The Effect of Broad-Scale Climate and Microclimate Conditions on Ixodes Scapularis (Acari: Ixodidae) Overwintering Survival and Distribution in Maine

Michelle Volk
University of Maine, michelle.volk@maine.edu

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THE EFFECT OF BROAD-SCALE CLIMATE AND MICROCLIMATE CONDITIONS ON *IXODES SCAPULARIS* (ACARI: IXODIDAE) OVERWINTERING SURVIVAL AND DISTRIBUTION IN MAINE

By

Michelle Volk

B.S. New York University, 2017

A THESIS

Submitted in Partial Fulfillment of the
Requirements for the Degree of
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August 2020

Advisory Committee:

Allison Gardner, Assistant Professor of Arthropod Vector Biology

Pauline Kamath, Assistant Professor of Animal Health

Danielle Levesque, Assistant Professor of Mammalogy and Mammalian Health
THE EFFECT OF BROAD-SCALE CLIMATE AND MICROCLIMATE CONDITIONS ON IXODES SCAPULARIS (ACARI: IXODIDAE) OVERWINTERING SURVIVAL AND DISTRIBUTION IN MAINE

By Michelle Volk

Thesis Advisor: Dr. Allison Gardner

An Abstract of the Thesis Presented in Partial Fulfillment of the Requirements for the Degree of Master of Science (in Ecology and Environmental Sciences) August 2020

Lyme disease is the most common vector-borne disease in North America, and human cases have increased substantially over the past few decades. Blacklegged ticks (Ixodes scapularis) are the primary vector for Borrelia burgdorferi, the causative agent of Lyme disease. Understanding the significance of climate and other ecological drivers of blacklegged tick survival will aid in predicting future distributions of blacklegged tick populations and identifying areas that pose a risk of Lyme disease to humans. This research explored whether winter climate facilitates or inhibits the spread of blacklegged ticks and the pathogens they transmit.

To determine the current range of blacklegged ticks in Maine, we systematically searched for and collected ticks across latitudinal and coastal-inland climate gradients across the state. We found higher densities of ticks in southern Maine (90.2 ticks/1000m²) than central Maine (17.8 ticks/1000m²) and no blacklegged ticks in northern Maine. To determine the effects of winter climate on blacklegged tick survival, we experimentally tested tick overwintering survival across the same gradients and assessed factors contributing to winter mortality both in locations where blacklegged tick populations are currently established and where the blacklegged tick remains
undetected. Overwintering survival was not significantly different between sites in southern Maine compared to northern Maine, which was likely due to sufficient snowpack that protected against low ambient temperatures in northern Maine. Snow cover and leaf litter contributed significantly to overwintering survival at sites in both southern and northern Maine. Our results suggest that winter climate is not constraining blacklegged tick populations in areas north of their current range and that additional mechanisms may be limiting the expansion of ticks into northern Maine thus far. The results of this study will aid in understanding how climate factors contribute to blacklegged tick overwintering survival, which has important implications for tick abundance and distribution during the spring and summer. This information can be used to inform future studies to assess the effects of climate change on the distribution of blacklegged ticks and tick-borne pathogens.
DEDICATION

I dedicate this thesis to my parents for their help and support.
ACKNOWLEDGEMENTS

I would first like to thank and acknowledge my advisor and mentor, Dr. Allison Gardner, for her unending support, encouragement, and guidance on my research and professional development. I would also like to thank my committee members, Dr. Pauline Kamath and Dr. Danielle Levesque, for dedicating their time, expertise, and advice to my research and academics at the University of Maine. I would also like to thank Chuck Lubelczyk at Maine Medical Center Research Institute for invaluable guidance, assistance, and field work during the development and execution of this project. I would also like to thank members of the Vector-Borne Disease Laboratory at MMCRI, especially Libby Henderson, for assistance in the field. I would also like to acknowledge and thank Jason Johnston at the University of Maine at Presque Isle for help in the initial stages of this project and for dedicating time and effort to coordinating field work in Presque Isle. I would like to thank my winter field technicians at Presque Isle, Alex McQuade and Alex Kimball, for their assistance monitoring field sites. In particular, I would like to thank and acknowledge Alyssa Marini for her dedication and hard work in establishing and monitoring the overwintering field experiment. I would like to thank and recognize Brianna Guy for being a tremendous and positive asset in the field and for providing maps for the tick distribution study. Thank you sincerely to all the members of the Gardner Lab for your constant support, feedback, and guidance at all stages of this project. Thank you in particular to Karla Boyd, Steph Hurd, Sara McBride, and Christine Conte for your friendship and support with research and classes at the University of Maine. Finally, I would like to thank and acknowledge my funding sources for making this research possible: the University of Maine Research Reinvestment Fund Graduate Research Assistantship, the Maine Outdoor Heritage Fund, the University of Maine Graduate Student Government, and Hatch fund #ME021905.
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CHAPTER 1
THE EFFECT OF BROAD-SCALE CLIMATE AND MICROCLIMATE CONDITIONS ON IXODES SCAPULARIS (ACARI: IXODIDAE) OVERWINTERING SURVIVAL AND DISTRIBUTION IN MAINE

Introduction

Global climate change is expected to facilitate emergences, re-emergences, and future distributions of infectious diseases including vector-borne diseases, which account for more than 17% of all infectious diseases worldwide (Githeko et al. 2000, Brownstein et al. 2005, Ogden and Lindsay 2016). Temperature and humidity, which are projected to change from micro to regional scales (Martens et al. 1995, Moore et al. 2011, Eisen et al. 2016), affect the survival, development, activity, phenology, and other life history and behavioral characteristics of arthropod vectors that then may influence disease transmission among wildlife reservoir hosts and to humans (Vail and Smith 1998, Ogden et al. 2004, Eisen et al. 2016). Thus, climate change has and is expected to continue to change the distribution of vectors and thus the distribution of the diseases they transmit. For instance, as climate warms in equatorial zones, there has been an increase in the transmission of mosquito-borne diseases such as malaria and Dengue fever (Githeko et al. 2000, McMichael et al. 2012). As temperate zones warm there has been a concomitant poleward spread tick-borne diseases such as Lyme disease as the optimal habitat of vectors expands northward (Brownstein et al. 2005).

Lyme disease is the most common vector-borne disease in North America and affects more than 30,000 people each year in the United States (Kugeler et al. 2015, CDC 2018). In the past few decades, Lyme disease has increased in prevalence and geographic distribution in the northeastern and midwestern United States (CDC 2018). One hypothesis for this trend is that an
increase in average temperature and precipitation is facilitating increased abundance and the northward expansion of the blacklegged tick (*Ixodes scapularis*), the primary vector of Lyme disease (Ogden et al. 2005). Blacklegged ticks transmit *Borrelia burgdorferi*, the bacterial pathogen that causes Lyme disease, in a complex system in which *I. scapularis* acquire the pathogen by feeding on infected reservoir hosts, such as white-footed mice (*Peromyscus leucopus*), eastern chipmunks (*Tamias striatus*), and deer mice (*Peromyscus maniculatus*), before transmitting the pathogen to a subsequent host (Ostfeld et al. 2006). Blacklegged ticks have three life stages (larval, nymphal, and adult) during a two-year life cycle (Figure 1). After questing (host-seeking) in the summer and fall, blacklegged ticks enter diapause and overwinter twice during their life cycle (as nymphs and adults) buried under snow and leaf litter, which improve insulation and provide moisture for overwintering ticks (Brunner et al. 2012). Although *I. scapularis* is the primary vector of *B. burgdorferi*, the pathogen has 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).
Figure 1. The life cycle of blacklegged ticks. Blacklegged tick larvae emerge in the mid-late summer to quest for a blood meal before molting into nymphs. Nymphs overwinter and emerge in the late spring and summer to quest for the second bloodmeal before molting into adults. Adults overwinter a second time and emerge in the spring to lay eggs (CDC 2011).

Blacklegged ticks spend the majority of their life off-host, and climate may interact with their life cycle through several mechanisms including impacts on off-host tick survival and behavior. First, temperatures less than -10° C and relative humidity less than 85% cause nymphal tick mortality in laboratory environments due to freezing injury or desiccation (Stafford 1994, Burks et al. 1996, Vandyk et al. 1996, Ogden et al. 2004). Relative humidity is very significant to tick survival due to the tick’s high body surface area to volume ratio which can lead to water loss during dry winter conditions, as well as the lack of intake of dietary water throughout the blacklegged tick’s life cycle (Ostfeld 2011). It is currently unknown whether sustained periods of low temperatures or fluctuations with sudden cold spells of shorter duration are more detrimental to blacklegged tick overwintering survival (Vandyk et al. 1996). Second, exceptionally cold or dry
conditions have been shown to decrease development rates in laboratory experiments (Ogden et al. 2004). Low relative humidity can cause quiescence and deter blacklegged ticks from leaving the high-humidity microclimate in leaf litter, preventing them from climbing vegetation and actively questing for a host (Randolph and Storey 1999, Vail and Smith 2002). Climate may further impact blacklegged tick phenology and disease transmission at both local and regional scales. Increasing temperatures are likely to result in an increase in tick abundance as well as an increase in disease prevalence due to a longer season of activity for feeding and increased opportunities for pathogen transmission (Gatewood et al. 2009, Wallace et al. 2019).

Because blacklegged ticks are particularly vulnerable to cold and dry conditions, it is likely that variations in climate affect overwintering survival and thus the number of ticks that emerge in the spring and summer (Estrada Peña 2002, Brownstein et al. 2003). Several studies have assessed the effects of cold and dry conditions on blacklegged tick survival using lab-based temperature and humidity experiments to find the lethal limits of low temperature and humidity (Stafford 1994, Burks et al. 1996, Randolph and Storey 1999, Ogden et al. 2004), but these laboratory studies are limited in several aspects. First, it is unlikely that ticks overwintering in natural conditions experience the lowest winter temperature and humidity because they typically occupy insulated microclimates (Burks et al. 1996). Second, because blacklegged ticks overwinter in insulated microclimates under snow and leaf litter, which are dependent upon local climate and vegetation, it is important not only to assess the effect of ambient temperature and humidity on tick survival but to also consider the interaction between ambient climate and microclimate and the significance of spatiotemporal variation in microclimate conditions on overwintering success. For instance, a northern climate with low ambient temperature and humidity may experience enough snowfall to allow ticks to survive under snow cover in an optimal microclimate despite inhospitable ambient
conditions (Lindsay et al. 1998, Lindsay et al. 1999). Tick overwintering survival also needs to be tested using field experiments to assess whether blacklegged ticks can survive winter conditions in areas where populations are not established. Because snow cover provides an insulated microclimate for overwintering ticks, it is possible that increased snowfall in northern areas will provide sufficient insulation for blacklegged tick survival (Brownstein et al. 2005). However, snowpack is predicted to decrease due to climate change (Hayhoe et al. 2007), which will result in decreased soil temperatures during the winter when ticks rely on snow as insulation for survival (Decker et al. 2003). Thus, due to the projected changes in winter temperature and precipitation, it is necessary to understand the effects of the interactions between ambient conditions and microclimate conditions in areas both in and outside of the blacklegged tick’s current range.

Maine has a high incidence of tick-borne diseases with over 2,000 human cases of Lyme disease reported in 2019 (Maine CDC 2020). Since the first human cases in Maine were reported to the CDC in 1986 (Maine CDC 2020), Lyme disease has been spreading from southern to northern Maine, and citizen-submitted tick data indicate that the greatest prevalence of Lyme disease occurs in southern and coastal Maine and few or no blacklegged ticks and incidences of tick-borne disease are reported in northern Maine (Rand et al. 2007, Maine CDC 2020). These passively-collected data may be biased by the distribution and density of individuals who submitted ticks, and the majority of Maine’s population resides in its southern half (Rand et al. 2007). Thus, a wide-scale active-surveillance effort is needed to determine the current distribution of blacklegged ticks in Maine in areas with both high and low human population densities.

Maine has two broad-scale precipitation and temperature gradients: snowfall increases south to north and west to east, while temperature decreases south to north and west to east. (Birkel 2018). These climate gradients provide a unique opportunity to conduct field experiments to study
the effects and interaction of both broad-scale climate and microclimate conditions on blacklegged tick survival. Additionally, because blacklegged ticks are currently increasing in abundance and distribution across Maine, we can assess the effects of the interaction of winter temperature and winter precipitation on blacklegged tick survival in areas where ticks are well established and in areas where blacklegged ticks have yet to be documented, thus determining whether or not winter conditions in northern Maine and similar areas limit the spread of blacklegged ticks.

First, to determine the current distribution of blacklegged ticks across broad-scale climate gradients in Maine using active surveillance, we systematically searched for and collected ticks throughout the state during their active summer months. Based on citizen-submitted tick distribution data, we did not expect to find high densities of blacklegged ticks in northern Maine. Second, to determine the effects of climate on blacklegged overwintering tick survival, we tested tick survival across broad-scale temperature and precipitation gradients in Maine and assessed factors contributing to winter mortality both in locations where blacklegged tick populations are currently established and where blacklegged ticks remain undetected. We hypothesized that blacklegged tick overwintering survival would be lower in northern Maine than southern Maine due to lower ambient temperatures in northern Maine. Finally, to determine the effects of microclimate on blacklegged tick survival, we tested tick overwintering survival under varying microclimate conditions (snow removal, leaf litter removal, both snow and leaf litter removal, and a control for which the insulation was not manipulated). Because blacklegged ticks are vulnerable to cold and dry conditions, we hypothesized that overwintering survival will decrease with decreasing insulation.
**Methods**

**Site Selection**

To test the hypothesis that broad-scale climate patterns impact overwintering survival and thus the abundance of blacklegged ticks that successfully emerge in the spring and quest throughout the summer, we drag sampled for blacklegged ticks at seven sites across Maine from June-August 2019 (Figure 2): Cape Elizabeth (York County), Kennebec Highlands Public Reserved Land (Kennebec County), Orono (Penobscot County), Duck Lake Public Lands (Hancock County), Seboeis Public Reserved Lands (Piscataquis County), Cutler Coast Public Reserved Lands (Washington County), and Presque Isle (Aroostook County). These sites were selected to cover temperature and precipitation gradients from north to south and inland to coastal Maine to assess broad-scale tick distribution in the state. Locations within each site were selected by the availability of mixed hardwood forest.

To assess tick overwintering survival as a potential mechanism to explain state-wide variation in tick densities, an overwintering field experiment was conducted over two winters (November-April) from 2018-2019 (Winter 2019) and 2019-2020 (Winter 2020). Enclosures containing nymphal blacklegged ticks were placed in the ground at five of the seven sites that were dragged during June-August 2019: Cape Elizabeth (York County), Orono (Penobscot County), Seboeis Public Reserved Lands (Piscataquis County), Cutler Coast Public Reserved Lands (Washington County), and Presque Isle (Aroostook County) (Figure 2). Cutler was omitted as a study site for Winter 2020 due to significant flooding during Winter 2019. At each site, the experiment was set up in an area isolated from human activities with high elevation to prevent flooding in the enclosures when snow began to melt.
Figure 2. The seven sites used in this study, selected to span the precipitation and temperature gradients in Maine. Each shape indicates the purpose for which the study site was used; triangles indicate that the site was only used for drag-sampling during the summer; squares indicate that the site was dragged during the summer and contained only control overwintering enclosures; circles indicate that the site was dragged during the summer and contained control and treatment overwintering enclosures.

Off-host tick collection

Ticks were collected using a standard “dragging” method (Zimmerman and Garris 1985). Questing ticks attach to a 1m² piece of denim attached to a dowel rod that is dragged across vegetation or leaf litter. Ticks were removed from the drag cloth and stored in 70% ethanol.
Because ticks do not quest during rain or wet conditions, dragging was not performed at any site during rain or when the vegetation or leaf litter was wet enough to soak the drag cloth. At each site, a 8,000 m² 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. Grids were delineated around areas of woodland that were not bisected by roads or trails. Eight 125-m transects were dragged across the grid for a total of 1000 m². Sites were dragged once every two weeks, for a total distance of 1,000 m² per month or 2,000 m² per month from June-August.

All *I. scapularis* life stages and other tick species found were collected and preserved in 70% ethanol and stored in a -4°C freezer. Larval ticks were removed from the drag cloth with a piece of adhesive tape and stored in a plastic bag in a -4°C freezer. All ticks were identified to life stage and species using standard keys (Keirans and Clifford 1978, Keirans and Litwak 1989, Durdan and Keirans 1996). Ticks were collected between 10 June and 23 August in 2019, which is the predicted activity period of nymphal *I. scapularis* in Maine (Rand et al. 2007).

**Ambient Climate Measurements**

To test the hypothesis that climate impacts blacklegged tick distribution, hourly temperature and relative humidity were measured at each site using iButton data loggers (DS1923-F5#, Maxim Integrated, Whitewater, WI.). To protect the iButton data loggers from precipitation and direct sunlight, ambient data loggers were secured inside an inverted 9 oz green Solo® cup attached to a stake. The stake was inserted in the ground so that the iButton was ~6 cm above the ground to reflect the microclimate of questing height.
Pathogen Prevalence Analysis

To determine the proportion of nymphal blacklegged ticks infected with pathogens, *I. scapularis* nymphs collected during the summer of 2019 were tested for *B. burgdorferi*, as well as *Anaplasma phagocytophilum*, and *Babesia microti*, two other emergent pathogens transmitted by blacklegged ticks in Maine. We tested 179 ticks out of 909 ticks collected from Cape Elizabeth, Orono, Kennebec Highlands, Presque Isle, Cutler Coast, and Duck Lake. Because very few *I. scapularis* were collected from Kennebec Highlands (15), Cutler Coast (three), and Duck Lake (two), all ticks were tested for pathogens. An additional 154 ticks were tested from Cape Elizabeth and Orono (i.e., 77 specimens from each location). Four *Ixodes cookei* (three nymphs and one adult) and one nymphal *Ixodes marxi* collected from Presque Isle were tested to determine if these pathogens are being transmitted in cryptic cycles where no *I. scapularis* were found.

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 (10µL) contained 5µL of Bio-Rad iQ Multiplex Powermix (Hercules, CA, USA), 3µL of primers and probes (Hojgaard et al. 2014, Xu et al. 2016) (Table 1), and 2µL of extracted DNA template. PCR reactions were completed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), and conditions were as follows: 3 minute Hot-start at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds. Each sample was run in duplicate to account for false positives. 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.

**Table 1.** Premixed primers and probes to test nymphal *I. scapularis* for *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, and *Babesia microti*

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer Name</th>
<th>Sequence (5’-3’)</th>
<th>Conc (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tick DNA Control</td>
<td>TickCon-F</td>
<td>AATACTCTAGGGATAACACGCTAATAATTTT</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>TickCon-R</td>
<td>CGGTCGGACTCAGATCAAGTGGAGA</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>TickDNA</td>
<td>AAA TAG TTT GCG ACC TCG ATG TTG GAT T</td>
<td>250</td>
</tr>
<tr>
<td><strong>Borrelia spp.</strong></td>
<td>Bburg-F</td>
<td>CGGGCTCTAAAGGCGATTTTGGAGA</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Bburg-R</td>
<td>GCTTCAGCTGCGCGCTAAATAG</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Borrelia</td>
<td>AGA TGT GGT AGA CCC GAA GCC GAG TG</td>
<td>250</td>
</tr>
<tr>
<td><strong>Anaplasma phagocytophilum</strong></td>
<td>Aphago-F</td>
<td>ATGGAAGGTAGTTGTTGGTTGATTTGATTT</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Aphago-R</td>
<td>TTGGCTCTGAAGGCGCTAGGTC</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Anaplasma</td>
<td>TGG TGG CAG GGT TGA GCT TGA GAT TG</td>
<td>250</td>
</tr>
<tr>
<td><strong>Babesia microti</strong></td>
<td>Hoj_Bab_18S-F</td>
<td>CGA CGG CGG TCC TGC CCT TTG</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Hoj_Bab_18S-R</td>
<td>ACG AAG GAC GAA TCC ACG TTT C</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Babesia</td>
<td>ACA CGG CCC GTC GCT CCT ACC G</td>
<td>250</td>
</tr>
</tbody>
</table>

**Overwintering Survival Experiment**

Tick overwintering 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.) and all-weather tape (SI-13786BL, Gorilla®, Cincinnati, OH.) to prevent wildlife from interfering with the vials inside the buckets (Figure 3). At each site, buckets were inserted into ~20 cm holes dug in the ground and filled with the excavated soil to ~five cm from the top. One polypropylene vial of 15 unfed, pathogen-free *I.*
*scapularis* nymphs was placed on top of the soil in each enclosure. *I. scapularis* nymphs were obtained from the Oklahoma State University Tick Rearing Facility (Stillwater, OK.). Using a tack, three holes were punched through the top of each vial and mesh was placed over the opening of each vial before the vial was sealed to prevent the release of ticks into the surrounding environment. Ticks were counted before being placed in the field and again upon retrieval to ensure that no ticks escaped during the winter.

To test the hypothesis that snow cover and leaf litter act as insulation and promote overwintering survival, four treatments were created to manipulate insulation in the tick enclosures. The treatments were snow removal (SR), leaf litter removal (LR), both snow and leaf litter removal (SLR), and a control for which there was no manipulation of insulation conditions (C). To represent 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 one cm, snow was removed from the tops of snow removal and snow/leaf removal enclosures. Snow was allowed to accumulate throughout the winter over leaf removal and control enclosures. The sites at Cape Elizabeth, Orono, and Presque Isle each contained four replicates of four treatments for a total of sixteen enclosures. Due to the remote location of the sites at Seboeis and Cutler (Winter 2019 only), it was not feasible to regularly remove snow after each snow event throughout the winter. Thus, each site contained four replicates of the control enclosures and no treatment enclosures. Due to flooding in the enclosures during Winter 2019 at Orono and Cutler, drainage holes were drilled into the bottom of each enclosure at Orono for Winter 2020, and Cutler was eliminated as a study site from the experiment.
During Winter 2020, one replicate was excluded from the Presque Isle data set because vials became waterlogged due to poor drainage in the enclosures. To test the hypothesis that snow cover and leaf litter provide insulation from low temperature and low humidity, iButton data loggers were also placed inside each enclosure next to the tick vials to record temperature (°C) and relative humidity (%) once every hour. Snow depth was measured weekly as well as after each snow event.

The ticks were placed in enclosures in late November before significant ground frost and snow cover accumulated. In 2018, ticks were placed in the ground at all sites between 24 November–28 November and retrieved in 2019 between 13 April and 26 April. In 2019, ticks were placed in the ground at all field sites between 26 November and 1 December and retrieved in 2020 between 2 April and 21 April. To evaluate ticks for survival, we held ticks at room temperature for at least 30 minutes and observed them for movement.

Because significant snow accumulation in northern sites buried ambient iButtons, ambient temperature data was obtained from the Maine Climate Office at the University of Maine Climate Change Institute (Climate Change Institute 2020) and relative humidity data was obtained from the NOAA National Centers for Environmental Information (National Oceanic and Atmospheric Administration 2020). Because of their proximity to each study site, data collected from weather stations located in Portland, Bangor, Millinocket, and Caribou were used as ambient data for Cape Elizabeth, Orono, Seboeis, and Presque Isle, respectively. Winter 2020 weekly precipitation data from Presque Isle was obtained from the NOAA National Centers for Environmental Information (National Oceanic and Atmospheric Administration 2020).
Figure 3. Ticks were placed in one-gallon buckets with mesh-covered holes cut into the sides and the lid. Buckets were filled with excavated soil and placed into the ground. Ticks were placed on top of the soil and the insulation treatment was applied (i.e., leaf litter was placed on top of the ticks or no leaf litter was included in the enclosure)

Statistical Analysis

Data analysis was conducted using R version 3.5.1 (R Core Team 2013). Tick survival data met assumptions for normality and was not transformed. To test the hypothesis that tick density varies by month along climate gradients, one-way ANOVAs were used to compare the densities of all life stages of *I. scapularis* collected from Cape Elizabeth and Orono. The predictor variables were month and location and the response variable was density. To test the hypothesis that overwintering survival will decrease with decreased microclimate insulation, both within locations and across locations, we used a one-way ANOVA to determine if microclimate treatment resulted in a significant difference in tick overwintering survival. The predictor variable was treatment and
the response variable was percent survival. To test the hypothesis that overwintering survival is different across broad-scale climate gradients, a one-way ANOVA was performed to determine if location resulted in a significant difference of survival of ticks overwintering in control enclosures. The predictor variable was location and the response variable was percent control survival. For both one-way ANOVA tests, we used a Tukey HSD test with an alpha value of 0.05 for post-hoc analysis using the multcomp package in R (Hothorn et al. 2008). To determine which abiotic factors influenced tick overwintering survival, a multiple linear regression model was created with mean temperature, mean minimum temperature, average snow depth, and snow maximum as predictor variables and percent tick survival as the response variable. Stepwise selection using the MASS package in RStudio (Venables and Ripley 2002) was performed to identify the most important predictors and the most predictive model of tick overwintering survival. To test the hypothesis that temperature and humidity are different across broad-scale climate gradients, a repeated measures ANOVA using the multcomp package in R studio (Hothorn et al. 2008) was performed to determine the effect of treatment and location on microclimate temperature and humidity. The predictor variables were treatment and location and the response variable was weekly temperature or humidity.

**Results**

*I. scapularis* distribution and pathogen prevalence in Maine

**Off-host tick collection.** Between 10 June and 23 August, 2019, a total of 1188 ticks of various species were collected off-host across Maine. A total of 909 *I. scapularis* were found at Cape Elizabeth, Kennebec Highlands, Orono, Duck Lake, and Cutler (Figure 4). Additional tick species were collected: *Ixodes cookei* (4), *Ixodes marxi* (1), *Dermacentor variabilis* (43), and
*Haemophysalis leporispalustris* (233). *Ixodes scapularis* were found at all sites except Seboeis, where only *H. leporispalustris* was collected, and Presque Isle, where only *I. cookei* and *I. marxi* were collected. *Haemophysalis leporispalustris* were collected at other locations (Duck Lake and Cutler), but *I. cookei* and *I. marxi* were not collected at any locations other than Presque Isle.

The total density of nymphs at Cape Elizabeth (90.2 ticks/1000m$^2$) was significantly higher than at Orono (17.8 ticks/1000m$^2$) ($F = 64.307; df= 1,2; p = 0.015$), while larval and adult densities were not significantly different (Table 2). There was no significant difference in densities of any life stage for each individual month (June, July, and August) between Cape Elizabeth and Orono.
Figure 4. The results of *I. scapularis* distribution in comparison to winter climate gradients: winter precipitation increases south to north and west to east, while temperature decreases south to north and west to east (Birkel 2018) 4a. The distribution of all *I. scapularis* life stages collected across Maine between June-August, 2019. No *I. scapularis* were discovered at Seboeis or Presque Isle. 4b. The average temperature (°F) for December, January, and February 2018-2019. 4c. The
average temperature (°F) for December, January, and February 2019-2020. 4d. The total precipitation (in) for December, January, and February 2018-2019. 4e. The total precipitation (in) for December, January, and February 2019-2020 (NOAA National Centers for Environmental Information 2020)

Table 2. Comparison of densities of *I. scapularis* (ticks/1000m²) collected at Cape Elizabeth, Kennebec Highlands, Orono, Duck Lake, and Cutler Coast for June, July, and August of 2019

<table>
<thead>
<tr>
<th>Site</th>
<th>June</th>
<th>July</th>
<th>August</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Life stage</td>
<td>Density (ticks/1000m²)</td>
<td>Total</td>
</tr>
<tr>
<td>Cape Elizabeth</td>
<td>Larva</td>
<td>5</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>Nymph</td>
<td>91</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>Adult</td>
<td>52</td>
<td>19</td>
</tr>
<tr>
<td>Kennebec Highlands</td>
<td>Larva</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Nymph</td>
<td>3.5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Adult</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>Orono</td>
<td>Larva</td>
<td>20</td>
<td>45.5</td>
</tr>
<tr>
<td></td>
<td>Nymph</td>
<td>18.5</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Adult</td>
<td>1.5</td>
<td>3</td>
</tr>
<tr>
<td>Duck Lake</td>
<td>Larva</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Nymph</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Adult</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cutler Coast</td>
<td>Larva</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Nymph</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Adult</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Pathogen Prevalence.

Overall, the infection prevalence for *B. burgdorferi* was highest in Cape Elizabeth and Orono, and *A. phagocytophilum* and *B. microti* were only detected in ticks collected from Cape Elizabeth (Table 3). Out of four *I. cookei* collected and tested from Presque Isle, one *I. cookei* nymph was positive for *B. burgdorferi.*
Table 3. Results of pathogen testing *I. scapularis* for *B. burgdorferi*, *A. phagocytophilum*, and *B. microti*

<table>
<thead>
<tr>
<th>Site</th>
<th>Number tested</th>
<th>Count <em>B. burgdorferi</em> (+)</th>
<th>Infection Prevalence</th>
<th>Count <em>A. phagocytophilum</em> (+)</th>
<th>Infection Prevalence</th>
<th>Count <em>B. microti</em> (+)</th>
<th>Infection Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cape Elizabeth</td>
<td>77</td>
<td>16</td>
<td>20.1%</td>
<td>11</td>
<td>14.2%</td>
<td>3</td>
<td>3.8%</td>
</tr>
<tr>
<td>Kennebec Highlands</td>
<td>15</td>
<td>1</td>
<td>6.6%</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Orono</td>
<td>77</td>
<td>7</td>
<td>9.1%</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Duck Lake</td>
<td>2</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Cutler Coast</td>
<td>3</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Presque Isle</td>
<td>5</td>
<td>1</td>
<td>2%</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0%</td>
</tr>
</tbody>
</table>

*I. scapularis* overwintering survival experiment

**Survival of ticks overwintering in control conditions.** During Winter 2019, there was no significant difference in survival of ticks overwintering in control conditions (i.e., leaf litter and snowpack present) between locations ($p = 0.085$) (Figure 5). Cutler Coast and Orono data were excluded from the 2018-2019 analyses because all vials became waterlogged due to poor drainage in the enclosures. Winter 2020 control tick survival was similar at Cape Elizabeth and Seboeis, lower at Orono, and lowest at Presque Isle ($F = 38.7; \text{df} = 3,4; \ p < 0.001$). Control tick survival was significantly lower during Winter 2020 than Winter 2019 at Presque Isle ($F = 12.60; \text{df} = 1,2; \ p = 0.002$), but not at Cape Elizabeth or Seboeis.
Figure 5. Survival of ticks overwintering in control (i.e., under snow cover and leaf litter) conditions

**Figure 5.** Percent survival of control ticks for Winter 2019 and Winter 2020. Asterisks indicate significant differences in control survival between Winter 2019 and Winter 2020. Significance letters indicate significantly different control survival between sites for within each year. Asterisks indicate significantly different percent survival between Winter 2019 and Winter 2020.

**Variability in climate conditions across sites and years.** During Winter 2019, the average statewide temperature was 2.38°C lower than Winter 2020 (Climate Change Institute 2020). This was reflected at the site-scale, with higher ambient temperatures recorded in Winter 2020 at Cape Elizabeth, Seboeis, and Presque Isle than in Winter 2019: ambient temperature at Cape Elizabeth was 0.88°C higher; ambient temperature at Seboeis was 0.78°C higher, and ambient temperature at Presque Isle was 4.12°C higher. Both average snow depth ($F = 97.86; \text{df} = 3,4; p < 0.001$) and maximum snow depth ($F = 51.14; \text{df} = 3,4; p = 0.001$) were significantly higher at Seboeis and Presque Isle than at Cape Elizabeth and Orono within each year. Within sites, neither average
snow depth nor maximum snow depth differed significantly between Winter 2019 and Winter 2020. (Table 4)

**Table 4.** Variability in climate conditions (average and maximum snow depth and mean temperature) across sites and years

<table>
<thead>
<tr>
<th>Site</th>
<th>Winter 2019</th>
<th></th>
<th></th>
<th>Winter 2020</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Max depth</td>
<td>Mean</td>
<td>Average</td>
<td>Max depth</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td>depth (cm)</td>
<td>(cm)</td>
<td>temperature (°C)</td>
<td>depth (cm)</td>
<td>(cm)</td>
<td>temperature (°C)</td>
</tr>
<tr>
<td>Cape Elizabeth</td>
<td>3.50</td>
<td>9.76</td>
<td>-0.83</td>
<td>2.30</td>
<td>11.20</td>
<td>0.063</td>
</tr>
<tr>
<td>Orono</td>
<td>5.93</td>
<td>15.20</td>
<td>-2.91</td>
<td>3.61</td>
<td>10.30</td>
<td>-2.73</td>
</tr>
<tr>
<td>Seboeis</td>
<td>35.41</td>
<td>79.10</td>
<td>-4.29</td>
<td>38.13</td>
<td>59.00</td>
<td>-3.52</td>
</tr>
<tr>
<td>Presque Isle</td>
<td>36.83</td>
<td>81.50</td>
<td>-6.43</td>
<td>44.05</td>
<td>81.28</td>
<td>-2.26</td>
</tr>
</tbody>
</table>

**Analysis of location as a predictor of ambient temperature conditions.** A repeated measures ANOVA indicated that mean ambient temperature and mean minimum ambient temperature varied significantly between Cape Elizabeth, Seboeis, and Presque Isle for Winter 2019, and both mean ambient temperature and mean minimum ambient temperature varied significantly between Cape Elizabeth, Orono, Seboeis, and Presque Isle for Winter 2020 (Table 5,6). A Tukey HSD test for Winter 2019 indicated that mean ambient temperature and mean minimum ambient temperature were significantly higher at Cape Elizabeth but similar at Seboeis and Presque Isle, while both mean and mean minimum temperature were highest at Cape Elizabeth, similar at Orono and Seboeis, and lowest at Presque Isle for Winter 2020. Mean ambient temperature and mean minimum ambient temperature varied significantly at Cape Elizabeth, Seboeis, and Presque Isle between Winter 2019 and Winter 2020 (Figure 6). Ambient temperatures recorded at Cape Elizabeth, Seboeis, and Presque Isle were higher in Winter 2020 than Winter 2019: ambient temperature at Cape Elizabeth was 0.88°C higher; ambient temperature at Seboeis was 0.78°C higher, and ambient temperature at Presque Isle was 4.12°C higher.
Table 5. Results of one-way ANOVA to test location as a predictor of ambient mean temperature. Significance letters assigned to each location indicate significantly different mean temperatures.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Df</th>
<th>F value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Winter 2019</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week</td>
<td>1,2</td>
<td>7.696</td>
<td>0.008</td>
</tr>
<tr>
<td>Location: (CEA, SEB, PI)</td>
<td>2,3</td>
<td>13.157</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Winter 2020</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week</td>
<td>1,2</td>
<td>15.24</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Location: (CEA, OR, SEB, PI)</td>
<td>3,4</td>
<td>14.49</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 6. Results of one-way ANOVA to test location as a predictor of ambient mean minimum temperature. Significance letters assigned to each location indicate significantly different mean minimum temperatures.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Df</th>
<th>F value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Winter 2019</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week</td>
<td>1,2</td>
<td>6.964</td>
<td>0.011</td>
</tr>
<tr>
<td>Location: (CEA, SEB, PI)</td>
<td>2,3</td>
<td>12.386</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Winter 2020</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week</td>
<td>1,2</td>
<td>8.162</td>
<td>0.006</td>
</tr>
<tr>
<td>Location: (CEA, OR, SEB, PI)</td>
<td>3,4</td>
<td>12.008</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Figure 6: The weekly mean ambient temperature (Winter 2019 and Winter 2020) and mean minimum ambient temperature (Winter 2019 and Winter 2020). Weekly means were calculated between 1 December and 13 April (Winter 2019) and 1 December and 4 April (Winter 2020).

Analysis of abiotic ambient conditions as predictors for control tick overwintering survival.

Using stepwise selection, a multiple linear regression model was used to explain the influence of ambient abiotic conditions on control tick survival for both Winter 2019 and Winter 2020. The predictors were mean ambient temperature, mean minimum ambient temperature, mean relative humidity, average snow depth, and maximum snow depth. Mean temperature and mean minimum temperature were retained by the model but not statistically significant in predicting control tick survival for Winter 2019, while mean temperature and mean minimum temperature were retained as significant predictors of control tick survival for Winter 2020 (Table 7). Neither average snow
depth nor maximum snow depth were retained by the model as significant predictors of overwintering survival.

Table 7. Results of one-way ANOVA for ambient abiotic conditions as retained or significant predictors of tick overwintering survival for Winter 2019 and Winter 2020.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Df</th>
<th>F value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Winter 2019</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean temperature</td>
<td>1,2</td>
<td>3.21</td>
<td>0.11</td>
</tr>
<tr>
<td>Mean minimum temperature</td>
<td>1,2</td>
<td>3.40</td>
<td>0.099</td>
</tr>
<tr>
<td><strong>Winter 2020</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean temperature</td>
<td>1,2</td>
<td>14.1835</td>
<td><strong>0.003</strong></td>
</tr>
<tr>
<td>Mean minimum temperature</td>
<td>1,2</td>
<td>4.8182</td>
<td><strong>0.049</strong></td>
</tr>
</tbody>
</table>

Analysis of microclimate treatment as a predictor for overwintering survival. Four microclimate conditions were tested at Cape Elizabeth and Presque Isle for Winter 2019: Snow removal, leaf removal, snow/leaf removal, and a control. Control ticks showed the greatest overwintering survival, followed by snow removal and leaf removal ticks at both Cape Elizabeth and Presque Isle. Snow/leaf removal ticks showed the lowest survival at both sites (Figure 7). A one-way ANOVA indicated that microclimate treatment had a significant effect on tick overwintering survival at both Cape Elizabeth ($F = 32.68, df = 3,4, p<0.001$) and Presque Isle ($F = 7.39, df =3,4, p= 0.0046$). A Tukey HSD test indicated that survival was highest in control ticks, similar in snow removal and leaf removal ticks, and lowest in snow/leaf removal ticks for both Cape Elizabeth and Presque Isle (Figure 7)
The same four microclimate conditions were tested at Cape Elizabeth, Orono, and Presque Isle for Winter 2020. At the Orono site, leaf removal ticks showed the greatest survival and snow/leaf removal ticks showed the lowest survival. A one-way ANOVA indicated that microclimate treatment had a significant effect on tick overwintering survival at the Orono site ($F = 4.33$, df = 3, $p = 0.028$). A Tukey HSD test indicated that, at the Orono site, survival was similar between control ticks and all other treatments, but that survival was higher in snow removal ticks than snow/leaf removal ticks (Figure 7). A one-way ANOVA indicated that, for Winter 2020, microclimate treatment did not have a significant effect on tick overwintering survival at Cape Elizabeth or Presque Isle.

A one-way ANOVA indicated that survival was significantly different between Winter 2019 and Winter 2020 at Cape Elizabeth ($F = 20.301; df = 1,2; p <0.001$) but not at Presque Isle.
Figure 7: Survival of ticks overwintering in different microclimate conditions

**Figure 7.** The mean survival of ticks overwintering at Cape Elizabeth (CE), Orono (OR), and Presque Isle (PI). The treatments are control (C), snow removal (SR), leaf removal (LR), and snow/leaf removal (SLR). Significance letters above each bar were obtained with the Tukey HSD test and indicate significant differences in tick survival between treatments within each location.

**Analysis of microclimate conditions as predictors of tick overwintering survival.** Using stepwise selection, a multiple linear regression model evaluating the influence of abiotic microclimate conditions (mean temperature, mean minimum temperature, mean relative humidity, average snow depth, and maximum snow depth) on tick survival at Cape Elizabeth and Presque Isle indicated that mean temperature and mean relative humidity were the significant predictors of overwintering survival in Winter 2019 and mean temperature was the only significant predictor of overwintering survival in Winter 2020. (Table 8).
Table 8. Results of one-way ANOVA to test abiotic microclimate conditions as predictors of tick overwintering survival for Winter 2019 and Winter 2020

<table>
<thead>
<tr>
<th>Variable</th>
<th>Df</th>
<th>F value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Winter 2019</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean temperature</td>
<td>1,2</td>
<td>20.87</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean relative humidity</td>
<td>6,7</td>
<td>10.53</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Winter 2020</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean temperature</td>
<td>12,13</td>
<td>6.044</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Analysis of treatment and location as predictors of microclimate conditions. A repeated measures ANOVA indicated that mean microclimate temperature varied significantly by week, location, and treatment for Winter 2019 (Table 9). A Tukey HSD test indicated that the mean temperature of control and leaf removal treatments was significantly higher than snow removal and snow/leaf removal treatments. For Winter 2020, treatment and location had a significant effect on mean temperature but week did not. A Tukey HSD test indicated that control temperature was higher than leaf removal and snow/leaf removal temperature and mean temperatures were highest at Cape Elizabeth and lowest at Presque Isle (Figure 8). Within sites (Cape Elizabeth and Presque Isle), mean temperature was significantly different between Winter 2019 and Winter 2020 ($F = 19.21; \text{df} = 1.2; p < 0.001$).
Table 9. Results of one-way ANOVA to test treatment and location as predictors of microclimate mean temperature. Significance letters assigned to each treatment and location indicate significantly different mean temperatures between treatments or locations.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Df</th>
<th>F value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Winter 2019</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week</td>
<td>1.2</td>
<td>5.871</td>
<td>0.016</td>
</tr>
<tr>
<td>Treatment: (CA, SRB, LRA, SLRB)</td>
<td>3,3</td>
<td>8.537</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Location: (CEA, PIB)</td>
<td>1,2</td>
<td>61.021</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Winter 2020</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week</td>
<td>1.2</td>
<td>0.076</td>
<td>0.784</td>
</tr>
<tr>
<td>Treatment: (CA, LRb, SRAB, SLRB)</td>
<td>3,4</td>
<td>5.410</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Location: (CEA, ORb, Plc)</td>
<td>2,3</td>
<td>192.511</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
**Figure 8**: The mean temperature of each treatment (Control, Snow Removal, Leaf Removal, Snow and Leaf Removal) and ambient temperature for Winter 2019 and Winter 2020 at Cape Elizabeth and Presque Isle.

A repeated measures ANOVA indicated that week and location had a significant effect on relative humidity throughout Winter 2019, but treatment did not (Table 10). For Winter 2020, treatment and location had a significant effect on relative humidity. A Tukey HSD test indicated that relative humidity was significantly higher at Presque Isle than Cape Elizabeth and Orono and that control enclosures had a significantly higher relative humidity than other treatments. Mean relative humidity was significantly different between Cape Elizabeth and Presque Isle for Winter 2019 and Winter 2020 ($F = 3.96; \text{df} = 1.2; p < 0.001$) (Figure 9).
**Table 10.** Results of one-way ANOVA to test treatment and location as predictors of mean microclimate relative humidity. Significance letters assigned to treatment and location indicate significantly different relative humidity between treatments and locations.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Df</th>
<th>F value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Winter 2019</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week</td>
<td>1,2</td>
<td>9.274</td>
<td>0.003</td>
</tr>
<tr>
<td>Treatment</td>
<td>1,2</td>
<td>0.469</td>
<td>0.494</td>
</tr>
<tr>
<td>Location: (CEA, PIB)</td>
<td>1,2</td>
<td>15.295</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Winter 2020</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week</td>
<td>1,2</td>
<td>3.346</td>
<td>0.069</td>
</tr>
<tr>
<td>Treatment: (CA, SRB, LRB, SLRB)</td>
<td>3,4</td>
<td>10.682</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Location: (CEA, ORA, PIB)</td>
<td>2,3</td>
<td>9.146</td>
<td>&lt;0.001</td>
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</table>
Figure 9: The mean relative humidity of each treatment (Control, Snow Removal, Leaf Removal, Snow and Leaf Removal) Winter 2019 and Winter 2020 at Cape Elizabeth and Presque Isle.

**Discussion**

We found that blacklegged ticks can successfully overwinter where blacklegged tick populations are not yet established in northern areas due to sufficient snow cover that provides insulation from ambient conditions. The removal of snow cover decreased overwintering survival more than the removal of leaf litter, suggesting that snowpack plays an important role in overwintering survival in northern locations. We found that ambient temperature had a significant effect on overwintering survival but ambient relative humidity did not. Despite detecting a higher prevalence of blacklegged ticks and tick-borne pathogens in southern Maine than northern Maine,
our field experiments suggest that overwintering survival is not the only mechanism constraining blacklegged ticks from expanding north of their current range.

As expected, we found a decreasing gradient of blacklegged tick density from southern to central Maine. We found no blacklegged ticks at our sites north of Orono (Seboeis or Presque Isle), while other tick species including *H. leporispalustris, I. cookei,* and *I. marxi* were found north of Orono. While we did not detect *I. cookei* and *I. marxi* at any sites 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).

Additionally, we found a higher prevalence of *B. burgdorferi* 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 prevalence for Lyme disease, anaplasmosis, and babesiosis; in 2019, a higher prevalence 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). Two other *Ixodes* species were collected in Presque Isle where no *I. scapularis* were found, and one *I. cookei* tested positive for *B. burgdorferi. Ixodes cookei* and *I. marxi,* while both vectors of Powassan encephalitis, are considered to be poor vectors of *B. burgdorferi* (Artsob 1984, Barker et al. 1993). We tested ticks for *B. burgdorferi* sensu lato, which includes non-pathogenic *B. burgdorferi* strains; thus, it is possible that the *B. burgdorferi* we detected at Presque Isle was non-pathogenic and not capable of causing Lyme disease in humans. Several studies have found that pathogenic *B. burgdorferi* is not efficiently transmitted between *I. cookei* and their primary hosts in locations where Lyme diseases cases were reported but *I. scapularis* were not well established (Barker et al. 1992, Ryder
et al. 1992). This suggests that *I. cookei*, although capable of being infected, would like not be a primary vector of *B. burgdorferi* in areas outside of the blacklegged tick’s range, such as northern Maine.

Location did not have a significant effect on overwintering survival during Winter 2019. Location had a significant effect on overwintering survival during Winter 2020 due to decreased survival at Orono and Presque Isle, but survival at Cape Elizabeth (southern Maine) and Seboeis (northern Maine) was not significantly different and did not differ between Winter 2019 and Winter 2020. As expected, mean ambient temperatures were higher in southern Maine and lower in northern Maine, and temperatures in northern Maine often decreased below -10°C. During both winters, control enclosure temperatures remained consistently above -10°C, suggesting that ticks overwintering at the northern sites did not experience the lowest ambient temperatures throughout the winter due to sufficient insulation and that reduced survival at Orono and Presque Isle during Winter 2020 may have been caused by flooding or the formation of ice inside the enclosures.

We found that degree and type of microclimate insulation had a significant effect on overwintering survival during Winter 2019, which was colder than Winter 2020. For both sites (Cape Elizabeth and Presque Isle) during Winter 2019, control ticks (i.e., ticks overwintering under both leaf litter and snow cover) had significantly higher survival than snow removal and snow/leaf removal ticks. The higher survival seen in control ticks during Winter 2019 was likely due to the insulation provided by snowpack and leaf litter, as indicated by the higher microclimate temperatures recorded in control enclosures compared to other treatment enclosures at both sites. Decreased survival in snow/leaf removal ticks during Winter 2019 was likely due to exposure to cold ambient temperatures, which was indicated by lower microclimate temperatures recorded in enclosures with no insulation during both Winter 2019 and Winter 2020. This suggests that
insulation provides a warmer, more humid microclimate, and that snow removal and a combination of both snow and leaf litter removal negatively impacted overwintering survival.

**Impacts of additional factors on blacklegged tick survival and distribution**

We found a higher abundance of blacklegged ticks and tick-borne pathogens in southern Maine than central Maine, and we discovered no blacklegged ticks at either of our northern sites despite determining that blacklegged ticks can successfully overwinter in northern Maine. The distribution of blacklegged ticks we found is consistent with previous passive-surveillance studies and the current known distribution of blacklegged ticks in Maine (Rand et al 2007, University of Maine Cooperative Extension 2019, Maine CDC 2020), and the lower pathogen prevalence we detected in Orono compared to Cape Elizabeth is consistent with the knowledge that nymphal tick abundance correlates with Lyme disease cases (Rand et al. 2007). We found that snow cover and leaf litter successfully insulated overwintering enclosures against low ambient temperatures in southern and northern Maine, which suggests that the interaction between broad-scale winter climate gradients and microclimate conditions are not limiting blacklegged tick survival in northern areas where questing blacklegged ticks were not detected.

Thus, the decreased blacklegged tick abundance in central and northern Maine may be due to additional abiotic and biotic factors other than winter climate, as well as the effect of winter climate on other blacklegged tick life stages (i.e., gravid female survival and fecundity). First, increases in blacklegged tick abundance have been linked to habitat change (i.e., deforestation, fragmentation, etc.) (Allan et al. 2002). Southern and coastal regions of Maine that have greater tick abundance are more heavily developed while northern Maine, where blacklegged tick abundance is low, remains largely forested (Plantinga et al. 1999, Ostfeld et al. 2006). Second, blacklegged tick expansion into northern Maine may be constrained by lower densities of white-
tailed deer (*Odocoileus virginianus*) compared to southern Maine (Elias et al. 2020). Adult blacklegged ticks feed primarily on white-tailed deer (Lane et al. 1991), and it has been demonstrated in some studies that the removal of deer from habitats reduces tick abundance (Daniels et al. 1993, Rand et al. 2004, Kilpatrick et al. 2014); however, other studies suggest that deer and tick abundances are only weakly related (Schulze et al. 1984, Amerasinghe et al. 1992, Rand et al. 2003). Third, while the risk of freezing or desiccation due to climate may constrain the survival of blacklegged ticks, migratory birds are thought to be largely responsible for the establishment of blacklegged ticks into new regions (Ogden et al. 2008), and in Maine migratory birds have been linked to the local establishment of ticks where tick populations are emergent but not established. (Rand et al. 1998). Thus, while winter temperatures less than -10°C do not appear to constrain the survival of blacklegged ticks in northern Maine, the introduction and establishment of new tick populations to an area relies more heavily on the activity of migratory birds. Tick expansion into northern Maine may further be constrained by the higher abundance of coniferous tree species in northern Maine compared to southern and coastal Maine, where there is a greater abundance of deciduous and mixed hardwood forests (Irland 1997). Fewer blacklegged ticks have been found associated with coniferous forests compared to deciduous forests (Adler et al. 1992, Ostfeld et al. 1995, Lindsay et al. 1999) which is likely due to the additional protection and insulation that deciduous leaf litter provides (Bunnell et al. 2003, Lubelczyk et al. 2004). Besides winter climate, additional climate factors may be limiting the spread of blacklegged ticks to northern Maine. Questing ticks are at risk of desiccation when high summer temperatures coincide with low precipitation and ticks leave the high humidity leaf litter to seek a host; thus, the risk of mortality due to climate may be higher during the summer than the winter (Berger et al. 2014). In contrast, overwintering ticks do not need to emerge from their insulated microclimate if the climate
conditions are not favorable. Finally, blacklegged tick distribution is limited by the accumulation of degree-days necessary for eclosion (egg-hatching) in May and ovipositon (egg laying) in July. A necessary threshold of degree-days for these events was not attained at sites in northern Maine, thus possibly preventing the establishment of blacklegged tick populations in northern Maine (Rand et al. 2004).

**Impacts of winter climate and microclimate on blacklegged tick overwintering survival**

There are many mechanistic factors that may influence the blacklegged tick distribution and range expansion and account for a higher abundance of blacklegged ticks and tick-borne pathogens in southern Maine compared to central and northern Maine. We tested one mechanism, which was the hypothesized effect of broad-scale winter climate and microclimate on overwintering survival. We found that the survival of blacklegged ticks overwintering under both snow and leaf litter was not significantly different in southern Maine and northern Maine during Winter 2019 (Cape Elizabeth and Presque Isle) or southern Maine and one northern Maine site during Winter 2020 (Cape Elizabeth and Seboeis) despite significantly lower ambient temperatures in northern Maine than southern Maine during both years. This is likely because leaf litter and snow cover provided sufficient insulation to prevent freezing or desiccation caused by ambient conditions at northern sites. Laboratory experiments suggest that blacklegged ticks are susceptible to death or desiccation at temperatures lower than -10°C and relative humidity lower than 85% (Stafford 1994, Vandyk et al. 1996). We found that all enclosures (both controls and treatments) at Cape Elizabeth and Presque Isle maintained temperatures above -10°C throughout both winters, even while ambient temperature dropped below -10°C. Snow/leaf removal enclosures that had no insulation still maintained higher temperatures than ambient conditions, which is likely due to their proximity to the ground and surrounding leaf litter (NOAA 2017). Relative humidity remained
close to 100% for many of the enclosures throughout the winter, but was significantly higher in control enclosures during Winter 2019. The occasional decreases to 0% humidity detected by the iButtons were likely due to the formation of ice or other debris around the iButtons inside the enclosures (DS1923 2019). Because ice was also found inside some vials upon the retrieval of ticks in April, it was evident that ice was able to form inside the enclosures as well as the vials; thus, the formation of ice and freezing conditions in the buckets (as indicated by the iButtons) were likely also experienced by the ticks inside the vials and likely contributed to tick mortality in enclosures that contained a significant amount of ice.

We found that snow removal and snow/leaf removal treatments resulted in the most significantly reduced overwintering survival during Winter 2019 at both Cape Elizabeth and Presque Isle. Consistent snow cover insulates soil against surface air temperatures and protects many soil-dwelling arthropods from freezing (Decker et al. 2003, Templer et al. 2012). Snow removal enclosure temperatures were significantly lower than control enclosure temperatures in Winter 2019, and snow/leaf removal enclosure temperatures were consistently the lowest, suggesting that snowpack insulates the soil and thus overwintering ticks from ambient conditions. Previous studies investigating the effect of insulation on overwintering survival found that snow removal did not have a significant effect on overwintering survival success while leaf litter did (Burtis et al. 2015, Linske et al. 2019). However, these studies were conducted in New York (Burtis et al. 2015) and Connecticut and southern Maine (Linske et al. 2019), all of which have high densities of blacklegged ticks and typically experience less snowfall and more intermittent snow cover than central and northern Maine. Perhaps more representative of the winter climate and low tick densities in northern Maine, Lindsay et al. (1995) assessed nymphal overwintering survival at sites in southern and northern Ontario and found that nymphal survival was higher at
northern sites than 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 Winter 2019, which was colder than Winter 2020, and that survival was higher at Presque Isle during Winter 2019. 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 in this study resulted in significant tick mortality in snow removal and snow/leaf removal treatments when snow was removed during Winter 2019. It is possible that the warmer ambient temperatures during Winter 2020 limited the effect of snow removal on overwintering survival because the ticks were not exposed to temperatures lower than -10°C as often as during Winter 2019.

We also found that control tick survival significantly decreased between Winter 2019 and Winter 2020 at Presque Isle, which may have been caused by higher overall temperatures during Winter 2020. 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). While ambient temperatures recorded at Cape Elizabeth, Seboeis, and Presque Isle were higher during Winter 2020 than Winter 2019, control enclosure temperatures at Presque Isle during Winter 2020 were lower than Winter 2019 and there were a greater number of 0% humidity values recorded during Winter 2020 at Cape Elizabeth and Orono (i.e., possibly indicating ice formation inside the enclosures). We made further observations that, upon retrieval of the ticks in April 2020, enclosures were encased in a greater degree of ice at Presque Isle than retrieval during April 2019. Additionally, inoculative freezing, which occurs when ticks are exposed directly to ice crystals, can occur at a few degrees below zero in saturated conditions when relative humidity is high (Burks et al. 1996). Inoculative freezing poses a greater risk to mortality than other cold tolerance
behaviors (Burks et al. 1996), which may have contributed to the lower survival at Presque Isle during Winter 2020.

Some limitations to this study may have affected the overwintering survival and distribution results. First, we used unengorged, lab-reared nymphal ticks for the overwintering experiment. Unengorged nymphs are the most cold-hardy out of all other blacklegged tick life stages (Vandyk 1996); thus, it is possible that repeating this experiment with engorged adult ticks, which overwinter before emerging in the spring to oviposit, may result in overall lower overwintering survival rates or reduced fecundity. Additionally, it has been demonstrated that blacklegged ticks infected with A. phagocytophilum may be more tolerant to cold temperatures and display higher overwintering survival than the lab-reared ticks used in this study (Neelakanta et al. 2010). Second, 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 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). It has been documented that ticks that have direct access to the soil or leaf litter have a higher overwintering 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). Finally, while drag sampling occurred during the predicted activity period of both nymphal and larval ticks, there were limitations of the timing and frequency of dragging due to the number of researchers sampling each site; we sampled some sites only once per month (i.e., 1000m² were sampled once in June, once in July, and once in August), which is likely not sufficient to gain insights into variation in blacklegged tick phenology across Maine. The same 8000m² grid was sampled at each
site during each visit, so it is possible that small populations of blacklegged ticks in northern Maine were not detected by this study.

The results of this study suggest interesting open problems to address in future research on the impacts of winter on blacklegged ticks and blacklegged tick overwintering. First, we determined that snow cover provided sufficient insulation to allow for overwintering survival in areas north of the blacklegged tick’s current range. However, as a result of climate change, snowpack is predicted to decrease in the northeastern United States (decreasing soil temperatures) while ambient temperatures are expected to increase overall (Hayhoe et al. 2007). This begs the question of whether future reduced snow cover will lead to uninhabitable microclimate conditions and prevent the northern spread of blacklegged ticks or if warmer winter temperatures will allow ticks to survive a winter with reduced snowpack. Second, we determined the overall overwintering survival of ticks under various conditions, but did not study overwintering survival at a finer temporal scale to determine which conditions (i.e., extended periods of low temperatures, sudden cold spells, etc.) are more likely to cause mortality. Extended cold periods can lead to cold hardiness in some arthropods, such as hard-bodied ticks, while short exposure to near-freezing temperatures leads to rapid cold hardening (Lee et al. 1987, Bale 1989). When exposed to cold temperatures, blacklegged tick mortality is more often a result of chill injury, which is the accumulation of physiological damage due to cold, while injury and mortality from inoculative freezing occurs when ticks are exposed to ice (Burks et al. 1996, MacMillan and Sinclair 2011). Additional studies should attempt to assess which winter conditions predicted by climate change models lead to different cold tolerance responses and whether sudden cold spells or extended cold temperatures result in greater tick mortality. Third, we studied overwintering survival in forests dominated by deciduous or mixed forests, but not forests dominated by coniferous trees. Leaf litter
depth is shallower in coniferous forests compared to deciduous forests and does not offer the same amount of insulation against environmental conditions (DeGraaf and Rudis 1990). Because a significant portion of Maine and other northern areas are dominated by coniferous forests, research is needed on the interaction between leaf litter type and climate.

The results of the study also indicate the necessity for additional research on the mechanisms of blacklegged tick and tick-borne pathogen spread. Our active surveillance distribution results corroborate previous and current passively-determined distributions of blacklegged ticks across the state (Rand et al 2007, University of Maine Cooperative Extension 2019, Maine CDC 2020). We determined that winter climate is not preventing tick survival in central and northern Maine, but tick densities in southern Maine were significantly higher than central Maine. Future studies are needed to assess what other factors may be preventing the spread of blacklegged ticks, such as the effect of summer temperatures and humidity on tick survival, behavior, and questing to determine if summer climate has a greater effect on tick survival and range expansion than winter survival (Berger et al. 2014). Because questing ticks spend more time above the refuge of the leaf litter, it is possible that the threat of desiccation is greater during the summer than during the winter due to the increased metabolic cost of walking and questing in a low humidity environment (Perret et al. 2003, Randolph 2004). We additionally determined that tick-borne pathogen prevalence was lower in central Maine compared to southern Maine, and future research is needed to assess the effect of climate on pathogen transmission, as well as how B. burgdorferi is being transmitted in areas such as Presque Isle where blacklegged ticks are not yet established. It is likely that the duration and seasonality of winter climate impacts blacklegged tick phenology and disease transmission on a local and regional scale. A shorter winter may increase tick abundance as well as disease prevalence due to a longer season of activity for host-
seeking, feeding, and pathogen transmission (Wallace et al. 2019), while a longer winter likely results in less synchronous host-seeking among larval and nymphal ticks, which is necessary for pathogen transmission may result in the more persistent strain of *B. burgdorferi* that is present in the northeastern United States compared to the southeastern United States (Randolph et al. 1999, Gatewood et al. 2009). While blacklegged ticks continue to expand north of their range, it will be necessary to study the impacts of the duration and seasonality of winter climate on tick phenology and pathogen transmission in areas where blacklegged ticks are emergent.

Our results showed 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 overwintering survival. It has been shown that removing leaf litter can reduce the abundance of questing nymphs during active periods; thus, it is possible that removing leaf litter before ticks enter diapause in the late fall might reduce overwintering survival as well (Schulze et al. 1995). The findings of this study indicate that blacklegged ticks can successfully overwinter in areas north of their current range, and that snow cover plays a significant role in overwintering 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 management practices to mitigate tick-borne disease risk as well as future studies assessing the effect of climate change and the interaction between decreasing snowfall and increasing temperatures on the spread of blacklegged ticks. This study can help improve the understanding of the spread of blacklegged ticks and Lyme disease and the northern limits of their distribution and aid in understanding the relationship between climate change and the spread of vector-borne diseases.
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BIOGRAPHY OF THE AUTHOR

Michelle Volk was born in Illinois on January 16, 1995. She was raised in Glen Ellyn, Illinois and graduated from Glenbard South High School in 2013. She attended New York University and graduated in 2017 with a Bachelor’s degree in Biology and an interest in vector-borne diseases. She entered the Ecology and Environmental Sciences graduate program at The University of Maine in the fall of 2018 under the guidance of Dr. Allison Gardner. Upon receiving her Master’s degree, Michelle will be entering the Fisheries and Wildlife doctoral program at Michigan State University to pursue research on the ecology and genetics of blacklegged ticks in Michigan. Michelle is a candidate for the Master of Science degree in Ecology and Environmental Sciences from the University of Maine in August 2020.