ECOLOGICAL FACTORS UNDERPINNING EXPANSION OF *BORRELIA*-INFECTED TICK POPULATIONS IN THE SOUTHEASTERN UNITED STATES

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ECOLOGICAL FACTORS UNDERPINNING EXPANSION OF *BORRELIA*-INFECTED TICK POPULATIONS IN THE SOUTHEASTERN UNITED STATES

A Thesis Presented for the

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Degree

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Janetta Renea Kelly

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Abstract

In the eastern United States, Lyme disease is caused by the bacterium *Borrelia burgdorferi* sensu stricto (*Bb*) and vectored by the blacklegged tick *Ixodes scapularis*. This tick is found in all eastern states, yet Lyme disease has been considered endemic only in the North. *Bb* infection was found only rarely in *I. scapularis* in southern states in past decades, but infected tick populations have recently expanded into southwestern Virginia. There is concern that further southwards spread of infected tick populations into Tennessee could occur either from Virginia, to the east of the Appalachians, or from Kentucky, to the west. There are, however, two hypotheses as to why there might be an ecological barrier to such spread: i) *I. scapularis* densities in eastern Tennessee may be too low to support *Bb* transmission cycles; or ii) immature ticks in eastern Tennessee may feed primarily on non-reservoir competent lizards. This study set out to assess whether or not *Bb*-infected tick populations have become established in eastern Tennessee and to investigate these hypotheses. Winter surveys for adult *I. scapularis* at 130 sites in Tennessee, Kentucky, and Virginia in 2017-2018 and 2018-2019 determined that most eastern Tennessee counties have established blacklegged tick populations. *Bb*-infected ticks were found in four of these counties. Comparisons of *Borrelia* strain types between states suggested that southwards movement of infected ticks into eastern Tennessee has been primarily from Virginia rather than Kentucky. Live-trapping at selected sites indicated that while some immature *I. scapularis* do feed on reservoir incompetent lizards, mice are abundant and are also serve as hosts. Stable Isotope Analysis supported predictions that *Bb*-positive ticks were more likely to have fed on herbivores (e.g., rodents) than on insectivores (e.g., lizards) and that nymphal *I. scapularis* were more likely to have fed on rodents than lizards in eastern Tennessee. I conclude, therefore, that neither low tick abundance nor immature tick host selection represent a significant barrier to...
*Bb* emergence in eastern Tennessee. Health practitioners and the public in this area should be alert to the potential for increasing Lyme disease risk in coming years.
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Chapter 1: Introduction, background, and objectives

Lyme disease is the most commonly reported vector-borne illness in the United States and poses an increasing public health risk to humans. According to the Center for Disease Control, there were approximately 36,500 officially reported cases in 2016, however the true number of cases may be ten times higher (Mead et al. 2015). Most cases are reported by early symptoms that include fever, chills, headache, muscle and joint aches, and most commonly an erythema migrans rash. However, if left untreated, Lyme disease can cause serious health issues such as arthritis, facial palsy, heart palpitations, inflammation of brain and spinal cord, and problems with short-term memory (CDC 2016). For a human to acquire Lyme disease, an individual must be bitten by an *Ixodes* spp. tick infected with *Borrelia burgdorferi* senso stricto (*Bb*), the bacterium that is causative agent for Lyme disease. The key North American vector ticks are *Ixodes pacificus* in the western US, and *I. scapularis* (the blacklegged tick) in the eastern US (Steere et al. 2004). There is no transovarial transmission of *Bb*, so the larval or nymphal stage of the tick must feed on an infected reservoir host, usually a wild animal, to become infected. Infected ticks then are able to pass the bacteria to other uninfected hosts (Barbour et al. 1996). This tick-host cycle maintains infection in areas considered ‘endemic’ for Lyme disease.

While most human cases of Lyme disease in the United States are reported from the Northeast and upper Midwest (Figure 1.1), the known range of blacklegged ticks extends throughout most of the eastern United States (Eisen et al. 2016). Therefore, there is opportunity for Lyme disease risk to grow in areas of the eastern U.S. that are not presently considered 'Lyme endemic'.
**Figure 1.1:** Confirmed cases of Lyme disease in the United States in 2015 (CDC 2017). One dot has been placed randomly within county of residence for each case.
Indeed, there is growing evidence that Lyme endemic areas are expanding. For example, Lyme disease is spreading northwards into eastern Canada (Ogden, et al. 2009), and in the U.S. The Midwestern and northeastern Lyme endemic areas appear to be merging through eastward spread of infected ticks into Michigan (Lantos et al. 2015) and westward spread of infected ticks into Pennsylvania and Ohio (Wang et al. 2014). There has also been rapid southward expansion of Lyme cases in inland Virginia over the past decade (Lantos et al. 2015), and a recent report of widespread Bb-infected blacklegged ticks in Kentucky (Figure 1.2). It is unclear what has facilitated this spread and whether or not spread will continue further south.

1.1 Infection status of southern I. scapularis populations

*Ixodes scapularis* is widespread in the Southeast as well as in the North (Eisen et al. 2016). In southeastern states, this tick is abundant in some coastal areas (e.g., Maestas et al. 2013), but inland it is often at much lower abundance, with patchy distributions. In northern Lyme-endemic areas, Bb prevalence is typically 40% or higher in adult *I. scapularis*, whereas zero or near-zero prevalence have been reported from studies in states such as Texas (Williamson et al. 2010), Florida (J. Tsao, unpublished data) and Tennessee (Rosen et al. 2012). There are competing hypotheses for why this is so (see section 1.3).

Given recent findings of epidemiologically-important differences in host-seeking behavior of northern and southern *I. scapularis* (Arnoe et al. 2016), there is growing concern that Lyme disease risk may increase if northern populations of *I. scapularis* expand into southern states (Kelly et al. 2014). Brinkerhoff et al. (2014) reported rapidly increasing Lyme disease incidence in counties to the east of Virginia’s Appalachian Mountains between 2001 and 2009. Soon thereafter, Herrin et al. (2014) reported a high prevalence of Bb infection in adult *I. scapularis* collected from southwestern Virginia. More recently, Lockwood et al. (2018) reported
Figure 1.2: Orange shading indicates counties where *I. scapularis* had been detected in Kentucky, prior to the research reported here. Black dots indicate counties where *B. burgdorferi* was detected in *I. scapularis* (from Lockwood et al. 2018).
an overall 11% prevalence of infection in *I. scapularis* collected from white-tailed deer in Kentucky (Figure 1.2). Virginia and Kentucky both border Tennessee, so these recent findings have raised the question of whether Tennessee will be affected by continued southward spread of infected ticks or, alternatively, whether there are biological or environmental factors that might prevent such spread.

1.2 *Ixodes scapularis* and *Borrelia burgdorferi* genetic variation

Population genetic studies of *I. scapularis* suggest that there are two distinct genetic lineages of this species in the eastern US. The southern clade includes ticks collected from the Southeast and Mid-Atlantic while the northern clade extends throughout the Northeast and upper Midwest (Rich et al. 1995). The northern clade has also been detected in southeastern states, however, suggesting that the southward spread of Lyme is associated with expansion of northern-clade ticks (Trout et al. 2009, Qiu et al. 2002). Although the two clades readily interbreed and are considered one species, differences in host-seeking behavior and other life-history traits are apparent. For example, host-seeking differences are apparent in both host-selection and the questing behavior. Southern *I. scapularis* tend to feed on incompetent hosts such as lizards (Oliver, et al. 1993) and in southern states, nymphaal ticks are difficult to collect through drag-surveys and are less likely to encounter humans because they typically host-seek low to the ground or within the leaf litter (Goddard et al. 2006, Brinkerhoff et al. 2014). These behavioral differences help explain why Lyme disease risk is lower in the southeastern US than in the northeast (Arsnoe et al. 2019).

There are numerous strains of *Borrelia burgdorferi* senso stricto (*Bb*) and several different typing systems to describe these strains. Strain identification is predominately based on DNA sequences of two genetic loci: the plasmid-borne, highly polymorphic outer surface protein
gene that encodes outer surface protein C, and the intergenic spacers (IGS) between the rrs and rrlA rDNA called IGS1 (Travinsky 2010). There are also multilocus sequence types (MSTs) that allow for the characterization of diverse bacterial populations by analyzing the genetic variation of multiple loci encoding proteins essential for cell maintenance (Margos 2012). Furthermore, there are rRNA intergenic spacer types (RSTs) that can help researchers and medical specialist understand the severity of Lyme disease in humans (Jones 2006). These typing methods can help us understand Lyme disease history, dispersal, and severity. For example, if Bb-infected ticks are now present in Tennessee, determining which Bb genetic types are present may provide clues as to their invasion route – are infected ticks arriving from the east of the Appalachians via Virginia or from the west via Kentucky?

1.3 Potential abiotic barriers and host barriers to southward spread of Lyme disease risk

Given rapid southward spread of Lyme disease in Virginia over the past decade (Lantos et al. 2015) the question facing residents of Tennessee and other southern states is whether this southward spread will begin to increase the risk of Lyme disease in areas where Bb-infected tick populations have not previously been found. The blacklegged tick itself is a generalist, ranging from Mexico to Canada, yet many counties in the Southeast have been considered essentially free of Bb-infected tick populations. Hypotheses as to why this has been so include:

**Hypothesis 1:** Although I. scapularis is present throughout the Southeast, tick population densities are too low to support endemic Bb transmission. Abiotic factors such as temperature and humidity may make Tennessee and adjacent states, too hot or too desiccating to sustain a high-density blacklegged tick population. Soil type, leaf litter or other habitat characteristics might also be unfavorable.
Hypothesis 2: In the South, immature *I. scapularis* feed primarily on lizards (which are poorly reservoir competent for *Bb*). This incompetency breaks the transmission cycle of *Bb*.

1.4 Research aim and objectives

The broad aim of this thesis was to collect up-to-date information on the status of the blacklegged tick populations in eastern Tennessee, and to compare those findings to data from adjacent, more northern tick populations in southeastern Kentucky and southwestern Virginia. The specific research objectives were:

1. To compare blacklegged tick abundance, *Bb* prevalence, and *Bb* genetics, in eastern Tennessee versus southwestern Virginia and southeastern Kentucky;
2. To investigate the hypothesis that abiotic or habitat factors create a barrier to spread of infected blacklegged ticks into Tennessee;
3. To investigate the hypothesis host-selection factors create a barrier to spread of infected blacklegged ticks into Tennessee.

Chapter 2 of my thesis addresses the first objective by describing the results of a multi-state, multi-county survey for adult ticks undertaken in fall/winter 2017/18 and 2018/19 in eastern Tennessee, southeastern Kentucky, and southwestern Virginia. The ticks collected in these surveys were subjected to pathogen testing and genetic sequencing to help address this objective.

Chapter 3 addresses the second objective by relating tick and *Bb* data to habitat and environmental measurements collected at replicate field sites in each of the three states.

Chapter 4 addresses the third objective by relating tick and *Bb* data to host abundance data collected via live-trapping and trail camera surveillance at the same field sites investigated in Chapter 3.
Chapter 5 uses Stable Isotope Analysis methods to test the prediction that \textit{Bb}-infected ticks are likely to have fed previously on rodents, and the prediction that lizard-feeding is more common in Tennessee than in more northern Kentucky and Virginia.

Chapter 6 summarizes the main conclusions of the research and makes recommendations for further investigation.
Chapter 2: Blacklegged tick abundance, and Lyme pathogen prevalence and genotypes, in eastern Tennessee and adjacent states

2.1 Introduction

*Ixodes scapularis* - the blacklegged tick - is the key vector of Lyme disease. This tick is found throughout the eastern United States (Eisen et al. 2016), however human cases of Lyme disease have historically been concentrated in the Northeast and upper Midwest (CDC 2017). In recent years, spread of *I. scapularis* populations infected with *Borrelia burgdorferi* senso stricto (*Bb*, the etiological agent of Lyme disease) has been occurring in multiple directions, including northwards into Canada (Ogden et al. 2009), eastward into Michigan (Lantos et al. 2015), and westward into Pennsylvania and Ohio (Wang et al. 2014). There is also strong evidence for recent spread into several southeastern states, including Virginia (Herrin et al. 2014), Kentucky (Lockwood et al. 2018), and Tennessee (Hickling et al. 2018). However, it is uncertain how many counties in this region have established populations of infected ticks.

In northern Lyme-endemic states, nymphal (second life stage) *I. scapularis* pose the greatest risk to humans in the Lyme disease cycle (Falco et al. 1999). This is because nymphs have already fed once, allowing for a possibility of *Bb* being acquired from the previous host and transmitted to the next host. Nymphal ticks are very small and thus less noticeable than adult ticks, resulting in a high chance of undetected attachment to a human and thus increased chance of *Bb* transmission.

In northern states, nymphs are easily collected in summer using drag-cloth surveys; drag-counts are commonly used as method for assessing Lyme disease risk (e.g., Diuk-Wasser et al. 2012). In the southern United States, however, nymphal *I. scapularis* are much more difficult to collect by this method (Goddard & Piesman 2006). Arsnoe et al. (2016) compared northern- and
southern-origin nymphs in outdoor mesocosms and found that southern nymphs are far less likely to feed above the leaf litter. This lack of questing above the leaf litter lowers their chance of attachment to large hosts such as humans and is consequently a likely cause of low Lyme disease incidence in the South (Arsnoe et al. 2019).

Because of their tendency to remain below the surface of the leaf litter, few nymphaI. scapularis are collected by drag-cloth surveys in southern states. In contrast, adult I. scapularis in the South are often collected on drag cloths or from hunter-killed deer, provided such surveys are undertaken during the adult ticks' cool-season activity period (October-March; see for example Rosen et al. 2012 and Lockwood et al. 2018). It should also be noted that unfed ("flat") adult ticks collected from vegetation have fed twice, increasing their chances of coming in contact with Bb and thus improving their usefulness as a target for detecting Bb in new areas. Engorged adults collected from deer and other wildlife have fed for a third time, however their pathogen status can be difficult to interpret, because some pathogens may be in the blood meal itself, rather than truly infecting the tick. Therefore, to estimate the distribution, relative abundance, and pathogen prevalence of blacklegged ticks in the South, it is particularly useful to conduct targeted drag surveys to collect flat adult ticks from vegetation during the winter.

Genetic typing of Bb collected from infected I. scapularis can provide information on the geographic origin of the bacteria and how infection may affect human health. Molecular typing of B. burgdorferi based on restriction fragment length polymorphisms in the 16S-23S ribosomal DNA (rDNA) spacer region has yielded three broad ribosomal spacer type (RST) groups: RST 1, 2, and 3 (Liveris et al. 1995). RST 1 spirochetes are more prevalent, relative to RST 2 and 3 spirochetes, in ticks from the Northeast versus in the Midwest (Gatewood et al. 2009). RST 1 is more common and more invasive than the other RST groups, so the proportion of Lyme disease
cases involving infection RST 1 bacteria is high. Based on analysis of clinical isolates, disseminated infections (such as spirochetemia and secondary erythema migrans) are more strongly associated with RST 1 than with RST 2 or 3 (Wormser et al. 1999). Infection with RST 1 also produces more severe disease (e.g., more severe arthritis and carditis symptoms) in experimentally infected animals when compared to RST 3 (Wang et al. 2002).

2.2 Objectives

The field survey described in this chapter aimed to assess whether blacklegged tick populations infected with \( Bb \) are now established in eastern Tennessee, southeastern Kentucky and/or southwestern Virginia, and if so to estimate \( Bb \) prevalence and RST type of the infected ticks.

2.3 Methods

2.3.1 Field sampling

In fall/winter of 2017/18 and 2018/19, we assessed tick abundance at multiple field sites in eastern Tennessee, southeastern Kentucky, and southwestern Virginia, using a standard drag-cloth survey method (Rullison et al. 2013). Sites were selected from satellite imagery using Google Earth (https://www.google.com/earth/). Selected sites consisted of forested public land, less than 800 m in elevation, with accessible trails or paths (including nature trails, bike trails, and powerline cuts). Dragging was conducted during the peak months (October-March) for adult \( I. \text{scapularis} \) activity. A 1-m\(^2\) corduroy cloth was dragged over the vegetation to collect host seeking ticks for 30-60 min per person at each site. The cloth was checked every 10 paces, with all adult ticks identified in the field using morphological characteristics and then placed in a 1.5ml microcentrifuge vial with 70% ethanol (EtOH). A different vial was used for each site;
each vial was labeled with a 4-letter site abbreviation, date, and a unique 6-digit vial number on a small paper tag was placed inside the vial. Start time, stop time, relative humidity (%RH) and temperature (°C) were measured at each site using a Kestrel™ 3000 Pocket Weather Meter. Upon returning to the lab, each site's geo-coordinates, elevation (m), and the distance dragged (m), were obtained from Google Earth.

All adult *I. scapularis* were sent to the Hamer lab at Texas A&M University to be screened for *Borrelia* spp. by DNA extraction and quantitative multiplex real time PCR using differential probes to target the 16S rDNA of Lyme group *Borrelia* and of relapsing fever group *Borrelia* (detailed methods are described in Tsao et al. 2004). Samples positive in the 16S assay (to a maximum of 6 per site), and a random subset of negative samples, were then subjected to PCR amplification of the 16S-23S rDNA intergenic spacer region followed by Sanger DNA sequencing to obtain species-level identification (methods in Bunakis et al. 2004).

2.3.2 Abundance and prevalence analysis

Raw counts of *I. scapularis* on the drag-cloths were converted to ticks collected per person-hour, as an index of tick activity. Mean ticks/hour were calculated for each state and compared statistically using 1-way ANOVA. Percent prevalence of *Bb* infection was calculated by pooling the results from all sites in each state; prevalence differences between states were assessed using Chi² tests of association using the *chitest* function in Excel 2013 (Microsoft®; Redmond, Washington). Blacklegged tick distribution at the county level was mapped by categorizing counties having as ‘detected’ or ‘established’ *I. scapularis* populations. Detection was defined as less than 6 of one life stage and less than two life stages of *I. scapularis* collected in a county. Established was defined as more than 6 of one life stage or more than two life stages collected in a county. These criteria were used by Eisen et al. (2016) to produce a national map
of *I. scapularis* distribution; we generated an updated version of the Eisen map for the counties we surveyed.

2.3.3 *RST* strain type analysis

Samples testing positive for *B. burgdorferi* senso stricto by 16s-23S rDNA intergenic spacer region and Sanger sequencing (described above) were assigned an RST type by using MEGA (Molecular Evolutionary Genetic Analysis, Penn State University) to align sample sequences with known RST sequences from GenBank®; we categorized each of our samples as RST 1, 2 or 3 based on which known RST sequence the sample clustered most closely with.

The locations of *Bb*-positive ticks, color-coded according to their RST type, were mapped using ArcMap 10.5 (ESRI, Redlands, CA).

2.4 Results

A total of 130 field sites were surveyed in the three states, with adult *I. scapularis* detected at 85 of those sites (Figure 2.1).

2.4.1 State-by-state results

**Kentucky**

Twenty-six dragging events occurred on 20 Kentucky sites from October 2017-January 2018, with 93 adult *I. scapularis* were collected. No other tick species were collected. Ticks per hour ranged from 0-30, with an average of 5 adult ticks collected per hour (Figure 2.2).

Eleven dragging events occurred on 7 Kentucky sites from November 2018-December 2018. A total of 52 adult *I. scapularis* were collected. No other tick species were collected during
Figure 2.1: Drag-cloth survey locations on a topographic map of the study area. Yellow dots indicate sites where no *I. scapularis* were collected, white dots indicate where uninfected *I. scapularis* were present, and red dots indicate sites where at least 1 tick positive for *Bb* was detected.
Figure 2.2: Mean number of adult *Ixodes scapularis*/hour at field sites in southeastern Kentucky, eastern Tennessee and southwestern Virginia, based on drag-cloth surveys undertaken in winter 2017/18. Means presented ± SE, number of sites surveyed in parentheses.
the drag-cloth surveys during either year. Ticks per hour ranged from 0-28 with an average 7 adult ticks collected per hour (Figure 2.3). Several *I. scapularis* were also collected from hunter-killed deer during one visit to a meat processor in November 2018. A subset of these ticks was submitted for *Bb* testing.

**Tennessee**

Seventy-two dragging events occurred on 66 Tennessee sites from October 2017-March 2018. A total of 597 adult *I. scapularis* were collected. Five other ticks were collected, one unidentified and 4 *A. americana*; these were excluded from the analysis. Ticks per hour ranged from 0-60, with an average of 11 adult ticks collected per hour (Figure 2.2).

Twenty-one dragging events occurred on 18 Tennessee sites from October 2018-December 2018. A total of 165 adult *I. scapularis* were collected. No other tick species was collected during this time. Ticks per hour ranged from 0-31, with an average of 10 adult ticks collected per hour (Figure 2.3).

**Virginia**

Fifteen dragging events occurred on 12 Virginia sites from October 2017-February 2018. A total of 380 adult *I. scapularis* were collected. No other tick species was collected during the drag-cloth surveys. Ticks per person hour ranged from 0-180, with an average of 50 adult ticks collected per hour (Figure 2.2).

Seven dragging events occurred on 7 Virginia sites in November of 2018. A total of 77 adult *I. scapularis* were collected. No other tick species was collected during the drag-cloth surveys. Several *I. scapularis* were also collected from one visit to a meat processor but were not
Figure 2.3: Mean number of adult *Ixodes scapularis*/hour at field sites in southeastern Kentucky, eastern Tennessee and southwestern Virginia, based on drag-cloth surveys undertaken in winter 2018/19. Means presented ± SE, number of sites surveyed in parentheses.
subject to *Bb* testing because they were out of our study area. Ticks per hour ranged from 0-20, with an average of 10 adult ticks per hour (Figure 2.3).

There was a significant difference in the number of ticks/hour dragged between states (1-way ANOVA, \( F_{1,153} = 6.00, P = .0031 \)).

### 2.4.2 Bb prevalence

From October 2017 to March 2018 a total of 1070 adult *I. scapularis* were collected across our three target states, of which 590 were tested for *Bb*; 78 of the tested ticks (13.2%) were *Bb*-positive. Positive ticks were detected at 22 of the 85 sites where adult blacklegged ticks were collected (Figure 2.1). Positive ticks were detected in all three states: 3 from Kentucky (4% prevalence), 29 from Virginia (45%), and 46 from Tennessee (10%) (Figure 2.4).

From October 2018 to December 2018, a additional total of 294 adult *I. scapularis* were collected across our three target states, of which 164 were were tested for *Bb*: 37 of the tested ticks (22.5%) were *Bb*-positive. Positive ticks were again detected in all three states: 10 of 47 in Kentucky (21% prevalence), 13 of 49 tested from Virginia (27%), and 14 of 68 in Tennessee (21% prevalence) (Figure 2.5). With data from both winter surveys pooled, *Bb* prevalence varied significantly between states (Chi-square test of association, \( P < 0.0001 \)).

*Updated I. scapularis distribution map for the study area*

Relative to the information mapped by Eisen et al. (2016), we identified additional counties with *I. scapularis* present in Kentucky (3 new counties), Tennessee (17 new counties) and Virginia (4 new counties). Most of these new counties were classified as having
Figure 2.4: Percentage of adult, unfed *I. scapularis* positive for *Bb* in winter 2017/18. Error bars are binomial 95% confidence intervals; number of ticks tested in parentheses.
Figure 2.5: Percentage of adult, unfed *I. scapularis* positive for *Bb* in winter 2018/19. Error bars are binomial 95% confidence intervals; number of ticks tested in parentheses.
"established" *I. scapularis* populations (Figure 2.6). We detected *Bb*-infected *I. scapularis* in 4 Kentucky counties, 4 Tennesse Counties, and 5 Virginia counties (Figure 2.6).

2.4.3 *Bb* strain types

All three RST types were found within the subset of *Bb*-positive ticks tested; the geographic distribution of the strain types is shown in Figure 2.7. The most common type found was RST 3 (15 *Bb*-positive ticks), followed by RST 2 (12 ticks) and RST 1 (7 ticks). Seven of the twelve RST 2 ticks were found in Kentucky; three were found on the Kentucky/Tennessee border, the remainder (2) of the RST 2 ticks were found in Virginia. RST 3 ticks were found in all three states.

2.5 Discussion

2.5.1 *Ixodes scapularis* abundance

We began surveillance of eastern Tennessee counties for *I. scapularis* due to recent increases in the abundance of this vector tick and of human Lyme disease incidence in southwest Virginia (Herrin et al. 2014, Lantos et al. 2015). We found that most eastern Tennessee counties now have established *I. scapularis* populations (Figure 2.6). Tick densities were variable, but sometime high; we collected ~60 ticks per hour at our most abundant *I. scapularis* site, which rivals tick numbers seen in Lyme-endemic counties in the Northeast. We also evaluated nearby counties in Kentucky and Virginia that represented potential routes of invasion of *Bb*-infected *I. scapularis* populations moving in from the north. We found that most of these surveillance counties also had established populations of *I. scapularis*. 
Figure 2.6: Comparison of county-level blacklegged tick population records for the study area cumulative to 2016 (from Eisen et al. 2016) versus cumulative to 2019 (this study). Reported ticks are classified by collecting less than 6 of one life stage and less than 2 life stages. Established ticks are classified by greater than 6 of one of life stage, or collection of 2 or more life stages.
Figure 2.7: Geographic distribution of \( Bb \) RST types among \( I. \) scapularis collection sites in winter 2017-2018 and 2018-2019. Different sized dots are used so that the presence of multiple RST types at a single site can be visualized.
There was a statistically significant difference in mean tick abundance across our three states, with highest numbers in Virginia and lowest numbers in Kentucky. This difference was primarily seen in 2017/18, with tick counts being more similar among states the following winter. This difference between the years could be related to differences in abiotic patterns between the two years, or to differences in our selection of sites between years. Tick numbers in our study area tend to be high near large rivers and lakes. Several sites sampled in Kentucky in the second winter were close to Laurel River Lake, at locations not sampled the previous year.

Understanding and predicting changes in tick population abundance can help public health officials determine whether or not populations are large enough to create a risk for pathogen transmission to the public. Tick abundance clearly fluctuates between years; some of these fluctuations are likely related to *I. scapularis*’ multi-year life cycle. Therefore, tick surveillance should be repeated as often as possible in areas at risk of disease emergence. There are multiple factors that could affect nymphal survival and development into adult ticks in one year but not in another, therefore, to properly understand tick abundance patterns in an area, multiple years’ abundance data, abiotic data, and host data need to be collected.

### 2.5.2 *Borrelia burgdorferi* infection

Because of public health concerns arising from detection of high *Bb* infection prevalence in wild questing ticks in southwest Virginia (Herrin et al. 2014) and more recently throughout Kentucky (Lockwood et al. 2018), we set out to evaluate *Bb* prevalence in *I. scapularis* in eastern Tennessee, and also in nearby counties in Kentucky and Virginia that might represent source populations for any infected ticks found. As we anticipated, the blacklegged ticks we collected from Virginia had the highest prevalence (45% and 27% in 2017-2018 and 2018-2019, respectively; Figures 2.4, 2.5). The drop in *Bb* prevalence in Virginia between years was
statistically significant (chi-square test of association; P=0.041) and could be related to somewhat lower tick numbers in the second winter or perhaps simply to different sites being surveyed. Tennessee and Kentucky both had increased $Bb$ prevalence in the second winter (Tennessee increased from 10% to 21%; Kentucky from 4% to 11%), in part because a higher proportion of survey sites in 2018-2019 were at low elevation and several were close to large rivers and lakes. Nevertheless, increased $Bb$ prevalence may also be due, in part, to increasing invasion of infected northern blacklegged ticks. For example, ticks from one site in Tennessee had been collected and tested for $Bb$ for 6 years prior to this study (see Hickling et al. 2018) with no $Bb$ infection found; in winter 2018/19 we made the first detection of an infected blacklegged tick at this site. We do not know if this single infected adult arrived as a chance drop-off of an infected nymph from a migrating host or represents an early stage of an endemic infection cycle at this site.

$Bb$ prevalence in questing ticks should be monitored yearly at selected reference sites to determine prevalence trends, so that proper precautions should be taken by the public. Repeated monitoring can also be helpful in determining how infected ticks are spreading and what factors are influencing their movement. Surveillance results can then be used as a basis for risk assessment in other similar areas where $I. scapularis$ numbers are increasing.

2.5.3 RST typing

DNA sequencing of $Bb$-positive samples was used to identify the $Borrelia$ species at our study sites and to determine the ribosomal spacer type (RST) of these $Borrelia$; RST type can help indicate the geographic origin of infected ticks.

Ticks typically avoid high elevations due to delayed development and delayed activation of molting at the lower temperatures associated with higher altitudes (e.g., Jauda et al. 2012).
The high elevation ranges of the Appalachian Mountains could potentially serve as a boundary for expansion of infected tick populations. However, these higher elevations may not be barriers to movement of some wildlife hosts, especially migratory birds that are known to be capable of transporting infected immature ticks (Ogden et al. 2008, Brinkerhoff et al. 2009). Birds are the most likely route of state-to-state transfer of infected ticks in our study area, since it seems unrealistic that host species such as rodents and deer would be moving infected tick populations over the large distances and high elevations between the Bb clusters mapped in Figure 2.6.

The eastern and western edges of the Appalachian Mountains could serve as potential travel routes for these hosts, therefore, we aimed to gain understanding of long-distance movement of infected tick populations by analyzing the geographic pattern of RST types across our study area. All three RST types were found in Tennessee ticks, with the geographic pattern of RST types (Figure 2.7) suggesting multiple routes of transfer. Firstly, RST 2, which is a strain associated with the upper Midwest (Gatewood et al. 2009), was found in all 3 states, but was most abundant in Kentucky and was only detected in Tennessee at 2 sites close to the Kentucky/Tennessee border.

In contrast, RST 1, which is associated with northeastern tick populations, was found in southwestern Virginia and northeastern Tennessee, but not in Kentucky. This pattern suggests a route of transport of infected northern I. scapularis from areas east of the Appalachians into eastern Tennessee, perhaps from the headwaters of the New River Valley in Virginia across into the headwaters of the Tennessee River Valley.

RST 3, which is associated with the upper Midwest, was found in all 3 states, and was the only type found in southeastern Tennessee. This distribution is difficult to interpret, but one possibility is that this is a widely dispersed endemic strain type that has been present in all three
states prior to more recent invasion of northern ticks. If so, the positive southern Tennessee ticks may simply be linked to the other RST 3 ticks found in northeastern Tennessee. Finer-resolution strain typing, to look at variation within the three broad RSTs, help to address this question. In order to better understand routes of transfer, RST types need to be tracked from northern Lyme endemic areas along likely dispersal routes to more recently discovered infected tick populations. That data could then be compared to home ranges and migration routes of hosts to assess how the infected tick populations are being transported.

2.5.4 Study limitations

A major limitation of the research reported in this chapter was the lack of consistent *Ixodes scapularis* dragging at the same sites two years in a row. Our second winter surveying adult ticks mainly focused on sites where wildlife hosts were being studied (see Chapters 3-5) and on new areas with habitat similar to sites where we had successfully detected ticks and *Bb* the previous year. This was due to lack of time and funding to repeat the large number of drag-cloth surveys we undertook in the first year (113 dragging events).

To properly monitor tick spread in a region, the same sites would need to be consistently drag-surveyed from year to year; doing so was beyond the scope of this 2-year study. In this study, our main objectives were firstly to identify counties with established populations of *Ixodes scapularis*, and secondly to focus on 9 areas where we could collect abiotic, habitat and host data, and gather ticks for Stable Isotope Analysis.

This study focused on sampling adult ticks, because they are the easiest life stage to drag sample, and the life stage with the highest expected *Bb* prevalence. Nevertheless, it is nymphal ticks that pose the biggest disease risk for humans because they are small and can be easily missed during tick checks. The nymphal life stage has also already fed on one host,
allowing for transmission of Bb from the host to the tick. In Tennessee and other southern states, it is usually difficult to collect questing southern nymphs using drag-cloth surveys (Diuk Wasser et al. 2012) because they exhibit different questing behaviors than northern I. scapularis, which are easily dragged (Arsnoe et al. 2019). Since southern nymphal ticks are mostly under the leaf litter (perhaps due to higher temperatures in the south), their risk to humans is low. Our findings suggest an invasion of Bb infected populations from Virginia may be occurring, however, so the behavior of the invading nymphs may be different from the current I. scapularis populations in Tennessee in ways that will pose a greater risk of human exposure to Lyme disease. Public health officials and medical professionals need to be aware that the risk of Lyme disease in eastern Tennessee is probably growing, which is differs from what older studies had concluded (e.g., Rosen et al. 2012). Anyone who is active outdoors in eastern Tennessee should be advised to do routine, thorough tick checks on themselves, their family, and pets.
Chapter 3: Influences of abiotic and habitat factors on *Borrelia burgdorferi* senso stricto infected tick populations in eastern Tennessee and adjacent states

3.1 Introduction

Lyme disease is the most reported human vector-borne disease in the United States, so interest surrounding the key vector, *Ixodes scapularis*, and the bacterium, *Borrelia burgdorferi* senso stricto (*Bb*) is increasing. *Bb*-infected populations of *I. scapularis* appear to be expanding into the southeastern United States (e.g., Lantos et al. 2015, Hickling et al. 2018), however, population numbers and pathogen prevalence vary greatly from state to state. Some states, like Virginia, have seen a recent influx of *I. scapularis* (Kelly et al. 2014) as well as an increase in human Lyme disease case rates (Lantos et al. 2015). Other southern states, such as Tennessee and Kentucky, have lower populations of *I. scapularis* (see Figure 2.2) and much lower incidence of Lyme disease ([https://www.cdc.gov/lyme/stats/tables.html](https://www.cdc.gov/lyme/stats/tables.html)).

Researchers have hypothesized that *I. scapularis* population densities in the Southeast may not be high enough to sustain an endemic *Bb* transmission cycle as is seen in the Northeast. Low tick density could be due to biotic factors, such as host availability, and/or abiotic and environmental factors that affect tick survival and reproduction. Past studies on *I. scapularis* population dynamics have identified a wide range of potentially-important factors, including habitat type (Maupin et al. 1991, Stafford and Magnarelli 1993, Ostfield et al. 1995, Ginsberg et al. 2004), temperature and humidity (Stafford et al. 1994, Vial and Smith 2002, Ogden et al. 2004, Berger et al. 2014), fire (Wilson et al. 1986, Stafford et al. 1998, Gleim et al. 2014), elevation (Guerra et al. 2002, Jouda 2012), and host selection (Apperson et al. 1993, Kerr et al. 2012, Arsnoe et al. 2016). Because so many different factors potentially influence tick
population abundance and geographic spread, it is challenging to predict areas of emerging Lyme disease risk.

Many studies have attempted to model the spread of *I. scapularis* using climatic variables, especially in the presence of climate change. Most research studies to date, both field and laboratory, have focused either on understanding *I. scapularis* population dynamics in the Northeast (where Lyme disease is endemic), or on the potential for climate change to trigger future spread. Some studies have focused on spread northwards into Canada (e.g., Soucy et al. 2018 and references therein) while others considered spread throughout the United States (e.g., Brownstein 2003, Hahn et al. 2016). Meanwhile, our understanding of factors affecting southwards spread remains limited. The goal of this study was, therefore, to investigate key abiotic and environmental variables that could potentially limit *I. scapularis* abundance in Tennessee, and thereby prevent or at least delay expansion of *Bb*-infected tick populations into this state. Particular attention was paid to the effect of site elevation on tick abundance, as studies in the Northeast have suggested that *I. scapularis* tends to avoid high elevations (Duik-Wasser et al. 2010, Hanh et al. 2016). If so, the Appalachian Mountains could act as a protective barrier against spread of northeastern ticks into southeastern Kentucky and northeastern Tennessee.

In addition to univariate analysis of key factors such as elevation, we also investigated climatic effects on *I. scapularis* distribution and abundance using a Maxent modeling approach. Maxent (http://biodiversityinformatics.amnh.org/open_source/maxent/) is an environmental niche model (ENM) that uses occurrence data in conjunction with environmental data to model the environmental conditions that meet a species’ ecological requirements and predict the suitability of habitat in a given area. Maxent uses the principle of maximum entropy on presence-only data to create the environmental niche model (Phillips et al. 2006), which can then be used
to model future predictions in areas where the species is not yet present, under different environmental conditions, and to better understand the suitability of habitat where the species already occurs (Merow et al. 2013). Maxent models can be especially useful when studying vector-borne disease risks because understanding the habitat suitability of the vectors and where potential spread can occur can help better target disease surveillance and other public health measures.

3.2 Objectives

- To determine whether elevation and other key environmental factors measured during our large-scale survey in TN, KY and VA in 2017-2018 (as described in Chapter 2) are statistically related to *I. scapularis* abundance and/or *B. burgdorferi* (*Bb*) prevalence.
- To determine whether abiotic and habitat factors collected at a finer spatial scale -- at 9 sites in these three states -- are statistically related to *I. scapularis* abundance or *Bb* prevalence at those sites.

3.3 Methods

3.3.1 Large-scale survey

As part of the large-scale survey reported in Chapter 2, we assessed *I. scapularis* abundance and *Bb* prevalence at 113 sites in eastern Tennessee, southeastern Kentucky, and southwestern Virginia. Ticks were collected in fall/winter of 2017/18 using the standardized dragging method described in Section 2.2. Relative humidity (%RH) and temperature (°C) were recorded at each site using a Kestrel™ 3000 Pocket Weather Meter at the start of each dragging session. When using the Kestrel™ device, the readings were recorded once the output readings
had stabilized for that location. Elevation (m) at each site location was obtained from Google Earth (https://www.google.com/earth/).

**Elevation analysis**

Linear regression models were run using the Stata 14_2 software package (StataCorp, College Station, Texas) to evaluate whether elevation had any effect on the mean number of ticks collected per site in each state. The number of adult *I. scapularis* collected per person-hour at each site visit was used as the dependent variable and elevation at the site was the independent variable. Records from sites where no ticks were collected were included. Repeated sites at different dates were also included. Four separate models were run - one for each state (Tennessee, Kentucky, and Virginia) plus one with the data for all states combined; the resulting regression lines-of-best-fit are presented graphically.

**Maxent model**

To investigate the effect of geographic differences in climatic variables on abundance, Maxent models were constructed using Maxent 3.4.1 and run with two separate datasets of tick selection points. One model included locations where 10 or more ticks per 1000 m were dragged (this was assumed to be a high enough density to potentially maintain *Borrelia burgdorferi* (*Bb*) transmission cycles; G. Hickling, pers. comm.); a second model was run using all occurrences where ticks were collected (i.e., including sites where ticks were present but the density was potentially too low to maintain a *Bb* transmission cycle). These models only included sites from our large-scale survey of adult *I. scapularis* in 2017.

The study area extent was determined by creating a minimum convex polygon around the locations of sites with '10 or more' *I. scapularis* per 1000 m, buffered by 2.5 decimal degrees,
which was the largest distance between sample sites, excluding the Virginia sites (if these had been included, the extent would have been greater than desired for this project). All climate and vegetation data were extracted using the Extract by Mask tool in ArcMap 10.5 to match the extent of our study area.

Environmental variable data layers for the Maxent model were prepared using ArcMap 10.5 (ESRI, Redlands, CA). Previous studies of tick life-cycles (e.g., Stafford and Magnarelli, 1993, Ogden et al. 2002, Vial and Smith, 2002, Berger et al. 2014) have suggested that temperature and humidity are important factors influencing blacklegged tick distribution and abundance. Therefore, four climate variables available from the PRISM climate database (http://prism.oregonstate.edu/) were chosen due to their hypothesized relevance:

1. Mean daily dew point - averaged over all days in the month. Dew point is defined as the temperature to which the air would have to cool in order to reach saturation.
2. Maximum daily temperature - averaged over all days in the month.
3. Minimum daily temperature - averaged over all days in the month.
4. Monthly total precipitation (i.e., rain plus melted snow).

The previous 6 months' data for these four variables was downloaded from PRISM as a raster file with 4.5 km resolution. Only the October-December component of these data were analyzed so as to match the period of tick field-sampling. The cell statistics tool in ArcMap 10.5 was then used to generate new raster layers representing the maximum and minimum of each climate variable. Vegetation cover variables from MODIS/Terra Vegetation Continuous Fields Yearly L3 Global 500m SIN Grid V051 (https://modis.gsfc.nasa.gov/) were also placed in the model. These variables used were % tree cover, % nontree vegetation, and % non-vegetation, all for year 2015. Three tiles from MODIS covered the area of our survey; these were mosaicked
together, re-projected to the correct coordinate system, and resampled at 4.5 km resolution to match the climate data.

A correlation analysis was run on the climate and environmental variables using the ‘remove highly correlated variables’ tool found in ArcMap's SDMToolbox. All climate and environmental variables were loaded into the tool and a csv file with all pairs of variables and corresponding r values was generated. Any pair of variables with a correlation >0.07 in the correlation matrix was removed from the relevant model. The basic settings in Maxent included cross validation with 5 replicates. For cross validation, the samples were divided into 5 replicate folds and each fold in turn was used as test data. Minimum training presence was also applied as a threshold rule in advanced settings to use the least suitable training presence record as the threshold. Since five replications were run, each Maxent model generated 5 threshold rasters. For each model output in Maxent, the rasters were summed in order to account for all of the environmental variables. Once the rasters were summed, they were then reclassified as 0,1 with 0 being less than 3 models in agreement regarding tick presence (= not likely to occur) and 1 being 3 or more models agreed (= likely to occur). This procedure was repeated for each Maxent output. Once outputs were summed and reclassified, the final products (sum of each model per dataset) were then summed together to generate a single agreement-of-presence map for each dataset.

3.3.2 9-site comparisons

In addition to the large-scale survey described in 3.3.1, 9 field sites (3 per state; Figure 3.1) were selected for more intensive study through wildlife host trapping and abiotic variable data collection. These sites are described below.
Figure 3.1. Map showing the Tennessee, Kentucky and Virginia counties where the 9 field sites selected for fine-scale comparisons were located.
Site 1: Oak Ridge (OAKR)

Starting Latitude, Starting Longitude: 36.003938, -84.216916

OAKR is located in Anderson County, near Oak Ridge, Tennessee. The study site is a part of the University of Tennessee's Forest Resources AgResearch and Education Center (UT FRAEC); 250 of 2,204 total acres are open to the public, while the rest is reserved as a research unit for University of Tennessee projects. Our study site was located in the northern part of the research unit, approximately 200 m south of Union Valley Road. The area is not actively managed unless forest management is part of the research project. The area is heavily forested with a few access roads.

Site 2: Carson Woods (CAWO)

Starting Latitude, Starting Longitude: 35.583754, -84.179329

Our Carson Woods site is part of Tennessee Valley Authority’s (TVA) Tellico Reservoir, near Highway 72 in Monroe County. It is a public area with some camping sites along the lake edge. The area has multiple uses including a cooperative wildlife habitat development between East Tennessee Bobwhite Chapter of Quail Unlimited and TVA. Tennessee Wildlife Resources Agency (TWRA) and Tennessee Department of Environment and Conservation (TDEC) also have interest in managing the property for wildlife habitat and scenic integrity. The forested study area was surrounded by water to the west and the south and by agricultural land and residential housing to the north and east.

Site 3: Chuck Swan Wildlife Management Area (PEAV)

Starting Latitude, Starting Longitude: 36.389201, -83.875161
Our Peavy Hollow site was located in Chuck Swan Management Area, which is a 25,000-acre Wildlife Management Area (WMA) managed by TWRA and Tennessee Division of Forestry (TDF). The WMA, located in Union County, forms a peninsula between the Powell and Clinch River and Norris Lake. The WMA is managed for wildlife habitat and is open to public, with several unpaved roads providing access for recreation and hunting.

Site 4: East Manchester (EMAN)
Starting Latitude, Starting Longitude: 37.1443, -83.7608

This site was located in forest adjacent to a public all-terrain vehicle (ATV) trail system located within the city limits of Manchester KY. The trail and forested area are open to the public year-round, with no obvious signs of habitat management.

Site 5: Manchester Exit (EXIT)
EXIT 1 Starting Latitude, Starting Longitude: 37.1379, -83.7744
EXIT 2 Starting Latitude, Starting Longitude: 37.1364, -83.7710

Located off of Hal Rogers Parkway and within Daniel Boone National Forest this site is also within Manchester KY city limits. The site is divided by a major highway, therefore half of our grid was labeled Exit 1 and the other half labeled Exit 2. The fields adjacent to our forested field site were being managed for pollinator habitat zones during the spring and summer months. However, throughout the year, some of the fields were regularly mowed for aesthetic purposes and road visibility. The two wooded edge areas that were surveyed had minimal public usage.

Site 6: Levi Jackson State Park (LEVI)
Starting Latitude, Starting Longitude: 37.0798, -84.0436
Levi Jackson State Park is located in Laurel County KY. The 896 acre park is open to the public and is a multiple use facility that focuses on preserving the history of the area. Our field site was located in forest near the public swimming pool and campground facilities and is surrounded by a multiple use trail system.

**Site 7: Resident Property (MAUR)**

Starting Latitude, Starting Longitude: 37.289743, -80.738433

This site was part of a forested private residential property in Giles County VA, located near Pulaski Giles Turnpike and Tranquility Drive. The property has one residential house located on it, with the forested land used mainly for hunting and outdoor recreation by the property owners. The area is not actively managed and only open to family and friends of the owner. The property is surrounded by other privately-owned forests and agricultural land.

**Site 8: Hunting Property (SAMY)**

Starting Latitude, Starting Longitude: 37.359256, -80.764543

This site was on a private hunting property in Giles County VA near Highway 460 and Clendennin Road. The property is managed for deer and bear hunting predominantly, with one small cabin used only during hunting seasons. The property is gated, and public access is not granted. The property is surrounded by forest except for some residential properties near its southern boundary.

**Site 9: High Street (HIGH)**

Starting Latitude, Starting Longitude: 37.3171, -80.7169

This site was in a privately-owned forested area adjacent to residential homes. The forested area is small (< 1 acre) and had little understory; it was bordered by High Street to the
west, homes to the north and south, and a field edge to the east. The area is private but is not gated; a trail traversing the site was used by property owners for dog walking.

Environmental variables

In April 2017, we attached one Hobo temperature/humidity data logger (model U23 Pro v2, Onset, Bourne MA) to the base of a tree at each field site and set it to record data at 1-hour intervals. Over subsequent months, each Hobo's data was unloaded periodically in the field onto laptop computers. Data collection ended, and the Hobos were recovered, in April 2019. HOBOWare software (Version 3.7.16; https://www.onsetcomp.com/hoboware-free-download) was used to obtain average, minimum and maximum temperature and humidity for immature and adult *I. scapularis* activity periods. Larval activity period was defined as 1 July-1 September and adult activity period was defined as 1 November-1 February; for brevity, we refer to these as each site's "summer" and "winter" periods, respectively. Data was pooled for these months and then analyzed to get averages, maximums and minimums for the active months.

Vegetation plots

At each site, a 60m x 75m live-trapping grid, consisting of 20 livetraps spaced at 15m intervals, was established for small mammal capture (see Section 3.2). Vegetation plots were evaluated at each corner and in the middle of these grids. Percent groundcover, percent canopy cover, species composition, soil type, and leaf litter depth were recorded on each plot. Percent groundcover was recorded by visually assessing the percentage of each groundcover type in a 0.25m² quadrat at five points within the grid (outer corners and in the middle). Groundcover was categorized as graminoids, forbs, woody vegetation, *Rubus* spp., or bare ground. Graminoids comprised any species of grass present in the groundcover, forbs included any other herbaceous
plant that was not a grass, woody vegetation referred to tree saplings, any plant in the genus *Rubus* was counted under the *Rubus* category, and bare ground included open soil and leaf litter.

Percent canopy cover was determined using a densitometer (Model C, Forest Densimeters Bartlesville, OK). Readings were taken from each cardinal direction over a fixed point, with the densitometer held approximately 12” in front of the body at elbow height. These recordings were averaged for each plot and then averaged by grid. Canopy species composition was determined using the penny method (https://www.clemson.edu/extension/publications/files/forestry-wildlife/fw02-homemade-devices-to-determine-basal-area.pdf), with species recorded for all trees counted as being “in” each plot (i.e., if the width of the device (the penny) is smaller than the width of the tree then the tree is counted as ‘in’). Leaf litter depth (cm) was recorded using a ruler at the base of each tree in the plot, and a ~50g soil sample was taken from each tree point. A ribbon test (NRCS, USDA Guide to Texture by Feel; https://www.nrcs.usda.gov/wps/portal/nrcs/detail/soils/edu/?cid=nrcs142p2_054311) was applied to categorize soil type.

One of our hypotheses was that areas with more leaf litter would have more ticks and increased *Bb* prevalence in those ticks. To test this, litter depth measurements were classified as < 2 cm or ≥ 2 cm, and two-sample t-tests were run using Stata 14.2 to determine if there was a significant association between leaf litter depth (cm) and either tick abundance or *Bb* prevalence at each site.
3.4 Results

3.4.1 Large scale survey

A total of 113 site visits were undertaken during the large-scale survey between October 2017-March 2018 (26 in Kentucky, 72 in Tennessee, and 15 in Virginia).

Elevation

Kentucky sites ranged in elevation from 252-433 m (mean = 321 m). Elevations of sites where I. scapularis was collected ranged from 257-433 m. There was no significant correlation between elevation and ticks/hour at the Kentucky sites (Figure 3.2; R² = 0.062, N = 26, P=0.218). Average temperature during our drag surveys was 18.6 °C and average relative humidity was 45.8% in winter 2017-2018. Average temperature during our surveys was 15.3 °C and average relative humidity was 45.2% in winter 2018-2019.

Tennessee sites ranged in elevation from 209-562 m (mean = 336 m). Ticks were collected at both the maximum and minimum range of elevation. There was no significant relationship between elevation and ticks/hour at Tennessee sites (Figure 3.3; R² = 0.025, N = 72, P=0.187). Average temperature during drag surveys was 15.2 °C and average relative humidity was 57.0% during our surveys in winter 2017-2018. Average temperature during our drag surveys was 14.3 °C and average relative humidity was 62.3% in winter 2018-2019.

Virginia sites ranged in elevation from 487-719 m (mean = 595 m). Ticks were collected at both the maximum and minimum elevation sites. There was a weak positive correlation between elevation and ticks/hour collected at Virginia sites (R² = 0.19, N = 15, P=0.105) (Figure 3.4). Average temperature was 13.1°C and average relative humidity was 68% during our
Figure 3.2: Relationship between elevation (m) and number of adult *Ixodes scapularis* collected per hour in southeastern Kentucky, based on dragging surveys undertaken at 26 sites in winter 2017/18. Dots indicated ticks/hour at each site and the line indicates the linear regression line of best fit.
Figure 3.3: Relationship between elevation (m) and ticks/hour in eastern Tennessee based on dragging surveys undertaken at 72 sites in winter 2017/18. Dots indicate ticks/hour at each drag location and the line indicates the linear regression line of best fit.
Figure 3.4: Relationship between elevation (m) and ticks/hour in southwest Virginia based on dragging surveys undertaken at 15 sites in winter 2017/18. Dots indicated ticks/hour at each drag location and the line indicates the linear regression line of best fit.
surveys in winter 2017-2018. Average temperature during our drag surveys was 15.1 °C and average relative humidity was 57.3% in winter 2018-2019.

When data from the three states were pooled, a highly significant positive relationship between elevation and tick abundance was apparent (Figure 3.5; $R^2 = 0.21$, $N=113$, $P<0.001$). However, when the elevation vs. tick abundance relationships were plotted separately for each state (Figure 3.6), it was apparent that Kentucky and Tennessee ticks had similar patterns of low overall tick abundance that tended to decrease with increasing elevation, whereas Virginia sites had much higher tick abundance that tended to increase with elevation (Figure 3.6).

**Maxent modeling**

Correlations among our climate and environmental variables are shown in Table 3.1. All pairs of variables with a correlation coefficient $>0.7$ in the correlation matrix were deemed “correlated” and were removed from the models. The retained variables were as follows: minimum dew point (mindew), maximum dew point (maxdew), minimum precipitation (minprecip), maximum precipitation (maxprecip), minimum minimum temperature(min_tmin), maximum minimum temperature(max_tmin), maximum maximum temperature(max_tmax), maximum minimum temperature(min_tmax), percent non-tree vegetation (%nontree), percent tree cover (%tree), and percent non-vegetation (%nonveg). Dew point was highly correlated with all the temperature variables (Table 3.1). Instead of making assumptions regarding the most useful combination of variables in our model, we chose to run models using subsets of the full correlation matrix shown above. The following subsets were used:
Figure 3.5: Relationship between elevation (m) and ticks/hour for all three states' data pooled based on dragging surveys undertaken at 113 sites in winter 2017/18. Dots indicate ticks/hour at a drag location and the line indicates the linear regression line of best fit.
Figure 3.6: Elevation vs. tick abundance linear relationships plotted separately for each state.
Table 3.1: Correlation matrix output for environmental variables run using the 'remove highly correlated variables' tool in ArcMap 10.5. Coefficients shaded blue were considered 'highly correlated' and were removed. See footnote for explanation of the variable names that were retained in the final model.

<table>
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<th>Layer</th>
<th>mindew</th>
<th>maxdew</th>
<th>minprecip</th>
<th>maxprecip</th>
<th>mintmax</th>
<th>mintmin</th>
<th>maxtmin</th>
<th>nontree</th>
<th>nonveg</th>
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<td>-0.181</td>
<td>-0.179</td>
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</tbody>
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1. Variables were as follows: minimum dew point (mindew), maximum dew point (maxdew), minimum precipitation (minprecip), maximum precipitation (maxprecip), minimum minimum temperature(min_tmin), maximum minimum temperature(max_tmin), maximum maximum temperature(max_tmax), maximum minimum temperature(min_tmax), percent non-tree vegetation (%nontree), percent tree cover (%tree), and percent non-vegetation (%nonveg)
1. Max_dew model includes:
   a. Max_dew
   b. Minprecip
   c. Maxprecip
   d. Nontree
   e. Tree

2. Min_tmin model includes:
   a. Min_tmin
   b. Minprecip
   c. Maxprecip
   d. Nontree
   e. Tree

3. Max_tmax model includes:
   a. Max_tmax
   b. Minprecip
   c. Maxprecip
   d. Nontree
   e. Tree

A total of six models using the above three subsets were run, three for sites with ≥10 *I. scapularis*, and three for sites with any *I. scapularis* (Table 3.2).
Table 3.2: List of the 6 models run using Maxent

<table>
<thead>
<tr>
<th>&gt; 10 ticks</th>
<th>All occurrences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max_dew model</td>
<td>Max_dew model</td>
</tr>
<tr>
<td>Min_tmin model</td>
<td>Min_tmin model</td>
</tr>
<tr>
<td>Max_tmax model</td>
<td>Max_tmax model</td>
</tr>
</tbody>
</table>
In the Maxent model where all tick occurances are considered, the model predicted that our drag-survey sites (n=113) would have *I. scapularis* present (i.e. either reported or established; see Chapter 2) based on the environmental characteristics used (Figure 3.7). However, the model where only locations wherever >10 ticks/hour were collected were used as occurances, the coverage of likely to occur habitat is more limited (Figure 3.8). Areas where the species is not likely to occur in high numbers include northeastern Tennessee and parts of southern Virginia.

3.4.2 9-site survey

The two sites with highest tick abundance were PEAIV in Tennessee and MAUR in Virginia. Both sites were oak dominated, had a high percentage of *Rubus* groundcover, and average leaf litter >2 cm deep. However, soil types were vastly different for the two sites. When analyzed by state, Virginia sites (considered most ‘at risk’ for Lyme disease due to state-wide increases in abundance and prevalence of *Bb*) had all loam soil types, a litter depth of 2.20 cm on average, a canopy coverage average of 70% and relatively low summer temperature and humidity (20.5°C and 68% relative humidity). Tennessee and Kentucky also had predominantly loam type soils but relatively lower average litter depth (1.66 cm and 1.69 cm, respectively). Mean *Bb* prevalence among ticks at sites with < 2 cm litter depth was 3.8 ± 2.4SE, compared with 41.0 ± 14.3SE ticks as sites with > 2 cm litter depth. Mean tick abundance at sites with < 2 cm litter depth was 1.40 ± 0.16 compared to 2.21 ± 0.03 at sites with > 2 cm litter depth. These findings support our *a priori* prediction that increased litter depth would favor increased tick abundance (Welch’s one tailed t-test assuming unequal variances, t= -4.90, 3.24df, P=0.0068).
Figure 3.7: Maxent model outputs summed to generate a map of model agreement of presence for all occurrences of I. scapularis; 0 indicates areas where less than 3 models were in agreement regarding tick presence. The green area indicates locations where 3 or more models were in agreement that I. scapularis were likely to occur. Red dots are locations where ticks were collected.
Figure 3.8: Maxent model outputs summed to generate a map of model agreement of presence using >10 ticks per 1000m occurrences; 0 indicates areas where less than 3 models were in agreement regarding tick presence. The red area indicates locations where 3 or more models were in agreement that *I. scapularis* was likely to occur. Yellow dots indicate areas where > 10/1000m were collected.
Bb prevalence (Welch’s one-tailed t-test assuming unequal variances, \( t = -2.58, 4.22 \text{df}, P=0.029 \)). However, relative humidity and temperature had little variation between sites and/or states (Appendix 1).

3.5 Discussion

*Ixodes scapularis* can be introduced to new areas in a variety of ways, including long-distance transport of immature ticks by birds (Klich et al. 1996, Hamer et al. 2011) and local transport of immature and/or adult ticks by deer (Watson and Anderson, 1976). Whether such ticks are then able to establish successfully in a new area will depend in part on the suitability of the habitat and abiotic conditions that the ticks encounter in that new area. Determining what factors allow a tick population to establish and increase is a difficult task, however, because many factors are involved, and those factors vary in different geographic locations.

3.5.1 Elevation

Elevation was an environmental factor of particular interest in this study because of potential for the Appalachian Mountains to block, or at least channel, the spread of ticks between Virginia, Tennessee and Kentucky. Previous studies have reached varying conclusions regarding the effect of elevation on *Ixodes* spp. distribution and abundance. For example, Jauda et al. (2012) indicated that for *I. ricinus* in Switzerland, tick abundance and Bb prevalence both decreased with increasing altitude. In contrast, another Swiss study (Cadenas et al. 2007) concluded that Ixodid ticks were more abundant at higher elevations and that aspect affected their ability to collect ticks.

In the U.S., some studies have concluded that elevation is not a significant factor in tick abundance. For example, Guerra et al. (2002) found elevation was not an important discriminator
in environmental models of *I. scapularis* distribution in the Upper Midwest. In contrast, Duik-Wasser et al. (2010) modelled *I. scapularis* distribution across the eastern United States and concluded that increasing elevation was strongly negatively correlated with nymphal activity. This relationship is important because the nymph is the life stage of *I. scapularis* most commonly associated with Lyme disease. In Diuk Wasser et al.'s (2012) study, elevation was not a statistically significant predictor of tick abundance when analyzed state by state, however it was statistically significant when the data from all three states were pooled.

Our results similarly suggest that abundance-elevation relationships may vary from state to state (Figure 3.6). A negative elevation-abundance relationship as seen in TN may also occur in Kentucky - the lack of statistical significance when we examine our data state-by-state is likely a consequence of the small sample size compared to the pooled data. In addition, we note some limitations to our survey coverage - due to time and travel limitations, sites at high elevations in TN and KY were not sampled intensively because of our low expectation of collecting *I. scapularis* at those sites.

In contrast to Tennessee and Kentucky, a very different abundance-elevation pattern was seen in southwestern Virginia. Tick abundance was much higher overall and tended to increase at higher elevations. This trend did not reach statistical significance (again, our sample size was small), but is consistent with recent survey work by the Virginia Department of Health (David Gaines, pers. com.) that also suggested that tick abundance was highest at high elevations. The practical significance of this finding is that the high elevation terrain of the Appalachian Mountains may not be as effective a barrier as might have been hoped for preventing southwards spread of infected *I. scapularis* populations into Tennessee.
The presence of abundant *I. scapularis* populations at high elevations in southwestern Virginia is surprising and raises the possibility of behavioral and/or physiological differences between Virginia ticks and Tennessee/Kentucky ticks. High elevations are generally thought to be more desiccating, colder, and therefore less suitable for ticks (Daniel et al. 1993). Low temperatures in early summer can cause delays in development (Randolph et al. 2002) and saturation deficits affect tick behavior and survival (Lees 1946; Gaede and Knulle 1997) due to increased energy loss (Randolph and Storey 1999). Therefore, the success of Virginia ticks at high elevation raises new questions about potential differences in rainfall and humidity patterns east vs west of the Appalachian Divide, and/or in the physiology of the ticks themselves on either side of the Divide.

### 3.5.2 Temperature and humidity

Temperature is an important environmental variable for tick populations because many developmental processes slow at low temperature (Ogden 2002, Randolph et al. 2002). Development and survival of a tick is critical in reproduction, therefore influencing abundance. Furthermore, Vial and Smith (2002) noted that temperature affects tick host-seeking behavior; specifically, movement and percentage of time spent questing was greater at 25°C and reduced at higher and lower temperatures.

Based on winter dragging data from our large-scale survey, our Virginia sites, where ticks were easily collected and *Bb* was common, had lower average winter temperature and a higher average winter humidity than our Kentucky and Tennessee sites. The relatively high winter humidity in southwestern Virginia may be an important explanation for why mean adult tick abundance was much higher there than in most of our Tennessee and Kentucky sites. However, the Virginia sites were drier in winter 2018-2019 (57.3% average RH as opposed to
the 68% RH in winter 2017-2018), which could explain the lowered adult tick abundance seen in winter 2018-2019.

In contrast to the winter data from the large-scale survey, climatic data from the Hobo data loggers at our 9 intensive sites indicated that year-round, all 9 sites had similar temperature and relative humidity averages. This demonstrates that data taken while dragging for ticks is winter is not a good indication of the weather conditions that immature ticks will experience at other times of year.

3.5.3 Maxent modeling

Climate modeling and environmental niche modeling are common in vector-borne disease surveillance. In particular, Maxent has been used in several studies to predict habitat suitability for *I. scapularis*. Maxent models do not consider host movement, habitat, or host selection, however they can help identify climatic variables that could influence tick populations. Hahn et al. (2016), using Eisen et al. (2006) data as their occurrences, used Maxent modeling to produce a potential future distribution map of *I. scapularis* for the contiguous United States. The output of this model is interesting because some areas deemed “unsuitable” included sites where our field surveys detected established populations of *I. scapularis*. Peterson et al. (2017) criticized the Hanh et al. (2016) for several assumptions used in the model and argued that the results underestimated the extent of suitable habitat for *I. scapularis*. Using the same occurrence data but different model methodology, Peterson et al. (2017) produced two new models indicating wider potential spread of *I. scapularis*. Both new models classified all 9 of our sites as being suitable for *I. scapularis* populations (see maps in Peterson et al. 2017).

Our Maxent model predicted that collecting >10 ticks/hour in most of the study area is unlikely, probably due to higher elevation leading to cooler temperatures that delay development,
which correlates with our drag surveys. The model also predicted that areas in northeastern Tennessee and southern Virginia were unlikely to have tick abundance of >10 ticks/hours. This again correlates with our drag survey results in northeastern Tennessee, including high elevation sites in Cherokee National Forest, where populations of *I. scapularis* are sparse or absent.

Our model predictions incorporated information on cover type variables and layers used (% tree, % non-veg, % non-tree). These variables were selected because previous research (e.g., Soucy et al. 2018) reported that heavily forested suburban and rural areas are more suitable tick habitat than are agricultural areas. Similarly, Maupin et al. (1991) found that most *I. scapularis* of all life stages are found in forested area or forested edge as opposed to residential lawns, probably due to reduced tick survival in areas exposed to direct sunlight (Ostfield et al. 1995). Therefore, we would have expected that the model would classify areas of urban and suburban development (% non-tree) in our study area as unsuitable habitat. However, some large cities in our study area (e.g. Knoxville TN, Chattanooga TN, and Lexington KY) were predicted to have >10 tick/hour tick densities. This could be due to the % tree cover in these cities still being sufficient to classify them as suitable tick habitat. Most of the area predicted to be unsuitable in northern Tennessee is high-elevation National Forest; if our model predictions hold true, that region might serve as a barrier to tick and pathogen movement from Virginia. However, even if that area is not suitable tick habitat, wide-ranging wildlife hosts (such as birds and perhaps deer) could potentially move infected ticks from a suitable habitat in Virginia to another suitable habitat in Tennessee, passing over or through the unsuitable habitat in between.

3.5.4 9-site survey

To assess *I. scapularis* habitat, we looked at a range of variables including dominant tree species, soil type, canopy cover, groundcover and amount of leaf litter. Dominant tree
species, canopy cover, and groundcover are important factors in host habitat that influence host abundance, which then contributes to tick abundance (see Chapter 4). Past studies have reported significant effects of leaf litter and soil type on tick abundance, as well as other climatic variables to be discussed later. Schulze et al. (1995) found that leaf litter can affect tick abundance and that the removal of leaf litter reduced the abundance of *I. scapularis* nymphs. In our 9-site study, areas with deeper leaf litter (>2 cm on average) seemed to favor more adult ticks in the winter months; we found a statistically significant correlation between litter depth (cm) and adult abundance (ticks/hour). *Ixodes scapularis* likely take advantage of deep and damp leaf litter in the summer months to avoid overheating and desiccation. Therefore, forests with tree species that produce long-lasting leaf litter, such as oaks (family *Fagaceae*), are favorable for these ticks. In addition, Guerra et al. (2002) found that sandy/loamy soils were associated with greater tick abundance. Our findings were consistent with this study; our sites with the highest adult tick abundance were predominately of the loam soil texture class.

### 3.5.5 Study limitations

A challenge for these kinds of analysis is that areas with low tick numbers may represent poor habitat for the species but alternatively could also be an indication of suitable habitats that have not yet been colonized. Furthermore, while tick abundance can be a good indicator of habitat suitability for the tick species in an area, what is optimal habitat for one life stage of *I. scapularis* may not be suitable for another life stage. For example, temperatures in winter at sites in the South may be suitable for adult questing, but those sites may be too hot and dry for immature ticks in summer.

Future research should aim to gather multiple years of data during different seasons, revealing population trends, which will be necessary fully understand the role of habitat
preferences in the geographic spread of Bb-infected I. scapularis. A larger scale study gathering more species-specific data for vegetation analysis would be useful in more fully understanding habitat preferences. Our anecdotal observations suggest that I. scapularis in eastern Tennessee often seek out damp microhabitats, such as roadside ditches filled with fallen leaves, so analysis on a smaller special scale may be needed to properly understand tick habitat preferences. Furthermore, analysis of environmental data to understand tick population abundance and Lyme disease risk must consider the entire life cycle of a tick. We focused mainly on what factors are suitable for adult ticks, future research should investigate larval and nymphal habitat relationships in our study area.
Chapter 4: Influences of the vertebrate host community on Borrelia burgdorferi senso stricto infected tick populations in eastern Tennessee and adjacent states

4.1 Introduction

Lyme disease is the most reported vector-borne disease in humans in the United States. It is vectored by the tick, *Ixodes scapularis*, and caused by the bacterium *Borrelia burgdorferi* senso stricto (*Bb*). Populations of *Bb*-infected *I. scapularis* are thought to be expanding throughout the southeastern United States and into areas where low tick abundance was thought to be a barrier to Lyme disease (Wang et al. 2014, Herrin et al. 2014, Lockwood et al. 2017, Hickling et al. 2018).

Host choice by *I. scapularis* affects not only tick abundance but also pathogen prevalence and therefore, human health. Studies in the Northeast on the competence vs incompetence of hosts to transmit *Bb* to *I. scapularis* have found that juvenile *I. scapularis* commonly feed on rodents, which are reservoir competent hosts (Piesman and Spielman, 1979). In the southeastern United States, however, it has been suggested that most juvenile *I. scapularis* feed on lizards, which are largely reservoir incompetent (Apperson et al. 1993). If so, this could lead to low *Bb* infection rates in southern tick populations and consequently low Lyme disease risk in southern states (Kerr et al. 2012, Moody et al. 2013). This suggested effect of lizards on Lyme disease dynamics is considered by some researchers to be an example of the “dilution hypothesis”, which suggests that increased host species diversity can help suppress disease transmission, provided the additional host species are less reservoir-competent (Ostfeld and Keesing 2000).

Meanwhile, other researchers have suggested that the key driver for the emergence of Lyme disease and other tick-borne diseases in recent decades has been increasing deer numbers throughout the eastern U.S. (Telford et al. 1997, Paddock and Yabsley 2007). White-tailed deer
are not reservoir-competent for *B. burgdorferi* (Telford et al. 1988) but they are a key host for adult female *Ixodes*, which turn their meal of deer blood into thousands of eggs that help populate each new tick generation.

Numerous studies performed in the Northeast aimed to better understand how host availability affects *I. scapularis* populations, with some studies emphasizing small mammal abundance (Van Buskirk and Ostfield 1995) and species richness (Ostfield and Keesing 2000, Schmidt and Ostfield 2001) while others have focused on deer abundance (e.g., Wilson et al. 1988, Deblinger et al. 1993, Rand et al. 2004). For example, in a modeling exercise based on data from their Northeastern study site, Van Buskirk and Ostfield (1995) concluded that *Bb* transmission dynamics were affected more by variation in small mammal abundance than by variation in deer abundance. It is unclear, however, whether the conclusions of these studies hold true in southeastern states.

**4.2 Objectives**

The two objectives of this chapter are:

1) To compare the availability of lizards (vs. small mammals) at our Tennessee field sites vs. our Kentucky and Virginia sites to the north;

2) To test Van Burskirk and Ostfield's (1995) prediction that Lyme disease dynamics are driven more strongly by small mammal abundance than by deer abundance.

**4.3 Methods**

Starting in spring 2018, lizards and small mammals were live-trapped on our 9 study sites (described in Section 3.3.2) to investigate potential host community differences between sites. Trail cameras were used to index the abundance of deer and other mesomammals.
4.3.1 Mammal trapping

Twenty Sherman live-traps (LFA Folding Live Capture Trap, H. B. Sherman Traps, Inc.) were placed at each field site on a 4 x 5 trapping grid. Traps were placed 15 m apart and baited with apple pieces and oats. Apple pieces served as a water source for captured mammals. The apple pieces and oats were changed each time the traps were checked. The traps were always checked within 24 hours of placing them and any individual caught was taken to a common processing area for biological sample collection.

Captured small mammals were removed from the traps, weighed (nearest 0.5g by Pesola spring scale) and identified to species based morphological characteristics, using a standard field guide (Kays and Wilson 2002). Small mammals were then briefly anesthetized using isoflurane. A Monel #1005-1 metal ear tag was then applied to one ear to identify recaptures. All visible ticks were collected from each small mammal using fine forceps and placed in 70% ethanol (EtOH). A tissue sample was collected from each ear using a Biopunch® (2 mm) and placed in 70% EtOH. Lastly, a whole blood sample was taken from a tail clip and then transferred into a microcentrifuge tube. The tail tip from the blood draw was also collected opportunistically as an additional tissue sample. Each mammal was then released at its capture location; the total number of small mammals captured per 100 trap nights (including recaptures) was tallied as measure of small mammal abundance. All vials were labeled with date, location, species, ear tag number, and sex. All collection methods were approved by UTIA’s IACUC committee (IACUC number 1846-0609).
4.3.2 Lizard trapping

Lizards were live-trapped using a burlap trapping method (Horn and Hanula, 2006). In May-July of 2018, burlap fabric squares (1 x 1 m) were folded in half and tied around tree trunks at each study site using a 1.5m lengths of polyester rope (Figure 4.1)

Burlaps were attached to suitable trees at each of our nine field sites; at each site 20 burlaps were placed on trees at 15m intervals to form a 4x5 grid. Burlaps were placed roughly 1.6m above ground level. Trees >10 inches in diameter breast height (DBH) were targeted so that the burlap's ends would not overlap on the tree. Over time, insects were attracted to the burlap, becoming an attractant for arboreal lizards, such as skinks. Lizards resting under burlaps were hand-caught and then moved to a central location for processing; a record was kept of any lizards seen but not captured while checking the burlaps.

Lizards caught were identified to species using a standard field guide (Conant and Collins 1998) and weighed (nearest 0.5g by Pesola spring scale). Any ticks located on the lizards were removed with fine forceps and placed in 70% EtOH. A lateral scale clip was performed on captured lizards to allow recaptures to be identified. Blood was taken from lizards by ventral tail vein blood draw using a 20-gauge needle and a 3 ml syringe. Tissue samples were opportunistically taken if lizards “dropped” their tails as an escape mechanism during capture. All lizards were released at their capture location; the total number of lizards seen or captured per 100 burlap checks at each site was tallied as a measure of abundance.

4.3.3 Biological sample testing

All ticks collected from mammals and lizards were counted and identified morphologically to species level. Blood samples from small mammals and blood/tissue samples from lizards were reserved for Stable Isotope Analysis (see Chapter 5).
Figure 4.1: Example of a burlap square hanging from rope attached to a tree trunk approximately 1.6m above ground level.
4.3.4 Trail camera photo surveillance

To monitor deer and mesomammal activity, two trail cameras - either a EnKeeo PH730S (https://www.enkeeo.com/) or a Bushnell 6MP Trophycam (Bushnell Outdoor Products, Overland Park KS) - were run continuously from June 2018 - September 2019 at each of our 9 sites. Cameras were set to photo-only, 3-image sequence, 10-sec minimum interval between sequences, with infrared flash enabled for night-time image capture. Animals photographed were identified to species where possible or were classified as "unknown". An activity index was calculated for each species based on the number of individuals of that species photographed, except that if a member(s) of the same species was seen within 1 hour of a previous image of that species, it was assumed to be the same animal and was not re-counted. If an individual of the same species was seen over 1 hour later, it was counted as a new individual. The number of "new" deer photographed per day was calculated based on the rules listed above, with days of coverage determined from the dates on the trail camera images. Deer photographed per day were then aggregated to obtain an index of deer seen per 100 camera-trap nights.

4.3.5 Statistical analysis

Linear regressions were run using Stata 14_2 to determine whether deer abundance (deer per 100 camera-trap night) and small mammal abundance (small mammals per 100 trap nights) were associated with adult *I. scapularis* abundance (adult ticks collected per hour) and the ticks' *Bb* prevalence (%). Capture rates were too low to allow for an equivalent analysis of the lizard catch-rate data.
4.4 Results

4.4.1 Host and tick abundance

Deer, small mammal, lizard and tick abundance indices for each of our 9 sites are summarized in Table 4.1. Small mammals were caught at all sites in all states. Two species of *Peromyscus* (*P. leucopus* and *P. maniculatus*) comprised the large majority (93.5%) of 70 captures in our small mammal traps followed by eastern chipmunks (*Tamias striatus*; 5.7%), and shrews (*Blarina* spp.; 1.4%).

Lizards were captured at 3 of our 9 sites and seen at 4 sites (Table 4.1), with very few lizards captured overall. Common five-lined skink (*Plestiodon fasciatus*; 66% of 6 lizard captures) were the most commonly captured species. White-tailed deer (*Odocoileus virginianus*) was the species most commonly recorded by our trail cameras (57.5% of 2642 photo-observations). Raccoons (*Procyon lotor*), Virginia opossums (*Didelphis virginiana*), and squirrels (*Sciurus* spp.) were other species frequently seen on the trail camera images (Table 4.2). American black bear (*Ursus americanus*), coyotes (*Canis latrans*), and foxes (*Urocyon* spp. and *Vulpes* spp.) were also seen occasionally at some sites.

There was no significant relationship evident between deer abundance and adult *I. scapularis* abundance among our 9 sites (Figure 4.2; $R^2=0.01$, $N=9$, $P=0.83$). Similarly, there was no significant relationship evident between small mammal abundance and adult *I. scapularis* abundance among sites (Figure 4.3; $R^2=0.038$, $N=9$, $P=0.62$).

4.4.2 *Ixodes scapularis* infestation rates

Juvenile ticks were collected from hosts in all three states (Table 4.2). Only larval *I. scapularis* were collected from small mammals and lizards in Tennessee. One larval
Table 4.1: Abundance indices for small mammals, deer, adult ticks, nymphaal ticks and lizards, and *Bb* prevalence for adult ticks, at each of our 9 sites.

<table>
<thead>
<tr>
<th>State</th>
<th>Site</th>
<th>Small mammals per 100 trapnights(^1)</th>
<th>Deer per 100 camera nights</th>
<th>Adult ticks per hour (in winter)</th>
<th>Nymphal ticks per hour (in summer)</th>
<th>Adult tick <em>Bb</em> prevalence</th>
<th>Lizards per 100 burlap nights(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHSW</td>
<td></td>
<td>6.4</td>
<td>1.6</td>
<td>31.8</td>
<td>8.0</td>
<td>67%</td>
<td>2.0</td>
</tr>
<tr>
<td>TN</td>
<td>MGCA</td>
<td>5.3</td>
<td>2.9</td>
<td>12.0</td>
<td>1.3</td>
<td>0%</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>OAKR</td>
<td>4.3</td>
<td>1.1</td>
<td>17.2</td>
<td>4.0</td>
<td>5%</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>EMAN</td>
<td>1.0</td>
<td>0.0</td>
<td>12.2</td>
<td>1.2</td>
<td>0%</td>
<td>1.0</td>
</tr>
<tr>
<td>KY</td>
<td>EXIT</td>
<td>16.7</td>
<td>9.8</td>
<td>5.1</td>
<td>1.5</td>
<td>10%</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>LEVI</td>
<td>2.0</td>
<td>8.0</td>
<td>13.5</td>
<td>3.0</td>
<td>7%</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>HIGH</td>
<td>0.0</td>
<td>18.1</td>
<td>5.3</td>
<td>16.0</td>
<td>13%</td>
<td>0.0</td>
</tr>
<tr>
<td>VA</td>
<td>MAUR</td>
<td>9.6</td>
<td>18.9</td>
<td>33.4</td>
<td>51.0</td>
<td>39%</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>SAMY</td>
<td>15.0</td>
<td>17.2</td>
<td>22.2</td>
<td>10.0</td>
<td>79%</td>
<td>0.0</td>
</tr>
</tbody>
</table>

1. All species of small mammals caught were pooled. This includes *Peromyscus* spp., *Tamias striatus*, and *Blarina* spp.

2. Includes lizards captured and seen. All species of skinks caught were pooled. This includes *Plestiodon fasciatus* and *Scincella lateralis*. 
Table 4.2: Trail camera tallies from May 2018 to November 2018 for the four most abundant species on our 9 targeted sites.

<table>
<thead>
<tr>
<th>Species</th>
<th>OAKR</th>
<th>PEAV</th>
<th>CAWO</th>
<th>MAUR</th>
<th>SAMY</th>
<th>HIGH</th>
<th>EMAN&lt;sup&gt;1&lt;/sup&gt;</th>
<th>LEVI</th>
<th>EXIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deer</td>
<td>33</td>
<td>37</td>
<td>28</td>
<td>433</td>
<td>104</td>
<td>559</td>
<td>.</td>
<td>193</td>
<td>132</td>
</tr>
<tr>
<td>Raccoon</td>
<td>125</td>
<td>5</td>
<td>13</td>
<td>51</td>
<td>2</td>
<td>44</td>
<td>.</td>
<td>79</td>
<td>44</td>
</tr>
<tr>
<td>Opossum</td>
<td>87</td>
<td>11</td>
<td>7</td>
<td>16</td>
<td>0</td>
<td>4</td>
<td>.</td>
<td>0</td>
<td>68</td>
</tr>
<tr>
<td>Squirrel</td>
<td>83</td>
<td>17</td>
<td>61</td>
<td>44</td>
<td>1</td>
<td>50</td>
<td>.</td>
<td>7</td>
<td>40</td>
</tr>
</tbody>
</table>

<sup>1</sup> Both trail cameras were stolen at this site, so no photographs were obtained.
Figure 4.2: Relationship between adult *I. scapularis* abundance and deer abundance at each of our 9 sites. Blue dots indicate sites where all ticks collected were *Bb*-negative; red dots indicate sites where at least one *Bb*-positive tick was detected.
Figure 4.3: Relationship between adult *I. scapularis* abundance and small mammal abundance at each of our 9 sites. Blue dots indicate sites where all ticks collected were *Bb*-negative; red dots indicate sites where at least one *Bb*-positive tick was detected.
Dermacentor variabilis collected from a Peromyscus maniculatis at SAMY in Virginia; no other ticks were collected from the small mammals at that site. One larval Dermacentor variabilis was also collected from a P. maniculatis at EXIT in Kentucky; I. scapularis was collected from other small mammals caught at that site.

Lizards from Tennessee had the highest number of ticks attached (Table 4.3). On average, small mammals were infested with 2.5 ticks per individual, 4.5 ticks per individual, and 7.5 ticks per individual in Tennessee, Virginia, and Kentucky respectively. Of the Peromyscus spp. caught in Tennessee, 66% had juvenile I. scapularis attached. Of the Peromyscus spp. caught in Virginia, 14% had juvenile I. scapularis attached. Of the Peromyscus spp. caught in Kentucky 63% had juvenile I. scapularis attached. Both Tamias striatus caught in Virginia had I. scapularis attached; one T. striatus caught in Kentucky did not. Both Plestiodon spp. skinks caught in Tennessee had I. scapularis attached; no skinks were caught in Kentucky or Virginia.

4.4.3 Host abundance and tick Bb prevalence

No significant correlation was seen between deer abundance and Bb prevalence in adult I. scapularis at our 9 sites (Figure 4.4; R²=0.13, N=9, P=0.33) although when one outlier site was removed (CHSW; 1.6 deer per 100 camera night, 67% Bb prevalence in ticks) the apparent positive relationship approached statistical significance (R²=0.48, N=8, P=.057).

Similarly, no significant correlation was seen between small mammal abundance and Borrelia prevalence among adult I. scapularis at our 9 sites (Figure 4.5; R²=0.25, N=9, P=0.17) although when one outlier site was removed (EXIT in Kentucky; 16.7 small mammals per 100 trap nights, 10% Bb prevalence in ticks) the positive relationship became statistically significant (R²=.64, N=8, P=.017)
Table 4.3: Average number of juvenile *Ixodes scapularis* per animal by state and genus of host species. (The number of individuals with ticks and the number inspected are shown in parentheses.) Dots in boxes indicate no individual of that species was caught in that state.

<table>
<thead>
<tr>
<th>Host species</th>
<th>Tennessee</th>
<th>Virginia</th>
<th>Kentucky</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Peromyscus spp.</em></td>
<td>2.5 (16/24)</td>
<td>4.5 (3/12)</td>
<td>7.5 (12/19)</td>
</tr>
<tr>
<td><em>Tamias striatus</em></td>
<td>.</td>
<td>1.5 (2/2)</td>
<td>0.0 (0/1)</td>
</tr>
<tr>
<td><em>Plestiodon spp.</em></td>
<td>6.5 (2/2)</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td><em>Blarina sp.</em></td>
<td>.</td>
<td>0.0 (0/1)</td>
<td>.</td>
</tr>
<tr>
<td><em>Scinella sp.</em></td>
<td>.</td>
<td>.</td>
<td>0.0 (0/1)</td>
</tr>
</tbody>
</table>
Figure 4.4: Relationship between deer abundance and % Bb prevalence in adult *I. scapularis* across 8 sites (CHSW was treated as an outlier and excluded from the analysis).
Figure 4.5: Relationship between small mammal abundance and % Bb prevalence in adult *I. scapularis* across 8 sites (EXIT was treated as an outlier and excluded from the analysis).
4.5 Discussion

Despite decades of research and hundreds of studies, many important aspects of Lyme disease ecology remain poorly understood. One of several key areas of uncertainty is the effect of host abundance on *I. scapularis* abundance and infection prevalence (Kilpatrick et al., 2016). Although *I. scapularis* feeds on many vertebrate hosts, most studies focused on northeastern ecosystems have emphasized two species as being key to the maintenance of *Bb*-infected *I. scapularis* populations: white-footed mice (*Peromyscus leucopus*) and white-tailed deer (*Odocoileus virginianus*) (Halsey et al. 2018). In the Southeast, however, researchers have emphasized that lizards may be the key host for immature *I. scapularis* (Apperson et al. 1993, Oliver et al. 1993, Kerr et al. 2012, Moody et al. 2013).

4.5.1 Lizards as hosts

In a North Carolina coastal plain study, Apperson et al. (1993) reported that *I. scapularis* was the only species of tick found feeding on lizards. Of 147 lizards caught and examined for ticks, 36.7% had ticks on them. Rodents were also trapped during their study, and 17.8% (23/129) had tick infestations. However, the rodents were predominantly infested with *Dermacentor variabilis*; only five *I. scapularis* were collected from rodent hosts. Other studies reporting that *I. scapularis* commonly parasitize lizards (primarily skinks) include Oliver et al. (1993), Giery and Ostfield (2007), and Kollars et al. (1999). We had difficulty capturing lizards, so our data was limited, but we did find immature *I. scapularis* on both skinks captured in Tennessee.

Lizards are viewed as important for Lyme disease ecology in the South due to their reservoir incompetence for *Bb*. Levine et al. (1997) argued that lizards are susceptible to *Bb* through multiple routes of transfer (needle or tick bite) however, after inoculating 146 skinks,
only 2 were infected with \textit{Bb}. Moody et al. (2013) fed laboratory infected nymphs on 25 uninfected skinks and found \textit{Bb} prevalence in the nymphs declined from 72\% before feeding to 7\% after feeding. This suggests that feeding on lizards will clear \textit{I. scapularis} of \textit{Bb} infection, which implies that lizards could serve as a potential barrier for \textit{Bb} spread in Tennessee and other southeastern states.

This hypothesis -- that lizards can potentially act as a barrier to \textit{Bb} transmission (Fish and Howard, 1999) – has been thought of as a ‘lizard filter.’ Since we did find juvenile \textit{I. scapularis} feeding on skinks in Tennessee, we cannot entirely discount the possibility of a lizard filter operating in this state. Nevertheless, mice were abundant on our Tennessee field sites, and larvae and nymphal \textit{I. scapularis} were found feeding on them as well as on lizards. Our detection of \textit{Bb}-infected ticks in four Tennessee counties (Chapter 2) suggests that the lizard filter, if present, has not been strong enough to stop \textit{Bb} transmission cycles from becoming established in parts of eastern Tennessee.

The biggest limitation to this part of our study was in our lack of a robust sample size of captured lizards that could be inspected for juvenile \textit{I. scapularis} and that could have helped us understand the relative abundance of lizards versus rodents on our sites. A larger sample of lizards would also have provided more data for our host bloodmeal analysis in Chapter 5.

4.5.2 Deer vs. small mammals

Given that lizards may not have as big a role in \textit{Bb}-transmission dynamics in Tennessee as we suspected initially, deer and rodent studies from the Northeast seem worth revisiting, since host-based interventions (for example, deer culling and rodent acaricide treatments) are possible tools that could be used to combat Lyme disease emergence in TN. A review by Speilman et al. (1985) emphasized the importance of deer in maintaining abundant tick populations, and since
then additional studies have reported links between deer abundance and tick abundance. For example, Rand et al. (2004) and several other similar studies (Wilson et al. 1985, Deblinger et al. 1993) have reported a decrease in *I. scapularis* abundance after deer were removed from an area. However, there was a subsequent rise in the number of questing adult *I. scapularis* for 2-3 years after the reduction due to the 2-year life cycle of the species and the ability of unfed larvae to overwinter and nymphs to diapause for two seasons (Yuval and Speilmen 1990). Nevertheless, these studies speak to the important role that deer play in the reproductive process of adult *I. scapularis*.

Although deer are important for feeding adult ticks, it is less clear how larval and nymphal tick abundance vary with deer density (Kilpatrick et al. 2017), since immature ticks mostly feed on smaller hosts such as *Peromyscus* spp., which are highly reservoir competent. In contrast, deer are largely reservoir incompetent for *Bb* and do not contribute directly to disease transmission cycles. *Ixodes scapularis* is a generalist species and has been detected on over 125 wildlife species (Keirans et al. 1996) but not all of the hosts parasitized are reservoir competent (LoGiudice et al. 2003). Host selection clearly plays a large role in *Bb* transmission dynamics, yet it remains challenging to determine which species juvenile ticks are parasitizing.

The complexity of this system has led to strong disagreements among researchers in the Northeast as to the relative important of deer vs. rodents for determining Lyme disease risk, which has typically been quantified as the density of infected nymphs (DIN) in a given area. To help address this question, Van Buskirk and Ostfeld (1995) developed a simulation model of host-tick-*Bb* interactions, using parameters appropriate for Northeastern forest communities. Their model suggested that increasing the abundance of hosts for juvenile ticks (i.e., rodents) caused tick abundance to increase continuously as rodent numbers increased and caused tick *Bb*
infection rates to increase until essentially all hosts were infected. In contrast, their model suggested that tick abundance and Bb-infection responded to adult host (i.e., deer) densities only when deer were very scarce. At normal deer densities the model assumed all adult ticks could find a host and so any further increase in deer numbers was inconsequential for Lyme disease risk. These relationships are summarized graphically in Figure 4.6.

We were interested in whether our data could shed any light on the applicability of Van Buskirk and Ostfeld's (1995) model to Lyme disease ecology in southern states. We found no statistically significant relationship between tick abundance and deer or rodent abundance on our 9 study sites. Other factors, including abiotic patterns and habitat features are also influencing I. scapularis abundance and Bb prevalence on our sites (Chapters 2 and 3), so these may be obscuring host abundance effects. Our field data did hint at possible positive relationships between Bb prevalence and our abundance measures of both deer and rodents, but outlier data-points meant these relationships were not statistically significant. We conclude that our 9 field site study design did not provide enough statistical power to properly assess the effect of host abundance on the tick/Bb dynamics.

4.5.3 Study limitations

A major limitation to our host study was a lack of understanding of habitat suitability for different host species. Vegetation and forest structure data was collected, but it was not feasible to evaluate whether or not sites were more suited for rodents versus lizards versus deer. It is likely that these three hosts occupy fairly similar habitat types, however, our results might have been clearer if we had been able to take this into consideration.
**Figure 4.6:** Hypothesized relationships between rodent and deer host abundance and density of *Bb*-infected *I. scapularis* nymphs (DIN) in Northeastern forests, based on modeling and parameter values proposed by Van Buskirk and Ostfeld (1995). Graphic courtesy of G. Hickling (University of Tennessee).
Chapter 5: Host selection of southern blacklegged ticks investigated using Stable Isotope Analysis: implications for Lyme pathogen transmission

5.1 Introduction

In northern Lyme-endemic areas, *Borrelia burgdorferi* (Bb; the causative agent of Lyme disease) prevalence in adult *Ixodes scapularis* (the blacklegged tick or ‘deer tick’) typically ranges from 20-40% (Wang et al. 2003). In contrast, in southern states *Bb* prevalence is typically an order of magnitude lower, and often zero (Stromdahl and Hickling 2012). The leading hypothesis for why this is so, is that immature ticks in the South feed primarily on reservoir-incompetent lizards, which prevents the establishment of endemic *Bb* transmission cycles between the ticks and their wildlife hosts (Apperson et al. 1993). If this hypothesis were correct, the tendency of immature *I. scapularis* to feed on lizards could act as a biotic barrier against southwards spread of *Bb*-infected *I. scapularis* populations that have recently become established in southwestern Virginia (Herrin et al. 2014, Lantos et al. 2015).

The importance of lizards as hosts for southern *I. scapularis* was first highlighted by Apperson et al. (1993) who found that 37% of lizards in a coastal North Carolina study were infested with immature *I. scapularis*, whereas only 13% of wild caught rodents were infested with *I. scapularis*. A limitation of this type of host infestation data, however, is that the proportion of ticks feeding on lizards versus other hosts depends on the relative abundance of the various hosts species; if lizard numbers are low, individual lizards may be heavily infested, yet the majority of immature ticks might feed on other, more abundant hosts.

To understand the consequence of lizard-feeding for *Bb* dynamics, it is important to estimate the proportion of ticks feeding on lizards and determine how that proportion varies geographically. One approach to answering this question would be to use chemical or molecular
techniques to determine what species of host each sampled tick last fed on. *Ixodes scapularis* is a three-host tick that feeds once during in each life stage and then drops into the leaf litter immediately after each blood meal. Engorged ticks are almost impossible to find in the litter, so blood meal analysis must be performed on ‘flat’ ticks once they resume host seeking (i.e., after they have digested their blood meal and molted into the next life stage). Analysis of the trace residues of previous bloodmeals in flat ticks collected from vegetation is extremely difficult; various methods have been tried with only limited success (e.g., Gariepy et al. 2012, Scott et al. 2012, Onder et al. 2013).

Stable isotope analysis (SIA) is a technique that has been used in wildlife management studies to determine diets of species such as black bears (*Ursus americanus*) (Hilderbrand et al. 1996), red fox (*Vulpes vulpes*) (Roth & Hobson, 2000), and gray wolf (*Canis lupus*) (Darimont and Reimchen, 2002). SIA has recently been proposed as a promising method to determine tick host preference (Hamer et al. 2015), because feeding on different hosts results in different ratios of carbon and nitrogen isotopes being ingested by the tick, and these isotopic signatures become incorporated into the tick’s tissues. Analysis of carbon and nitrogen isotopes can potentially be used to determine if the host animal was a herbivore, carnivore, and omnivore (Tieszen et al. 1983). SIA is unlikely, however, to be able to reliably distinguish between ticks that have fed on ecologically similar host species (LoGiudice et al, 2017).

A key question is whether immature *I. scapularis* in the South are primarily feeding on lizards (insectivores) as opposed to rodents (granivore/herbivores). The distinctly different diets of these two species groups may result in isotopic signatures that are distinct enough for an SIA analysis to be useful answering this host preference question.
5.2 Objectives

The goal of this chapter is to evaluate whether Stable Isotope Analysis is a useful tool for evaluating geographic differences in host selection by immature *I. scapularis*.

Specific objectives:

1) To supplement published data on nitrogen and carbon isotopic signatures of rodent vs lizard blood with additional data from our Tennessee, Virginia and Kentucky study sites;

2) To test the prediction that *Bb*-positive adult ticks from our field sites are more likely than *Bb*-negative ticks to have isotopic signatures characteristic of having fed on rodents rather than lizards;

3) To test the prediction that adult ticks from Tennessee are more likely to have isotopic signatures characteristic of lizard-feeding than are ticks from Kentucky or Virginia.

5.3 Methods

5.3.1 Tick and host collection for Stable Isotope Analysis

Ticks, small mammals, and lizards were collected from three field sites in each of Tennessee (TN), Kentucky (KY), and Virginia (VA), as described in Chapter 4. These states were chosen due to infection status/infection prevalence in ticks and how they could serve as invasion routes of infected populations of blacklegged ticks into southern states.

*Tick sampling*

An initial sample of ‘flat’ host-seeking adult blacklegged ticks was collected from the 9 field sites in winter 2017-2018 using standard drag-cloth sampling methods (described in Section 2.3). This preliminary sample was shipped to Texas A&M University for SIA (described below)
to determine if there was any detectable variation in isotopic signatures among ticks from different states. Additional ticks were collected in winter 2018-2019 and tested for *Bb* infection as well as SIA.

*Small mammal blood and tissue sampling*

Small mammal capture and processing was undertaken at each of the 9 sites from May 2018 until July 2018, as described in Section 4.3.1. A tissue sample was collected from both ears of each mammal caught using a Biopunch® (2 mm) and placed in 70% ethanol (EtOH). In addition, a whole blood sample (up to 50 µl) was taken using the tail clip method with a capillary tube and then transferred into a microcentrifuge tube. The tail clip from the blood draw was also collected opportunistically as an additional tissue sample.

*Lizard blood sampling*

Lizard capture and processing was undertaken from May 2018 until July 2019, as described in Section 4.3.2. Blood samples (up to 50 µl) were taken from lizards using the ventral tail vein blood draw method with a 20-gauge needle and a 3 ml syringe. Lizards would sometimes “drop” their tails as an escape mechanism during capture; in those cases, tail tissue samples were taken opportunistically.

All methods were approved by UTIA’s IACUC committee (IACUC number 1846-0609).

5.3.2 Stable Isotope Analysis

Ticks collected from vegetation in 2018-2019, and blood and tissue collected from live-trapped lizards and small mammals, were sent to the Stable Isotope Geosciences facility at Texas A&M University. Samples were dried, homogenized, and weighed into capsules. An elemental analyzer combusted samples at 1,200°C and the resulting CO₂ and N₂ gases were separated and
analyzed by isotope ration mass spectrometry (IRMS), Results are presented in standard delta (δ) notation as parts per thousand and are referenced to the Vienna Pee Bee Delemnite (VPDB) carbonate standard for ratios of C\textsuperscript{13}:C\textsuperscript{12} and relative to air for ratios of N\textsuperscript{15}:N\textsuperscript{14} (Hamer et al. 2015).

The winter 2017-2018 sample of ticks was analyzed using the above methods and compared to host isotopic signatures obtained from our own study and from the literature. The winter 2018-2019 sample of ticks was analyzed using both Stable Isotope Analysis and also RT-PCR for Bb. This was done by bisecting each adult tick with a sterile scalpel. Each tick was stored in the microcentrifuge tube and cut so that all internal parts were retained. One half of the bisected tick was then transferred to a second microcentrifuge tube. Each tube was given a vial code tag that assigned it to either SIA or Bb testing. After each tick was bisected, the scalpel was sterilized using 70% EtOH and flaming. SIA followed the protocol as listed above; the RT-PCR testing procedure is described in Section 2.3

5.3.3 Statistical analysis

Isotopic signatures of rodent vs lizard blood

δ\textsuperscript{13}C and δ\textsuperscript{15}N isotope values for rodent and lizard blood samples were averaged and compared to values for rodents and skinks published by Hamer et al. (2015). Host blood carbon and nitrogen differences between our three states were tested by ANOVA. To evaluate whether tissue samples could be used as an alternative to blood samples in future studies, scatter plots and Spearman’s correlation coefficients were calculated for isotopes of δ\textsuperscript{15}N and δ\textsuperscript{13}C in blood vs tissue, for individual rodents where both samples were collected.
**Isotopic signatures of ticks vs host blood**

Mean carbon and nitrogen signatures of host blood were compared graphically with the equivalent measurements of adult ticks collected in 2018-2019. A two-way ANOVA was performed using Stata 14.2 to determine if carbon and nitrogen signatures varied between states and/or between *Bb*-positive and *Bb*-negative ticks.

### 5.4 Results

#### 5.4.1 Isotopic signatures of rodent vs lizard blood

A total of 38 mice across the three states (KY, VA, TN) were analyzed using SIA; one specimen did not provide a carbon level under combustion (Table 5.1). Collecting sufficient blood from captured lizards proved problematic: only two lizard blood samples were large enough to be analyzed using SIA.

δ\(^{13}\)C isotope signatures in rodent blood varied significantly between states (F\(_{2,34}\)=6.04, P=0.0057) but δ\(^{15}\)N isotope signatures did not (F\(_{2,35}\)=0.42, P=0.66). Too few lizard blood samples were obtained to test for differences between states. Our rodent and lizard δ\(^{13}\)C estimates, and our lizard δ\(^{15}\)N estimate, were slightly higher than the values published by Hamer et al. (2015) (Table 5.1). Our rodent δ\(^{15}\)N estimate (5.08 ± 0.39SE) was much higher than Hamer et al.'s (2015) value (2.25 ± 0.12SE).

Isotope signatures estimated from rodent tissue samples were only weakly positively correlated with the equivalent estimates from rodent blood samples (Figures 5.1 a and b. Spearman’s r = 0.33, P = 0.081 for δ\(^{13}\)C; r = 0.44, P = 0.021 for δ\(^{15}\)N). (Note that the rodent δ\(^{15}\)N data had two very low tissue estimates that were treated as outliers.) Too few estimates were available to attempt an equivalent analysis for lizard tissue vs blood.
Table 5.1: Comparison of mean (±SE) δ¹⁵N and δ¹³C isotope values for rodent and lizard blood by state, and values previously published by Hamer et al. (2015). Sample size in parentheses.

<table>
<thead>
<tr>
<th>Source</th>
<th>δ¹⁵N rodent blood</th>
<th>δ¹³C rodent blood</th>
<th>δ¹⁵N lizard blood</th>
<th>δ¹³C lizard blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>VA</td>
<td>4.55 ± 0.54</td>
<td>-23.21 ± 0.50</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td></td>
<td>(10)</td>
<td>(10)</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>KY</td>
<td>5.74 ± 0.89</td>
<td>-25.86 ± 0.27</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td></td>
<td>(11)</td>
<td>(10)</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>TN</td>
<td>4.97 ± 0.53</td>
<td>-25.01 ± 0.09</td>
<td>7.42 ± 0.05</td>
<td>-24.25 ± 1.67</td>
</tr>
<tr>
<td></td>
<td>(18)</td>
<td>(18)</td>
<td>(2)</td>
<td>(2)</td>
</tr>
<tr>
<td>All 3 states pooled</td>
<td>5.08 ± 0.39</td>
<td>-25.28 ± 0.16</td>
<td>7.42 ± 0.05</td>
<td>-24.25 ± 1.67</td>
</tr>
<tr>
<td></td>
<td>(39)</td>
<td>(38)</td>
<td>(2)</td>
<td>(2)</td>
</tr>
<tr>
<td>Literature¹</td>
<td>2.25 ± 0.12</td>
<td>-24.50 ± 0.27</td>
<td>6.92 ± 0.98</td>
<td>-21.10 ± 0.81</td>
</tr>
<tr>
<td></td>
<td>(65)</td>
<td>(25)</td>
<td>(5)</td>
<td>(5)</td>
</tr>
</tbody>
</table>

¹ Hamer et al. (2015)
Figure 5.1: Relationship between a) tissue $\delta^{13}C$ and blood $\delta^{13}C$, and b) tissue $\delta^{15}N$ and blood $\delta^{15}N$, for rodents sampled during this study. Two readings (in orange) were treated as outliers when calculating the N trend line.
5.4.2 Isotopic signatures of ticks vs host blood

In winter 2018-2019, our tick isotope signatures did not closely match any of the host signatures published by Hamer et al. (2015). Our δ^{13}C values were closer to mouse than skink, whereas our δ^{15}N values were closer to skink than to mouse (Figure 5.2). The tick signatures were somewhat better match to the isotope signature data from hosts collected on our study sites (Figure 2.3); in particular, Tennessee ticks had very similar δ^{15}N signatures to our rodent blood samples.

5.3.3 Isotopic signatures of Bb-positive vs Bb-negative ticks, by state

Ticks from VA and KY in winter 2018-2019 had enriched δ^{15}N values than those from TN; in addition, in all three states the Bb-positive ticks had lower δ^{15}N signatures than the Bb-negative ticks (Figure 5.4). Two-way ANOVA indicated that both these factors were highly statistically significant (Table 5.2). In contrast, there was no statistically significant effect of either state or Bb-status on the ticks’ δ^{13}C signatures (Table 5.3).

5.5 Discussion

5.5.1 Stable Isotope Analysis as bloodmeal identification tool

Our results suggest that SIA has potential as a tool for studying tick host preference, which is valuable because host selection plays a large role in vector-borne disease transmission cycles. Other bloodmeal analysis methods have many limitations including inability to identify previous hosts, human DNA contamination, and lack of blood spectral libraries for comparison (Gariepy et al. 2012, Scott et al. 2012, Onder et al. 2013). Pulling engorged ticks from known hosts is a valuable way to understand host tick loads but does not provide information on host bloodmeals of previous life-stages, which is important for understanding infection status.
Figure 5.2: Mean $\delta^{13}C$ and $\delta^{15}N$ signatures (±SE) for adult ticks from TN, KY and VA collected in 2018-2019, compared with host blood values published by Hamer et al. (2015).
Figure 5.3: Mean $\delta^{13}C$ and $\delta^{15}N$ signatures for adult ticks from TN, KY and VA collected in 2018-2019, compared with host blood values for rodents and skinks collected on our study sites.
Figure 5.4: Effect of state of origin and Bb-infection status (Neg or Pos) on $\delta^{15}$N isotope signatures of adult *Ixodes scapularis* collected from vegetation. Means presented ± SE.
Table 5.2: Two-way ANOVA table for the effect of state of origin, and *Bb*-status, on $\delta^{15}N$ isotope signatures of adult *Ixodes scapularis* collected from vegetation.

<table>
<thead>
<tr>
<th>Source</th>
<th>Partial SS</th>
<th>df</th>
<th>F</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Model</td>
<td>115.7</td>
<td>5</td>
<td>6.75</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>State</td>
<td>87.0</td>
<td>2</td>
<td>12.70</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>$Bb$-status</td>
<td>23.1</td>
<td>1</td>
<td>6.95</td>
<td>0.0093</td>
</tr>
<tr>
<td>State * Status</td>
<td>2.5</td>
<td>2</td>
<td>0.36</td>
<td>0.69</td>
</tr>
<tr>
<td>Residual</td>
<td>318.7</td>
<td>93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>434.5</td>
<td>98</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5.3: Two-way ANOVA table for the effect of state of origin, and Bb-status, on $\delta^{13}$C isotope signatures of adult *Ixodes scapularis* collected from vegetation.

<table>
<thead>
<tr>
<th>Source</th>
<th>Partial SS</th>
<th>df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>3.58</td>
<td>5</td>
<td>1.28</td>
<td>0.27</td>
</tr>
<tr>
<td>State</td>
<td>2.94</td>
<td>2</td>
<td>2.63</td>
<td>0.078</td>
</tr>
<tr>
<td>Bb-status</td>
<td>0.009</td>
<td>1</td>
<td>0.02</td>
<td>0.90</td>
</tr>
<tr>
<td>State * Status</td>
<td>0.019</td>
<td>2</td>
<td>0.02</td>
<td>0.98</td>
</tr>
<tr>
<td>Residual</td>
<td>51.46</td>
<td>92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>55.06</td>
<td>97</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Therefore, with these challenges, finding new ways to accurately identify previous host bloodmeals in an efficient and accurate manner is important for understanding disease risk.

SIA was assessed in this study because of the promising results recently reported by Hamer et al. (2015). We were interested in evaluating tick host differences between states because this study included bordering states with high Bb infection prevalence in ticks (Virginia), low prevalence (Kentucky), and suspected zero prevalence (Tennessee). Our goal was to evaluate the hypothesis that immature ticks in the high infection state were more likely to have fed on reservoir competent hosts (rodents), compared to immature ticks in the suspected zero-prevalence state, which were hypothesized to be feeding on non-competent hosts (lizards).

Because they are insectivorous, lizards were expected to have a enriched δ^{15}N signatures than rodents because of their higher trophic level (Kelly, 2000), so we hoped that lizard-feeding vs. rodent-feeding would be detectable using isotopes of δ^{13}C and δ^{15}N. We found that there was a significant difference in tick δ^{15}N signatures by state and also by Bb infection status; ticks that were positive for Bb had a depleted δ^{15}N signatures as opposed to ticks that were negative, which matched our prediction. However, Virginia (our high infection prevalence state) had the most δ^{15}N on average in the ticks collected there, which was opposite of the pattern we predicted. We had assumed Virginia ticks would have a lower nitrogen signatures because more ticks were Bb positive and positive ticks would be more likely to have fed on a rodent than a lizard. One possible explanation for this unexpected result is that we did not consider hosts other than lizards and rodents in our analysis. For example, the enriched δ^{15}N content in Virginia ticks could be due to the ticks feeding on reservoir-competent insectivorous species, such as shrews and some birds (Baugh et al. 2004). Nevertheless, an important finding of the study was that the δ^{15}N signatures of most Tennessee ticks was a closer match to rodents than to lizards; this is counter
to suggestions by earlier researchers than immature ticks in a southern state like Tennessee would most likely be feeding on lizards. Higher than expected use of rodents by Tennessee ticks may help explain why $Bb$-infected tick populations are emerging in this state (Chapter 2).

Kentucky and Virginia ticks appear to be more inclined to feed on insectivorous species. We speculate, however, that these species are shrews or birds, not lizards, because lizards were rarely seen at our Kentucky and southwestern Virginia sites (Chapter 4).

As well as using host isotope signature data from previous studies in other regions of the United States, we collected our own vertebrate host blood, and evaluated whether or not mice from different states (KY, VA, TN) had different isotopic ratios. Our analysis showed a significant difference in carbon signatures between mice from different states, but nitrogen signatures did not differ significantly between states. It is not clear why carbon signatures varied; this needs further investigation.

We could not determine whether lizards’ signatures differed between states because of low capture rates and lack of success in collecting large enough blood samples for successful SIA. We were able to collect tail tissue from some lizards opportunistically however rodent blood and tissue isotopic signatures were poorly correlated (Figure 5.1), so we did not feel justified in using lizard tissue as a substitute for lizard blood. Nevertheless, our lizard blood isotopic ratio was similar to skink isotopic ratio in the Hamer et al. (2015) study, so we remain confident that the premise of our study - that lower nitrogen signature values are indicative of a rodent-fed rather than lizard-fed tick - is valid.

It is unclear why our rodents had more enriched $\delta^{15}N$ signatures than the *Peromyscus* mice in Hamer et al.'s (2015) study. The host blood used in that study was collected from wild-
caught hosts, so we have discounted the possibility that an artificial diet might have skewed their results.

5.5.2 Study limitations

While SIA seems a promising method for identifying previous host bloodmeals, a significant limitation to our study was our lack of success capturing lizards. In retrospect, burlaps should have been installed in much earlier in the spring to allow insects and arboreal lizards more time to find and use them; we caught most of our lizards late in the season. Pitfall traps should be considered as an alternative trapping method for lizards, although setting up and monitoring pitfall traps at nine locations across three states would be a major logistical challenge.

Our difficulties collecting an adequate amount of lizard blood in the field setting was a further limitation that could perhaps be addressed by using a different blood draw method or by collecting biological material other than blood for SIA. A potential problem with the latter solution, however, is lizard blood and tissue samples may not be well-correlated, as was the case with our rodent samples. If the correlation is poor, sampling blood seems preferable since that is the host material that ticks actually consume.

Another important limitation of the study is that we assayed only *Peromyscus* and lizard blood. Chipmunks, other small rodents, and shrews should all be considered, as should mesomammals, deer and birds (all of which could potentially feed nymphs). If any of the species that are feeding significant numbers of immature tick share similar diets, SIA is unlikely to be able to discriminate which of them ticks have parasitized.
Future studies that attempt SIA for tick bloodmeal analysis should consider the limitations addressed above and should anticipate that host diet signatures may vary significantly from one area to another. Collecting and analyzing adequate blood data from hosts in multiple study areas may prove difficult and time-consuming.
Chapter 6: Conclusions

6.1 Hypotheses

In the eastern United States, Lyme disease is caused by the bacterium *Borrelia burgdorferi* sensu stricto (*Bb*) and vectored by the blacklegged tick, *Ixodes scapularis*. Historically, Lyme disease cases have been concentrated in the Northeast and upper Midwest (CDC 2017). The vector tick is widespread throughout the eastern United States (Eisen et al. 2016) but *Bb* infection has rarely been found in southern ticks. Over the past decade, however, *Bb*-infected tick populations have expanded as far south as southwestern Virginia (Kelly et al. 2014) There has been concern that spread could continue south into Tennessee. There have been, however, two broad hypotheses suggesting that this spread might not occur:

1. *I. scapularis* densities in eastern Tennessee may be too low to support *Bb* transmission cycles;
2. Immature ticks in eastern Tennessee may feed primarily on non-reservoir competent lizards.

This thesis set out to determine whether *Bb*-infected ticks are now in eastern Tennessee and investigated these two hypotheses.

6.2 Tick abundance

We performed drag cloth surveys of 130 sites in winter 2017-2018 and 2018-2019 in Tennessee, Kentucky and Virginia. (We evaluated nearby counties in Kentucky and Virginia because they were potential routes of invasion for *Bb*-infected ticks moving into Tennessee.) From this survey work, we determined that most eastern Tennessee counties have established populations of *I. scapularis*. Tick densities were variable, but lower overall than in southwestern Virginia, and higher than in southeastern Kentucky. Some sites in eastern Tennessee had high
tick densities, similar to densities known to support robust *Bb* transmission cycles in southwestern Virginia and in northern Lyme-endemic areas. We conclude that low tick densities are not a barrier to transmission of *Bb* in parts of eastern Tennessee.

### 6.3 *Bb* prevalence and RST typing

Adult *I. scapularis* collected from the large-scale winter drag survey were subjected to RT-PCR for detection of *Bb*. *Bb*-infected ticks were detected in four Tennessee counties; these are the first records of infected ticks in eastern Tennessee. *Bb* prevalence in Tennessee was lower than in Virginia but higher than in Kentucky. *Bb*-positive ticks were then subjected to DNA sequencing to identify *Borrelia* strain types in each state. The typing results suggest that movement of northern infected ticks into Tennessee is primarily from Virginia rather than from Kentucky. It also appears that the spread of ticks may be bird-mediated due to the large distance and unfavorable terrain between southwestern Virginia and the *Bb*-positive counties identified in eastern Tennessee (Figure 2.6). Based on these findings, we conclude that *Bb* is endemic in some eastern Tennessee *I. scapularis* populations and that Lyme disease prevention precautions should be taken by individuals who are spending time in tick habitat.

### 6.4 Abiotic and habitat factors

*Ixodes scapularis* in Tennessee and Kentucky were mostly found at low elevations, in forested sites with substantial leaf litter. Maxent modeling of weather and habitat data suggest that most of eastern Tennessee provides suitable habitat for *I. scapularis*, whereas areas south of Tennessee are less suitable. More detailed analysis of factors such as temperature, relative humidity and habitat structure at selected sites provided no evidence of significant barriers to tick establishment in eastern Tennessee forests.
6.5 Host abundance and infestation

Host live-trapping at our targeted 9-sites indicated that immature *I. scapularis* in eastern Tennessee are feeding on both lizards and rodents. Our trail camera index of deer abundance was weakly positively correlated with both tick abundance and *Bb* prevalence. Similar weak correlations were found between small mammal abundance and both tick abundance and *Bb* prevalence. There were outlier sites in both datasets, however, so our study design lacked sufficient power to reach any firm conclusions about the importance of rodents vs. deer for Lyme disease dynamics in Tennessee.

6.6 Stable Isotope Analysis and host selection

Blood samples from hosts and adult *I. scapularis* were assayed by Stable Isotope Analysis (SIA) to determine if we could predict the previous host bloodmeal of adult *I. scapularis*. We conclude that SIA has potential as a tool for studying tick host preference over other bloodmeal analysis methods that pose some limitations. A preliminary analysis supported the prediction that *Bb*-positive ticks were more likely to have fed on granivores (e.g. rodents) than on insectivores (e.g. lizards). The SIA results also suggested that nymphal *I. scapularis* in Tennessee were more likely to have fed on rodents than on lizards. We suggest that extent of lizard feeding in eastern Tennessee is not sufficient be a barrier to *Bb*-emergence in this region.

6.7 Future directions

Future studies of *I. scapularis* and *Bb* in southern states should focus on large-scale, multi-state surveillance. Surveillance at sites where *Bb* has been detected at low prevalence should aim to determine whether *Bb*-positive ticks at these sites are drop-offs from migratory hosts or arise from recently-established endemic transmission cycles.
*Ixodes scapularis* were usually concentrated in small areas within our overall sites, so more information is needed on microhabitats for *I. scapularis* in southern states. Studies should also further investigate whether there is a correlation between large bodies of water and increased *I. scapularis* abundance, and if so, why this relationship exists. For example, are migratory birds using major rivers as migratory corridors and dropping *Bb*-infected ticks off during stop-overs? Or is it that water bodies increase local humidity in summer, when immature *I. scapularis* may be heat-stressed?

Host selection at southern sites needs to be evaluated more thoroughly. Although 9 sites across 3 states was a larger-scale study than previously attempted, our study design still seemed inadequate given the large number of interacting abiotic and biotic factors that affect Lyme disease dynamics. Larger studies, perhaps with multi-state collaborators, should be considered.
List of References


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Guerra, Martin; Walker, Edward; Jones, Carl; Paskewitz, Susan; Cortinas, Manuel Roberto; Stancil, Ashley; Beck, Louisa; Bobo, Matthew; and Kitron, Uriel, Predicting the Risk of Lyme Disease: Habitat Suitability for *Ixodes scapularis* in the North Central United States (2002). *Faculty Publications: Department of Entomology*. Paper 236.


Appendix
Appendix 1: 9 Site environmental variables calculations organized by state and site collected from May 2018-March 2019

<table>
<thead>
<tr>
<th>State</th>
<th>Site</th>
<th>Dominant Species</th>
<th>% Canopy Cover</th>
<th>% Groundcover</th>
<th>Leaf Litter (cm)</th>
<th>Nymphal Temperature (June-August)</th>
<th>Adult Temp (Nov-January)</th>
<th>Nymphal Min-Max &amp; Average humidity</th>
<th>Adult Min-Max &amp; average Humidity</th>
<th>Soil Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>VA</td>
<td>MAUR</td>
<td>White Oak</td>
<td>58%</td>
<td>2.125</td>
<td>Min: 10.5 Max: 28.7 Average: 20.5</td>
<td>Min: -13.4 Max: 26.4 Average: 3.4</td>
<td>Min: 65.7 Max: 100 Average: 96.1</td>
<td>Min: 26.3 Max: 100 Average: 90.1</td>
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<tr>
<td>VA</td>
<td>HIGH</td>
<td>Chinkapin Oak</td>
<td>77.75%</td>
<td>2.23625</td>
<td>Min: 10.5 Max: 28.9 Average: 20.5</td>
<td>Min: -14.72 Max: 21.6 Average: 3.27</td>
<td>Min: 32.3 Max: 100 Average: 91.7</td>
<td>Min: 29.3 Max: 100 Average: 82.7</td>
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<tr>
<td>VA</td>
<td>SAMY</td>
<td>White Oak</td>
<td>75.40%</td>
<td>2.225</td>
<td>Min: 11.2 Max: 30.5 Average: 21.2</td>
<td>Min: -14.6 Max: 20.2 Average:38.9</td>
<td>Min: 40.1 Max: 100 Average: 88.6</td>
<td>Min: 29.0 Max: 100 Average: 84.3</td>
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<tr>
<td>Location</td>
<td>Site</td>
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<tr>
<td>KY</td>
<td>EXIT</td>
<td>Red Maple</td>
<td>73.54%</td>
<td>Graminoid: 36.67</td>
<td>Forbs: 25</td>
<td>Woody: 0</td>
<td>Bareground: 0</td>
<td>Rubus: 42.5</td>
<td>1.7125</td>
<td>Min: 12.8</td>
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<td></td>
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<td>Min: 41.8</td>
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<tr>
<td></td>
<td>EMAN</td>
<td>White Oak</td>
<td>77.65%</td>
<td>Graminoid: 0</td>
<td>Forbs: 10</td>
<td>Woody: 3</td>
<td>Bareground: 1.4</td>
<td>Rubus: 85.6</td>
<td>1.0875</td>
<td>Min: 12.7</td>
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<td>Min: *</td>
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<td>Rubus: 72</td>
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<td>Forbs:</td>
<td>Woody:</td>
<td>Bareground:</td>
<td>Rubus:</td>
<td>Min:</td>
<td>Max:</td>
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<td>TN</td>
<td>PEAV White Oak</td>
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</table>

* Indicates no data (HOBO data logger failed)
Vita

Janetta Kelly is originally from Manchester, Kentucky and attended the University of Tennessee where she earned her Bachelor of Science Degree in Wildlife and Fisheries Science with minors in both Forestry and Watershed Science in Spring 2017. During her time as an undergraduate student, she worked in many seasonal technician positions and her own undergraduate research project where she found her passion for wildlife disease ecology and zoonoses. She served as Vice President of the UTK Student Chapter of the Wildlife Disease Association during her senior year as an undergraduate and served as President during her time as a graduate student. Upon graduation with her Bachelor’s degree, Janetta began a Master of Science degree in Wildlife and Fisheries Science at University of Tennessee, Knoxville. She will be graduating with her Master’s degree in May 2019.