Adult ticks (*Ixodes scapularis*) infected with *B. burgdorferi* have increased overwintering survival, with implications for tick population growth.

Short title: Infected ticks (*I. scapularis*) have increased overwintering survival

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The tick, *Ixodes scapularis*, vectors pathogens such as *Borrelia burgdorferi*, the bacterium that causes Lyme Disease. Over the last few decades *I. scapularis* has expanded its range, introducing a novel health threat into these areas. Warming temperatures appear to be a major cause of this northward expansion. However, other factors are also involved. We show that adult ticks infected with *B. burgdorferi* have greater overwintering survival than uninfected ticks. Adult ticks were placed in individual microcosms and allowed to overwinter in both forest and dune grass environments. In the spring we collected the ticks and tested both dead and living ticks for *B. burgdorferi* DNA. Infected ticks had greater overwintering survival compared with uninfected ticks every winter for three consecutive winters in both forest and dune grass environments. The increased winter survival of adult female ticks could have a large impact on tick population growth, as we demonstrate. Our results suggest that, in addition to climate change, *B. burgdorferi* infection itself may be promoting the northern range expansion of *I. scapularis*. Our study highlights how pathogens could work synergistically with climate change to promote host range expansion.

Introduction

Winter is a stressful time for arthropods. Low temperatures can cause tissue injury and death, limiting the northern ranges of many insects and their relatives, such as ticks (Williams et al., 2015). The medically-important tick, *Ixodes scapularis*, dies at temperatures below -10° to -12°C (Burks et al. 1996; Vandyk et al. 1996), and even relatively mild winter temperatures (e.g. -3°C) may be lethal when humidity is high (i.e. due to inoculative freezing, Burks et al., 1996). This cold intolerance may have historically excluded *I. scapularis* from northern regions of North America (Estrada-Pena, 2002). Nevertheless, factors other than temperature play a role in determining overwintering survival (e.g. Lindsay et al., 1995, Lindsay et al., 1998, Brunner et al., 2012, Burtis et al., 2016; Burtis and Pfleuger, 2017; Clow et al., 2017; Kilpatrick et al., 2017; Hammond-Collins et al., 2022; Volk et al., 2022). For example, *I. scapularis* can successfully overwinter in regions that are below their thermal limits based on winter air temperatures (e.g. Northwestern Ontario, Lindsay et al., 1995; Upstate New York, Burtis et al., 2019; Maine, Volk et al., 2022). Ticks can survive in these areas because they have adaptations, such as burrowing into leaf litter, that protect them from lethal temperatures (Brunner et al., 2012; Linske et al., 2019). However, overwintering survival is highly variable across years (e.g. Lindsay et al., 1998; Volk et al., 2022), and our understanding of the various factors that determine overwintering success remains incomplete. We examine the possibility that infection with the bacterium *Borrelia burgdorferi* (the causative agent of Lyme Disease, Burgdorfer et al., 1982) is also a factor in determining overwintering survival. We focus on the impact of infection on the overwintering survival of adult ticks, especially adult female ticks, because the overwintering survival of adult females has an impact on the size of the upcoming year's tick population. Despite their importance, few studies (e.g. Lindsay et al., 1995, 1998) examine overwintering in

adult females. We test whether infected adult ticks have greater overwintering survival than non-infected ticks, and what this effect may mean for its northward expansion.

Winter is a vulnerable time for adult *I. scapularis*. They have limited capacity for movement below 4ºC (Duffy and Campbell, 1994), making them unable to find a host or escape predators. Although the lack of questing (i.e. searching for a host) in winter means that they are without food for the season, it does allow them to remain in in their insulated spaces and avoid the relatively lower ambient air temperatures (Burtis et al., 2019). Leaf litter provides insulation, resulting in warmer temperatures and higher relative humidity beneath it compared to the ambient air (Linske et al., 2019). However, overwintering survival can be low, even when ticks are provided with leaf litter (e.g. Lindsay et al., 1998). Overwintering survival of unfed adult females varied from 27% to 78% in Long Point, Ontario, Canada, depending on the year (Lindsay et al., 1998). Infection may be an additional, previously overlooked factor that also increases tick overwintering survival.

Pathogens can affect host survivability at low temperatures in arthropods (Ferguson et al., 2016). In the related tick *Ixodes ricinus*, nymphs infected with *B. burgdorferi* were more likely to survive stressful environmental conditions (i.e. low relative humidity, Herrmann and Gern, 2010), including cold temperatures (Herrmann and Gern, 2013) than uninfected ticks. *B. burgdorferi* may increase *I. ricinis* survival under difficult conditions (Herrmann & Gern, 2015) because infection increases lipid production in the host (Herrmann et al., 2013). In *I. scapularis*, infection with the pathogen *Anaplasma phagocytophilum* induces the expression of the protein IAFGP (*Ixodes* anti-freeze glycoprotein, Neelakanta et al., 2010). This protein has both anti-microbial and cryoprotectant properties (Heisig et al., 2014). It enhances cold tolerance in the short-term under laboratory conditions (Neelakanta et al., 2010). However, it has not been

demonstrated that infection with *B. burgdorferi* (or any other pathogen) increases overwintering success in *I. scapularis* under natural winter conditions.

If infected ticks are more cold-tolerant, then this could increase their ability to survive the winter. This increased winter survival could contribute to the increased northward expansion of *I. scapularis* populations.

Results

Infected ticks were more likely to survive the winter than uninfected ticks.

Adult ticks were collected locally and placed in individual microcosms topped with maple/oak leaf litter (approximately 2 g). The microcosms were placed in the ground to the depth of the leaf litter (about 5 cm). Microcosms were placed in either: a) a coastal forest containing a mix of oak (*Quercus rubra*), maple (*Acer rubrum*) and spruce (*Picea rubens* and *Picea glauca*) or, b) within the dune grass next to a sand beach. Both areas harbour *I. scapularis* ticks in the spring, summer and fall. Microcosms were collected in the spring, and living and dead ticks were tested for the presence of *B. burgdorferi* DNA using qPCR.

Ticks infected with *B. burgdorferi* had higher overwintering success than uninfected ticks in each of the 3 years (Three-way Loglinear analysis for each year) (Fig. 1). In year 1 (N=70) the final model demonstrated significant interactions between infection and survival (X^2 (1)=9.8, p=0.002) as well as site and survival (X^2 (1)=11.1, p<0.001). In year 2 (N=288), the final model demonstrated significant interactions between infection and survival (X^2 (2)= 37.4, p<0.001) but not between site and survival (X^2 (1)=0.72, p=0.4). In year 3 (N=240) the final model demonstrated a significant interaction between infection and survival (X^2 (1)= 9.5. p=0.002), but marginal effects for site and survival (X^2 (1)=3.13, p=0.08).

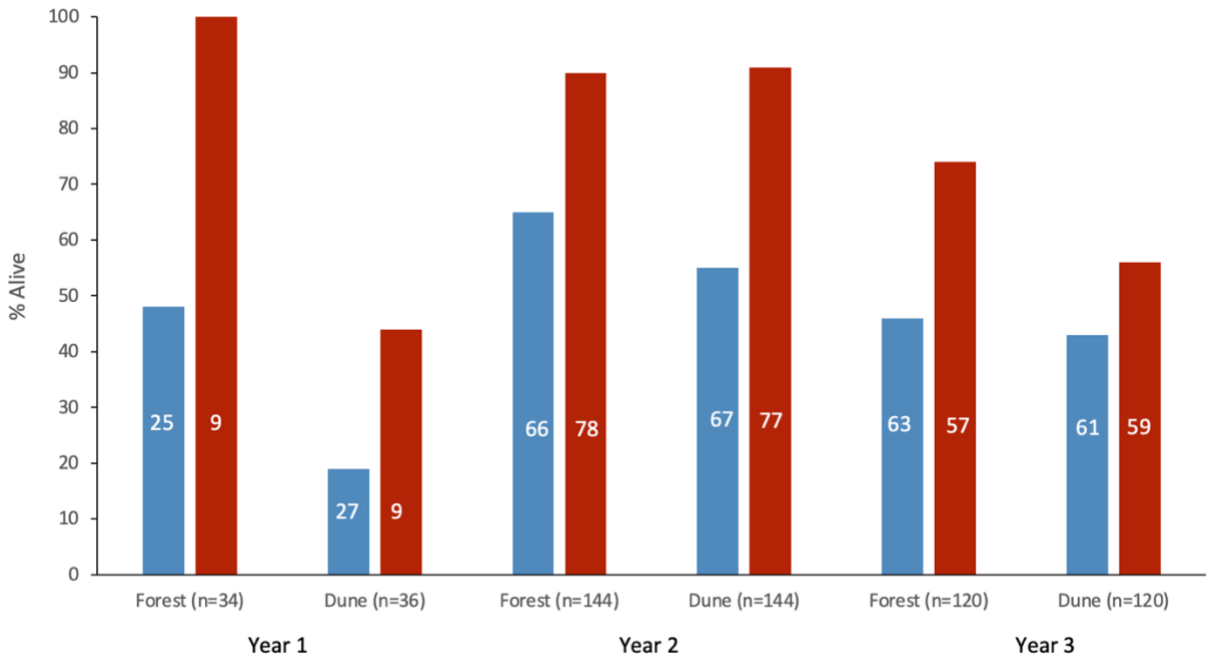

Figure 1. Percentage of *I. scapularis* infected with *B. burgdorferi* that survive the winter compared with uninfected ticks at each study site over the three winters. Numbers in white indicate the sample size. Blue bars represent uninfected ticks, while red bars denote infected ticks.

This positive relationship between infection and survival was true for both sexes (Fig 2. Factorial X^2 (1)=55.45, p<0001, n=598 ticks; males, X^2 (1)=29.9, p<0.0001, n=297; females, X^2 (1)=24.25, p<0.0001, n=301). Overall, the relative risk of death during overwintering for uninfected ticks (50.8% dead, n=289) vs infected ticks (21.5% dead, n=309) was 2.1 (95% Confidence interval 1.7 – 2.7). In other words, uninfected ticks were about twice as likely to die during the winter than infected ticks.

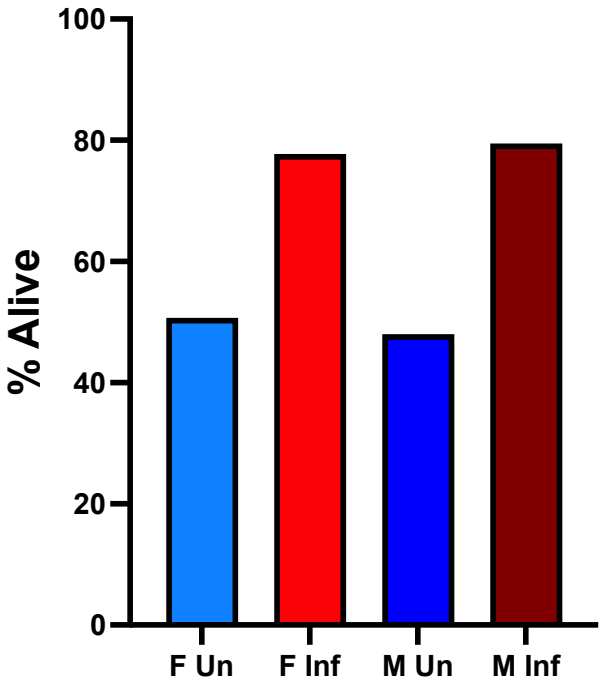

Figure 2. Infected females (F Inf, n=167, red bar) and males (M Inf, n=122, dark red bar) are more likely to survive the winter than uninfected females (F Un, n=134, light blue bar) and males (M Un, n=175, dark blue bar).

Tick overwintering success varied significantly across years (Year 1: 43% (n=30/70, Year 2: 76% (n=219/288), Year 3: 54% (n=130/240), post hoc X^2 (2)= 29.7, P<0.001). The infection rate also varied across years (Year 1: 26% (18/70), Year 2: (54% (155/288), Year 3: 48% (116/240), post hoc X^2 (2)=17.8, p<0.0001.

Temperature and Relative Humidity conditions

The forest sites had higher minimum temperatures than the dune sites (Table S3). Dune grass sites had a greater number of days with minimum temperatures below -5ºC, a temperature

at which ticks are unlikely to show movement in the field (Clark, 1995; Carroll and Kramer, 2003). 29.7% days were below -5ºC in the dune grass (106 days/357 days over 3 winters) compared with 14.5% of days in the forest sites (1 days/357 days over 3 winters)(X^2 (1)=24.7, p<0.0001). Year 1 had the lowest temperatures at the forest and dune grass sites (Table S3), and this year also had the lowest rate of winter survival.

Implications for tick population growth

Although *B. burgdorferi* does not appear to be vertically transmitted (Gray and Kahl, 2022), an increase in the survival of infected adult females could lead to an increase in the number of tick larvae in the next generation. Each additional infected female that survives the winter could supply up to 3,000 extra larvae (Mount et al., 1997) to the next generation. We estimated how infection could impact tick population growth (see Methods for assumptions). After five winters, an area in which 100% of the ticks are infected could have more than 6 times as many ticks as an area with no infected ticks (Fig. S1).

Discussion

Overwintering survival varied across the three years (Fig. 1), as has been observed in other field studies (e.g. Lindsay et al., 1998). Microclimate had little impact on survival, except in the first year in which a higher proportion of ticks survived in the forested site compared to the dune grass site. Other studies have also found that whether ticks have greater overwintering survival in a particular microclimate varies across the years (e.g. Maine, Volk et al., 2022). The effect is possibly most pronounced during the most severe winters. Despite the variability in overwintering survival across years and sites, infected ticks of both sexes were more likely to

survive the winter than uninfected ticks in each of the three winters at both sites. Therefore, this effect is robust enough to be detectable across both years and microclimates. The impact of this effect on tick population growth could be substantial (however see caveats below). The enhanced winter survival of infected ticks could be important during harsh winters, or for ticks in marginal areas. In some regions, it may make the difference between the local extinction of a population and its growth.

The impact of infection on tick population growth depends on whether there is a causal link between infection and overwintering survival. We did not infect our ticks, but collected them from the field. It is possible that the differential survival was caused, not by infection, but by some pre-existing difference(s) between infected and uninfected ticks. For example, it is possible that infected ticks must be in good condition to survive the infection, while in the uninfected ticks, there may be a mix of ticks in good and poor condition. This difference in initial condition between the two groups could explain the difference in host mortality. However, the evidence from the literature strongly suggests that *B. burgdorferi* causes the increase in winter survival. First, *B. burgdorferi* infection appears to have minimal fitness costs to ticks (Kurokawa et al., 2020), in fact it appears to decrease mortality under challenging environmental conditions (Herrmann and Gern, 2015; Benelli, 2020). Second, the concept that infection might enhance overwintering survival is plausible given that *B. burgdorferi* infection is known to cause physiological and behavioural changes in *I. scapularis* (Benelli, 2020). Perhaps, most convincingly, the bacterium *Anaplasma phagoctyophilum* has been shown to induce the expression of an anti-microbial peptide (IAFGP) that also has cryoprotectant properties (Heisig et al., 2014), resulting in better cold tolerance in infected *I. scapularis* (Neelankanta et al, 2010). The dual properties of IAFGP provide a causal mechanism for the positive effect of an infection

on overwintering survival. The ability of molecules to have dual defensive roles is common and is called cross-tolerance (Sinclair et al., 2013). In insects, the ability of immune responses to enhance cold tolerance is known (i.e. Krams et al., 2011).

A recent survey of ticks on the south shore of Nova Scotia found that about 4% of ticks were infected with *A. phagocytophilum* (Guillot et al., 2020). Co-infections of *A. phagocytophilum* and *B. burgdorferi* are typically rare, and occur at the rate expected if the pathogens were assorting randomly (Steiner et al., 2008). Therefore, it is unlikely that the enhanced overwintering survival we observed was due to *A. phagocytophilum* infection, not *B. burgdorferi*. Moreover, although our focus was on *B. burgdorferi*, we did run primers for *A. phagocytophilum*, and we did not find it in our samples (see supplementary information). Finally, ticks infected with this pathogen could have been in either the *B. burgdorferi* infected or uninfected group. Such a random assortment of *A. phagocytophilum* infected ticks would not produce the large increase in winter survival that we found in ticks infected with *B. burgdorferi*.

Initial tick populations are often *B. burgdorferi* free (Ogden et al., 2010). Tick population size needs to reach a certain threshold for *B. burgdorferi* to become endemic (Norman et al., 1999). Our study suggests that infection itself may increase the likelihood of this event by boosting tick population size, especially during winters with low overwintering survival. Additionally, it is unknown whether ticks vary genetically in their susceptibility to *B. burgdorferi*. However, if they do, then not only will the increased survival of infected females boost population growth, they will boost the population growth of individuals who are likely to be susceptible to infection. An increase in the proportion of susceptible hosts would benefit *B. burgdorferi* and also encourage the development of an endemic population of the bacterium.

The potential real world impact of *B. burgdorferi* infection on tick populations urges further study of this phenomenon. If increased overwintering survival of adult females leads to greater population growth, then tick population numbers should grow more rapidly once *B. burgdorferi* infects the population. There is some evidence for this pattern (e.g. Elias et al., 2022, Fig. 4a). But this pattern will only exist where other factors (such as the host population) are conducive to a rapid increase in tick numbers (see Ogden et al., 2005; Eisen et al., 2016; Wallace et al., 2019; Winter et al., 2020, Ogden et al., 2021 for a discussion of some of these factors). Therefore, the true impact on tick numbers will vary from region to region. It will also be important to establish whether the effect exists for all life stages. We examined the effect on unfed adult male and female ticks. However, engorged adult females also overwinter (Lindsay et al., 1998). If their survival is also enhanced, then the impact of *B. burgdorferi* on subsequent tick numbers will be increased. Similarly, whether infected nymphs also receive a boost in overwintering survival should be assessed. The increase in IAFGP production upon infection has been shown only in adult *I. scapularis* (Neelakanta et al,. 2010), and a survivorship benefit of infection under adverse conditions was found in adults, but not in nymphs, of the related tick *I. ricinis* (Herrmann and Gern, 2010). Therefore, it cannot be assumed that the same benefit exists in infected nymphs.

Regardless of exact nature of the link between *B. burgdorferi* and the increased overwintering survival of infected adult ticks, the results suggest that adult ticks found in the early spring are more likely to be infected than in the late fall. Unfortunately, the data to show whether this occurs in areas with endemic *Ixodes* populations are rare. Few studies examine the infection prevalence in late fall (e.g. November) and again in the same area in early spring (e.g. March/early April). If infected nymphs do not enjoy the same survival benefit, then newly

molted adults will not show a preponderance of infected individuals in the population. Whether adult ticks are more likely to be infected with *B. burgdorferi* by mid-spring appears to vary by year (Roome et al., 2018). Despite the lack of data, it would be prudent to take precautions against tick bites in the early spring when overwintering ticks become increasingly active.

B. burgdorferi and *I. scapularis* have co-existed for at least 20,000 years (Walter et al., 2017). Despite their long evolutionary history, whether *B. burgdorferi* actively manipulates its host to increase its overwintering survival (e.g. by increasing host lipid content or production of IAFGP) remains unclear. The ability of an antimicrobial protein to also function as a cryoprotective molecule (Neelakanta et al, 2010) makes the issue difficult to entangle. A variety of stressors can increase antimicrobial gene expression in arthropods (Adamo, 2022), including exposure to cold (e.g. *Drosophila*, Zhang et al., 2011). Therefore the fact that both cold and infection increase *IAFGP* transcription (Neelakanta et al., 2010) is not surprising. Therefore, even though increased cold tolerance benefits *B. burgdorferi*, it also benefits the host, and may not require active manipulation by *B. burgdorferi*.

Work on the related tick, *I. ricinis*, has demonstrated that ticks infected with *B. burgdorferi* are better able to withstand a variety of environmental challenges (Herrmann and Gern, 2015), such as desiccation (Herrmann and Gern, 2010). If the same is true in *I. scapularis*, infection may be a positive factor in the spread of these ticks into a range of marginal areas (e.g. those that are drier and hotter), as well as into colder areas. Once *B. burgdorferi* enters a population, infection could have a synergistic interaction with other environmental changes (e.g. the availability of the appropriate small mammal hosts), leading to boosted population numbers in previously marginal areas for *I. scapularis*.

Materials and Methods

Field Study

Field work was conducted at the Harrison Lewis Coastal Discovery Centre (HLC), Port L'Hebert, NS (HLC43°49'04.2" N 64°53'40.4" W), on the south shore of Nova Scotia. This area is experiencing increasing tick populations (Hatchette et al., 2015). The work was conducted over three winters (2018-2019, 2019-2020, and 2020-2021). Studies were approved by the University Committee on Laboratory Animals at Dalhousie University (protocol number: I18-06).

Ixodes scapularis were collected by dragging (Clow et al., 2017) from the HLC and Thomas Raddall Provincial Park, located adjacent to the HLC. Following collection, ticks were placed in individual microcosms using a design modified from Burtis (2017) as described in Adamo et al., 2022, and repeated here. The microcosm was an open plastic tube (15.24 cm in length x 1.27 cm in width), with mesh glued to each end, secured by a plastic cap. The plastic caps had a central hole that allowed rain and snow to enter in the top and connected the soil with the open bottom of the tube. The tubes contained sand and vermiculite (75% w/w sand and 25% w/w vermiculite) to a depth of 5 cm in the tube. The tubes were placed 5 cm into the ground. On top of the sand/vermiculite was 1 cm (approx. 2 g) of cut oak (*Quercus rubra* L.) and maple (*Acer rubrum* L.) leaves. A single tick was placed into each microcosm and then the microcosm was placed under a coarse netting (NuVue® (78.7cm L x 78.7 cm W x 83.8 cm H). A data logger (HOBO MX2301A) was placed in each netting enclosure with a probe 5 cm underground between the microcosms; the logger was covered with a solar radiation shield (Onset RS3-B) and the logger automatically recorded temperature and relative humidity every hour. In 2018, tick vials and data loggers were placed in in either dune grass (N=38) or in a mixed maple/oak/spruce

stand of trees (N=32). In years 2 and 3, we placed six microcosms under a single netting rather than placing all microcosms for one study site under the same netting enclosure. Additionally, an extra study location was created for each site, both of which were also located at the HLC; as a result, there were two forest sites (forest 1 and forest 2) and two dune grass sites (dune grass 1 and dune grass 2) in years 2 and 3. In year 2, there were 288 ticks placed into individual microcosms; the ticks were divided evenly between sites (n = 72 for each site). Therefore, there were 12 netting enclosures containing six microcosms placed at each site. Similarly, in year 3, there were 240 ticks placed into individual microcosms and were divided evenly between the four sites (n = 60 for each site), with 6 microcosms per netting enclosure.

In Year 1 only, ticks were added to the microcosms as they were collected (from October 25, 2018 to Nov. 15, 2018). In years 2 and 3, ticks were held at 4° C and then all ticks were placed in microcosms in the field on the same day (Year 2, Nov 7, 2019; Year 3 Nov. 28, 2020). Ticks were retrieved on February 8, 2019 in Year 1. In year 2, ticks were placed in the field on November 7, 2019, and retrieved on March 21, 2020, while in year 3 they were placed in the field on November 28, 2020, and were retrieved on March 23, 2021.

At the end of the field season, ticks were recovered by carefully sieving the tubes. Ticks that were immobile after collection were exposed to both a gentle exhalation by the experimenter (AEN) and probing with a paint brush. Ticks that showed no movement were tested again after 24 h at room temperature (approximately 22°C). If they still showed no movement they were determined to be dead. Both dead and living ticks were tested for the presence of *B. burgdorferi* DNA using qPCR, following the procedure modified from Courtney et al., 2004, as described in Adamo et al., 2022 (see Supplementary Information). We determined that we could accurately

measure infection in ticks that had been dead for 10 weeks (Table S2 and Supplementary Information).

Potential Effect of infection on tick population growth

Although the fitness cost (if any) of *B. burgdorferi* infection on adult females is unknown, in other arthropods, infection results in a reduction in fecundity (Schwenke et al., 2016). To estimate the possible effect of infection on tick population growth, we assumed that adult female ticks pay a 10% fecundity cost, based on data from *Drosophila* (McKean et al., 2008). We assumed that each female produces 1000 larvae if uninfected, (ticks can lay up to 3,000 eggs, but not all of those survive to produce larvae, Ogden et al., 2005), and 900 larvae if infected. *I. scapularis* has a complex life history, with multiple hosts, and a number of factors impact its population growth (e.g. see discussion in Ogden et al., 2005). For our estimate of the maximal effect of infection on population growth, we assumed that infection will only change overwintering survival and will not impact other aspects of tick survival (e.g. the likelihood of finding a host). Using our data, we assumed that infected females have an 80% chance of overwintering survival and uninfected females have a 50% chance of survival. We estimated tick numbers assuming that one population started with 10 infected females and 100% *B. burgdorferi* prevalence and compared it to a population that started with 10 uninfected females and 0% *B. burgdorferi* prevalence. We calculated the relative growth of the two populations (Fig. S1). We assumed all adult females died after laying eggs (Kilpatrick et al., 2017). We also assumed that ½ of larvae will be female, and we further assumed that all larvae will survive to overwinter as adult females.

Statistical Analysis

Because of methodological differences between years 1 and years 2 and 3, we analyzed the data using three 3-way Log-Linear analyses. Data from all 3 years fit the assumptions for a log-linear model. Post hoc tests were controlled for a false discovery rate using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995; Benjamini–Hochberg calculator (www.biostathandbook.com/benjaminihochberg.xls). Tests were performed using SPSS (ver. 27) and Prism (GraphPad, ver. 9.4.1).

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Supplementary Information

Adult ticks (*Ixodes scapularis*) infected with *Borrelia burgdorferi* have increased overwintering survival, with implications for tick population growth.

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Determination of tick infection status

The procedure used to determine tick infection status was as described in Adamo et al., 2022, and is repeated here. Ticks were identified using standard keys (Keirans and Litwack, 1989). DNA was extracted from *I. scapularis* following the Public Health Agency of Canada (PHAC) protocol, adopted from Courtney et al. (2004). We used a Qiagen QIAamp© DNA Mini Kit with an extra bead tube step (MN Bead Tubes Type D (Macherey-Nagel)) to improve the DNA extraction process. MN Bead Tubes Type D (Macherey-Nagel) were filled with 200 µl of Qiagen ATL buffer. Individual ticks were crushed thoroughly using a Bel-Art® Disposable Pestle. Bead tubes were vortexed for 30 minutes. 20 µl of Qiagen Proteinase K was then pipetted into each bead tube. Bead tubes were incubated at 56°C for 60-90 minutes. 200 µl of Qiagen AL buffer was added to each bead tube and tubes were vortexed for 15 seconds and then incubated at 70°C for 10 minutes. Finally, 200 µl of 100% ethanol was pipetted into each bead tube and then tubes were vortexed for 15 seconds. DNA extraction followed QIAamp© DNA mini kit instructions.

DNA was then screened for evidence of *B. burgdorferi* infection using a multiplex real-time PCR targeting the 23S rRNA of *B. burgdorferi*. *B. burgdorferi* infection was then confirmed in positive samples using primers targeting the ospA gene. Each sample was tested in triplicates and all plates included: a no template control (5 Microliters of milliQ water), a 25 Cq value positive control, and a 30 Cq value positive control. The thermocycling conditions occurred as follows: activation of enzyme AmpErase at 50°C for 2 minutes, denaturation of AmpErase and activation of AmpliTaq Gold® Polymerase at 95°C for 10 minutes, followed by 40 cycles of amplification at 95°C for 15 seconds and annealing at 60°C for 1 minute.

The integrity of DNA samples was assessed using gel electrophoresis using a Gel Doc™ XR+ Digital Gel Imaging System® from Bio-Rad® (Courtney et al., 2004). DNA quantity was determined using a Qubit fluorometer.

Table S1. Primer sequences used in Real-time PCR (Courtney et al., 2004).

	Primer or Probe	Sequence 5' to 3'
Borrelia burgdorferi	Bb 23Sf	cgagtcttaaaagggcgatttagt
	Bb 23Sr	gcttcagcctggccataaatag
	Bb 23S Probe FAM-TAMRA	agatgtggtagacccgaagccgagtg
	*ospA*f	ctggggaagtttcagttgaac
	*ospA*r	ttggtgccatttgagtcgta
	ospA Probe FAM-BBQ	ctgcagcttggaattcaggcactt
Anaplasma phagocytophilum	Ap *msp2*f	atggaaggtagtgttggttatggtatt
	Ap *msp2*r	ttggtcttgaagcgctcgta
	Ap *msp2* Probe VIC-TAMRA	tggtgccagggttgagcttgagattg

Detecting B. burgdorferi in Dead Ticks

Our experimental design depended on our ability to detect *B. burgdorferi* in ticks that may die at various times throughout the field experiment. As a result, we performed experiments

to determine whether *B. burgdorferi* would be detectable in a dead tick for up to 10 weeks, which matched the length of the field experiments. We cut field collected *I. scapularis* ticks in half longitudinally using a sterile razor. The first half was used immediately for DNA extraction. The second half of the tick was submerged in an aliquot of Vaseline to prevent dehydration, and was stored in a fluctuating thermal regime incubator (-5°C to 10°C) in 12-hour increments for 10 weeks. We chose this variability in temperatures to introduce freeze and thaw cycles, which assisted in determining the length of time the DNA is still measurable in *Ixodes scapularis* under Nova Scotian winter conditions. After the 10 week incubation period was completed, we extracted the DNA as previously described and tested for *B. burgdorferi* using qPCR.

Table S2 shows the high concordance of the results for the two tick halves. For adult females it was 100%.

Table S2. Concordance between the two halves of each tick tested 10 weeks apart.

Females	Infected 2nd 1/2	Uninfected 2nd 1/2
Infected 1st 1/2	10	0
Uninfected 1st 1/2	0	20

Males	Infected 2nd 1/2	Uninfected 2nd 1/2
Infected 1st 1/2	9	2
Uninfected 1st 1/2	0	19

Males were smaller than females, and cutting some of them in half may have reduced the amount of *B. burgdorferi* to below our detection limit.

We also compared the Cq values of the two halves of each infected tick. The Pearson correlation was $r^2=0.996$, $p<0.0001$, $n=20$ pairs, demonstrating the consistency in the results. These results

show that it is possible to reliably determine *B. burgdorferi* infection in the dead ticks we collected.

Results

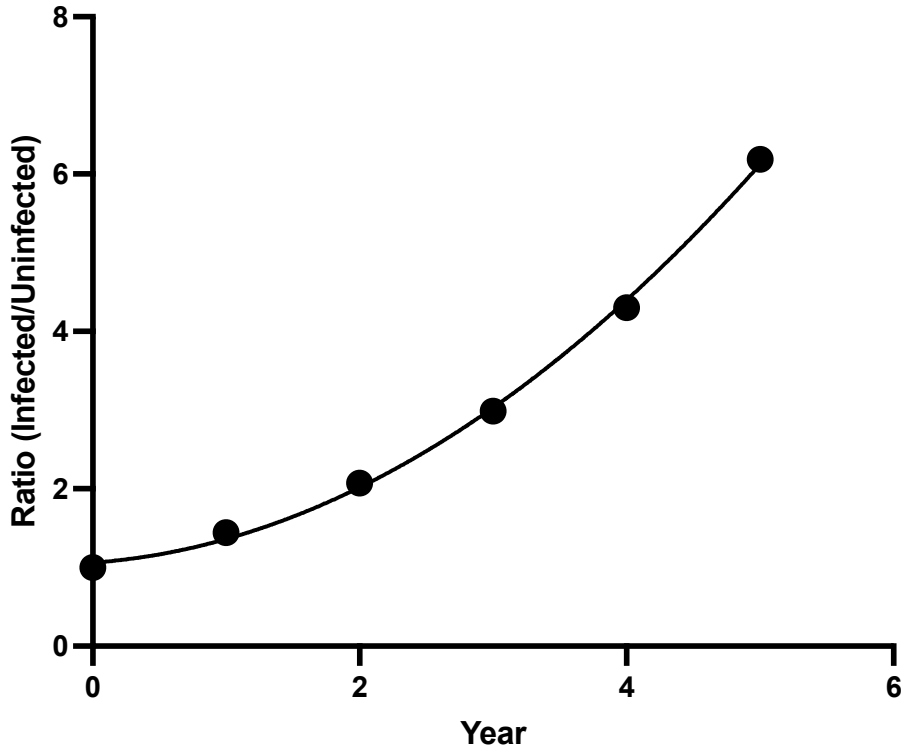

Figure S1. The ratio of the number of infected/uninfected ticks assuming that one population has a 100% infection rate, and the other population has a 0% infection rate. The boost in overwintering survival experienced by infected females could translate into a more rapid increase in tick numbers in infected populations compared to uninfected populations.

Weather Data

Table S3. Analyzed temperature at the forest and dune grass sites over the 2018-2019 (year 1), 2019-2020 (year 2), and 2020-2021 (year 3) winter seasons. Data from year 2 are the average of the two dataloggers at the two different locations at the forest and dune grass sites.

	Year 1, n=107 days		Year 2, n=135 days			
	Forest	Dune Grass	Forest 1	Forest 2	Dune Grass 1	Dune Grass 2
# of days temperature was <-5°C	39	47	3	0	31	18
# of days, temperature change >9°C	19	20	5	4	60	37
Average change in temperature daily, ±STD (°C)	6.91 ±3.79	6.80 2.90	4.16 2.35	4.05 2.27	8.08 3.99	7.26 3.50
Max daily change in temperature (°C)	29.4	16.1	10.8	11.6	18.85	16.3
Min daily change in temperature (°C)	1.46	1.40	0.25	0.90	0.07	0.77
Lowest night temperature (°C)	-14.10	-13.50	-6.09	-4.29	-12.80	-9.78
Average temperature in the night (°C)	-2.71	-3.86	-0.68	0.49	-2.96	-1.78
Highest daily temperature (°C)	15.3	15.8	14.04	13.70	16.17	16.13
Average temperature in the day (°C)	4.20	2.96	3.48	4.54	5.12	5.47

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