Host-pathogen associations inferred from bloodmeal analyses of *Ixodes scapularis* ticks in a low biodiversity setting

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ABSTRACT

Tick-borne pathogen emergence is dependent on the abundance and distribution of competent hosts in the environment. *Ixodes scapularis* ticks are generalist feeders, and their pathogen infection prevalence depends on their relative feeding on local competent and non-competent hosts. The ability to determine what host a larval life stage tick fed on can help predict infection prevalence, emergence, and spread of certain tick-borne pathogens and the risks posed to public health. Here, we use a newly developed genomic target-based technique to detect the source of larval bloodmeals by sampling questing nymphs from Block Island, RI, a small island with a depauperate mammalian community. We used previously designed specific assays to target all known hosts on this island and analyzed ticks for four human pathogenic tick-borne pathogens. We determined the highest proportion of larvae fed on avian species (42.34%), white-footed mice (36.94%), and white-tailed deer (20.72%) and occasionally fed on feral cats, rats, and voles, which are in low abundance on Block Island. Additionally, larvae that had fed on white-footed mice were significantly more likely to be infected with *Borrelia burgdorferi* and *Babesia microti,* while larvae that had fed on white-footed mice or white-tailed deer were significantly more likely to be infected with, respectively, mouse- and deer-associated genotypes of *Anaplasma phagocytophilum*. The ability to detect a nymph's larval host allows for a better understanding of tick feeding behavior, host distribution, pathogen prevalence, and zoonotic risks to humans, which can contribute to better tick management strategies.

IMPORTANCE

Tick-borne diseases, such as Lyme disease, babesiosis, and anaplasmosis, pose significant public health burdens. Tick bloodmeal analysis provides a noninvasive sampling method to evaluate tick-host associations and combined with a zoonotic pathogen assay, can generate crucial insights into the epidemiology and transmission of tick-borne diseases by identifying potential key maintenance hosts. We investigated the bloodmeals of questing *Ixodes scapularis* nymphs. We found that avian hosts, white-footed mice, and white-tailed deer fed the majority of larval ticks and differentially contributed to the prevalence of multiple tick-borne pathogens and pathogen genotypes in a low biodiversity island setting. Unraveling the intricate network of host-vector-pathogen interactions will contribute to improving wildlife management and conservation efforts, to developing targeted surveillance, and vector and host control efforts, ultimately reducing the incidence of tick-borne diseases and improving public health.

KEYWORDS: white-footed mouse, white-tailed deer, avian, lyme disease, Borrelia, Babesia, Anaplasma

INTRODUCTION

Tick-borne disease incidence and geographic range continue to increase worldwide due to geographical expansion of pathogens, vectors and hosts, climate change, and increased awareness and testing for tick-borne pathogens (1–4). In the United States, *Ixodes scapularis* ticks transmit pathogens of human and veterinary concern including *Anaplasma phagocytophilum*, *Babesia microti*, *Borrelia burgdorferi*, *Borrelia mayonii*, *Borrelia miyamotoi*, *Ehrlichia* spp., and Powassan viruses (5–7). *Ixodes scapularis* is a three-host tick, where each life stage feeds on a different host and may become infected during immature life stages (8). Hosts vary in their reservoir competence for different pathogens, defined as an integrated measure of the host's susceptibility to an infection, the pathogen's ability to evade the host's immune system and survive in the host, and the pathogen's ability to be transmitted to new susceptible vectors during bloodmeal uptake (9, 10).

The relative abundance and reservoir competence of vertebrate species in the local host community strongly impact pathogen transmission, as *I. scapularis* have limited feeding selectivity (11). White-footed mice (*Peromyscus leucopus*) are competent reservoir hosts for many tick-borne pathogens (12, 13), and other vertebrate hosts (i.e., birds, reptiles, mesomammals) have varying levels of reservoir competence (14–18). In the eastern United States, some small mammals [e.g., Eastern chipmunk (*Tamias striatus*), meadow vole (*Microtus pennsylvanicus*), shrews (*Sorex* spp.), white-footed mice] and some avian species [e.g. American robin (*Turdus migratorius*), Carolina wren (*Thryothorus ludovicianus*)] are competent reservoir hosts for *B. burgdorferi* sensu stricto, a causative agent of Lyme disease (12, 19–26), while other vertebrates show low or no competence for this pathogen [e.g., raccoons (*Procyon lotor*), Virginia opossum (*Didelphis virginiana*), white-tailed deer (*Odocoileus virginianus*), gray catbirds (*Dumetella carolinensis*), etc.] (24, 27–31). Additionally, *B. microti*, a causative agent of human babesiosis, has a limited range of competent hosts, primarily rodent species (15, 32–35). White-tailed deer play a dual role in the system; for adult *I. scapularis*, white-tailed deer are the primary reproductive host (36–38), but deer are not competent hosts for *B. burgdorferi* or *B. microti* and do not support the transmission of these pathogens (31, 39, 40). However, white-tailed deer are reservoir hosts for the non-human pathogenic (deer) variant 1 (Ap-V1) of *A. phagocytophilum*, but not the

pathogenic human-active variant (Ap-ha); both variants can be transmitted by *I. scapularis* nymphs and adults (41–44). Previous studies have shown that *A. phagocytophilum* is not transovarially transmitted in ticks and multiple genetic variants are maintained in the environment in different host species, with white-footed mice the most common reservoir host for Ap-ha (41–49). Some avian species may also serve as competent hosts for tick-borne pathogens (23–25, 29, 50–54); however, there is limited data on their contribution in maintaining these pathogens in natural populations in the United States. Therefore, determining what host a larval tick fed upon is vital to predicting patterns of pathogen prevalence in questing nymphal ticks in the environment, which constitutes the highest risk factor for humans (55).

While the reservoir competence of multiple host species for *B. burgdorferi* and other *I. scapularis*-borne pathogens has been assessed by field sampling or experimental estimates, the relative contribution of these species to the enzootic cycle based on their abundance and tick burdens is rarely known. Geographic areas characterized by low vertebrate diversity provide an opportunity to assess the role of a subset of previously proposed hosts in the ecology of tick-borne pathogens. White-footed mice and white-tailed deer have been recognized as the main contributors to the enzootic cycle of *B. burgdorferi* and as amplifying hosts of *I. scapularis*, respectively; however, the role of other vertebrate hosts as tick and pathogen hosts is poorly understood, as is the role of white-tailed deer in feeding *I. scapularis* larvae. In a previous study, we measured host density, larval tick burdens, and infection prevalence of *B. burgdorferi* in white-footed mice and multiple bird species on Block Island, RI. Because sampling white-tailed deer for larval ticks is challenging, as larvae are primarily only active during the non-hunting season, we estimated the contribution of larvae feeding on deer using a mathematical back calculation (25). We now employ a bloodmeal analysis method (34, 56) as a direct metric of larvae- and pathogen-host associations for *B. burgdorferi* and to assess host associations with *B. microti* and *A. phagocytophilum* in the same study area. Of particular interest is assessing *B. microti* and *A. phagocytophilum* association with birds. Some previous studies have reported no association of *B. microti* with avian species (57–60). While limited studies reported *B. microti* positive derived ticks from birds worldwide (15, 61, 62), these studies did not differentiate pathogenic from non-pathogenic *B. microti*-like species or had extremely small samples sizes, therefore, the available data supporting *B. microti* infection in avian species is unconvincing. We also aim to confirm *P. leucopus* as a key host for Ap-ha and white-tailed deer for Ap-V1 [given the absence of most rodents, shrews, and mesomammals at our study location (25, 63, 64)] and quantify the role of white-tailed deer as non-competent hosts for *B. burgdorferi* and *B. microti* infection.

To assess tick-host feeding association and specific host contributions to tick-borne pathogen prevalence, we collected questing nymphs from multiple locations on Block Island, RI, used the retrotransposon-based qPCR bloodmeal analysis for host identification (34, 56), and a multiplex quantitative real-time PCR (qPCR) assay for pathogen detection (65). We utilized specific primer pairs for all known hosts on Block Island to identify host DNA in field-collected *I. scapularis* nymphs. These data provide direct evidence of tick feeding behavior and host contributions to the enzootic transmission cycle.

MATERIALS AND METHODS

Sample collection

Questing *I. scapularis* nymphs were collected from seven locations across Block Island, RI, from late May to early July 2019 (27 May–3 July). These sites included Boy Scout campground

(BS), Clayhead Trail (CH), East Island private property (EI), Hodge Family Wildlife Preserve (HP), the Maze Trail (MZ), Block Island National Wildlife Refuge (NR), and Rodman's Hollow (RH). Ticks were collected by dragging a 1 m^2 corduroy cloth along the leaf litter in a mostly deciduous forested habitat, removing attached ticks every 10 m. Ticks were placed in live collection tubes and kept frozen at −80°C until molecular processing.

DNA extraction, pathogen detection, and bloodmeal analysis

DNA was extracted from nymphs using a modified HOTSHOT protocol described elsewhere (34). Ticks were screened for the presence of *A. phagocytophilum*, *B. microti*, *B. burgdorferi*, and *B. miyamotoi* using a multiplex quantitative real-time PCR (qPCR) protocol [see reference (65) for primers, probes, and cycling conditions]. Positive *A. phagocytophilum* samples were further investigated for type (Ap-ha and Ap-V1) using a previously published PCR assay that differentiates the two types by amplicon size due to a 26 bp deletion in the deer variant (Ap-ha = 196 bp and Ap-V1 = 170 bp) (49). Samples that tested positive with initial screening were reamplified using the published primers and then run on a 1% agarose gel to visualize amplicon size. Bloodmeal identification was used to determine the host type a nymph previously fed on using qPCR targeting mammalian retrotransposons as previously described (34, 56, 66, 67). Ticks for this study were analyzed using specific assays to identify bird, cat, rat, vole, white-footed mouse, and white-tailed deer. The rat primers (RatLINE369F: 5′-ATCCCCATCA AAATACCAATCC-3′ and RatLINE 422R: 5′-GCAAATTGTTCTGTCTAACTCTT-3′) were newly developed and tested for sensitivity and specificity as previously described (66). Briefly, retrotransposon sequences from rats were downloaded from GenBank, aligned with similar sequences from other rodents, and specific primers were designed using Geneious (BioMatters Ltd. Auckland, New Zealand). Primers were tested for specificity against a panel of DNA from other mammals that are possible bloodmeal hosts at New England field sites [see reference (34) for a complete list] as well as human DNA. Sensitivity was determined using serial dilutions of positive control DNA. All larval ticks were tested in duplicate and only those that were repeatable were determined positive.

Data analyses

Pathogen prevalence was calculated for each host type and collection site as the number of infected individuals/total individuals. We used Fisher exact tests to assess whether the host type was significantly different among collection sites, whether prevalence for each pathogen was significantly different among collection sites, whether prevalence for each pathogen was significantly different among host types, and whether *A. phagocytophilum* variants were significantly different among collection sites and host types. We then used pairwise and row-wise *post hoc* Fisher tests to assess comparisons between all pairs of collection sites and host types. Analyses were completed using R (rstatix). Additionally, we used the Mantel-Haenszel chi-square odds ratio test to assess whether infection prevalence with each of the top three pathogens was significantly different in each of the top three most prevalent host types compared to the other host types; the latter analyses were conducted using GraphPad Prism (version 9.3.1; GraphPad Software Inc.). For statistical analyses, nymphs with mixed bloodmeals were included in each host or pathogen category.

RESULTS

We collected a total of 424 questing nymphs from seven collection sites on Block Island, all of which were analyzed for the presence of four tick-borne pathogens: *A. phagocytophilum*, *B.*

microti, *B. burgdorferi*, and *B. miyamotoi*. Of these, 341 nymphs were analyzed for previous host bloodmeal using qPCR targeting retrotransposons. We detected six different host types from 227 of these nymphs from the seven collection sites (66.57% success rate) and detected tick-borne pathogens from 117 questing nymphs.

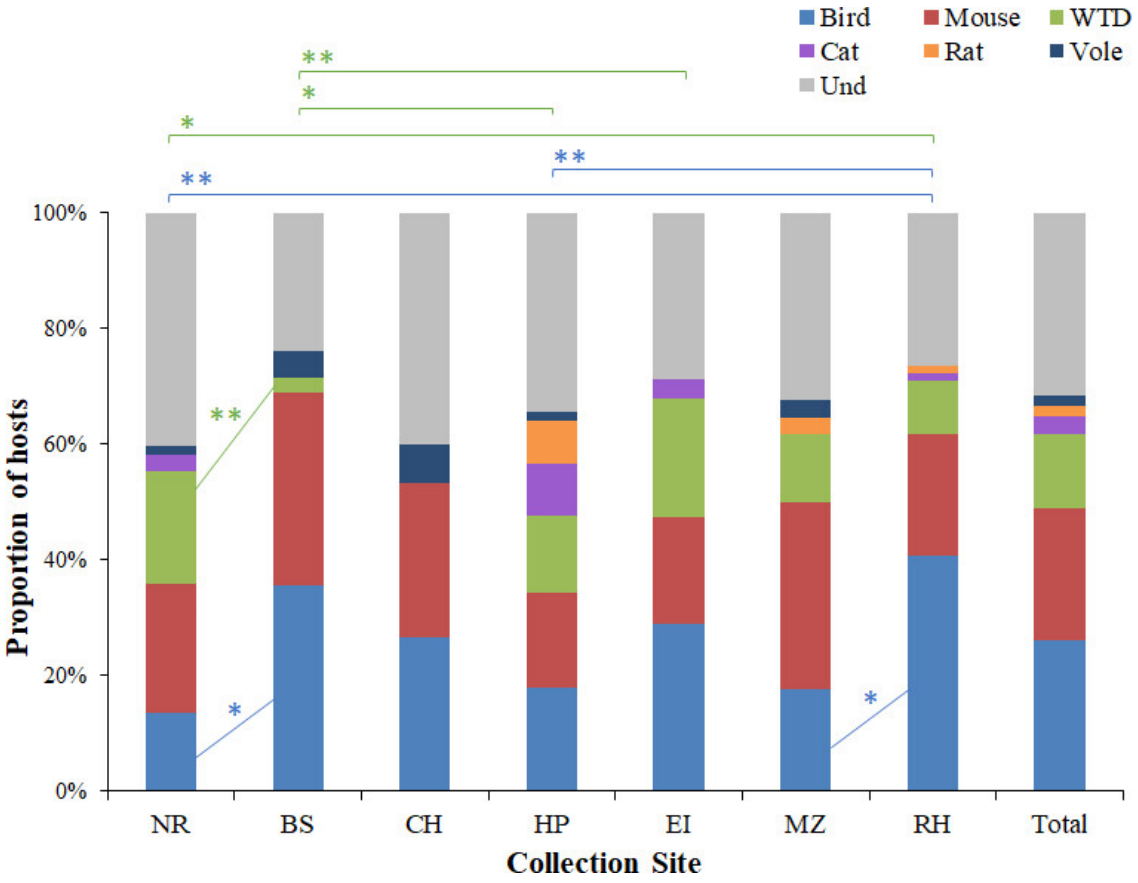

Fig 1 Proportion of hosts (number of specific host types/total hosts at a specific collection site) fed on by *Ixodes scapularis* larvae as determined using bloodmeal analysis from questing nymphs at each of the seven collection sites. Significant differences among host types and collection sites were calculated using Fisher exact tests and then *post hoc* pairwise comparisons between each host type. Colored lines/brackets correspond to significant differences observed between a host type at the indicated collection site, and asterisks denote the level of significant differences (*$P \leq 0.05$, **$P \leq 0.01$).

Host identification from nymphal bloodmeal

Based on bloodmeal analyses, the hosts most commonly fed upon by larval ticks on Block Island were birds, white-footed mice, and white-tailed deer (Table S1; Fig. 1). Cats, rats, and voles were also detected in questing nymphs; however, these hosts were uncommon and not observed at all collection sites (Table S1; Fig. 1). Mixed bloodmeals, resulting in more than one host detected, were observed in 5.28% of the total nymphs tested (n = 18), with one tick being positive for three hosts (bird, white-footed mouse, and white-tailed deer). The most common mixed bloodmeal combination was white-footed mice and white-tailed deer (n = 7). Of the mixed bloodmeals, 11 were infected with at least one pathogen; nine of these infected nymphs contained white-footed mouse as one of the combinations and the remaining two infected nymphs contained birds as one of the combinations (Table S2). One *B. miyamotoi* positive nymph was found as a mixed bloodmeal from a bird and cat. Two nymphs that fed on

white-tailed deer were positive for *B. burgdorferi*, one of these was in a mixed bloodmeal with a white-footed mouse and the other with a bird. Only including the most abundant hosts (and only samples where a host was detected), 42.34% of larvae fed on avian species, 36.94% on white-footed mice, and 20.72% on white-tailed deer.

Host type-collection site associations

HP exhibited the highest host richness, having at least one positive nymph from each host type investigated, while CH displayed the least host richness, where only three host types were detected (Fig. 1). Including all host types and excluding undetermined hosts, no significant differences were observed for the proportion of larvae that previously fed on white-footed mice, voles, or cats across collection sites ($P = 0.2700$, $P = 0.1020$, $P = 0.1080$, respectively); however, significant differences were observed for birds, white-tailed deer, and rats among collection sites ($P = 0.0145$; $P = 0.0095$; $P = 0.0335$, respectively). For pairwise analyses between collection sites, significantly more larvae from BS fed on birds compared to NR ($P = 0.0438$) and significantly more larvae from RH fed on birds compared to NR, HP, and MZ ($P = 0.0016$, $P = 0.0078$, $P = 0.0252$, respectively; Fig. 1). Additionally, significantly more larvae from NR fed on white-tailed deer compared to BS and RH ($P = 0.0020$, $P = 0.0226$, respectively) and significantly more larvae at HP and EI fed on white-tailed deer compared to BS ($P = 0.0382$, $P = 0.0048$, respectively; Fig. 1). While the overall proportion of larvae that fed on rats was significantly different among all collection sites ($P = 0.0335$), no significant pairwise comparisons were observed. Based on site, EI and MZ produced the highest number of mixed bloodmeals ($n = 4$ each) (Table S3).

Pathogen-collection site associations

Of the 424 questing nymphs that were screened for pathogens, 18.87% ($n = 80$) were positive for *B. burgdorferi*, 8.49% ($n = 36$) were positive for *B. microti*, 6.84% ($n = 29$) were positive for *A. phagocytophilum*, and 0.24% ($n = 1$) were positive for *B. miyamotoi* (Table S4). The HP site showed the highest pathogen richness, having at least one positive nymph from each pathogen tested and was the only site with a nymph positive for *B. miyamotoi* (Fig. 2; Table S4). CH showed the least pathogen richness, but the sample size at this site was relatively small compared to other sites. No significant differences were observed for the proportion of nymphs infected with *B. burgdorferi* or *B. microti* among any collection sites ($P = 0.2440$, $P = 0.6950$, respectively), while the proportion of nymphs infected with *A. phagocytophilum* was significantly different among collection sites ($P = 0.0330$). *Post hoc* pairwise analysis revealed that the proportion of nymphs collected from NR had a significantly higher infection prevalence of *A. phagocytophilum* compared to BS, HP, and RH ($P = 0.028$, $P = 0.0389$, $P = 0.0269$, respectively; Table S4; Fig. 2).

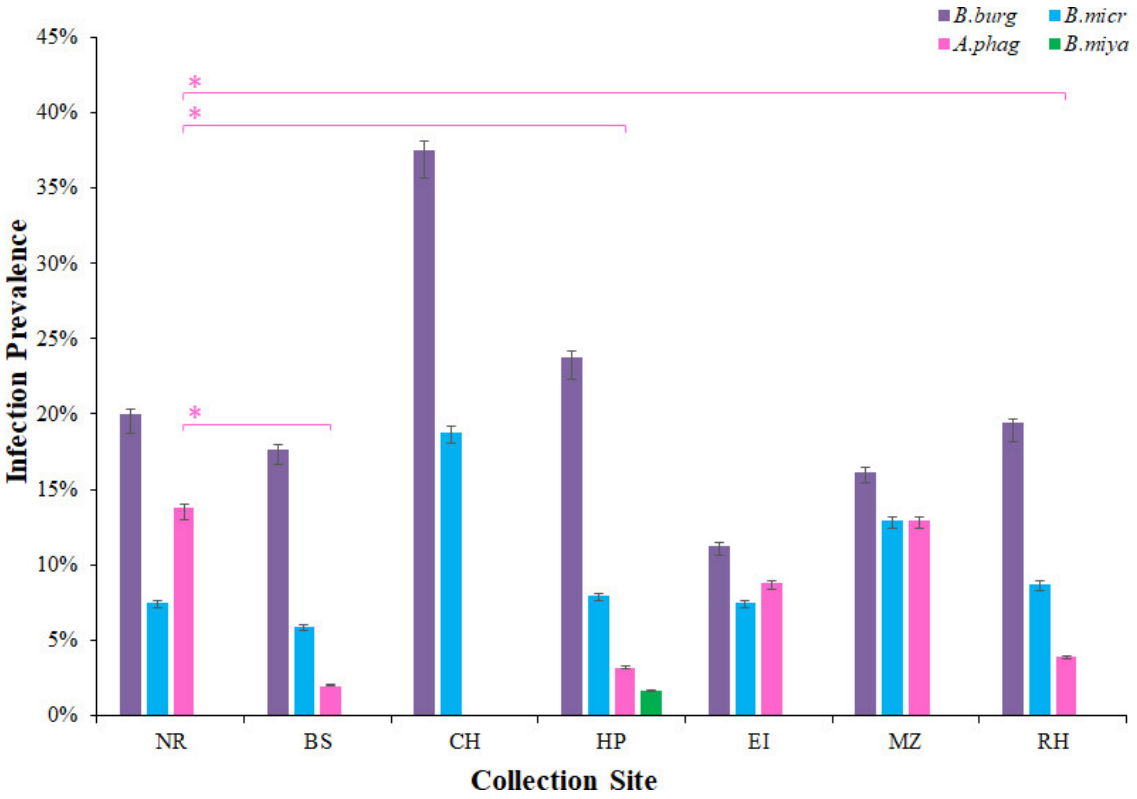

Fig 2 Pathogen infection prevalence of each pathogen at each collection site from questing *Ixodes scapularis* nymphs from Block Island, RI. Significant differences among pathogen infection prevalence and collection sites were calculated using Fisher exact tests and then *post hoc* pairwise comparisons between each pathogen at each collection site. Bars indicate 95% confidence limits, and colored brackets and asterisks denote significant differences between specific pathogens at different collection sites (*$P \leq 0.05$).

Pathogen-host associations

The association of pathogens with hosts was inferred via nymph bloodmeal analysis (Table 1). Significant differences were observed in the frequency of *B. burgdorferi*, *B. microti*, and *A. phagocytophilum* among all host types ($P = 0.0005$, $P = 0.0005$, $P = 0.0025$, respectively), excluding undetermined hosts. For *B. burgdorferi*, nymphs that previously fed on white-footed mice had significantly higher infection prevalence compared to nymphs that had previously fed on birds or white-tailed deer ($P = 0.0006$, $P = 0.0013$, respectively; Fig. S1). The proportion of nymphs that previously fed on rats showed significantly higher infection prevalence compared to those that fed on birds and white-tailed deer ($P = 0.0215$, $P = 0.0104$, respectively; Fig. S1). Additionally, the proportion of nymphs that had previously fed on voles exhibited significantly higher infection prevalence compared to those that fed on white-tailed deer ($P = 0.0372$; Fig. S1). For *B. microti*, the proportion of nymphs that had previously fed on white-footed mice exhibited significantly higher infection prevalence compared to birds and white-tailed deer ($P < 0.0001$, $P = 0.0064$, respectively; Fig. S1). Furthermore, the proportion of nymphs that had previously fed on voles had significantly higher infection prevalence compared to those that fed on birds and white-tailed deer ($P = 0.0001$, $P = 0.0082$, respectively; Fig. S1). Finally, for *A. phagocytophilum*, the proportion of nymphs that had previously fed on white-footed mice and white-tailed deer exhibited significantly higher infection prevalence compared to birds ($P = 0.0099$, $P = 0.0088$, respectively; Fig. S1). These results highlight the

potential importance of voles and rats in maintaining tick-borne pathogens in the enzootic cycle; however, the sample sizes for voles, cats, and rats were small (Table 1), and additional data are needed to estimate infection prevalence more accurately in these host types.

TABLE 1 The number of questing *Ixodes scapularis* nymphs (infection prevalence %) infected with each pathogen calculated by host type or undetermined hosts (none) based on bloodmeal analysis and qPCR[a]

Host	*B. burgdorferi*	*B. microti*	*A. phagocytophilum*	*B. miyamotoi*	Total
Bird	13 (13.83)	0	1 (1.06)	1 (1.06)	94
Mouse	34 (41.46)	24 (29.27)	12 (14.63)	0	82
WTD	4 (8.70)	2 (4.35)	8 (17.39)	0	46
Cat	2 (18.18)	1 (9.09)	0	1 (9.09)	11
Rat	5 (71.43)	0	0	0	7
Vole	4 (66.67)	3 (50.00)	0	0	6
None	15 (13.16)	6 (5.26)	7 (6.14)	0	114
Total	77 (21.39)	36 (10.00)	28 (7.78)	2 (0.56)	360

[a]WTD, white-tailed deer. Mixed bloodmeals are counted in each host category. The total column is the number of nymphs tested from each inferred host species.

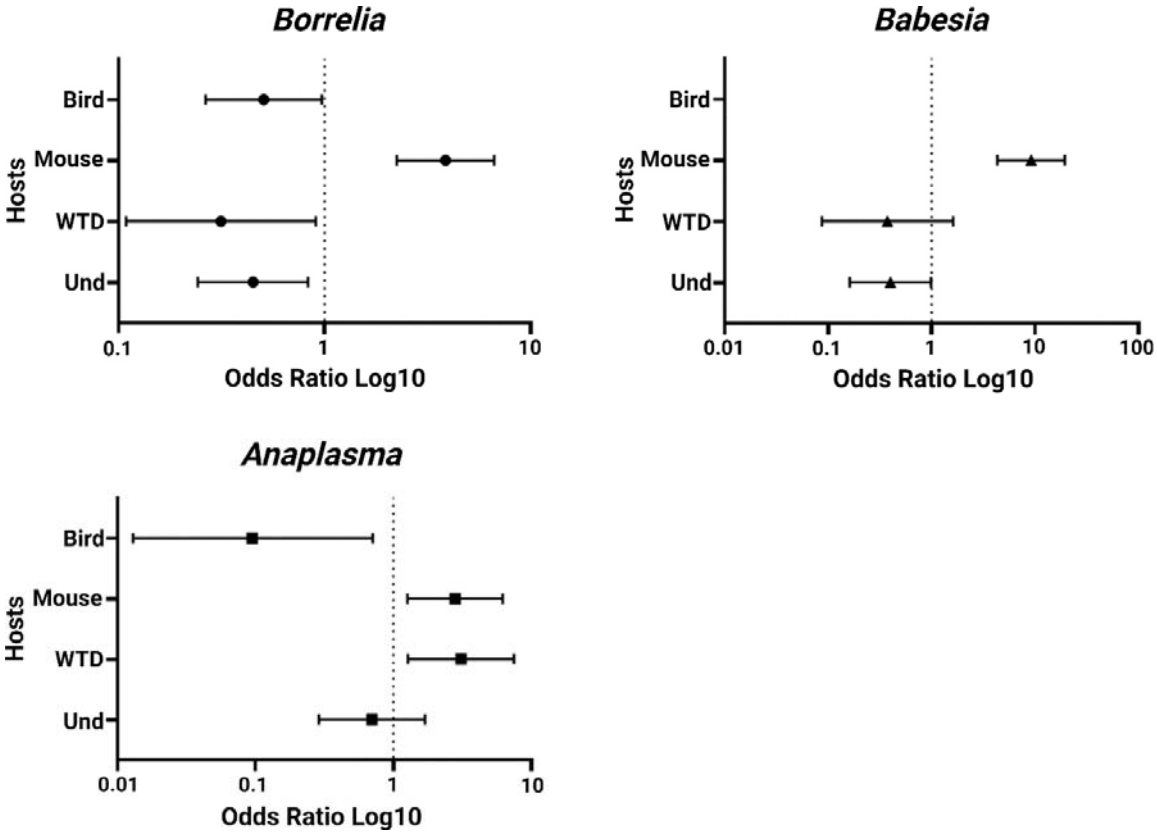

Fig 3 Odds ratios (Log_{10}scale) and range of the three most common inferred host species and undermined hosts (und) infected with each pathogen (excluding *Borrelia miyamotoi*). No bird species in this study were positive for human pathogenic *Babesia microti*. A Mantel-Haenszel chi-square odds ratio test was used to determine the degree of association of the most abundant pathogen types among each of the most prevalent host types. Bars signify the 95% confidence limits.

Excluding hosts with a small sample size (cats, rats, voles), the Mantel-Haenszel chi-square odds ratio test was used to determine the overall degree of association for the most abundant pathogen types (excluding *B. miyamotoi*) among each of the most prevalent host types (birds, white-footed mice, white-tailed deer). The proportion of nymphs that had previously fed on white-footed mice had significantly higher infection prevalence compared to all other abundant host types for each of the three pathogens [*B. burgdorferi:* $P < 0.0001$, $X^2 = 25.38$, df = 1, Odds Ratio (OR) = 3.87; *B. microti:* $P < 0.0001$, $X^2 = 43.68$, df = 1, OR = 9.17; *A. phagocytophilum:* $P = 0.0080$, $X^2 = 6.94$, df = 1, OR = 2.81] and the proportion of nymphs that previously fed on white-tailed deer had significantly higher *A. phagocytophilum* compared to the other prominent host types ($P = 0.0090$, $X^2 = 6.78$, df = 1, OR = 3.10; Fig. 3).

The variant type of *A. phagocytophilum* positive samples was also assessed. White-footed mice ($n = 9$), white-tailed deer ($n = 6$), birds ($n = 1$), undetermined hosts ($n = 7$), and two mouse/deer mixed bloodmeals were positive for *A. phagocytophilum*. Both Ap-ha and Ap-V1 exhibited significant differences in prevalence among host types ($P = 0.0040$, $P = 0.0055$, respectively). From pairwise comparisons, Ap-ha was significantly more prevalent in nymphs that had previously fed on white-footed mice compared to white-tailed deer ($P = 0.0188$), while Ap-V1 was significantly more prevalent in nymphs that had previously fed on white-tailed deer compared to white-footed mice ($P = 0.0188$; Fig. 4A). From row-wise comparisons, Ap-ha was significantly more prevalent in nymphs that had previously fed on white-footed mice compared to Ap-V1 ($P = 0.0332$), while Ap-V1 was significantly more prevalent in nymphs that had previously fed on white-tailed deer compared to Ap-ha ($P = 0.0133$; Fig. 4A). No significant differences were observed between the two variant types among collection sites (Ap-ha $P = 0.7900$; Ap-V1 $P = 0.7860$; Fig. 4B).

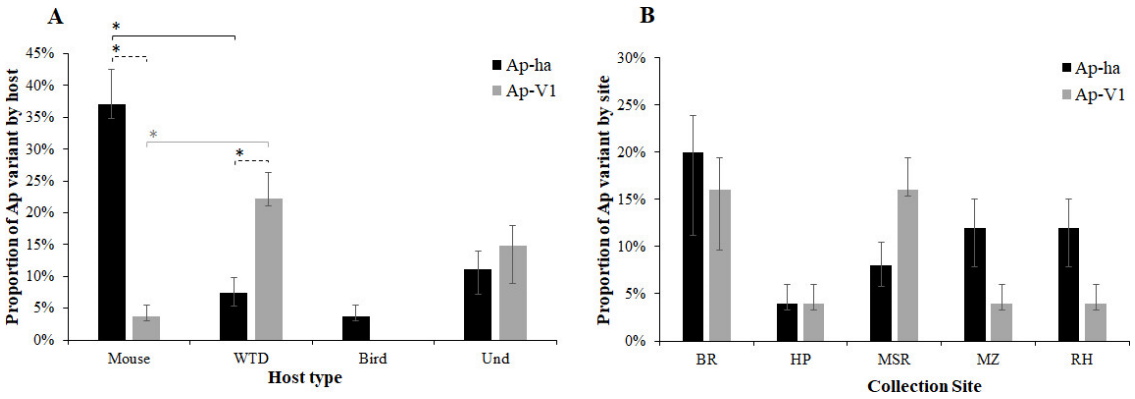

Fig 4 *Anaplasma phagocytophilum* human pathogenic (Ap-ha) and nonpathogenic "deer" variant 1 (Ap-V1) variant types based on host type (**A**) and collection site (**B**). Significant differences between the variant types for each host type and site were assessed using Fisher exact tests and then *post hoc* pairwise and row-wise comparisons (*$P \leq 0.05$). Bars signify 95% confidence limits, solid black brackets denote Ap-ha comparisons, solid gray brackets denote Ap-V1 comparisons, and dashed brackets denote comparisons between Ap-ha and Ap-V1 within the same host type.

Coinfection associations

Coinfection of two or more pathogens was observed in 27 nymphs with two nymphs showing infection with three pathogens. The most common coinfection was between *B. burgdorferi* and *B. microti* ($n = 17$) followed by *B. burgdorferi* and *A. phagocytophilum* ($n = 7$), while coinfection between *B. microti* and *A. phagocytophilum* was least observed ($n = 1$). *Borrelia miyamotoi* was never found coinfected with other pathogens. The greatest number of

coinfections were observed at collection sites RH and NR (Table S5) and in white-footed mice (Table S6).

DISCUSSION

Combined analyses of the bloodmeal source and infectious status of questing *I. scapularis* nymphs from Block Island, RI, provided insights into host-feeding behavior and host-pathogen associations in a low biodiversity setting. Host-pathogen associations of avian hosts, white-footed mice, and white-tailed deer were similar to those from other geographic locations, namely, *B. burgdorferi* and *B. microti* were more commonly associated with white-footed mice, while *A. phagocytophilum* was more commonly associated with white-footed mice and white-tailed deer (6, 13, 26, 51, 68, 69). More specifically, we validated our previous estimates based on back calculations [27% of larvae fed on birds, 44% on white-footed mice, 29% fed on white-tailed deer (25)] that the three most common host types in our study area contributed more than 20% each to larval bloodmeals (42% fed on birds, 37% on white-footed mice, 21% on white-tailed deer; findings from this study). The differences in host contributions in feeding the larval population between these studies possibly reflect yearly variations in host and vector abundance and distribution as well as methodological biases. Bloodmeal analyses, thus, provide a potential alternative to direct sampling from hosts for assessing the level of host-pathogen associations. We caution, however, that assessing patterns of host abundance, estimating infection, and tick burdens directly from trapped hosts would still be necessary for a full mechanistic understanding of tick population and infection dynamics, including whether ticks feed on hosts according to host availability or whether they display preferences for certain hosts.

Our results highlight the significance of white-footed mice in the enzootic cycles of three medically important tick-borne pathogens (34, 41–49, 70–72) and provide a target species for public health intervention programs to reduce human risk. Ticks that fed on white-footed mice were significantly more likely to be infected with *B. burgdorferi*, *B. microti*, and *A. phagocytophilum* human infectious Ap-ha variant compared to avian species and white-tailed deer. Consistent with our previous study (25), some avian hosts and white-tailed deer may act as "dilution hosts" for *B. burgdorferi* infection, reducing infection prevalence in nymphs even in low biodiversity settings.

Nymphs that had previously fed on white-tailed deer were significantly more likely to be infected with the Ap-V1 variant compared to Ap-ha, which was associated with white-footed mice. While these Ap variant-host associations were consistent with most previous studies, two nymphs that had previously fed on white-tailed deer were positive for Ap-ha (7%). A previous study that inoculated white-tailed deer with Ap-ha demonstrated that they are susceptible to infection with Ap-ha (47), while another study was unable to isolate Ap-ha from the blood of naturally infected wild white-tailed deer (41), suggesting that white-tailed deer, while susceptible, are not a significant host for this variant; however, more research is needed to solidify the Ap variant-host association. To our knowledge, this is the first report that investigated *A. phagocytophilum* in questing nymphs collected from Block Island, and our results are consistent with positive serological assays for *A. phagocytophilum* infection in human residents on Block Island (Krause, pers. comm.).

Bird species fed the highest number of ticks (26%) of all host types on Block Island; however, none of these ticks were positive for human pathogenic *B. microti*. These results suggest that birds are not important hosts for maintaining *B. microti* infection in this island environment

and may act as dilution hosts for this pathogen. The absence of *B. microti*-bird bloodmeal associations is consistent with previous bloodmeal analyses (67) but contradicts one study finding birds infected with *B. microti* (15). However, this later study did not differentiate between pathogenic *B. microti* and other *B. microti*-like agents which are not associated with human disease. The qPCR primers used in this study (65) are specific for pathogenic *B. microti*. It may be that birds are competent for the non-pathogenic *B. microti*-like parasites, but to date, this has not been investigated (15, 57–62). Low competence of avian hosts for *B. microti* may lead to lower overall prevalence of this pathogen in the nymphal ticks. However, because *B. microti* can utilize a non-vector-mediated vertical transmission strategy to maintain high levels of infection in the white-footed mouse and vole populations (73, 74), this alternative pathway can contribute to *B. microti* persistence.

Bloodmeal analysis identified a small number of hosts that are not typically sampled to assess *I. scapularis*-host associations in the field (rats, cats, and Block Island meadow voles), mainly because they are less common in deciduous forested habitats. Nymphs that fed on cats and rats as larvae were mostly sampled at Hodge Family Wildlife Preserve (HP) which is characterized by mostly prairie grasses, isolated forest patches, and residential properties. This site is also located close to the New Shoreham Transfer Station (the only waste disposal site on the island) where removal of waste from the island by ship traffic and close proximity to residential properties may lead to higher populations of rats and feral cats than in other parts of the island. Nymphs that fed on rats were only infected with *B. burgdorferi* (71.43%), suggesting they may be a competent host for *B. burgdorferi* on Block Island; however, our bloodmeal sample size for rats was small (n = 7) and additional samples are needed for further examination of their potential contribution in maintaining this pathogen on the island. Block Island meadow voles (*Microtus pennsylvanicus provectus*) were detected in five out of seven collection sites. This was not expected since our tick sampling design focused on forested habitats, and the endemic vole is found in meadow habitats (75, 76). The presence of forest-meadow edges in our study area may explain the occasional detection of this species. Three of the six voles had mixed bloodmeals with the second host being white-footed mice, indicating potential presence of both hosts in edge habitat. Of the three nymphs that only fed on voles, two were coinfected with *B. burgdorferi* and *B. microti*, suggesting these hosts are competent for both pathogens and may be important in the enzootic cycle and maintaining these pathogens, as has been shown in previous studies (73, 77–79).

A recent bloodmeal analysis study focusing only on white-footed mice and white-tailed deer found a proportion of unidentifiable bloodmeals from different sampling locations and assumed this to be partially a result of tick age (34). In this previous study, the researchers demonstrated that as the age of a tick increased the bloodmeal assay was less sensitive at detecting a host type, likely from continued digestion of the host bloodmeal. Cumulative host-finding success for larvae that survived their hatching year on Block Island was estimated at 77% (80), suggesting that most larvae in the current study molted and overwintered as nymphs and were subsequently over 6 months old when collected the following spring, resulting in host material that was too digested to reliably identify. In our study, host bloodmeal was not detected in 31.67% of nymphs which was consistent with a recent study using the same method (34). Our method was more successful compared to other studies which showed approximately 50%–60% undetected hosts (81, 82), confirming that our bloodmeal analysis method provides adequate sensitivity and accuracy for determining previous hosts. The inability to detect a host may also have occurred if larvae fed on a host we did not test for (domestic dogs, muskrats, reptiles, etc.); however, given the depauperate vertebrate fauna on Block Island, undetermined hosts are more likely the result of a hemolyzed and digested bloodmeal by the tick.

Additionally, mixed bloodmeals were observed in ~5% of questing nymphs which may have resulted from a larva being removed from their first host via grooming or host death, requiring the larva to find a second host to complete a full bloodmeal (34, 82).

From our bloodmeal analysis, mouse-targeted interventions for reducing larvae on mice (e.g., mouse fipronil bait boxes) would be advantageous in reducing the prevalence of all three pathogens (83, 84). Furthermore, a recent study provided evidence that a rodent-targeted vaccine (RTV) against *B. burgdorferi* effectively reduced the prevalence of *B. microti* via coinfection reduction (85), suggesting that even single pathogen interventions may be able to reduce the prevalence of other pathogens in a natural setting. Future studies should also perform bloodmeal analyses on other tick species in the environment, such as the newly discovered invasive tick species (*Haemaphysalis punctata* and *H. longicornis*) on Block Island (86), to aid in determining tick-host associations, predicting potential increases in tick-borne disease risk to humans, and to target specific host species for tick management strategies against invasive species (87–91).

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