SE ± 6) infected across all parks (Table 4). Infection prevalence of *Bo. burgdorferi* s.s. ranged between sites from 61/78 (78%; 68–86%) of rodents infected at DF to 11/31 (35.5%; CI = 21–53%) at MPEA. *Babesia microti* was detected at MPEA in 1/31(3.2%; CI = 0.7–4%) of the rodents suggesting a site prevalence of 3%. *Anaplasma phagocytophilum* was detected in rodents from 3 out of 7 study sites, including Blandair (3/88; 3.4%; CI = 28–48), MPEA (2/31; 6.5%; CI = 2–21), and David Force (33/78; 42.3%; CI = 32–53). However, none of *A. phagocytophilum* infection in mice existed as single pathogen infection. Coinfections of *A. phagocytophilum* and *Bo. burgdorferi* s.s. in rodents accounted for the largest proportion 37/38 (97%; CI = 87–99%) across sites. Moreover, *Bo. miyamotoi* was found in 3.4% of rodents (3/88; CI = 1–10%) at Blandair, 9.3% (4/43; CI = 4–22%) at Cedar Lane, 2.4% (1/42; CI = 1–12) at Centennial, and 2.6% (2/78; CI = 1–9%) at David Force (Table 4).

Seasonal distributions of *Bo. Burgdorferi* s.s. prevalence in *Peromyscus* spp. showed a significant difference in infection when comparing the spring and summer to fall ($F = 6.2$; df = 2, 21; $P = 0.007$). Across all parks, landscape-level infection prevalence was highest in the spring (49%; SE ± 7.7) and summer (52%; SE ± 4.0), while lowest in the fall (18%; SE ± 9.7).

Discussion

High incidence of Lyme disease is typically associated with high densities of *I. scapularis* nymphs (Eisen and Eisen 2018). Densities of questing *I. scapularis* nymphs have also been shown to be greater in northern states compared to states toward southern latitudes (Diuk-Wasser et al. 2010) where nymphal questing behavior has been shown to differ (Arsnoe et al. 2019). Interestingly, Maryland ranks 7th in states with the highest incidence of Lyme disease in the United States (CDC 2020), although Maryland has relatively low nymph densities compared to other areas in the northeastern United States (Diuk-Wasser et al. 2010). Maryland is also geographically located in the mid-Atlantic region along the latitudinal gradient where *I. scapularis* questing behavior could be varied (Arsnoe et al. 2015) making the assessment of Lyme disease risk from questing *I. scapularis* nymphs alone challenging (Eisen and Eisen 2018).

Previous studies in Maryland have reported higher densities of *I. scapularis* nymphs, although the historical prevalence of *Bo. burgdorferi* s.l. has remained relatively similar. Swanson and Norris (2007) reported a mean density of 0.4 *I. scapularis* nymphs per 100 m^2 (SE ± 0.3) with 14.7% (51/348) found to be positive for *Bo. burgdorferi* s.s. in 2003. Approximately a decade later, Johnson et al. (2017) drag-sampled 3 Maryland parks in 2014–2015 and found host-seeking *I. scapularis* nymphs in still higher densities (mean density per 100 m^2 = 3.6, SE ± 1.8), though site prevalence of *Bo. burgdorferi* s.s. infection (10–36%) was similar to our *Bo. burgdorferi* s.l. infection of 21% (23/110; CI = 15–30%). However, when we consider *Peromyscus* individuals positive for *Bo. burgdorferi* s.s., our mean site prevalence (53%; SE ± 6%) was much higher than in the 2014–2015 study. This suggests *Bo. Burgdorferi* s.s. can persist in *Peromyscus* hosts in areas with lower nymph densities (Arsnoe et al. 2019).

Infection-risk can be estimated from monitoring the densities of host-seeking *I. scapularis* nymphs. Although our sites show a high prevalence (53%; SE± 6%) of *Bo. burgdorferi* s.s. circulating in *Peromyscus* hosts, other pathogens, including *A. phagocytophilum*, *Ba. microti*, and *Bo. miyamotoi*, were also present at sites where rodent relative densities were high. We found on the landscape level, *I. scapualris* nymphs collected from *Peromyscus* spp. with the highest burden were more likely to carry infected nymphs. This would suggest a higher probability of encountering infected nymphs in areas of high *Peromyscus* presence. Although questing nymph densities are low at our sites compared to Hofmeister et al. (1999) or Swanson and Norris (2007) rodent tick burdens seem to be at levels where pathogens are maintained in rodent hosts and parasitizing ticks.

Rodent tissue sampling can provide further insight into infection probabilities and the persistence of tick-borne infections in rodent hosts. Monitoring these levels of infection over

time can be helpful in monitoring changes in tick-borne pathogen prevalence for a given geographic space. For example, Hofmeister et al. (1999) sampled *P. leucopus* rodents (n = 202) from 1991 to 1993 and found that 26% of rodents were infected with *Borrelia* across 3 yr and 42% were *Borrelia*-positive concurrent cross-sectional studies in Baltimore County, Maryland. A second study in 2001 determined 25% of 173 *P. leucopus* in the Maryland coastal plains to be infected with the ospA or ospC *Borrelia burgdorferi* variants (Anderson and Norris 2006). Though the previous study (Anderson and Norris 2006) focused on specific surface proteins for detection in rodent tissues, our study showed a higher *Bo. burgdorferi* s.s. mean prevalence (64%; Table 2) in rodents from 2017. Further, Zawada and others (2020) recently published tissue-specific detections of *Borrelia* infection in rodents from Fairfax, Virginia and report that 43% of rodent ear tissues were positive for *Bo. burgdorferi* s.l. (Zawada et al. 2020). We found 50.7% (194/382; CI = 46–56%) of overall Howard County *Peromyscus* at our study sites were infected with *Bo. burgdorferi* s.s.

Considering coinfections with other pathogens, we found 15/110 (13.6%; CI = 8.5–21.4%) of host-seeking nymphs to be infected with *Bo. burgdorferi* s.l. and overall the nymphs had an *A. phagocytophilum* prevalence of 2/110 (1.8%; CI = 0.6–6.2%), which is much higher than an earlier report of 0.3% *A. phagocytophilum* prevalence in questing *I. scapularis* nymphs (Swanson and Norris 2007). Given that coinfections with tick-borne pathogens can have mutualistic relationships (Diuk-Wasser et al. 2016, Cabezas-Cruz et al. 2018), the reduction of transmission probabilities where coinfections may occur will be an important facet of strategies aimed at reducing Lyme disease risk through nymphal burden reduction.

It is also important to consider the influence of non-target species (e.g., chipmunks, shrews, and squirrels) may have on not only estimates of infection prevalence, but also their contribution to pathogen maintenance and transmission. For example, Alghaferi et al. (2005) included small mammals such as eastern chipmunks (*Tamias striatus*) when determining prevalence of ospC-specific *Borrelia* in Maryland and Pennsylvania. The authors found 60% (71/118; CI = 5–69%) of small mammals contributed to overall prevalence. In our study, we opportunistically sampled 7 *T. striatus* which were captured and sampled. We found 4/6 (66.7%) *T. striatus* to be infected with *Bo. burgdorferi* s.s. When we include these mammals in the assemblage, prevalence at Centennial and David Force sites increase by 0.2% and 1%, respectively. Though it is not a substantial increase in our case, nonetheless, the increase suggests non-target host species can carry tick-borne pathogens in our study area and are likely contributing to enzootic pathogen maintenance.

Targeting stages where coinfection transmission events are more likely to occur can help reduce the potential for pathogen maintenance. Studies using 4-poster treatments or fipronil treated bait boxes have been successful at reducing tick abundances on the landscape (Schulze et al. 2017, Williams et al. 2018b). These two approaches target tick reduction at points where *I. scapularis* feed on their mammalian hosts (Fig. 4). Rodents and deer are the main contributors of *A. phagocytophilum* and rodents are commonly associated with *Ba. microti* and/or *Bo. burgdorferi* s.l. coinfections (Diuk-Wasser et al. 2010). *Borrelia miyamotoi* exhibits vertical as well as horizontal transmission (Rollend et al. 2013) and may co-occur with *Ba. microti* and/or *Bo. burgdorferi* s.l. (Diuk-Wasser et al. 2010, Hersh et al. 2014). Reducing opportunities for vectors, hosts, and pathogens to interact may help

reduce tick-borne pathogen maintenance and eventually reduce the incidence of tick-borne infections in Maryland.

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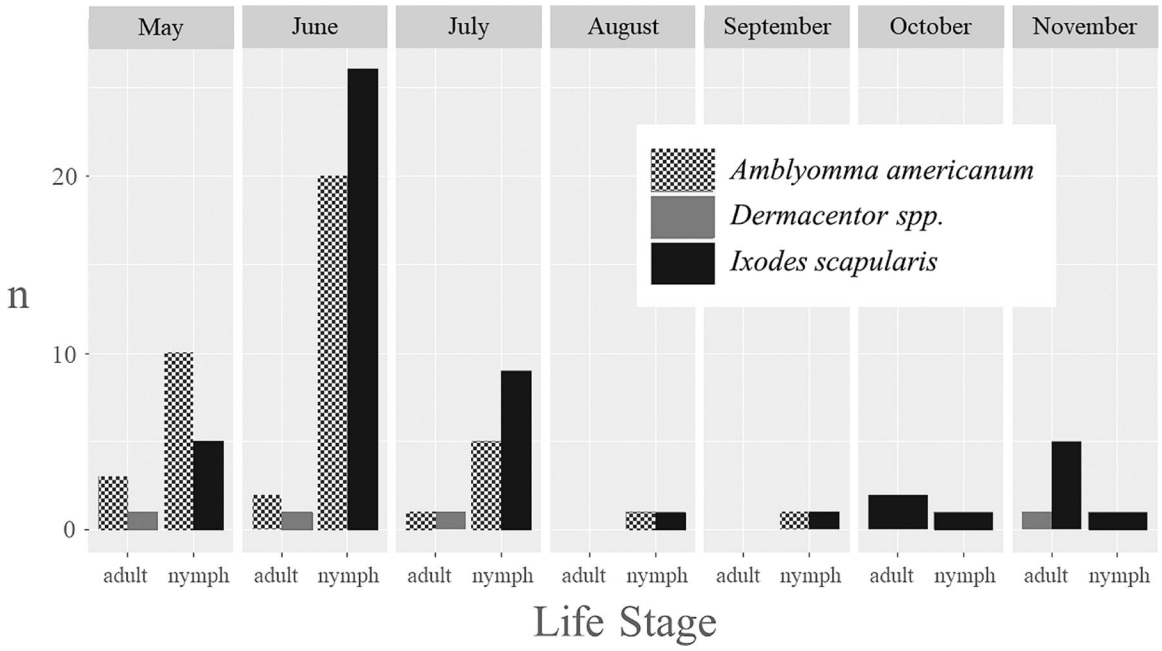

Fig. 1.
Tick species collected from drag-sampling parks in Howard County, Maryland, 2017.

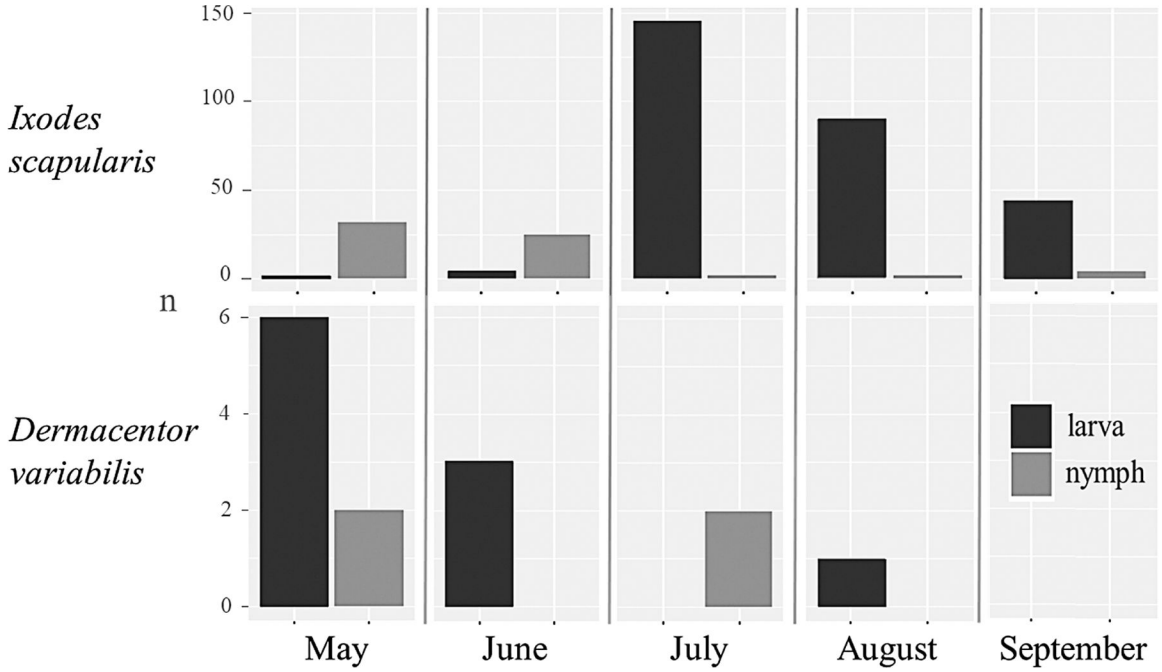

Fig. 2.
Tick species collected from *Peromyscus* spp. rodents at parks in Howard County, Maryland, 2017.

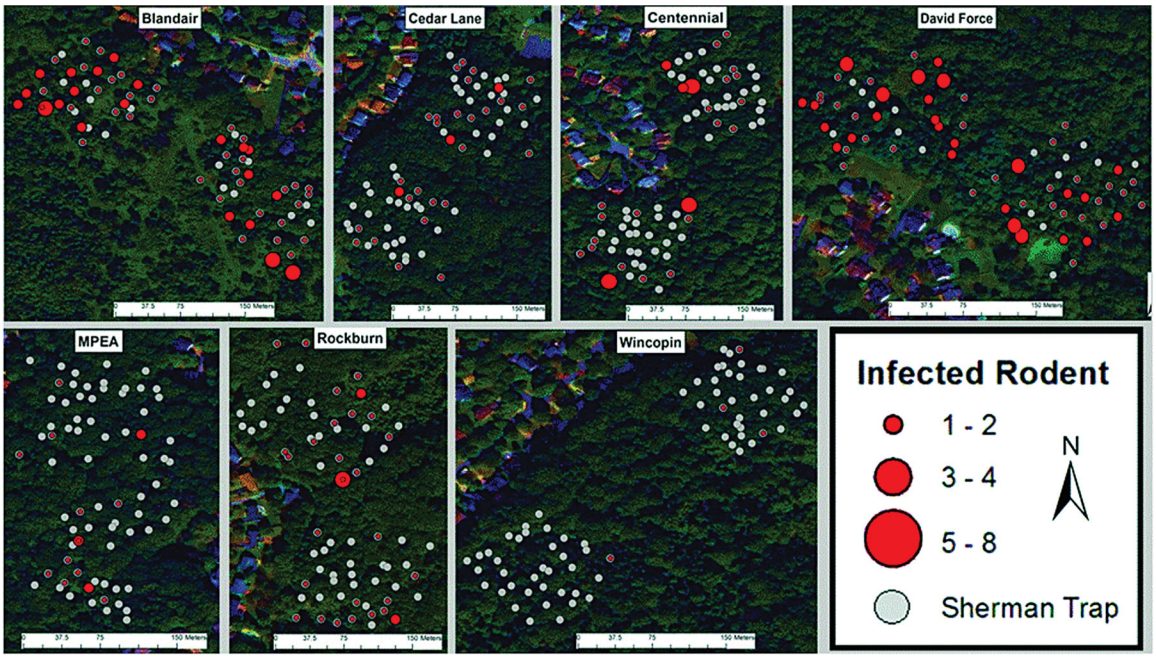

Fig. 3.

Distribution and capture frequency of *Borrelia burgdorferi* sensu stricto-infected *Peromyscus* spp. rodents at suburban parks in Howard County, Maryland, 2017.

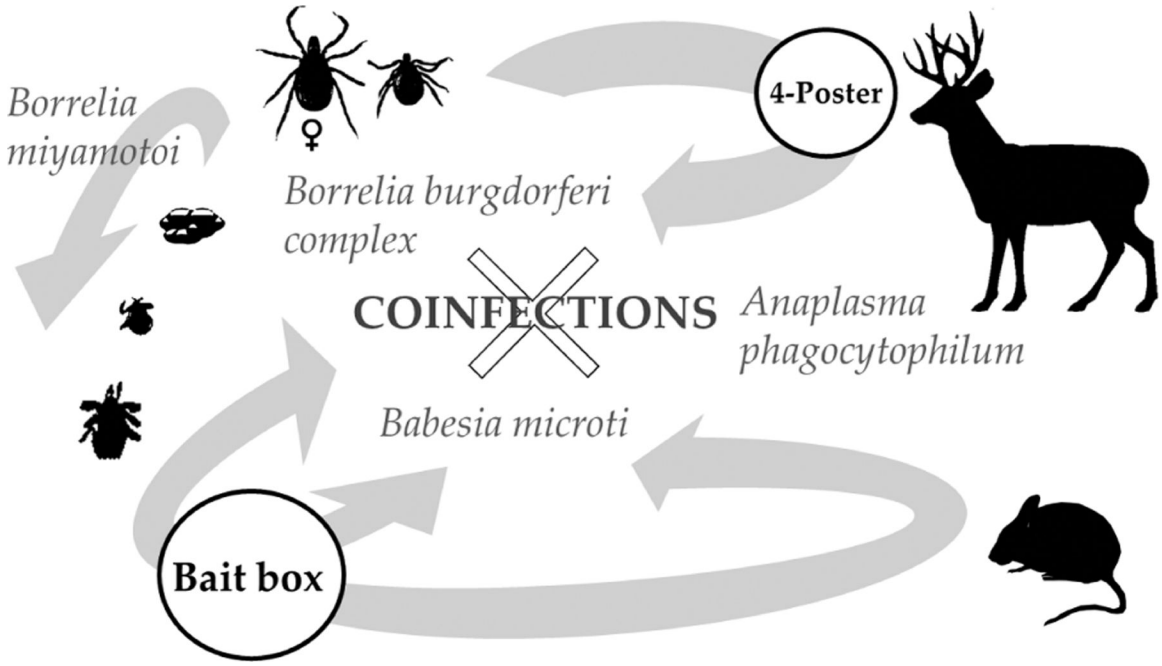

Fig. 4.

Fipronil treated bait boxes and four-poster acaricide applicators target critical steps in tick-borne pathogen circulation among *Ixodes scapularis* ticks, *Odocoileus virginianus* deer, and P*eromyscus* spp. rodent hosts.

Table 1.

Primers and probes included in the in-house multiplex PCR master mixes to detect *Borrelia* pathogens in rodent ear tissue

Target	Primers and probes	Sequence 5′−3′	Size (bp)	Reference	Final concentration (μM)
M76 master mix					
Bo. burgdorferi sensu stricto (*oppA2* gene)	Bb-F	AATTTTTGGTTCCATACCC	162	Graham et al. 2018	0.45
Bo. mayonii (*oppA2* gene)	Bmayo-F	GCCCGATTTAATCAAAGA	144	Graham et al. 2018	0.45
	Bb/Bmayo-R	CTGTCAATAGCAAGAGTTAA		Graham et al. 2018	0.9
	Bb-Probe	HEX-CGTTCAATACACACATCAAACCACT-BHQ1		Graham et al. 2018	0.2
	Bmayo-Probe	FAM-ACACGCACATTAAACCGCTTGAT-BHQ1		Graham et al. 2018	0.2
Bo. miyamotoi (*purB* gene)	purB_F	TCCTCAATGATGAAAGCTTTA	121	Graham et al. 2016	0.3
	purB_R	GGATCAACTGTCTCTTTAATAAAG		Graham et al. 2016	0.3
	purB_Probe	CalRD610-TCGACTTGCAATGATGCAAAACCT-BHQ2		Graham et al. 2016	0.2
M78 master mix					
Bo. burgdorferi sensu lato (chromosomal DNA)	Bbsl_F	CCCAAAGCAGGTGCCTTAGC	78	This study	0.3
	Bbsl_R	TCTGTAGGTTTTAGGTTCGAGTCC		This study	0.3
	Bbsl_probe	AGGCCACATCCCGAATGAAGCGCA		This study	0.2
Bo. miyamotoi (*glpQ* gene)	glpQ_F	GACCCAGAAATTGACACAACCACAA	108	Graham et al. 2016	0.3
	glpQ_R	TGATTTAAGTTCAGTTAGTGTGAAGTCAGT		Graham et al. 2016	0.3
	glpQ_Probe	CalRd610-CAATCGAGCTAGAGAAAACGGAAGATATTACG-BHQ2		Graham et al. 2016	0.2
GAPDH				Rodent GAPDH Control Reagents kit	0.2/0.2

BHQ1 and BHQ2: Black Hole Quencher 1 and 2, respectively; CalRd610: CalFluor Red 610; FAM, 6-Carboxyfluorescein; HEX, Hexachloro-Fluorescein Phosphoramidite.

Table 2.

Pathogen infection status of ticks collected by drag-sampling at parks in Howard County, Maryland, 2017

Site	Total ticks	*Amblyomma americanum*		*Dermacentor* spp.	*Ixodes scapularis*				Pathogen prevalence (%; CI)[a]		
		Nymphs	Adults (♂/♀)	Adults (♂/♀)	Nymphs	Adults (♂/♀)	NIP (±SE)	*D*	*Borrelia burgdorferi* s.l.	*Anaplasma phagocytophilum*	*Ehrlichia chaffeensis*
Blandair	10	1	-	1 (1/0)[a]	7	1 (1/0)	2 (0.3)	0.00001	-	-	-
Cedar Lane	19	2	-	1 (1/0)[v]	9	7 (3/4)	3 (0.3)	0.00003	6/16 (38; 18–62)	-	-
Centennial	19	0	-	1 (0/1)[a]	15	3 (2/1)	3 (1.3)	0.00003	5/18 (28; 13–51)	1/18 (6; 1–26)	-
David Force	12	0	-	1 (1/0)[a]	10	1 (1/0)	2 (0.3)	0.00002	3/10 (30; 11–61)	1/11 (9; 2–38)	-
MPEA	26	0	1 (0/1)	-	18	7 (4/3)	4 (1.4)	0.00004	2/25 (8; 3–25)	-	-
Rockburn	58	15	2 (2/0)	-	34	7 (5/2)	6 (2.3)	0.00006	7/41 (17; 9–31)	1/58 (2; <1–9)	-
Wincopin	63	37	7 (4/3)	-	17	2 (1/1)	4 (0.9)	0.00003	-	1/63 (2; <1–8)	1/44 (2; <1–12)

NIP = mean number of nymphs collected per sampling date during May–July; D = nymphal density during May–July per 100 m^2.

[a] *A. americanum* were tested for *A. phagocytophilum* and *Ehrlichia* spp.; *D. albipictus* (a) and *D. variablilis* (v) were tested for *Rickettsia* spp.; *I. scapularis* were tested for *A. phagocytophilum*, *Ba. microti*, and *Bo. Burgdorferi* s.l.

Table 3.

Ixodes scapularis ticks removed from *Peromyscus* spp. rodents at Howard County, Maryland parks, 2017

Site	No. mice examined	Infested mice[a] (%)	Tick burden[b]	No. nymphs collected	Infection prevalence in nymphs (%; CI)	
					Anaplasma phagocytophilum	***Borrelia burgdorferi* s.l**
Blandair	88	39/88 (44)	2.3 (89/39)	15	2/15 (13; 4–39)	12/15 (80; 54–93)
Cedar Lane	43	25/43 (58)	5.0 (126/25)	4	-	-
Centennial	42	17/42 (40)	2.9 (49/17)	6	-	3/6 (50; 19–82)
David Force	78	55/78 (70)	4.2 (229/55)	39	4/39 (10; 4–23)	26/39 (67; 51–79)
MPEA	31	13/31 (41)	1.5 (48/31)	7	-	5/7 (71; 35–91)
Rockburn	40	20/40 (50)	2.8 (56/20)	6	-	5/6 (83; 45–96)
Wincopin	8	3/8 (38)	1.7 (5/3)	3	-	3/3 (100; 47–100)

[a]% of rodents with *I. scapularis* ticks.

[b]Mean number of ticks per rodent host; pathogens tested include *Anaplasma phagocytophilum* and *Borrelia burgdorferi* s.l.

Table 4.

Parameter estimates for a generalized linear mixed-effects model estimating the likelihood of captured rodents to carry a *Borrelia burgdorferi* s.l.-infected *Ixodes scapularis* across an edge-forest gradient

Parameter	Coefficient	SE	*z*	*P*
Intercept	0.71	0.78	0.91	0.36
Spring	−0.75	0.76	−0.98	0.33
Summer	−1.33	0.71	−1.87	0.06
Mid-forest	−0.64	0.38	−1.68	0.09
Deep forest	−0.54	0.37	−1.48	0.14
Burden	0.15	0.06	2.36	0.02
Nymph	1.31	0.47	2.79	<0.01

Table 5.

Infection prevalence of *Peromyscus* spp. rodents captured at Howard County, Maryland parks, 2017

Site	n_m	n_r	Infection prevalence (%; CI)				Coinfection prevalence (%; CI)		
			Anaplasma phagocytophilum	*Babesia microti*	*Borrelia burgdorferi* s.l.	*Borrelia miyamotoi*	*Anaplasma* and *Bo. burgdorferi*	*Babesia* and *Bo. burgdorferi*	*Bo. Burgdorferi* and *Bo. miyamotoi*
			3/88 (3; 1–9)	-	55/88 (63; 52–72)	3/88 (3; 1–10)	3/88 (3; 1–10)	-	3/88 (3; 1–10)
Cedar Lane	43	37	-	-	18/43 (42; 28–57)	1/43 (2; <1–12)	-	-	1/43 (2; <1–12)
Centennial	42	35	-	-	23/42 (55; 40–69)	1/42 (2; 1–12)	-	-	1/42 (2; 1–12)
David Force	78	66	33/78 (42; 32–53)	-	61/78 (78; 68–86)	2/78 (3; 1–9)	31/78 (40; 30–51)	2/78 (3; 1–9)	-
MPEA	31	23	2/31 (6; 2–21)	1/31 (3; <1–17)	11/31 (35; 21–86)	-	2/31 (6; 2–21)	1/31 (3; 1–17)	-
Rockburn	40	28	-	-	23/40 (58; 42–72)	-	-	-	-
Wincopin	8	0	-	-	3/8 (38; 14–70)	-	-	-	-

n_m = total number of individual mice examined; n_r = number of recaptured individuals examined.