

Original article

Microclimate conditions alter *Ixodes scapularis* (Acari: Ixodidae) overwinter survival across climate gradients in Maine, United States

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ABSTRACT

The incidence and geographic range of vector-borne diseases have been expanding in recent decades, attributed in part to global climate change. Blacklegged ticks (*Ixodes scapularis*), the primary vector for multiple tick-borne pathogens in North America, are spreading rapidly beyond their historic post-colonial range and are thought to be constrained mainly by winter temperature at northern latitudes. Our research explored whether winter climate currently limits the distribution of blacklegged ticks and the pathogens they transmit in Maine, U.S.A., by contributing to overwinter mortality of nymphs. We experimentally tested tick overwinter survival across large-scale temperature and snowfall gradients and assessed factors contributing to winter mortality in locations where blacklegged tick populations are currently established and locations where the blacklegged tick has not yet been detected. We also tested the hypothesis that insulation in the tick microhabitat (i.e., by leaf litter and snowpack) can facilitate winter survival of blacklegged tick nymphs despite inhospitable ambient conditions. Overwinter survival was not significantly different in coastal southern compared to coastal and inland northern Maine, most likely due to sufficient snowpack that protected against low ambient temperatures at high latitudes. Snow cover and leaf litter contributed significantly to overwinter survival at sites in both southern and northern Maine. To further assess whether the current distribution of blacklegged ticks in Maine aligns with patterns of overwinter survival, we systematically searched for and collected ticks at seven sites along latitudinal and coastal-inland climate gradients across the state. We found higher densities of blacklegged ticks in coastal southern Maine (90.2 ticks/1000 m²) than inland central Maine (17.8 ticks/1000 m²) and no blacklegged ticks in inland northern Maine. Our results suggest that overwinter survival is not the sole constraint on the blacklegged tick distribution even under extremely cold ambient conditions and additional mechanisms may limit the continued northward expansion of ticks.

1. Introduction

Within recent decades, numerous hard-bodied tick species have spread dramatically beyond their historical ranges and increased in density within those ranges, leading to the concomitant expansion of the pathogens they transmit and the emergence and re-emergence of dozens of tick-borne diseases in humans and animals (e.g., Gubler, 1998; Randolph, 2001; Lindgren and Gustafson, 2001; Parola et al., 2005; Jaenson and Lindgren, 2011; Jaenson et al., 2012; Kilpatrick and Randolph, 2012; Mansfield et al., 2017; Nah et al., 2020). The most important

disease vector ticks in northeastern North America are nymphal and adult blacklegged ticks, *Ixodes scapularis* (Eisen et al., 2017; Adams et al., 2015), a species that may transmit a diverse array of pathogens, including *Borrelia burgdorferi* sensu stricto (the primary causative agent of Lyme disease in North America), *Anaplasma phagocytophilum* (human granulocytic anaplasmosis), and *Babesia microti* (human babesiosis). The blacklegged tick first was detected in the northeastern U.S.A. in small, isolated populations in the 1920s (Larrouse et al., 1928), restricted by deforestation and availability of habitat and key vertebrate hosts, and increased in range and density both northwards and westwards across

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the U.S.A. substantially throughout the latter half of the 20th century (Ogden et al., 2008; Diuk-Wasser et al., 2014; Lantos et al., 2017; Bisanzio et al., 2020; Gardner et al., 2020). Since 1998, the number of U. S. counties in which the blacklegged tick reportedly is established nearly has doubled (Dennis et al., 1998; Eisen et al., 2016), and the blacklegged tick also is spreading rapidly in southern Canada, advancing at rates of 46 km/year in Ontario (Clow et al., 2017).

Understanding the ecological mechanisms that constrain the establishment and maintenance of tick populations at the margins of their current spatial distributions may enhance our ability to predict future geographic range expansion of medically significant ticks and target public health interventions accordingly, as well as devise effective adaptive management strategies. Stimulated by change in photoperiod, blacklegged ticks enter diapause over winter as unfed or fed larvae, unfed nymphs, or unfed or fed adults (Smith and Cole, 1941; Yuval and Spielman, 1990; Rand et al., 2007; Gray et al., 2016). Diapause is terminated in response to increasing rising temperatures and increasing day-length (Belozzerov, 2009; Gray et al., 2016). Because laboratory studies have shown that exposure to temperatures below -10°C for as little as two hours kills blacklegged ticks (Burks et al., 1996; VanDyk et al., 1996), it long has been hypothesized that low winter temperatures constrain their northern distribution. However, this assumption has met with mixed support in field experiments because microhabitats experienced by overwintering ticks often become decoupled from ambient conditions (Boehnke et al., 2017). Although a study conducted in Canada indicates that low temperatures can limit overwinter blacklegged tick survival severely across multiple tick life-stages (Lindsay et al., 1995), additional experiments in New York suggest that tick behavioral avoidance of adverse conditions may result in overwinter temperature having little impact on tick survival (Brunner et al., 2014), or that other microhabitat conditions, such as the presence of natural predators of ticks, may impact tick overwinter survival more strongly than cold temperatures alone (Burtis et al., 2016). At least one field experiment

revealed that tick mortality during overwinter diapause is higher in early spring than during the coldest depths of winter due to tick depletion of lipid reserves as temperatures warm (Burtis et al., 2019). Finally, multiple field studies have revealed that insulation provided by snowpack, leaf litter, or both (Lindsay et al., 1998; Lindsay et al., 1999; Brownstein et al., 2005; Dautel et al., 2016; Linske et al., 2019) can greatly enhance tick survival in the soil in an optimal microclimate despite inhospitable ambient conditions. However, all these studies took place in locations in which blacklegged ticks already occur at detectable densities. Additional field experiments north of the blacklegged tick's current range offer the opportunity to assess whether ticks can survive winter conditions in regions where populations are not established and thus provide an explicit mechanistic link between overwinter survival and tick distributions.

The state of Maine in the northeastern U.S.A. is an ideal location in which to study climate impacts on blacklegged tick overwinter survival for multiple reasons. Maine is characterized by latitudinal and coastal-inland temperature and snowfall gradients (Fig. 1, Table 1) that provide a unique opportunity to conduct field experiments to study the interacting effects of broad-scale ambient climate and fine-scale microclimate conditions on blacklegged tick survival. Annual temperatures in the northeastern U.S. are increasing faster than in any other region in the

Table 1
Mean temperature and precipitation from December-February, 2021 across Northern, Central, and Coastal regions of Maine, U.S.A. Data obtained from Climate Change Institute, University of Maine.

Region (Maine, U.S.A)	Mean temperature December-February (°C)		Mean precipitation December-February (cm)	
	2018-2019	2019-2020	2018-2019	2019-2020
North	-9.6	-10.4	26.2	29.0
Central	-6.2	-6.5	32.5	30.7
Coastal	-4.4	-4.6	36.3	34.0

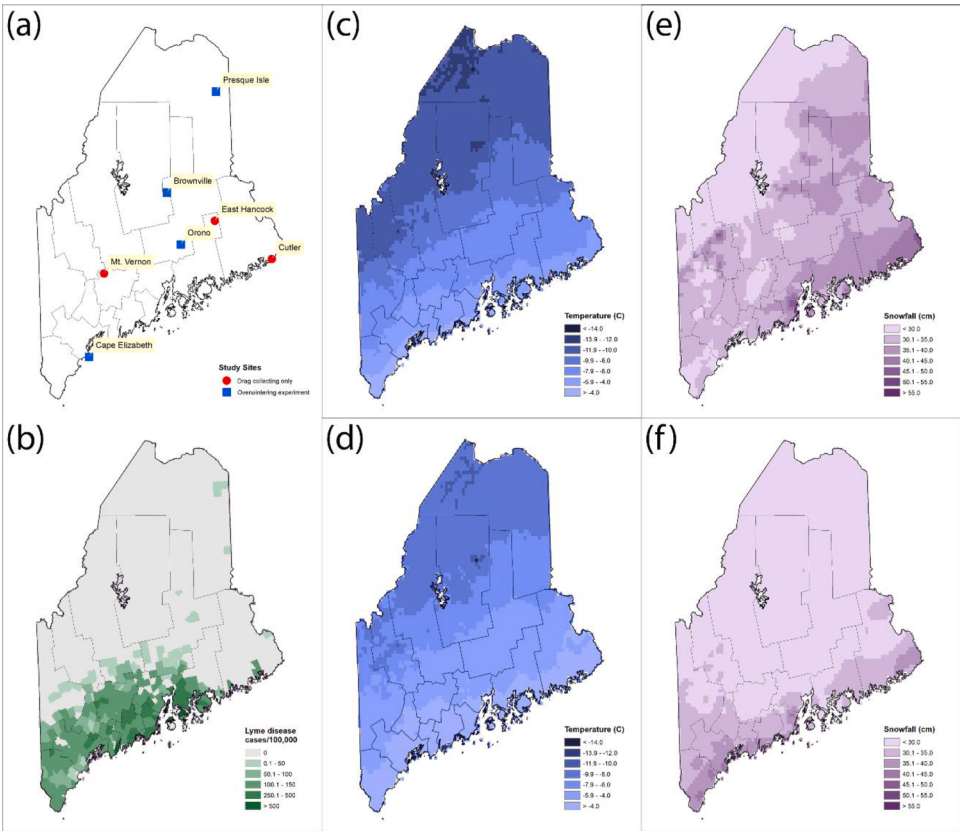


Fig. 1. Maps of (A) study sites used for tick collections (red) and both tick collections and overwinter survival experiments (blue) in Maine, United States; (B) town level Lyme disease cases/100,000 people in Maine, 2019; mean temperature (°C) from December to February in (C) 2018-2019 and (D) 2019-2020; and total snowfall (cm) from December to February in (E) 2018-2019 and (F) 2019-2020. Data were obtained from PRISM (<https://prism.oregonstate.edu/>). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

country (Karmalker and Bradley, 2017). Across Maine, the average annual temperature has increased significantly since 1960, with the greatest magnitude of change occurring during the winter, accompanied by increased frequency and intensity of rainfall and snowfall (Fernandez et al., 2020). Maine also is an epidemiologically significant state, with over 2,000 human cases of Lyme disease reported in 2019 (Maine CDC, 2020), and the geographic range expansion of the blacklegged tick is ongoing. Since the first human cases of Lyme disease in Maine were reported to the CDC in 1986 (Anderson et al., 1987; Ginsberg and Ewing 1988), the blacklegged tick and the pathogens it transmits have been spreading north and east across the state (Rand et al., 2007; Elias et al., 2021; Rounsville et al., 2021).

The goal of this study was to assess ecological pathways by which climate may currently constrain the northern distribution of blacklegged ticks. First, to determine the effects of climate on blacklegged tick overwinter survival, we tested nymphal tick overwinter survival across broad-scale temperature and snowfall gradients in Maine and assessed factors contributing to overwinter mortality over two years in locations where blacklegged tick populations are currently established and those where populations have not yet been detected. Second, to determine the effects of microclimate on blacklegged tick survival, we leveraged the experimental design and methods of Linske et al. (2019) to test nymphal tick overwinter survival in different microhabitats (i.e., snow removal, leaf litter removal, both snow and leaf litter removal, and a control in which insulation was not manipulated) spanning ambient temperature and snowfall gradients. We hypothesized that overwinter mortality decreases with increasing insulation, facilitating tick survival under harsher ambient conditions than considered by previous field experiments. Finally, to assess whether our overwinter survival findings align with the current distribution of blacklegged ticks with consequences for pathogen transmission in Maine, we conducted active surveillance of ticks during the summer when nymphs, the most epidemiologically significant life stage, are most likely to be active (Piesman et al., 1987) and screened ticks for tick-borne pathogens commonly reported in Maine (*B. burgdorferi* sensu lato, *B. microti*, and *A. phagocytophilum*).

2. Methods

2.1. Site selection

Enclosures containing nymphal blacklegged ticks were established at four sites across Maine in Cape Elizabeth [Cumberland County], Orono [Penobscot County], Brownville [Penobscot County], and Presque Isle [Aroostook County] (Fig. 1). These sites were selected to span the state's broad-scale temperature and snowfall gradients. Enclosures were established in mixed hardwood stands dominated by oak (*Quercus* spp.), maple (*Acer* spp.), birch (*Betula* spp.), and pine (*Pinus* spp.), which provide suitable habitat for blacklegged ticks (Ginsberg and Ewing, 1989; Adler et al., 1992; Ostfeld et al., 1995; Lindsay et al., 1999). Tree species composition and understory structure were comparable across sites, and efforts were made to maintain consistent site-level factors such as vegetation, soil type, and topography. We conducted off-host tick collections during the summer at these sites and three additional sites in Cutler, Mt. Vernon, and East Hancock (Fig. 1).

2.2. Overwinter survival experiment

To assess overwinter survival as a potential mechanism to explain state-wide variation in tick densities, we conducted a two-year field experiment from 2018 to 2020. To test the hypothesis that ambient climate and microclimate affect overwinter survival, tick enclosures were constructed from one-gallon plastic buckets. Three holes were cut in the lid of each bucket and four holes were cut around the side of each bucket to allow for air flow and drainage. Each hole was covered with a small square of mesh and secured with PVC Cement (#30894, Oatey®, Cleveland, OH, USA) and all-weather tape (SI-13786BL, Gorilla®,

Cincinnati, OH, USA) to prevent wildlife from interfering with the vials inside the buckets (Fig. 2). At each site, buckets were inserted into ~20 cm holes dug in the ground and filled with the excavated soil to ~5 cm from the top. In compliance with the Maine Department of Inland Fisheries and Wildlife regulations, which prohibited seeding soil cores directly with ticks (as was done in Bertrand and Wilson (1996)) to reduce the probability of the escape of ticks into the environment in areas where they are not currently established, one polypropylene vial containing 15 unfed, pathogen-free *I. scapularis* nymphs was placed on top of the soil in each enclosure. Nymphal ticks were used for this experiment because they are the most epidemiologically significant life stage (Piesman et al., 1987). Pathogen-free *I. scapularis* nymphs (three months post-molt) were obtained from the Oklahoma State University Tick Rearing Facility (Stillwater, OK, USA), which maintains a colony that originated and is refreshed from the natural population of *I. scapularis* in Stillwater, OK. Three holes were punched through the top of each vial using a tack to increase tick exposure to the temperature and humidity of the surrounding environment, and mesh was placed over the opening of each vial to prevent the accidental release of ticks. Ticks were counted before being placed in the field and again upon retrieval to ensure that no ticks escaped. At each site, the enclosures were established in an area isolated from human activities and slightly elevated relative to the surrounding area to prevent flooding in the enclosures during snowmelt.

To test the hypothesis that snow cover and leaf litter provide insulation and promote overwinter survival, four treatments were created to manipulate insulation in the enclosures based on the experimental design developed by Linske et al. (2019). The treatments were snow removal, leaf litter removal, both snow and leaf litter removal, and a control for which there was no manipulation of insulation conditions (referred to as SR, LR, SLR, and C, respectively, in figures and tables). To reflect the type and amount of leaf litter present in the surrounding area, leaf litter was obtained directly from the site and placed on top of each vial in snow removal and control enclosures before the enclosures were sealed. Leaf litter was replaced before the start of the second winter. No leaf litter was added to leaf removal and snow/leaf removal enclosures. After each snow event which resulted in snow cover greater than 1 cm, snow was removed from the tops of snow removal and snow/leaf removal enclosures. We allowed snow to accumulate throughout the winter over leaf removal and control enclosures.

The sites in Cape Elizabeth, Orono, and Presque Isle each contained four replicates of each of the four treatments for a total of 16 enclosures at each site. The enclosures were the experimental units of the study. Due to the remote location of the site in Brownville, it was not feasible to regularly remove snow after each snow event throughout the winter. Therefore, we established four replicates of the control enclosures and no manipulated enclosures at this site. Due to flooding in the enclosures during the first winter in Orono, data from this site were excluded from analyses during the first year of the study. Prior to placement in the field, ticks were kept in a plastic bag containing a damp paper towel for humidity and stored at -4°C for storage until conditions were appropriate for placing the ticks in enclosures. The ticks were placed in enclosures in late November before significant ground frost and snow cover accumulation. The duration of the experiment was from 24 November 2018 to 26 April 2019 and from 29 November 2019 to 21 April 2020. To evaluate ticks for survival at the conclusion of the experiment, we held ticks at room temperature for at least 30 min and observed them for movement. Efforts to stimulate movement such as applying human breath or gentle prodding with forceps were applied if the ticks did not start to move after 30 min at room temperature. The ticks were considered dead if they did not respond to stimulation efforts after 30 min.

2.3. Temperature and snowfall measurements

To test the hypothesis that snow cover and leaf litter provide

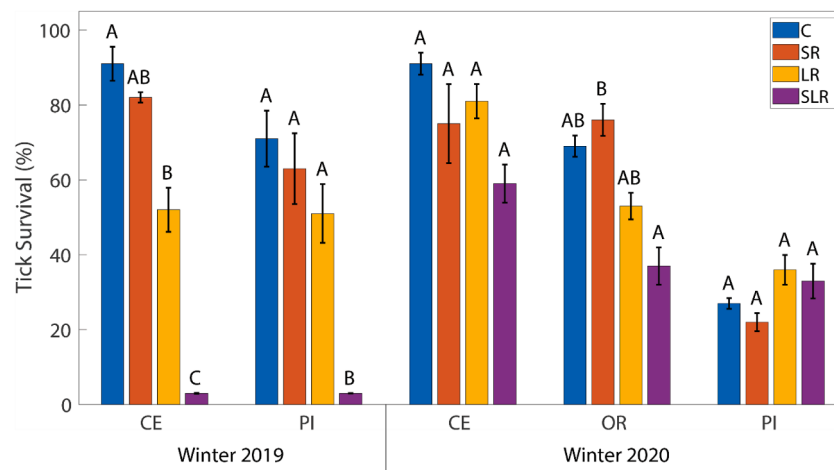


Fig. 2. Percent overwinter survival of *Ixodes scapularis* nymphs in Cape Elizabeth (CE), Orono (OR), and Presque Isle (PI) across microhabitat treatments. Microhabitat treatments include a control with leaf litter and snow present (C), snow removal (SR), leaf removal (LR), and snow/leaf removal (SLR). Grouping letters indicate significant differences between means at $\alpha = 0.05$ within each year and location.

insulation from temperature extremes, iButton data loggers (DS1922L Thermochron; Maxim Integrated, San Jose, CA, USA) were established inside each enclosure next to the tick vials to record temperature ($^{\circ}\text{C}$) once per hour. Snow depth was measured weekly and after each snow event. Because significant snow accumulation in northern sites buried ambient iButtons, ambient temperature data were obtained from the Maine Climate Office at the University of Maine Climate Change Institute (Climate Change Institute, 2020). Due to travel restrictions in 2020, weekly snowfall data in Presque Isle were obtained from the NOAA National Centers for Environmental Information (National Oceanic and Atmospheric Administration, 2020).

2.4. Off-host tick collection

To test the hypothesis that the current distribution of blacklegged ticks in Maine reflects our overwinter survival findings, we collected host-seeking blacklegged ticks at seven sites across Maine in Cape Elizabeth, Mt. Vernon, Orono, East Hancock, Brownville, Cutler, and Presque Isle (Fig. 1). Collections were conducted between 10 June and 23 August in 2019, the peak activity period of nymphal *I. scapularis* in Maine (Rand et al., 2007). At each site, an 8,000 m^2 grid was established in an area with favorable vegetation for blacklegged ticks (i.e., mixed hardwood and significant leaf litter) to maximize the possibility of finding blacklegged ticks. Ticks were collected using a standard “dragging” method (Zimmerman and Garriss, 1985), where questing ticks are collected from a 1 m^2 corduroy cloth attached to a dowel rod that is dragged across vegetation or leaf litter and checked for ticks every 10 m. Eight 125-m transects were dragged across the grid for a total of 1,000 m^2 per dragging session. Sites were dragged once every two weeks from early June to mid-August and dragging was not performed during rain or when the vegetation or leaf litter was wet enough to soak the drag cloth.

All *I. scapularis* life stages and other tick species found were collected and preserved in 70% ethanol and stored at -4°C . Larval ticks were removed from the drag cloth with a piece of adhesive tape and stored in plastic bags. All ticks were identified to life stage and species using standard keys (Keirans and Clifford, 1978; Keirans and Litwak, 1989; Durden and Keirans, 1996; Egizi et al., 2019).

2.5. Pathogen detection

To determine nymphal infection prevalence across the state, *I. scapularis* nymphs collected during the summer of 2019 were screened for *B. burgdorferi* sensu lato (s.l.), as well as *A. phagocytophilum* and *B. microti*, two emergent pathogens transmitted by blacklegged ticks in

Maine. We tested ticks collected in Cape Elizabeth, Orono, Mt. Vernon, Presque Isle, Cutler, and East Hancock. We tested all *I. scapularis* nymphs collected in Mt. Vernon ($n = 15$), Cutler ($n = 3$), and East Hancock ($n = 2$) and a randomly selected subset of 77 nymphs collected in both Cape Elizabeth and Orono. We also screened four *Ixodes cookei* (three nymphs and one adult) and one *Ixodes marxi* nymph collected in Presque Isle to determine whether the three pathogens of interest may be maintained in cryptic cycles in the absence of *I. scapularis* (Hamer et al., 2010).

Before DNA extraction, nymphal ticks were cut in half longitudinally and adult ticks were quartered to disrupt the exoskeleton and expose the salivary glands. DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA) using a modified extraction protocol (Hojgaard et al. 2014). PCR testing was completed at the University of Maine Cooperative Extension Diagnostic and Research Laboratory. Each PCR reaction (10 μL) contained 5 μL of Bio-Rad iQ Multiplex Powermix (Bio-Rad, Hercules, CA, USA), 3 μL of primers and probes (Hojgaard et al., 2014; Xu et al., 2016; Rounsville et al., 2021), and 2 μL of extracted DNA template. PCR reactions were completed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad), and conditions were as follows: 3 min Hot-start at 95°C , followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. Each sample was run in duplicate to account for false positives; samples were determined to be positive if DNA amplified at a critical threshold ($\text{Ct} \leq 35.0$). Due to a high frequency of hybridized primers, samples that initially tested positive for *B. microti* were rerun a third time replacing the Bio-Rad iQ Powermix for PowerUp SYBR Green Master Mix to eliminate false positives.

2.6. Statistical analysis

Data analysis was conducted using the lme4 package (Bates et al., 2015) in R version 3.5.1 (R Core Team, 2013). First, we used general linear mixed models (GLMMs) to test the hypotheses that tick overwinter survival rates vary across broad-scale climate gradients in control enclosures (i.e., snow and leaf litter present) and across microhabitat treatments. Second, we used GLMMs with repeated measures to test the hypotheses that temperature and snowfall vary across study locations in control enclosures and temperature varies across manipulated microhabitat treatments. Tukey mean separation tests were carried out to detect significant differences among treatment levels ($\alpha = 0.05$) using the emmeans package in R (Lenth, 2021). Finally, we used multiple linear regression to test for associations between ambient and microclimate conditions (i.e., mean and minimum temperature, mean and maximum snow depth) and tick overwinter survival. Year was included

as a random effect in all models. Tick survival data (calculated as a percentage of 15 nymphs per replicate enclosure) met assumptions for normality and homoscedasticity of variances and were not transformed.

3. Results

3.1. Effect of climate across large spatial scales on overwinter survival

During the first year of the study, there was no significant difference in survival of nymphal blacklegged ticks overwinter in control conditions (i.e., snow and leaf litter present) between locations (Fig. 3, Table 2). Percent survival in Cape Elizabeth, Brownville, and Presque Isle was 91%, 93%, and 71%, respectively. During the second year of the study, tick survival was highest in Cape Elizabeth (91%) and Brownville (93%), lower in Orono (69%) and lowest in Presque Isle (27%). Tick survival in control treatments was significantly lower during the second year than the first year in Presque Isle, but not in Cape Elizabeth or Brownville.

Overall, lower ambient temperatures were recorded during the first year of the study compared to the second, though neither average snow depth nor maximum snow depth was statistically different between years (Table 2, 3). Mean ambient temperature and minimum ambient temperature were highest in Cape Elizabeth and lowest in Presque Isle (Fig. 4), while average snow depth and maximum snow depth were significantly higher in Brownville and Presque Isle than in Cape Elizabeth and Orono (Table 2). Multiple linear regression identified minimum and mean ambient temperature as climate variables that may explain the influence of location on tick survival in control treatments (Table 2).

3.2. Effect of microhabitat treatment on overwinter survival

Microhabitat treatment affected tick overwinter survival in both Cape Elizabeth and Presque Isle during the first year of the study but not the second (Fig. 3, Table 4). At Cape Elizabeth, tick survival was highest in the control treatment followed by snow removal and leaf removal treatments, which resulted in the lowest survival (Fig. 3). At Presque Isle, control, snow removal, and leaf removal treatments were similar and tick survival was lowest in the snow/leaf removal treatment (Fig. 3). Similarly, microhabitat treatment affected tick survival in Orono; survival was highest in the leaf removal treatment and lowest in the snow/leaf removal treatment. Survival was significantly higher between the first and second years in Cape Elizabeth but not in Presque Isle.

During both years of the study, treatment and location had significant effects on mean and minimum microclimate temperature (Table 4). Overall, temperatures of control and leaf removal treatments were

Table 2

Summary of statistical tests concerning tick (*Ixodes scapularis*) survival and climate conditions in control treatments (i.e., leaf litter and snowpack present). Asterisks indicate statistically significant tests at $\alpha = 0.05$.

Effect	df	F	P
a) Tick survival in control treatments (i.e., leaf litter and snowpack present)			
Location	3, 20	21.39	< 0.01*
Year	1, 20	6.08	0.02*
b) Tick survival predicted by ambient conditions, December–April			
Mean temperature	1, 20	11.67	< 0.01*
Mean minimum temperature	1, 20	17.63	< 0.01
Mean snow depth	1, 20	0.01	0.92
Maximum snow depth	1, 20	1.35	0.26
Year	1, 20	1.28	0.27
c) Differences in ambient conditions across locations			
Mean temperature	3, 20	24.14	< 0.01*
Mean minimum temperature	3, 20	20.64	< 0.01*
Mean snow depth	3, 20	86.89	< 0.01*
Maximum snow depth	3, 20	57.13	< 0.01*

significantly higher than snow removal and snow/leaf removal treatments, and mean temperatures were highest in Cape Elizabeth and lowest in Presque Isle (Fig. 5). Multiple linear regression identified minimum and mean temperature as microclimate variables that may explain the influence of microhabitat conditions on control tick survival (Table 4).

3.3. Off-host tick collection and pathogen prevalence

We collected a total of 1,188 off-host ticks across Maine. A total of 909 *I. scapularis* were collected across the state in Cape Elizabeth, Mt. Vernon, Orono, East Hancock, and Cutler (Table 5). Additional tick species included *I. cookei* (n = 4), *I. marxi* (n = 1), *Dermacentor variabilis* (n = 43), and *Haemaphysalis leporispalustris* (n = 233) (Table 6). *Ixodes scapularis* were detected at all sites except in Brownville, where only *H. leporispalustris* was collected, and Presque Isle, where only *I. cookei* and *I. marxi* were collected. *Haemaphysalis leporispalustris* also were collected in East Hancock and Cutler, but *I. cookei* and *I. marxi* were not collected in any locations other than Presque Isle.

Overall, *B. burgdorferi* s.l. prevalence was highest in Cape Elizabeth (20%) and Orono (9.1%), and *A. phagocytophilum* and *B. microti* were only detected in ticks collected from Cape Elizabeth (Table 7). Among four *I. cookei* collected and tested from Presque Isle, one nymph was positive for *B. burgdorferi* s.l.

4. Discussion

Winter climate long has been hypothesized to constrain the

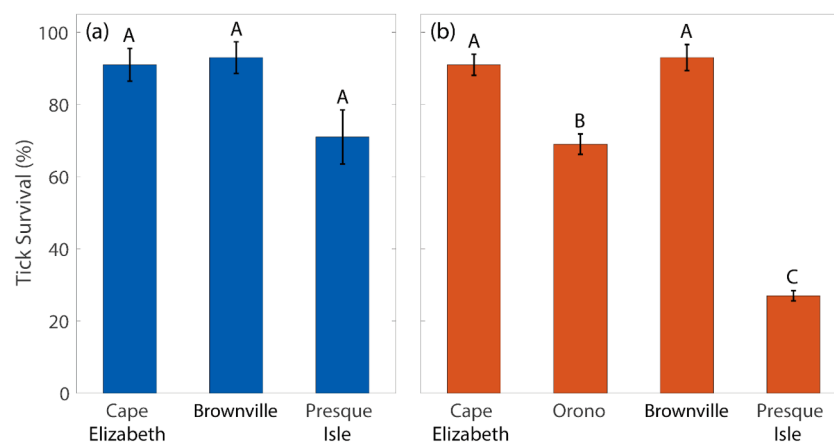
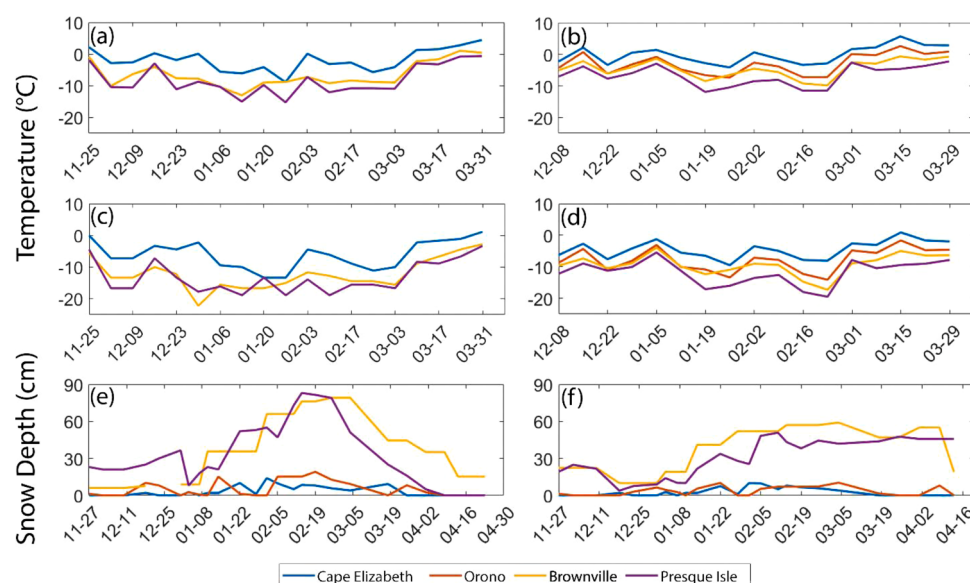


Fig. 3. Percent overwinter survival of *Ixodes scapularis* nymphs exposed to control conditions (i.e., leaf litter and snowpack present) in (A) 2018–2019 and (B) 2019–2020. Grouping letters indicate significant differences between means at $\alpha = 0.05$ within each year.

Table 3

Ambient climate conditions across overwinter survival study field sites during the first year (Winter 2019) and second year (Winter 2020) of the overwintering study.

Site	Winter 2019 Mean snow depth (cm)	Max snow depth (cm)	Mean temp (°C)	Winter 2020 Mean snow depth (cm)	Max snow depth (cm)	Mean temp (°C)
Cape Elizabeth	3.50 ± 0.87	9.76	-0.8 ± 0.1	2.30 ± 1.45	11.20	0.1 ± 0.1
Orono	5.93 ± 1.39	15.20	-2.9 ± 0.9	3.61 ± 0.72	10.30	-2.7 ± 0.1
Brownville	35.41 ± 8.39	79.10	-4.3 ± 0.1	38.13 ± 5.90	59.00	-3.5 ± 0.4
Presque Isle	36.83 ± 5.22	81.50	-6.4 ± 0.1	44.05 ± 3.32	81.28	-2.3 ± 0.5

**Fig. 4.** Weekly mean ambient temperature (°C) by study site in (A) 2018-2019 and (B) 2019-2020, minimum ambient temperature in (C) 2018-2019 and (D) 2019-2020, and snow depth (cm) in (E) 2018-2019 and (F) 2019-2020.**Table 4**Summary of statistical tests concerning tick survival and climate conditions in different microhabitat treatments. Asterisks indicate statistically significant tests at $\alpha = 0.05$.

Effect	df	F	P
a) Tick survival among microhabitat treatments by location			
Treatment	3, 69	14.60	< 0.01*
Location	2, 69	9.58	< 0.01*
Year	1, 69	1.35	0.25
b) Tick survival predicted by microclimate conditions (December-April)			
Mean temperature	1, 69	31.14	< 0.01*
Mean minimum temperature	1, 69	6.39	0.01*
Year	1, 69	0.57	0.57
c) Differences in mean temperature across microhabitat treatments and location			
Treatment	3, 69	13.08	< 0.01*
Location	3, 69	147.15	< 0.01*
Year	1, 69	23.41	< 0.01*

geographic distribution and spread of vector tick species, yet no previous studies have tested this hypothesis using field experiments both in areas where ticks are abundant and in areas where ticks currently do not occur at detectable densities (Elias et al., 2020). We found that cold ambient temperature may reduce blacklegged tick overwinter survival, but ticks nonetheless can survive over winter in northern regions where reproductive populations apparently are not yet established because snow cover provides insulation from ambient conditions. Temperatures in control enclosures (i.e., leaf litter and snowpack present) remained above -10°C consistently while temperatures in northern Maine often

were far lower, suggesting that ticks overwintering at the northern sites were not exposed to the lowest ambient temperatures throughout the winter because leaf litter and snow cover provided sufficient insulation to prevent freezing or desiccation. The removal of snow cover decreased tick overwinter survival more than the removal of leaf litter, demonstrating that snowpack may play an especially important role in overwinter survival in northern locations. Although we detected higher density of blacklegged ticks and prevalence of tick-borne pathogens in southern Maine compared to northern Maine, our field experiments suggest that overwinter survival of nymphs is not the primary mechanism constraining the current range of blacklegged ticks.

We found that increased insulation improved the survival of overwinter ticks in microhabitats and that insulation provides a warmer, more ideal microclimate. Snow and snow/leaf removal enclosure temperatures consistently were lower than control enclosure temperatures, suggesting that snowpack insulates the soil and thus prevented overwinter ticks from freezing despite low surface air temperatures (Decker et al., 2003; Templer et al., 2012). This finding is consistent not only with other field studies of blacklegged ticks (Lindsay et al., 1995; Burtis et al., 2015; Linske et al., 2019), but with several prior studies that assessed the importance of snowpack and snow depth on the regulation of soil temperature and frost. Hardy et al., (2001) conducted a snow removal experiment in New Hampshire, a state that, like Maine, has permanent snowpack during the winter, and found that snow removal results in more extensive soil frost than unmanipulated control conditions. Templer et al., (2012) tested the impacts of snow removal on the abundance, richness, and diversity of soil-dwelling arthropods in New Hampshire and found that a decrease in snowpack and snow depth may

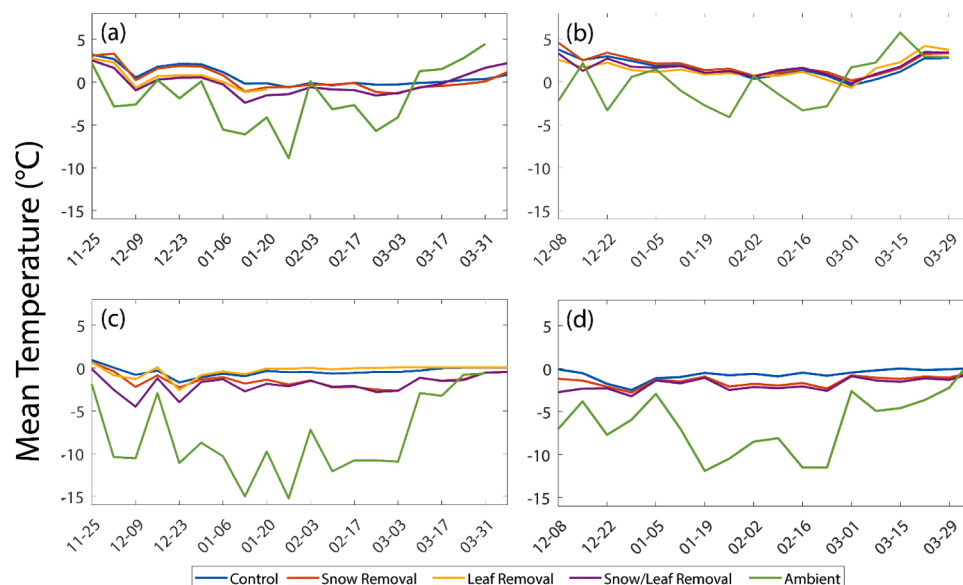


Fig. 5. Weekly mean temperature (°C) across treatments in Cape Elizabeth in (A) 2018-2019 and (B) 2019-2020 and weekly mean temperature across treatments in Presque Isle in (C) 2018-2019 and (D) 2019-2020.

Table 5

Ixodes scapularis densities (per 1000 m²) in Cape Elizabeth, Mt. Vernon, Orono, East Hancock, and Cutler, in Maine, United States, June – August 2019. A total area of 1000 m² was dragged at each site once every two weeks.

Site	Life Stage	Density (per 1000 m ²)		
		June	July	August
Cape Elizabeth	Larva	5	53	63.5
	Nymph	91	84	95.5
	Adult	52	19	7
Mt. Vernon	Larva	0	0	0
	Nymph	3.5	3	8
	Adult	0.5	0	0
Orono	Larva	20	45.5	4.5
	Nymph	18.5	29	6
	Adult	1.5	0	0
East Hancock	Larva	0	0	0
	Nymph	0	1	0
	Adult	0	0	0
Cutler	Larva	1	0	0
	Nymph	0	1	1
	Adult	1	0	0

reduce the abundance and diversity of arthropod communities. Previous tick overwinter studies conducted in New York, Connecticut, and southern Maine investigating the effect of insulation on overwinter tick survival found that snow removal did not decrease overwinter survival while leaf litter removal did (Burtis et al., 2015; Linske et al., 2019). This research extended the latter study, which was conducted in areas of Connecticut and southern Maine that support high densities of black-legged ticks, into central and northern Maine where blacklegged ticks

largely remain undetected. Perhaps more representative of the winter climate and low tick densities in northern Maine, Lindsay et al., (1995) assessed nymphal overwinter survival in southern and northern Ontario and found that nymphal survival was higher at northern sites compared to southern sites due to more continuous snow cover in the north despite lower ambient temperatures. We found that the effect of reduced insulation was more pronounced during the first year of the study in Presque Isle, in which ambient temperatures were lower but survival was higher than the second year. Thus, it is possible that the interaction between temperature and snow depth is more significant in colder climates, and the combination of colder ambient temperatures and greater average snow depth resulted in significant tick mortality in snow removal treatments during the colder winter. Warmer ambient temperatures during the second year may have limited the effect of snow removal on overwinter survival because the ticks were not exposed to temperatures lower than -10°C as often as during the first year.

Tick survival under the most ecologically realistic conditions (i.e., snow and leaf litter present) decreased between the first and second years of the study in Presque Isle, which may have been caused by higher overall temperatures and reduced snowpack during the second winter. Warmer winters, which typically result in more intermittent snow cover than colder winters, can cause a greater degree of soil freezing than colder winters (Decker et al., 2003). Although ambient temperatures were higher during the second winter, control enclosure temperatures in Presque Isle were lower than during the first winter. Upon retrieval of the ticks after the second winter, we noted that several enclosures were encased in more ice in Presque Isle than the first winter, which may also have contributed to mortality. Alternatively, it is possible that the

Table 6

Tick counts by species and site, June – August 2019 in Maine, United States.

Site	<i>Ixodes scapularis</i>	<i>Ixodes cookei</i>	<i>Ixodes marxi</i>	<i>Dermacentor variabilis</i>	<i>Haemaphysalis. leporispalustris</i>
Cape Elizabeth	635	0	0	30	0
Cutler	6	0	0	1	8
East Hancock	2	0	0	0	187
Mt. Vernon	16	0	0	12	0
Orono	250	0	0	0	0
Presque Isle	0	4	1	0	0
Brownville	0	0	0	0	45
Species Totals	909	4	1	41	233
Total	1,188				

Table 7Infection prevalence of questing *Ixodes scapularis* nymphs for tick-borne pathogens, June – August 2019 in Maine, United States.

Site	Number tested	Number and (%) of nymphs testing positive <i>Borrelia burgdorferi</i> sensu lato (+)	<i>Anaplasma phagocytophilum</i> (+)	<i>Babesia microti</i> (+)
Cape Elizabeth	77	16 (20.1%)	11 (14.2%)	3 (3.8%)
Mt. Vernon	15	1 (6.6%)	0	0
Orono	77	7 (9.1%)	0	0
East Hancock	2	0	0	0
Cutler	3	0	0	0
Presque Isle	5	1 (2%)	0	0

warmer spring temperatures during the second year resulted in lower survival. Warm spring temperatures induce higher energy use by ticks, which may cause increased mortality compared to low energy use during the winter (Burtis et al., 2019).

As expected, we documented a gradient of decreasing blacklegged tick density from southern to central Maine, which is consistent with previous passive surveillance studies (Rand et al., 2007; Elias et al., 2021; Rounsville et al., 2021). We detected no blacklegged ticks at our northern sites in Brownville and Presque Isle, yet other tick species including *H. leporispalustris*, *I. cookei*, and *I. marxi* were detected north of Orono. While we did not detect *I. cookei* and *I. marxi* in any locations except Presque Isle, both species are considered rare but widely distributed throughout Maine, and the distribution of these *Ixodes* species did not appear to change with the initial expansion of *I. scapularis* (Smith et al., 1992; Rand et al., 2007). However, *I. cookei* and *I. marxi* were not detected even after intensive surveillance conducted on Mt. Desert Island, Maine, where *I. scapularis* are now well-established (A.M. Gardner unpublished data) and these species historically were endemic, suggesting that the changing distributions of tick species in endemic areas warrants additional research. Elias et al., (2021) recently reported that passively collected nymphal submission rates decreased along a longitudinal gradient in Maine where average winter temperatures were below -13°C, which is consistent with our finding of longitudinally decreasing tick density. The lower pathogen prevalence we detected in Orono compared to Cape Elizabeth is consistent with well-established correlations between nymphal tick abundance and Lyme disease cases (Rand et al., 2007; Gardner et al., 2020).

Because we found that snow cover and leaf litter insulated overwinter enclosures against low ambient temperatures in southern and northern Maine, we suggest that cold ambient temperatures do not limit blacklegged tick survival in northern areas where questing blacklegged ticks were not detected. Thus, blacklegged ticks may be limited in central and northern Maine by other biotic mechanisms such as densities of white-tailed deer (*Odocoileus virginianus*), which are crucial hosts for adult blacklegged ticks (Wilson et al., 1990). There is a correlation between deer and blacklegged tick densities in New England (Rand et al., 2004b), and several studies have suggested that lower deer densities in northern Maine may inhibit the establishment of blacklegged tick populations (Rand et al., 2004b; Telford 2007; Elias et al., 2021). Second, the greater density of coniferous forests in northern Maine compared to greater densities of deciduous forest in southern Maine (Ireland 1997) may limit the size of northern tick populations, as the increased insulation caused by deciduous leaf litter compared to coniferous leaf litter creates a more favorable habitat that can increase off-host survival (Adler et al., 1992; Ostfeld et al., 1996; Lindsay et al., 1999). Maine's climate may further constrain the distribution of ticks, as the lesser duration of time available for host-seeking over the summer may limit tick population size in northern climates (Vail and Smith 2002; Ogden et al., 2005; Diuk-Wasser et al., 2014), and colder temperatures may impact other stages of the tick life cycle (i.e., development and fecundity) (Lindsay et al., 1995; Peavey and Lane 1996; Lindsay et al., 1998; Ogden et al., 2004).

We detected higher *B. burgdorferi* s.l. prevalence in blacklegged ticks collected from southern Maine than central Maine, and *A. phagocytophilum* and *B. microti* were only detected in ticks collected in

southern Maine. This is consistent with patterns in human case incidence for Lyme disease, anaplasmosis, and babesiosis; in 2019, higher incidence of Lyme disease and anaplasmosis was reported in southern Maine compared to northern Maine, and no babesiosis was reported in northern Maine (Maine CDC 2020). Although blacklegged ticks are the primary vectors of *B. burgdorferi* s.l., these pathogens have been detected in other *Ixodes* species such as *I. cookei*, *I. dentatus*, and *I. auritulus* (Levine et al., 1991; Barker et al., 1993; Morshed et al., 2005). Two other *Ixodes* species were collected in Presque Isle where no *I. scapularis* were found, and one *I. cookei* tested positive for *B. burgdorferi* s.l. Pathogen detection, however, does not imply vector competence; *Ixodes cookei* and *I. marxi* are both vectors of Powassan encephalitis virus, but experimental vector competence for *B. burgdorferi* s.l. has not been confirmed for *I. cookei* despite several studies being done and has never been evaluated for *I. marxi* (Artsob 1984; Barker et al., 1993). We tested ticks for *B. burgdorferi* s.l., which includes both pathogenic species (e.g., *B. burgdorferi* sensu stricto) and species not known to be pathogenic (e.g., *Borrelia andersonii*); thus, it is possible that the *B. burgdorferi* s.l. we detected at Presque Isle was non-pathogenic and not capable of causing Lyme disease in humans. Several studies have found that human-pathogenic *B. burgdorferi* sensu stricto is not efficiently transmitted between *I. cookei* and their primary hosts in locations where Lyme diseases cases were reported but *I. scapularis* are not well established (Barker et al., 1992; Ryder et al., 1992). Furthermore, *I. cookei* and *I. marxi* may aggressively parasitize humans under the right conditions (Lubelczyk et al., 2010) but they are not commonly reported as feeding on humans regularly (Hall et al., 1991; Lee et al., 2019). This suggests that *I. cookei*, although capable of being infected, may not be a primary vector of *B. burgdorferi* sensu stricto in areas outside the range of the blacklegged tick.

Some limitations to this study may have contributed to our overwinter survival and distribution result. First, ticks overwintered in polypropylene vials that were placed in leaf litter or soil instead of directly in the microcosm. This may have influenced humidity and temperature that the ticks were exposed to (i.e., condensation collected in the vials and temperature in the vials may have been different from the immediate surroundings in the soil or leaf litter) (Bertrand and Wilson, 1996; Brunner et al., 2014). It has been documented that ticks that have direct access to the soil or leaf litter have a higher overwinter survival than ticks that are housed in containers due to the fact that ticks placed directly in the soil or leaf litter are able to move or relocate to a more favorable microclimate with higher relative humidity or temperature, while ticks in vials are limited in mobility (Padgett and Lane, 2001). Alternatively, although we did not measure humidity directly inside the vials, increased moisture and relative humidity inside the vials may have increased tick survival compared to ticks overwintering directly in soil or leaf litter. Second, while we made efforts to select sites with similar tree and vegetation composition, survival may also be related to site level factors such as vegetation, soil type, and topography. Third, the laboratory-reared tick colony originated in Oklahoma and belongs to the southern clade of *I. scapularis* found in North America. Due to the population genetic and behavioral differences between northern and southern ticks (Ginsberg et al., 2014; Arsnoe et al., 2015), ticks originating from northern areas may be better adapted to surviving northeastern winter conditions, and our data thus represent an estimate

of the survival rate of southern ticks exposed to a northern climate. Finally, while we conducted a comparative study between latitudinally-distinct regions, our results are not necessarily a representation of an overall overwinter survival estimate of *I. scapularis* across its entire range.

Our study revealed that manipulating microclimate insulation can reduce tick survival, which can be used to inform small-scale or local management practices to reduce leaf litter or snow cover throughout the fall and winter to potentially reduce overwinter survival. Removing leaf litter can also reduce the abundance of questing nymphs during active periods. It has been shown that active forest management can additionally alter the amount of leaf litter present (Conte et al., 2021), with potential consequences for overwinter tick survival. Thus, while labor and time intensive, removing leaf litter on a localized scale before ticks enter diapause in the late fall may reduce overwinter survival as well (Stafford and Magnarelli 1993; Schulze et al., 1995; Linske et al., 2019). In the Northeast, homeowners are most likely to encounter *I. scapularis* ticks on residential properties and peridomestic risk of tick-borne disease can be high in endemic areas (Falco and Fish 1988; Maupin et al., 1991; Feldman et al., 2015). Therefore, we suggest that small-scale management practices such as raking leaves in one's backyard may reduce individual risk of exposure to ticks (Jordan and Schulz, 2019). The findings of our study indicate that blacklegged ticks can successfully overwinter in areas north of their current range, and that snow cover plays a significant role in overwinter survival and thus the number of ticks that might emerge in the spring and summer with the potential to transmit pathogens to humans. Ultimately, our results can be used to inform future studies assessing the relationship between climate change and the spread of blacklegged ticks, as current predictions of increasing temperatures and decreasing snowfall may affect not only overwinter survival, but tick development and reproduction. The current widening geographic range and increasing densities of blacklegged ticks portend a higher risk of tick-borne diseases to endemic and susceptible areas. As winter temperatures and snowfall are predicted to increase in the northeastern U.S.A and other areas of North America (Karmalkar and Bradley, 2017; Fernandez et al., 2020), the ability to predict how climate change will impact the changing distribution of blacklegged ticks, posing a major public health concern. Understanding how climate affects blacklegged tick survival at the northern limits of their distribution can aid in predicting the future spread of ticks and tick-borne diseases.

5. Author Statement

M.R.V., A.M.G., and C.B.L. conceptualized research questions and designed the study, M.R.V., C.B.L., and J.C.J. collected data, M.R.V. and A.M.G. analyzed data, A.M.G., C.B.L., and D.L.L. acquired funding, A.M.G. supervised the research, M.R.V. and A.M.G. wrote the original draft, and all authors contributed to review and editing of the manuscript.

Competing Interests Statement

The authors declare no competing interests.

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