GARSHONG, REUBEN AKWEI. Ph.D. Role of topographic corridors and small mammals in facilitating the spread of Lyme disease from southwestern Virginia to northwestern North Carolina. (2022)
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Lyme disease is the most important vector-borne disease in the United States. It is caused by the bacterium, *Borrelia burgdorferi* and transmitted by blacklegged ticks, *Ixodes scapularis*. An estimated 30,000 cases are reported to the CDC yearly from across the United States. Lyme disease cases in the Appalachian and western Piedmont foothills in northwestern North Carolina are rising, suggesting that there is an invasion of the disease in northwestern North Carolina. This study therefore set out to (1) evaluate if there are evidence for an invasion, and (2) understand how the invasion works and if northwestern North Carolina is a permissive area for Lyme disease establishment. Specifically, we do not know (1) how certain geographic features along the route of invasion may be influencing the spread of the disease, (2) whether the host community structure, and (3) seasonal tick lifecycle, are suitable for the establishment of Lyme disease enzootic cycle in northwestern NC. Hence, my specific goals were to: (1) determine the role of the New River as a potential route facilitating the spread of the pathogen and vector. (2) characterize the local and regional rodent community within northwestern North Carolina region, and (3) investigate the phenology of the life stages of the blacklegged tick vector within the region. For **aim 1**, I determined the role of the New River as a putative corridor for the spread of *I. scapularis* and *B. burgdorferi* by sampling ticks along a north-to-south gradient from southwestern Virginia to northwestern North Carolina using two 10-12 site flagging transects: one along the New River and a parallel one in the western NC Piedmont. My results showed (1) about thrice more *I. scapularis* density and 8% higher *B. burgdorferi* infection along the New River compared with the western Piedmont, (2) a more southern extent of the tick and pathogen

along the New River compared with the western Piedmont, although the tick extended further southern than the pathogen in both the New River and western Piedmont. These results suggested that the New River is acting as a corridor that is facilitating the spread of Lyme disease from southwestern Virginia into northwestern North Carolina. The mechanism of invasion can be (1) tick-first (when the tick precedes the pathogen), (2) dual-invasion (when the tick and pathogen invade simultaneously), or (3) spirochete-first (when the pathogen already exists, awaiting the invasion of the tick). My result was indicative of the tick-first hypothesis. In **aim 2**, I trapped rodents in selected sites along the New River and the Western Piedmont, inspected them for attached ticks, and collected ear tissue samples for *B. burgdorferi* screening. Out of the 174 rodents captured, 89.14% of them were *P. leucopus*, the competent reservoir host of *B. burgdorferi*, with 74% more individuals in the western Piedmont than the New River. Out of the 172 rodents tested, 38 of them were positive for *B. burgdorferi* of which 63.2% were from the New River. Of the 38 rodents that tested positive, two were not *P. leucopus* (one eastern gray squirrel and one pine vole) All the 98 *I. scapularis* ticks on rodents were collected from *P. leucopus* with 91.8% of them from the New River. Ninety-two of the *I. scapularis* ticks from the rodents were tested with 26 out of the 30 of them that tested positive for *B. burgdorferi* coming from the New River sites. These results provide a further support for the role of the New River as a potential spread corridor and showed that the rodent community structure in the mountains and western Piedmont area is suitable for the establishment of an effective enzootic transmission system of *B. burgdorferi*. To evaluate the phenology of the tick and of the transmission cycles, in **aim 3**, I flagged the two sites that showed highest tick densities in my first aim (i.e., the Alleghany and Ashe County sites), each month for 12 months to obtain seasonal information on the life cycle of the *I. scapularis* ticks. The results showed a phenology pattern that was typical

to that of the Lyme disease hyper-endemic regions in northeastern US. In this phenology pattern, the adults have two peaks (a lower one in early spring and a higher one in fall), and nymphs emerge in early spring before the emergence of larvae in mid-summer. Such a phenology is suited for an effective transmission of the pathogen among the wild rodents and humans, indicating that northwestern North Carolina is a suitable geographic region for the establishment of Lyme disease.

Put together, these findings indicated that western North Carolina, specifically the New River valley area, is a hotspot for the establishment of Lyme disease and could serve as a focus from where the disease can further spread to neighboring counties. To control the spread of the *B. burgdorferi* from wild animals to susceptible hosts such as humans, there is the need for state regulated programs that will ensure that regular monitoring through enhanced active surveillance for *I. scapularis* within the region (and possibly statewide) and their control using acaricides, and periodic *P. leucopus* vaccinations in the northwestern North Carolina area. This control measure will ensure that the prevalence of the pathogen in wild rodents is kept low to reduce Lyme disease risk. Public health officials also need to educate people who live and visit areas in and around northwestern North Carolina on proper tick control such as the wearing of permethrin-treated clothes when conducting outdoor activities, frequent checking of self for attached ticks when out in the woods and staying on demarcated paths when hiking in the woods. Information on what the early symptoms of Lyme disease are may also help to reduce the risk of Lyme disease becoming chronic in affected individuals. Future studies should include sampling ticks on hunter-harvested deer since this approach is the easier way to locate the ticks and usually show high *I. scapularis* detectability rate even when their densities are low. Also, other adjoining counties around the New River and its tributaries require investigation. It may also be important

to aim at identifying other possible natural and artificial events around the northwestern North Carolina that may be influencing the disease invasion.

ROLE OF TOPOGRAPHIC CORRIDORS AND SMALL MAMMALS IN

FACILITATING THE SPREAD OF LYME DISEASE FROM

SOUTHWESTERN VIRGINIA TO NORTHWESTERN

NORTH CAROLINA

by

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Approved by

_____
Dr. Gideon Wasserberg
Committee Chair

DEDICATION

I dedicate this dissertation to my wife, Agnes Kolog, and children for their unrelenting support and understanding that I needed to be away sometimes. I also dedicate it to my parents, Alfred and Leticia Garshong, and guardians, Stephen Adjei-Mensah and Vivian Garshong, who have supported me all throughout my life's journey up to this point. My brothers and sister, I dedicate this dissertation to you too. You all made this happen. I also dedicate this dissertation to all my lovely friends and loved ones, other extended family members, and all my teachers from preschool to the university. Finally, I dedicate this dissertation to the Almighty God. He surely makes all things beautiful in His time.

APPROVAL PAGE

This dissertation written by Reuben Akwei Garshong has been approved by the following

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CHAPTER I: GENERAL INTRODUCTION

Disease Niche Concept

An infectious disease, like species, has its own range of environmental conditions required to exist. Within that range of environmental conditions, the components of the disease must also be present. The components are the pathogen (i.e., an etiologic agent), and its reservoir host (i.e., an organism capable of acquiring and maintaining the pathogen in its system long enough to transmit it to another host). In the case of a vector-borne disease, a third component which is the vector (i.e., an organism that transmits the pathogen among members of the host) is also required. Every organism has its own ecologic niche which is defined as the set of conditions required by a species to maintain its populations without the need for immigration of other individuals from other areas (Peterson, 2006). Therefore, a disease niche (Figure 1) is the permissive environment within which the components of the disease (i.e., the pathogen, host, and vector) interact (Pavlovsky & Pious Jr., 1966). Within the disease niche, the pathogen cycles naturally among the individuals that constitute the reservoir host(s), which may be through a vector component as in the case of vector-borne transmissions. This way, the disease niche is established without necessarily causing the emergence of a disease.

An infectious disease emerges when a pathogen, introduced into a new host population, becomes established, and spreads (Morse, 2009). The frequency of contact that a new host population establishes with the pathogen through the natural reservoir host or vector causes the emergence of the disease. Ecological changes are one of the major drivers of disease emergence (Morse, 2009). Ecological changes in an environment can be naturally- or human-induced. Natural phenomena such as climate change [e.g., one caused by El-Nino-Southern Oscillations (e.g., Nicholls, 1993)], or anthropogenic disturbances such as agriculture or landscape

fragmentation may cause ecological changes that provide selective advantage to the pathogen through behavioral/physiological changes in the reservoir host, affording it an opportunity to invade new host populations (Morse, 1995; Rogers & Packer, 1993). When a pathogen spills from its primary host system into a new host population, it is termed a spillover event (Ellwanger & Chies, 2021). Zoonotic spillovers occur when pathogens move from an animal host system to infect humans, resulting in zoonotic diseases (also called zoonoses). Not all spillovers lead to disease emergence (Morse, 2009). However, when they do, they can be perennial, periodic, seasonal or a one-time event. When infectious diseases are widespread so that their continuous occurrences are only limited to particular geographic areas, they are described as endemic to those prescribed areas. Endemics can serve as sources that support the development of new endemic areas. Unexpected increases in the number of cases of a disease in a particular geographic area leads to an epidemic.

Figure 1. The epidemiologic triad and disease niche concept. Each circle represents suitable environmental conditions for a specific component of the disease niche. The square represents the environment, which includes external factors that allow disease transmission, both anthropogenic and natural environmental risk factors. The arrows represent the interactions between the niches. The nexus of the pathogen, host, and vector represents the set of conditions suitable for the establishment of the disease niche. Modified from Kaur et al. (2022) and Teague, (2018).
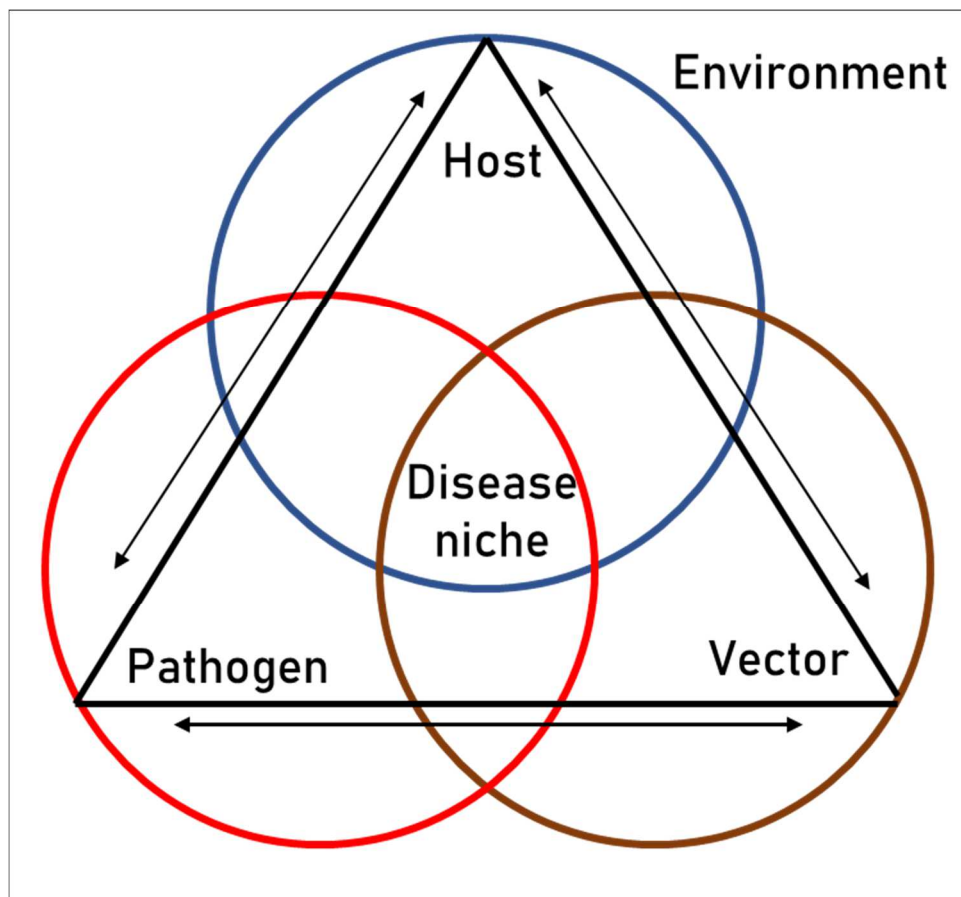
History of Lyme Disease

Lyme disease (LD), also known as Lyme borreliosis (LB) is an infectious vector-borne disease that has been inflicting humans for at least 5,300 years (Gannon, 2014; Keller et al., 2012). The longevity of the disease was made evident when scientists discovered the genetic

material of the LD pathogen in a 5,300-year-old ice mummy named Ötzi in the Eastern Alps of Italy in September, 1991 (Gannon, 2014; Keller et al., 2012). In the United States (US), the disease was first described in Old Lyme, New London County, Connecticut, in 1975 where the pathogen was misdiagnosed as causing juvenile rheumatoid arthritis among inhabitants (Steere et al., 1978; Walter et al., 2017). However, genetic analysis of museum specimens has revealed the existence of the pathogen in a reservoir host and the vector in different parts of the United States since the 1890s and 1940s, respectively (Gannon, 2014; Marshall et al., 1994; Persing et al., 1990; Walter et al., 2017).

Epidemiology of Lyme Disease

As of 2015, LD is the most widespread vector-borne disease and the seventh Nationally Notifiable infectious disease in the US (Adams et al., 2017). An estimated 30,000-40,000 cases of the disease are reported to the Centers for Disease Control and Prevention (CDC) by state health departments annually (Kugeler et al., 2021). Using insurance claims data from 2010 to 2018, it was found that an estimated 476,000 human cases were diagnosed and treated each year across the United States (Kugeler et al., 2021).

LD is an inflammatory tick-borne zoonosis caused by *Borrelia burgdorferi* senso lato group of bacteria (Mead, 2015). Hard ticks of the genus *Ixodes* transmit the pathogen (Burgdorfer et al., 1982; Gray, 1998; Stanek et al., 2012). Humans are infected with the pathogen when bitten by infected ticks. The disease is multisystemic and causes dermatologic (in about 72% of cases), rheumatologic (in about 27.5% of cases), neurologic (in about 12.5% of cases) and cardiac (in about 1.5% of cases) symptoms (Feder et al., 1993; Rubin, 2017). The most common early-phase clinical manifestation that characterizes LD is Erythema Migrans (EM). EM is the initial reddish skin rash, resembling a bull's eye, that develops at the site of the tick

bite (Rubin, 2017). EM reflects the localized reproduction of spirochetes within the skin and

usually begins 3-14 days after an infective tick bite (Mead, 2015). When left untreated, the

spirochetes disseminate to other parts of the body resulting in post-treatment Lyme disease

syndrome (Steere et al., 2004). Other symptoms of the disease aside for EM include fever,

headache, chills, mild stiff neck, arthralgia (inflammation of joints), myalgia (inflammation of

muscles), and lethargy (Feder et al., 1993; Mead, 2015). It is hypothesized that the different *B.*

burgdorferi senso lato strains may be responsible for the various levels of infectivity and

pathogenicity in infected humans across the world (Oliver, 1996). This is because different

strains migrate at variable frequencies to different parts of the body and vary in their ability to

stay in those infected sites (Steere et al., 2004).

Ecology of Lyme Disease

To understand the ecology of the transmission cycle and persistence of *B. burgdorferi*,

research has focused on understanding the interaction of the basic components of the disease: the

pathogen, the tick vector, and the vertebrate host species.

The pathogen: *Borrelia burgdorferi*

Borrelia burgdorferi belongs to the Order Spirochaetales, and Family Spirochaetaceae

(Wiltske et al., 2006). *Borrelia burgdorferi* senso lato has been categorized into 37 genospecies

based on variations in the 5S-23S and 16S-23S intergenic spacer regions of ribosomal genes

(Bauerfeind et al., 2016; Gray et al., 2002). Three of these genospecies, namely *B. burgdorferi*

senso stricto (hereafter may be referred to as Bbss), *B. garinii* and *B. afzelii* are known to

commonly infect humans (Bauerfeind et al., 2016). In Eurasia, all three genospecies exist

although most of the cases are caused by *B. garinii* and *B. afzelii* (Bauerfeind et al., 2016). In the

US, *B. burgdorferi* senso stricto is the pathogen accounting for almost all LD cases. A rare

genospecies of the pathogen, *B. mayonii,* was recently detected in six patients from Minnesota, Wisconsin, and North Dakota (Pritt et al., 2016), and in wild *P. leucopus* in Minnesota (Johnson et al., 2017).

The Vector: The Blacklegged Tick

From the epidemiologic point of view, a hematophagous vector such as a female *Anopheles* mosquito or ixodid tick, must feed on infectious vertebrates, acquire the pathogen during the blood feeding process, maintain it through one or more life cycles (i.e., transtadial passage), and pass it on to other hosts when next it feeds (Gray et al., 2002). LD is transmitted to humans by hard ticks of the genus *Ixodes* (members of the arachnid group of arthropods) (Burgdorfer et al., 1982; Gray, 1998; Stanek et al., 2012). Different *Ixodes* species transmit the disease in various parts of the globe (Figure 2). In the United States, *I. scapularis* transmits most of the *B. burgdorferi* infection compared to its sister species *I. pacificus* due to the relative distribution of reservoir hosts, which probably makes *I. pacificus* rarely infected compared to *I. scapularis* (Steere et al., 2004). *Ixodes scapularis* also transmits *Anaplasma phagocytophilum*, which causes anaplasmosis (e.g., Holman et al., 2004), *B. miyamotoi*, which causes relapsing fever (e.g., Han et al., 2016; Krause et al., 2015), *Ehrlichia muris eauclarensis*, which causes ehrlichiosis (e.g., Pritt et al., 2011), *Francisella tularensis*, which causes tularemia (e.g., Maestas, 2019), Powassan encephalitis virus, which causes tick-borne encephalitis (e.g., Ebel, 2010), and *Babesia microti*, which causes babesiosis (e.g., Holman et al., 2004).

Figure 2. Global distribution of the Ixodid ticks that transmit *Borrelia burgdorferi* senso lato (Schotthoefer & Frost, 2015).
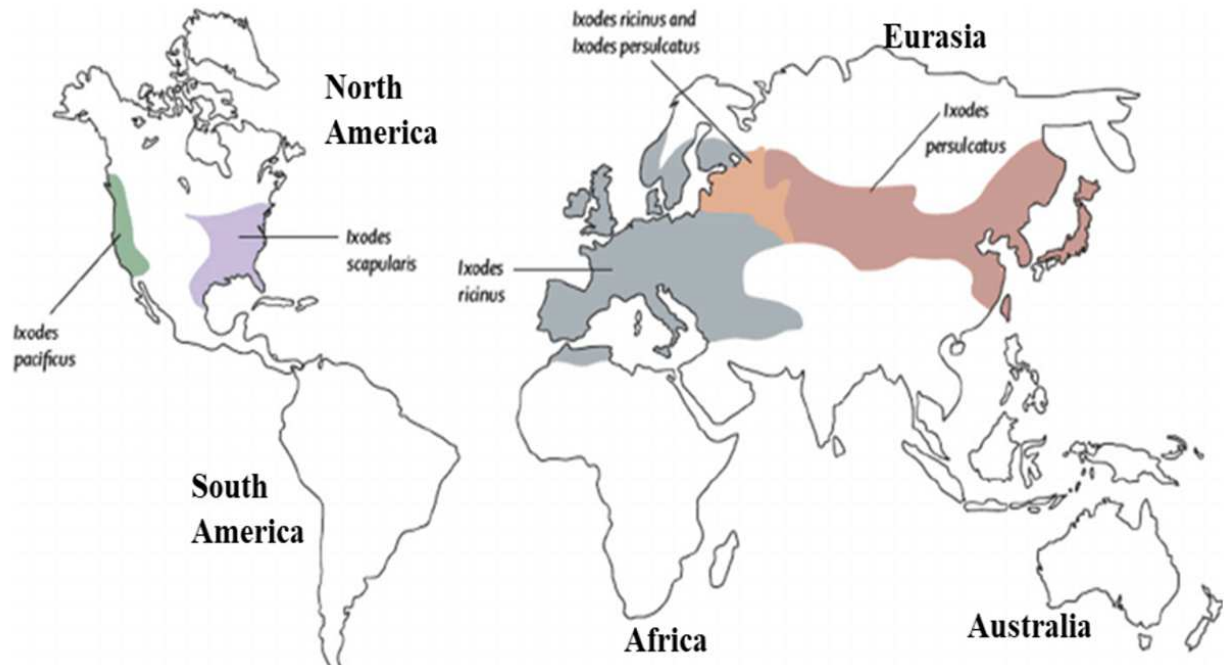
Blacklegged ticks are commonly found in forested areas with leaf litter and forest cover (Gray, 1998; Pfäffle et al., 2013). During their active periods, such areas provide high humidity and mild temperatures needed for their survival (Pfäffle et al., 2013). However, mortality rates and oviposition failure of *I. scapularis* are high when temperatures are above 31°C (Needham & Teel, 1991; Ogden et al., 2004). Under laboratory conditions, engorged female *I. scapularis* laid eggs at 21°C, > 95% relative humidity and 16 (light):8 (dark) hour photoperiod (Arsnoe et al., 2019). *Ixodes scapularis* also inhabit ornamental and lawn areas but in fewer numbers (Frank et al., 1998). Tick sampling to investigate increasing prevalence of *B. burgdorferi* in *I. scapularis* populations in the Tennessee valley, Tennessee, was halted during periods of rain, strong wind, low temperature of < 8°C (i.e., 46.4 F), and low relative humidity of < 40% (Hickling et al., 2018). This was because *I. scapularis* would usually not quest under such weather conditions.

The Vertebrate Hosts System

Ixodes scapularis is a generalist in terms of host choice, acquiring blood meals from at least 52 different mammal species, 60 species of birds, and 8 reptile species (Anderson & Magnarelli, 2008). Different host species of the spirochete differ in their degree of reservoir competence (i.e., the ability to acquire, maintain and transmit infection to a vector). *Peromyscus leucopus* has been shown to demonstrate higher *I. scapularis* burden and greater infectivity relative to other sympatric small mammal species, at least in Lyme disease endemic areas (Mather et al., 1989). A study in the northeast of the US showed that *P. leucopus* infected over 90% of *I. scapularis* that blood fed on them compared to chipmunks that infected about 70% of *I. scapularis* (Schmidt & Ostfeld, 2001). Meadow voles were also reported to be competent hosts with a prevalence of 5.5% (Mather et al., 1989). A study on *I. affinis,* a sibling species to *I. scapularis,* in three southeastern states (i.e., Georgia, Florida, and South Carolina) showed that enzootic transmission of *B. burgdorferi* was maintained by cotton mouse (*Peromyscus gossypinus*), hispid cotton rat (*Sigmodon hispidus*), and the eastern woodrat (*Neotoma floridana*) based on their relative abundances and infection rates (Oliver et al., 2003). The white-tailed deer, *Odocoileus virginianus*, is the preferred reproductive host of *I. scapularis* (Figure 1.3). However, the white-tailed deer is an incompetent reservoir host of *B. burgdorferi.* Some other incompetent reservoir hosts include, the five-lined skink (*Eumeces fasciatus*), eastern fence lizard (*Sceloporus undulatus*), and ovenbirds (*Seiurus aurocapillus*) (Magnarelli et al., 1992).

Tick questing periods might have evolved to coincide with host availability (Yuval & Spielman, 1990), so that the abundance of vertebrate hosts affects the density of *I. scapularis* that would survive within a given habitat. The infection rate of the *I. scapularis* ticks within a habitat depends on the availability of reservoir hosts. For example, improved acorn production in the

northeastern US was found to boost the abundance of both *P. leucopus* and white-tailed deer (Ostfeld et al., 2001a). The consequent effect was the increased abundance of both *I. scapularis* larvae (from eggs laid by *I. scapularis* females that blood fed on deer) and *P. leucopus* (due to increased reproductive success caused by food abundance) during spring-summer of the first year of *I. scapularis* life cycle. The higher densities of *P. leucopus* and the emergence of nymphs before larvae in this system ensured that the many *P. leucopus* are infected before the emergence of larvae in order to effectively perpetuate the Bbss transmission cycle. The overall effect was increased rate of *Bbss* infection in *I. scapularis* nymphs in the second year which correlated with heightened human LD risk in the second year (Ostfeld et al., 2001a, 2006).

Life Cycle of *Ixodes scapularis* in Relation to the Transmission Cycle of the Pathogen

The life cycle of *I. scapularis*, which generally lasts two years, involves an inactive egg stage, and three active life stages comprising of the six-legged larva, eight-legged nymph and adult. The duration of diapause (a period of hormonally controlled arrested development, which enables ticks to avoid entering questing phases at unfavorable times of the year) and the periods of questing (i.e., host-seeking) activity, which are controlled by climate and photoperiod, determines the rate of development of *I. scapularis* from one life stage to the next (Estrada-Peña & de la Fuente, 2014; Gray, 1998). Diapause may be (a) behavioral, involving a form of quiescence (i.e., a state of torpor, which is a sudden response to unsuitable environmental conditions, usually low temperature), or (b) developmental (also called morphogenetic), involving the cessation in development of (i) the laid eggs, (ii) the engorged larvae or nymphs or (iii) oviposition by engorged females (Gray et al., 2016).

Each active stage of *I. scapularis* requires a blood meal from a different host. The life cycle of *I. scapularis* is therefore termed a three-host life cycle (Figure 1.3). The engorged

female *I. scapularis* lays 1000-2000 eggs in the leaf litter, where relative humidity is high enough to ensure the survival of the eggs (Gray, 1998; Mead, 2015). Egg laying spans about 2-3 weeks and can occur anytime during the adult tick activity: fall (i.e., September to November), winter (if conditions are suitable), or spring (i.e., March to May), when adult *I. scapularis* are active (Mead, 2015). However, spring is the time when temperatures are suitable enough to support the development of the eggs. The female *I. scapularis* dies after oviposition while the male dies after mating. Eggs hatch in mid-summer (around July) and the larvae begin questing (host seeking) for animal hosts (mostly small to medium-sized vertebrates such as rodents, birds, or reptiles) in the vegetation until late fall (around September) of the first year (Yuval & Spielman, 1990). Larvae that acquire blood meal early in the summer molt promptly into nymphs (Nuttall & Labuda, 2008; Paesen et al., 1999). Larvae that fail to acquire blood meals and newly molted nymphs overwinter until spring of the second year (Yuval & Spielman, 1990). From spring through early summer of the second year (i.e., May through early July but can be as early as March and as late as November depending on the weather conditions), the nymphs become active, questing for suitable hosts (Anderson, 1989). The nymphs take their blood meal from the same range of hosts as the larvae and attach to their hosts for about the same amount of time, that is, 3-7 days (Anderson, 1989). Nymphs that successfully acquired blood meal molt into adults. However, the newly emerged adults remain inactive until fall (i.e., September to November, but can still quest if winter conditions are favorable) (Anderson, 1989; Yuval & Spielman, 1990). Females acquire blood meals from the host by feeding for about 8-11 days (Anderson, 1989). Females that could not attain blood meals in fall overwinter and then quest again during early spring (i.e., around March) of the following year (Anderson, 1989). Adults that fail to acquire blood meals die by the summer of the second year (Yuval & Spielman, 1990).

Figure 3. The life cycle of *I. scapularis* showing the times of emergence of each life stage and their probable hosts.
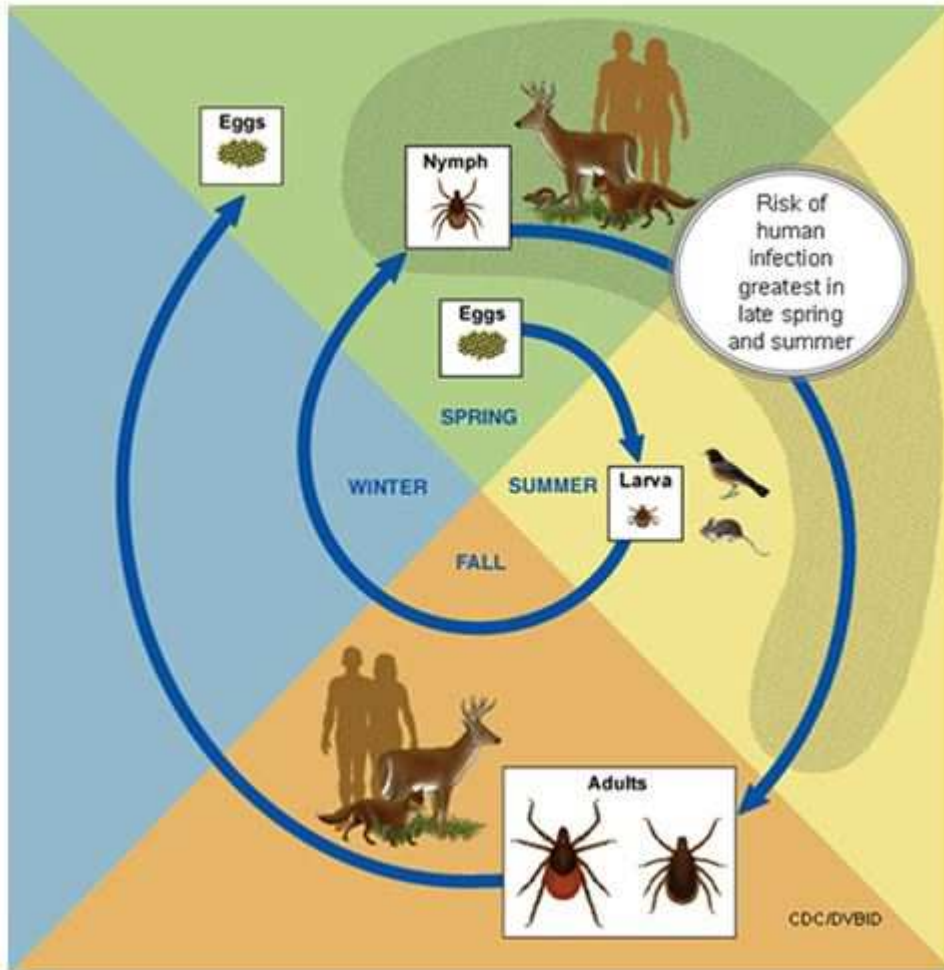
www.cdc.gov/ticks/

Host Seeking, Biting, and Feeding

Heights at which ticks quest increase with age and is based on their ability to withstand different levels of water loss (Estrada-Peña & de la Fuente, 2014). Larvae usually quest in the lower layers of the leaf litter, explaining why smaller vertebrates that crawl on the forest floor, such as small mammals, fossorial reptiles and ground feeding birds, are usually their preferred hosts (Anderson & Magnarelli, 2008). The nymphs, which quest relatively higher than the larvae, are usually limited to small- to medium-sized vertebrates while the adults usually attach

to large mammals such as *Odocoileus virginianus* (white-tailed deer) because they can quest even higher (Anderson & Magnarelli, 2008).Once a tick finds a host, it actively searches for a spot on the host to attach and start blood feeding. A favorable spot on the host is where the tick cannot easily be seen or groomed off. This is important because the tick must latch onto the host for days to become replete. When a favorable spot is found, the tick probes the skin and inserts its mouthparts (called hypostome) by cutting, ripping, and tearing the host's skin using its toothed chelicerae (called denticles) found on the ventral side of the hypostome (Sonenshine et al., 2014). The first step in the blood feeding process involves the secretion of a cement-like substance that binds the hypostome of the tick tightly to the host's skin (Gray, 1998).

The feeding tick begins a series of peristaltic movements that inject its saliva through the mouthparts into a "feeding cavity" (Gray, 1998), which is created as the tick cuts, rips, and tears the host's skin Simultaneously, a series of biochemical changes occur in the cells of the salivary glands, adapting its physiology and pharmacological properties to the feeding status of the tick (Nuttall & Labuda, 2008). The presence of many pharmacologically active compounds in the tick's saliva including antihistamines to prevent inflammation, analgesics to prevent pain, and anticoagulants to allow the sustained flow of blood into the feeding cavity, enable the tick to blood feed successfully. There is also break down of the cells surrounding the feeding site, and evasion of the host's immune response by the secretion of immuno-suppressive compounds (Nuttall & Labuda, 2008). If the blood feeding tick is infected with a pathogen (could be a co-infection), the infected saliva that it injects into the feeding cavity may cause an infection.

Emergence of Lyme Disease in North America

Borrelia burgdorferi, which is an ancient pathogen of forested habitats, has its history connected to human land use (Wood & Lafferty, 2013). European settlement into North America

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during the 17th and 18th centuries caused the deforestation of much of North American forests in a complex east-to-west gradient of disturbance (Hall et al., 2002). Large-scale deforestation continued to occur during the green revolution era (between 1950 and late 1960s) when agricultural production dominated globally (Tilman, 1999). Further, agricultural intensification, intensive hunting pressure and logging continued to reduce suitable habitats for deer and, consequentially, *I. scapularis* populations (Daszak et al., 2001). The remnants of *I. scapularis*, together with their hosts and reservoir hosts of *B. burgdorferi* became restricted to isolated forest refuges (e.g., remote areas of eastern Long Island, New York). Reforestation of much of the Unites States in the 20th century (Daszak et al., 2001), and reduction in hunting pressure enabled forests and wildlife to expand (Fish & Childs, 2009). The expansion increased forest habitats and caused *B. burgdorferi* hosts and *I. scapularis* to abound again. The later re-colonization of abandoned lands (Fish & Childs, 2009), increased the proximity between humans and forested areas, leading to re-emergence of LD in the United States. Wood & Lafferty (2013) mentioned that rural areas which have more forests than suburban and urban areas have the highest incidences of LD. On the other hand, differences among forest types may cause differences in *I. scapularis* abundance, hence, LD incidence (Wood & Lafferty, 2013).

Lyme Disease in the Piedmont Region

Lyme disease cases in Virginia were once restricted to the northern parts of the state, but are recently reported to be expanding southward (Diuk-Wasser et al., 2006; Heimberger et al., 1990). Before 2006, most studies of *I. scapularis* ticks in Virginia were distributed in the eastern and southeastern parts of the state and found in a decreasing in abundance of *I. scapularis* ticks and their *B. burgdorferi* infection with increasing distance from the coast (Sonenshine et al., 1995). A similar trend was found in North Carolina where a 5-year study (1983 to 1987) of ticks

on hunter-harvested deer found *I. scapularis* to be most common in the Coastal Plains and absent

or rare in the Piedmont and Appalachian Mountains (Apperson et al., 1990a). The same was the

case in Maryland in 1989 and 1991, including a very low detection (on only 1-5% of deer) in the

Appalachian Mountains (Amerasinghe et al., 1992, 1993).

However, in the winters of 2012 and 2013, *B. burgdorferi*–infected adult *I. scapularis*

ticks were detected in Pulaski County, Virginia (Herrin et al., 2014). This prompted a study in

Tennessee to investigate a possible invasion of these ticks into the upper Tennessee valley area

in 2017 (Hickling et al., 2018). The authors dragged in 70 sites spanning 26 counties and

collected 479 adult *I. scapularis* in 49 out of the 70 sites. Following the tick classification status

in Eisen et al., (2016), they found that *I. scapularis* was established in 23 out of 26 counties with

more than 3-fold increase in *I. scapularis* abundance since 2012 (Hickling et al., 2018). They

also detected *B. burgdorferi* infection in 46 out of the 479 adult ticks. Recent review of LD cases

in Virginia and North Carolina (from 2000 to 2014) showed that Lyme disease cases in Virginia

have increased since 2007 with most notable area of expansion occurring towards southwestern

Virginia along the Appalachian Mountains and predicted possible rising cases in North Carolina

along the same route (Lantos et al., 2015). From October 2015 to July 2017, Teague (2018) used

tick flagging and hunter-harvested deer tick collection techniques to characterize the

entomological risk for LD along a north-to-south gradient from VA (Fairy Stone State Park) to

NC (Lake Norman State Park). Teague (2018) found that *I. scapularis* and *B. burgdorferi*

infection were decreasing from Virginia towards the south. This finding suggested a southward

spread of the vector and the pathogen into North Carolina (Figure 4A & B). The southernmost

counties of *I. scapularis* distribution were Alexander and Iredell whereas that of *B. burgdorferi*

were Forsyth and Yadkin (Fig. 4B). *Ixodes scapularis* were found on deer in the south but those
detected ticks were not infected with *B. burgdorferi* (Figure 4B: middle and bottom charts).

**Figure 4. North to south distribution pattern of I. scapularis (A) by flagging and (B) from
hunter-harvested deer.** Map insert shows the categorization of sites into north, central and
south.
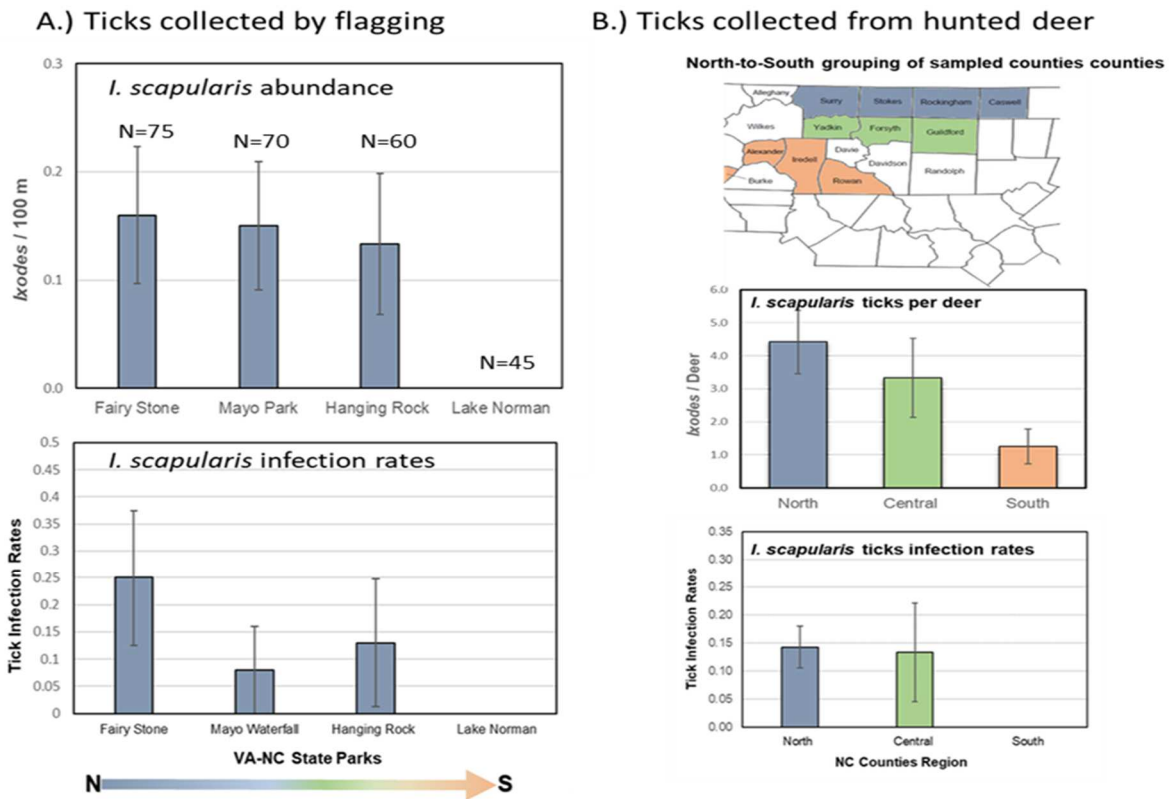
Teague's study was restricted to the western Piedmont region and did not consider factors
that may be facilitating the spread of the ticks and the pathogen they carry. Furthermore, his
work did not entail the potential of vector-pathogen establishment after invasion

GENERAL GOALS OF STUDY

The spread of infectious diseases to a new area requires the (1) the pathogen to invade
into the new area through its host and/or vector organism, and (2) the establishment of the

disease niche which involves the establishment of the vector and host and pathogen in the new environment (Lederberg & Relman, 2009). Therefore, my general goals were to: (1) investigate where the *I. scapularis* and *B. burgdorferi* spread is occurring from southwestern Virginia to northwestern North Carolina, and whether there are certain geographic features responsible for facilitating their spread, and (2) investigate whether there are favorable host and environmental conditions to cause the establishment of a LD niche after the successful invasion. Specifically, I developed three aims for this study.

Aim 1: Investigate the North-to-South Pattern of *Ixodes scapularis* Invasion and the Role of the New River Corridor in Facilitating This Spread

Lyme disease cases are expanding southward, along the Appalachian Mountains (Lantos et al., 2015). In addition, LD cases in the New River valley area of Virginia are reported to be on the rise (Rife, 2017). Current confirmed LD cases in North Carolina likewise shows an increase in the number of Lyme disease cases in northwestern North Carolina (NC Division of Public Health, 2019).

Accordingly, I hypothesized that *I. scapularis* and *B. burgdorferi* were spreading from southwestern VA to northwestern NC and that this north-to-south spread is being facilitated by the New River, which is acting as a dispersal corridor for white-tailed deer since deer are known to disperse along river corridors (Cortinas & Kitron, 2006).

Approach: I sampled for *I. scapularis* ticks in sites along the New River (Pulaski County, Virginia to Burke County, North Carlina) and corresponding western Piedmont (i.e., from Bedford County, Virginia to Iredell County, North Carolina) by flagging along 100m transects (10 – 12 transects per site). Ticks were identified and characterized by life stage. A proportion of

sampled *I. scapularis* ticks were sent to CDC for *B. burgdorferi*, *B. miyamotoi*, *A. phagocytophilum*, and *Babesia microti* screening, using multiplex qPCR assay.

Predictions: I predicted: (1) a north-to-south gradient in *I. scapularis* tick densities and *B. burgdorferi* infection rates, and (2) that the *I. scapularis* tick densities and *B. burgdorferi* infection rates would be higher and extend further south along the New River compared with the western Piedmont region. In terms of the invasion mechanism, I predicted the tick-first scenario. The tick-first scenario is observed when uninfected *I. scapularis* are detected first in a previously uninvaded area through their dispersal by incompetent reservoir hosts before the later detection of *B. burgdorferi* through slower secondary invasion by competent reservoir host (Hamer et al., 2010). The other two possible scenarios are dual-invasion (occurs when both the tick and the pathogen are detected simultaneously in a previously uninvaded site), and spirochete-first (this is when *B. burgdorferi* is already being circulated enzootically in an area by non-human biting vectors so that the invasion by the human-biting vector, *I. scapularis*, initiates the spread of the disease).

Aim 2: Characterize the Local and Regional Rodent Community and Its Role in Lyme Disease Spread and Persistence

The presence of suitable hosts for ticks would mean that they can get established after the invasion. The pathogen's establishment, on the other hand, is dependent on the distribution and abundance of competent reservoir hosts. Biodiversity affects the LD risk (Schmidt & Ostfeld, 2001). In an environment where there is high biodiversity, the relative abundance of *P. leucopus*, the principal reservoir host of the pathogen, is reduced (or diluted) because the chances of a tick encountering a reservoir host is reduced. This phenomenon is termed the dilution effect hypothesis. Conversely, rodent abundance relative to other rodent increases the chances of a

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questing tick encountering a generalist reservoir host like *P. leucopus*, that is, the amplification effect.

Prediction: Locations with high relative frequencies of *P. leucopus* would have higher tick burdens and *B. burgdorferi* infection rates. The presence of *B. burgdorferi*-infected rodents in areas where *I. scapularis* was undetected will indicate that the invasion was a spirochete-first scenario and not tick-first as previously predicted in Aim 1.

Approach: Small mammals were live-trapped in selected sites in northwestern North Carolina. For sites along the New River, corresponding sites in the western Piedmont were also selected. Captured rodents were identified and had ear tissue samples collected to test for *B. burgdorferi* using qPCR analysis. Ticks collected off the rodents were also identified and individually tested for *B. burgdorferi*. Nested PCR and DNA sequencing was performed on 10 randomly selected positive samples as a confirmatory test. Sampling sites were flagged during trapping to increase the chances of finding ticks that may otherwise not be found on the rodents.

Aim 3: Determine the Phenology and Patterns of Infection in the Invading *Ixodes scapularis* Ticks in The Northwestern North Carolina Corridor

The two-year life cycle of *I. scapularis* ticks in the Northeast is one that has the nymphs emerging before the larvae (called asynchronous phenology). This pattern ensures that the nymphs that may have been infected during the larval feeding time will infect newly recruited reservoir hosts before the feeding time of the larvae. That way, the tendency of larvae picking up the infection is increased so that the pathogen cycle can continue. The life cycle also has nymphal activity peaking in the summer months when human outdoor activities are high thereby increasing LD risk. The. *I. scapularis* ticks are likely to be invading from southwestern Virginia area. This means that the invading ticks are likely to be northeastern United States *I. scapularis*

tick populations. Since there are invading along the Appalachian Mountains, the climate may be providing suitable psychrophilic conditions necessary to maintain their two-year life cycle. Therefore, I predicted that the life cycle of the *I. scapularis* ticks in northwestern North Carolina would not only be comparable to the typical two-year life cycle as observed in the northeastern United States but also follow a similar seasonal variation in the life stages of the *I. scapularis* ticks. I also predicted that infection rates in *I. scapularis* would higher in adults than nymphs since the adults have undergone two feeding cycles as against one for the nymphs. Therefore, *B. burgdorferi* infected rates should be higher in mid-fall to early winter than in early spring to mid-summer.

Approach: Two tick hotspot sites were chosen and flagged monthly for 12 to 14 months. The collected ticks were identified. *Ixodes scapularis* ticks were categorized according to life stage for each month. A proportion of the *I. scapularis* ticks were sent to CDC for pathogen screening using the multiplex qPCR approach. The proportion of ticks of a particular life stage that was questing was calculated for each month to show the relative differences in tick emergence by month. In addition, stage-specific monthly activity was determined to understand the variations in yearly tick activity by life stage.

GENERAL METHODS

Tick flagging for **Aim 1** involved the establishment of 10-12 100-m transects. In this aim, I paired selected sites along the New River to corresponding latitudinally parallel sites along the western Piedmont. The New River sites ran from Pulaski County in Virginia to Burke County in North Carolina (NC) while the western Piedmont sites ran from Bedford County of Virginia to Iredell County of NC, creating a north to south gradient for comparison of *I. scapularis* distribution and *B. burgdorferi* infection rates. Ticks collected from the same transect were put

into the same vial containing 95% ethanol, labeled, and stored at -20°C in the laboratory for later analysis. The ticks were identified using appropriate keys and *I. scapularis* ticks were sent to CDC's Division of Vector-Borne Diseases in Fort Collins, Colorado for pathogen testing. The pathogen testing at CDC involved the use of multiplex PCR following the protocol as outlined in Graham et al. (2016, 2018). The sites in Aim 1 were categorized from north to south as UpperVA, LowerVA, BorderNC, UpperNC, MidNC, LowNC, and LowerNC. Using R software, tick density and *B. burgdorferi* infection rates was modeled with the New River and western Piedmont regions denoting the Site predictor, and north-to-south gradients denoting the Gradient predictor using generalized linear model under the negative binomial function for tick abundance and weighted logistic regression for *B. burgdorferi* infection rates.

Small mammal sampling for **Aim 2** was set up using Sherman collapsible traps (HB Sherman Traps Inc., Tallahassee, FL, USA) and Longworth traps (Anglian Lepidopterist Supplies, Norfolk, England) to help determine the distribution and abundance of rodents and their *B. burgdorferi spread* pattern. Traps were baited in the first year with sunflower seeds and later changed to peanut butter (Great Value Creamy Peanut Butter, Walmart Stores Inc., Bentonville, AR, USA) mixed with oatmeal (Great Value Old Fashioned Oats, Walmart Stores Inc., Bentonville, AR, USA) in succeeding years. The latter bait was used because it increased trapping success. Each trapped rodent was identified using standard morphometric measurements with the help of a field guide (Reid, 2006). *Borrelia burgdorferi* diagnostic test was performed for each sample using qPCR analysis as outlined in Tsao et al. (2004). I selected 20% of the positive samples and performed nested PCR, ran gel electrophoresis, and sent samples for Sanger sequencing. The molecular work was done at North Carolina State University following Tsao et al.'s (2004) protocols.

For **Aim 3**, I flagged for ticks in Alleghany and Ashe Counties for 12 to 14 months using twelve 100 m transect for each site every month. Laboratory protocols were similar to that of aim 1. Each sites' tick abundance for the month was determined and used to plot graphs of the proportion of ticks by life stage for each month and stage-specific tick activity by life stage for the year to understand their times of emergence and determine how similar the phenology pattern is to that of the Northeast.

CHAPTER II: INVESTIGATE THE NORTH-TO-SOUTH PATTERN OF *IXODES SCAPULARIS* INVASION AND THE ROLE OF THE NEW RIVER CORRIDOR IN FACILITATING THIS SPREAD

ABSTRACT

Lyme disease (LD) cases in Virginia are expanding southward into North Carolina. Recent studies have shown that the disease is following the southern Appalachian and Piedmont Mountain ranges, possibly because they provide suitable psychrophilic environments comparable to what is experienced in the Northeast. Although LD cases are rising in the New River Valley, there is little information on the vector and pathogen components associated with the rising cases in this area. Also, the role of geographic features along the path of the southward descent that may be facilitating the spread of the disease is unknown. This study investigated the role of the New River as a potential corridor that may be facilitating Lyme disease spread from southwestern Virginia to northwestern North Carolina. *Ixodes scapularis* ticks were collected along the New River from Pulaski County in Virginia to Burke County in North Carolina, and at corresponding sites along the western Piedmont region from Bedford County in Virginia to Iredell County in North Carolina. A representative number of the collected ticks were screened for *Borrelia burgdorferi* to determine infection rates. The results indicated that Alleghany County in North Carolina and its neighboring Ashe County were the current spots of peaked *I. scapularis* abundance and *B. burgdorferi* infection. The results indicated a hump-shaped north-to-south distribution of the *I. scapularis* ticks as well as their *B. burgdorferi* infection rates. Non-infected *I. scapularis* ticks were detected further south than *B. burgdorferi*-infected *I. scapularis*, suggesting that the tick-first invasion mechanism is occurring. Higher densities of *I. scapularis* nymphs were found in the New River region compared to western Piedmont and had spread

further south along the New River compared to western Piedmont. Overall, the New River is facilitating the southward spread of *I. scapularis* and *B. burgdorferi* into western North Carolina.

INTRODUCTION

Lyme Disease Invasion

Lyme disease (LD) is a multisystemic, inflammatory disease that is caused by the spirochete, *Borrelia burgdorferi,* and transmitted by *Ixodes scapularis* ticks. In the United States, it is reported to be spreading into areas beyond its original endemic foci (Schwartz et al., 2017). The invasion of the disease into new areas is primarily due to the spread of its vector, *Ixodes scapularis*. This is supported by a passive tick surveillance study in Maine (from1989 to 2006 when Lyme disease cases in the state were now emerging). Tick surveillance was based on submissions of ticks by residents. The tick submissions were classified into counties. The results of the study showed that counties where *I. scapularis* nymphs were submitted the more had higher number of Lyme disease cases (Rand et al., 2007). Recent data suggest that *I. scapularis*, over the past two decades, has expanded from its northeastern focus northward into upstate New York, Vermont, New Hampshire, and northern Maine; westward across Pennsylvania, eastern Ohio, and New York; and south- and southwestward into West Virginia, Virginia, and North Carolina (Eisen et al., 2016).

Transmission Mechanisms of Lyme Disease

The mechanism of Lyme disease invasion was suggested to follow any of the following three scenarios: "tick-first," "dual-invasion," or "spirochete-first". Whichever scenario occurs depends on whether the vector and/or pathogen is at the leading edge of the invasion wave (Hamer et al., 2010).

Hamer et al. (2010) studied the mechanisms of *I. scapularis* invasion from the Upper Peninsula of Michigan into the Lower Peninsula area. Before their study from 2004 to 2008 (in May and June of each year when larva, nymph, and adult were active), established populations of *I. scapularis* were restricted to Menominee County of the Upper Peninsula, adjacent to endemic foci in Wisconsin. This afforded them the opportunity to study the invasion mechanism of *I. scapularis* and/or *B. burgdorferi* into Michigan's Lower Peninsula in real time (Hamer et al., 2010). They tested the three invasion scenarios by assessing (1) ticks attached to mammals and birds, (2) ticks on the forest floor collected by dragging, and (3) mammal and bird community (by live-trapping and mist-netting, respectively) within the Lower Peninsula area where no *I. scapularis* ticks had been detected yet. They had two sampling transects that extended from the area of no *I. scapularis* detection in the southwestern end of Michigan's Lower Peninsula toward the north. One of the transects extended up north along Lake Michigan (called the coastal transect with sites C1 to C4). The other transect extended inland (called inland transect with sites I1 to I4) in the northeast direction. Ticks were collected off animals and by dragging. They also collected ear biopsies from the mammals they trapped. All samples collected were identified and screened for *B. burgdorferi*. They observed all the three mechanisms of tick and/or *B. burgdorferi* invasion at different sites.

At one site, Hamer et al. (2010) detected no *B. burgdorferi* despite *I. scapularis* infestation on mice in the last two years of the study, supporting the "tick first" scenario. The "tick-first" hypothesis suggests that the tick vector leads the invasion before the slower secondary invasion by mammalian or avian hosts who introduce the pathogen (Hamer et al., 2010). Specifically, it is proposed that *I. scapularis* ticks invade new areas via long range dispersal of adult *I. scapularis* by white-tailed deer. The white-tailed deer is a non-reservoir host

(i.e., does not harbor *B. burgdorferi*), hence they only introduce non-infected ticks. The deer is also the host on which the adult *I. scapularis* mate (reason behind deer being termed as reproductive hosts). The later invasion by the pathogen is through the attachment of infected ticks to dispersing reservoir hosts such as certain rodents or birds (Hamer et al., 2010). However, rodents are the main agents of transmission of *B. burgdorferi* (Dumas et al., 2022).

At another site, Hamer et al. (2010) observed the dual-invasion mechanism occurring when they detected *B. burgdorferi* in *I. scapularis* in an area where they had previously found no *I. scapularis* nor *B. burgdorferi*. In that case, *I. scapularis* and *B. burgdorferi* got established concurrently. The "dual-invasion hypothesis" suggests that infected immature *I. scapularis* are locally dispersed or transported by reservoir hosts into new areas (Hamer et al., 2010). In the new area, the infected ticks detach from their reservoir hosts, molt, and seek for their next bloodmeal, infecting potential reservoir hosts in the new area with *B. burgdorferi* during the blood feeding process. This begins an enzootic (relating to non-human animals) transmission cycle in the new area if the reservoir host community is stable. Under the dual-invasion scenario, *B. burgdorferi* enzootic transmission cycle will become established at the edge of the invasion wave because the vector, the competent host, and the pathogen will co-occur (Hamer et al., 2010).

Yet again, Hamer et al. (2010) detected, in two of their four study sites, *B. burgdorferi* in *Dermacentor variabilis* (from rodent), *I. texanus* (from rodents), *I. cookei* (from rodents), *I. dentatus* (mainly from birds), *I. marxi* (from rodents), and *Haemaphysalis leporispalustris* (mainly from birds) before detecting the pathogen in the human-biting, *I. scapularis* in later years. They concluded that (1) it was either the enzootic transmission of the spirochete was maintained by ticks other than *I. scapularis,* or (2) that *I. scapularis* densities at those sites were

too low to be capable of maintaining the spirochete. This observation exemplified the "spirochete-first" scenario. The "spirochete-first" hypothesis suggests that the *B. burgdorferi* spirochete is already present in the area and is being circulated enzootically by cryptic vectors (e.g., *I. dentatus, I. texanus,* and *I. neotomae*). Cryptic vectors are non-human biting vectors (Telford and Spielman, 1989), probably because they feed particularly on non-human hosts, or do not quest above-ground, hence, hardly encounter humans. The later arrival of the human-biting vector (termed "bridging vector"), *I. scapularis*, amplifies the enzootic transmission but, even more importantly, establishes a zoonotic spill-over system from the rodents to humans (Hamer et al., 2010).

The Role of Topographic Features in Lyme Disease Spread

Patterns and mechanisms responsible for the spread of *B. burgdorferi* across landscapes are poorly understood. *Ixodes scapularis* by itself does not move far from the point of detachment from host or place of hatching or molting (Khatchikian et al., 2012). Rather, their spatial movement is driven by the movement of their hosts. Host movement can either be short- or long-ranged. Small-sized terrestrial vertebrates (such as rodents) have small home ranges and do not move far away from their natal site (Keane, 1990). Short night travels by adult rodents with established home ranges can also disperse ticks over short ranges (Garman et al., 1993). On the other hand, large terrestrial vertebrates, such as deer, are thought to be responsible for the long-range dispersal of *I. scapularis*. For example, the white-tailed deer (*Odocoileus virginianus*) can transport *I. scapularis* 23-45 km in 31 to 356 hours (i.e., 21 – 18.6 km/day) (Nelson et al., 2004). Many studies have associated the dispersal of *I. scapularis* with deer population increase and dispersal (e.g., Barbour & Fish, 1993; Daniels et al., 1993; Rand et al., 2003; Spielman et al.,

1985; Wilson et al., 1988). Birds, like mammals, can also spread ticks and *B. burgdorferi* over short, medium, and long ranges (Sparagano et al., 2015; Wilhelmsson et al., 2020).

Aside from deer being capable of translocating ticks over long distances, their movement across the landscape has been described as directional and along river corridors (Kilgo et al., 1996; Sparrowe & Springer, 1970). Tick burdens on deer have an association with river valleys. Cortinas & Kitron (2006) mentioned that forests along rivers are continuous, hence providing corridors for the dispersal and migration of animals, and possibly a channel for the spread of *I. scapularis*. Kitron et al. (1992), using hunter-harvested deer from a 3-year study in Ogle County in northwestern Illinois, found that the proximity of infested deer to the Rock River increased over three years. They also found that tick-infested deer were significantly closer to the river than non-infested deer. Another study in the Missouri River area revealed that white-tailed deer used the river valley as a travel corridor and most yearling males that dispersed stayed in the river valley and established adult home ranges in woodlots adjacent to the river (Clements et al., 2011; Long et al., 2010). A similar study in Maine (from 1989 – 2006) showed that in inland areas, *I. scapularis* densities first increased along major river valleys before moving further inland (Rand et al., 2007). The dispersal or permanent movement of animal hosts away from original sites of dwelling could drive ecological processes including pathogen transmission and vector range expansion (Nathan, 2001).

Southern Spread Patterns of *Ixodes scapularis* and *Borrelia burgdorferi*

Lyme disease cases in Virginia have expanded beyond the endemic foci in the northern parts of the state toward the south with a shift in geographic spread pattern from the Coastal Plain to the Appalachian Mountain and Piedmont regions (Brinkerhoff et al., 2014). A spatio-temporal cluster analysis of Lyme disease cases in Virginia and North Carolina, from 2000 to

2014, showed that Lyme disease cases had significantly expanded toward southwestern Virginia along the Appalachian Mountains approaching northwestern North Carolina (Lantos et al., 2015; Figure 5). In 2009, confirmed and probable Lyme disease cases in North Carolina were sparsely distributed with more cases in the Coastal Plain than the Piedmont and Mountain regions (Figure 6). A decade later, a clear geographic shift from a relatively more eastern distribution to the current distribution with most human Lyme disease cases now concentrated in the northwestern parts of the state occurred (Figure 6).

Figure 5. Spatio-temporal cluster analysis of Lyme disease cases along the Appalachian Mountains of Virginia and North Carolina.
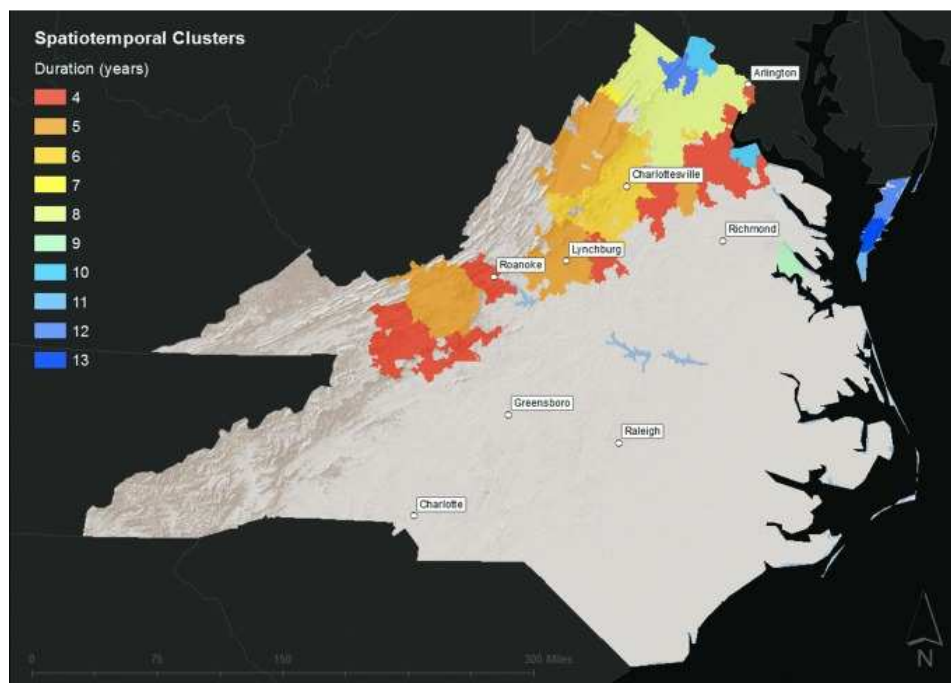
Figure 6. Maps of 2009 and 2019 of LD cases in North Carolina showing a shift in the geographical distribution of confirmed and probable LD cases from a more eastern-central distribution to a clear cluster in northwestern counties of North Carolina (NC Division of Public Health, 2019).
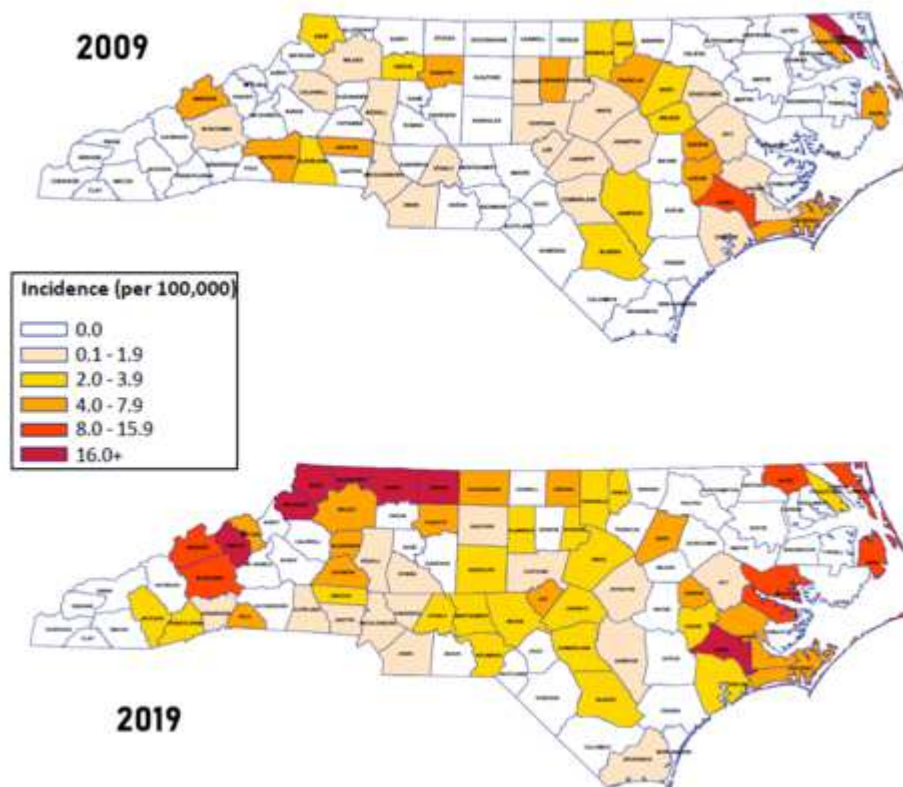
The initial concentration of Lyme disease cases in 2009 to the Coastal Plain in North Carolina was supported by a statewide surveillance on *I. scapularis* infesting deer across North Carolina (from September to December during 1983 to 1987) (Apperson et al., 1993). Apperson et al. (1993) found highest tick infestation rates in the Coastal Plain and absent or uncommon on deer harvested in the Appalachian Mountains and Piedmont regions of North Carolina.

The later shift in human case distribution suggested a change in the underlying tick vector distribution or an introduction of the pathogen. For that purpose, the North Carolina Department

of Health and Human Services (NCDHHS) commissioned several university partners to conduct active tick surveillance to better map *I. scapularis* distribution throughout the state. Teague (2018) conducted a series of tick flagging surveys in several state parks going from southern Virginia (north) through northern NC (Central) and down to the Charlotte area (south). His finding showed a clear north-to-south decrease in tick densities and in their respective *B. burgdorferi* infection rates, with southernmost ticks and infections observed in Hanging Rock State Park but not in the southern Lake Norman State Park (Figure 7A). Based on collection from hunter-harvested deer from a northern and a southern deer processing stations (where deer identification was limited to county level), Teague (2018) similarly observed a clear a north-to-south gradient in both *I. scapularis* density and *B. burgdorferi* infection rates (Figure 7B).

Figure 7. North-to-south gradient of *I. scapularis* (by flagging (A) and deer hunting (B) and their infection with *B. burgdorferi* in the Piedmont region of North Carolina (Teague, 2018).
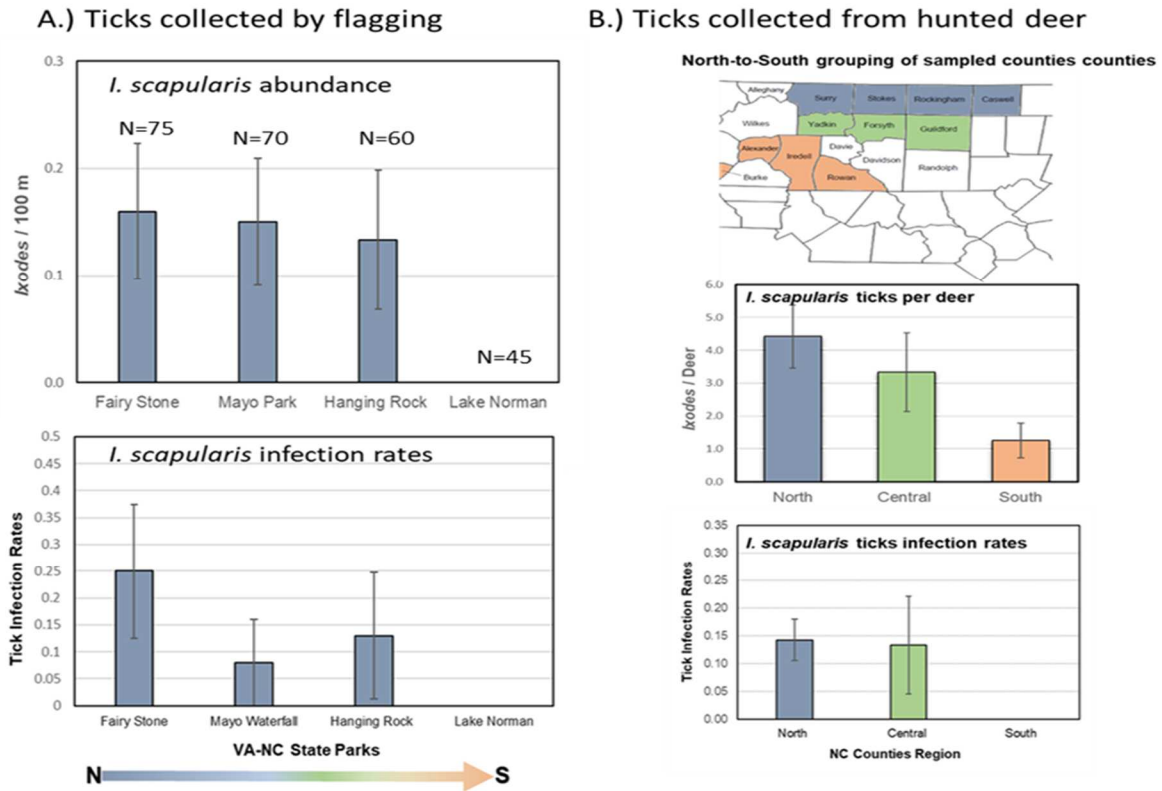
Teague's (2018) study focused on the western Piedmont area of North Carolina and southern Virginia but did not cover the northwestern counties with the highest human case incidence (Fig. 2.2). The general goal of this dissertation was to better characterize *I. scapularis* distribution and *B. burgdorferi* infection patterns in ticks and rodents within northwestern North Carolina and to try to elucidate the underlying ecological processes. In this chapter, specifically, I wanted to determine whether there are certain geographic features (such as a river valley) that may be facilitating the southward spread of *I. scapularis* and the *B. burgdorferi* pathogen into western North Carolina.

Study Aim

Lyme disease cases in the New River Valley area of southwestern Virginia are rising (Rife, 2017). The New River which flows from North Carolina through Virginia appears to link the high Lyme disease incidence counties in Virginia with the high incidence counties in North Carolina. This suggests that the New River Valley may be acting as a corridor that is facilitating *I. scapularis* and *B. burgdorferi* spread, possibly through the movements of deer and/or carrier rodents. This study aimed to investigate the role of the New River as a corridor that is facilitating the spread of *I. scapularis* and *B. burgdorferi* from southwestern Virginia to northwestern North Carolina.

Hypotheses

The New River is a geographic feature that connects both areas of high incidence of Lyme disease in southwestern Virginia and northwestern North Carolina. Therefore, I hypothesized that *I. scapularis* and *B. burgdorferi* are spreading from southwestern Virginia to northwestern North Carolina with the New River acting as a corridor for their hosts, hence, facilitating their southward spread through that route.

Approach

To test this hypothesis, I used a "natural experiment" approach, in which I sampled sites along a north-to-south gradient going from Virginia to North Carolina: one along the New River Valley and a parallel one along the western Piedmont (approximately 15-20 miles east of the New River valley). These paired sites were latitudinally parallel, allowing me to compare tick density and tick infection rates between parallel sites in the New River versus the reference site

in the western Piedmont. The pattern of the distribution of the ticks and their infection rates would indicate the mode of invasion.

Predictions

Based on the hypothesis that the New River acts as a putative spread corridor for the tick and the infection they carry, I made the following predictions:

1. New River versus western Piedmont pattern: I predicted that *I. scapularis* densities and *B. burgdorferi* infection rates will be higher along the New River Valley compared to the western Piedmont.

2. North-to-south pattern: I predicted that *I. scapularis* tick density and *B. burgdorferi* infection rates would decrease from southwestern Virginia (the hypothesized source of vector and pathogen invasion) towards the south in both the New River and western Piedmont regions. Since the New River is facilitating the spread, I predicted that *I. scapularis* ticks and their *B. burgdorferi* infection would be detected further south along the New River gradient compared to the western Piedmont region.

3. Differential north-to-south pattern between the New River and the western Piedmont: I predicted that although the density of *I. scapularis* would decrease following a north-to-south gradient, both along the New River and western Piedmont; *I. scapularis* tick density and *B. burgdorferi* prevalence would be more along the New River compared to the western Piedmont region.

METHODS

Sampling Sites

Sampling sites were categorized into two primary regions, those sites along the New River and those along the adjacent western Piedmont region. The western Piedmont transect sites had the northmost site in Bedford County, Virginia and the southmost site in Iredell County, North Carolina (Fig. 8). The New River transect sites, on the other hand, ran from Pulaski County, Virginia to Burke County, North Carolina (Fig. 8). The sites in Caldwell and Burke Counties were not along the New River. They were located further south beyond the Watauga County sites (Figure 8). I wanted to establish sites of no *I. scapularis*, hence needed to extend the transect further south beyond Boone. I grouped the site(s) along the New River and corresponding site(s) in western Piedmont into sampling tiers (Table 1). Sites in the New River and corresponding western Piedmont were grouped together as belonging to the same tier. When two sites were close enough to each other and were in the same county, I considered them as belonging to the same tier. The sites at the border were left as single sites because they sat at the edge of the border between Virginia and North Carolina, serving as ports of entry for the tick and pathogen. Therefore, they were likely to provide vital information about the tick and pathogen invasion if left to stand alone. Moreover, spreading the sites out into many tiers provided a finer spatial resolution than when aggregated. This also provided a better statistical power. Parallel sites were about 20 miles apart. Those that exceeded 20 miles were because they were the closest suitable sites. There were seven distinct bands: Upper Virginia (UpperVA), Lower Virginia (LowerVA), Border between North Carolina and Virginia (BorderNC), Upper North Carolina (UpperNC), Mid North Carolina (MidNC), Low North Carolina (LowNC), and Lower North Carolina (LowerNC) (Table 2.1).

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Figure 8. Tick sampling sites in the New River (blue) and western Piedmont (red) regions. Insert in the upper left corner is the location of the sampled counties in Virginia and North Carolina. New River sites comprised of: Claytor Lake State Park (CLPSP), New River Trail State Parks in Hiwassee (NRTSPH), New River Trail State Park in Byllesby, (NRTSPB), New River State Park in Alleghany (NRA), New River State Park on Hwy 221 (NR221), New River State Park, Wagoner access (NRW), Clawson-Burnley Park (CLBP), Rocky Knob Park (RKP), Mortimer (MTMR), Tuttle Educational State Forest (TTESF), and Lake James State Park (LJSP). Western Piedmont sites were Smith Mountain Lake State Park (SMLSP), Rocky Knob Recreational Area (RKRA): Fairy Stone State Park (FRSSP), Cumberland Knob, (CBK): Camp Raven Knob (CRK), Stone Mountain State Park (SMSP), Rendezvous State Forest (RSF), Smithey's Creek Wildlife Management Area (SCWMA): Rocky Face Mountain Recreational Area (RKFM), Dusty Ridge Park (DRP), and Lake Norman State Park (LKNSP). The "New River transect" extended beyond the New River in Watauga County to include MTMR, TTESF and LKSP
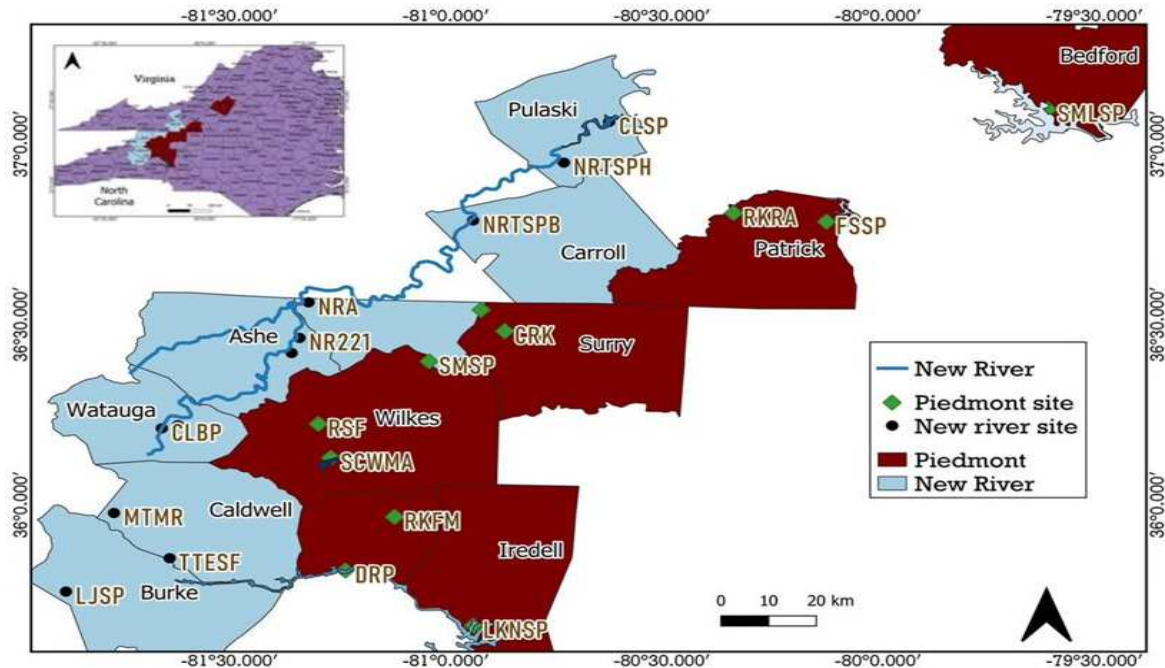
Table 1. Description of the seven tick sampling tiers. The ten site pairs depicted above (Fig. 2.4) were aggregated into seven regional tiers going from north to south. Names of the sites as it corresponds to Figure 8 above, provides brief site features. Sites were aggregated based on their proximity to each other and/or belonging to same county.

Sampling tiers	New River Site	Western Piedmont Site	Site features
Upper Virginia (UpperVA)	Claytor Lake State Park	Smith Mountain Lake State Park	CLSP: Mature oak-hickory-poplar forest; Hardwood, pines, and coves
	New River Trail State Park, Hiwassee		
Lower Virginia (LowerVA)	New River Trail State Park, Byllesby	Rocky Knob Recreational Area	Poplar, magnolia, and oak forest; Oak-Hickory Forest; Pine Forest
		Fairy Stone State Park	
Border of North Carolina (BorderNC)	New River State Park, Alleghany Access	Cumberland Knob Recreational Area	Oak-Hickory Forest, pines, shrubs
Upper North Carolina (UpperNC)	New River State Park, 221 Access	Camp Raven Knob	Oak-Hickory Forest, pines, shrubs; Scarlet, oak, maple, hickory
	New River State Park, Wagoner Access	Stone Mountain State Park	
Mid of North Carolina (MidNC)	Clawson-Burnley Park	Rendezvous State Forest	Acidic cove and montane seeps; Mountain hardwoods; Sycamore; poplar, oak-hickory
	Rocky Knob Mountain Bike Park	Smithey's Creek Wildlife Area	
Low North Carolina (LowNC)	Mortimer	Rocky Face Mountain Rec. Area	Oak-Hickory, Pine; Acidic cove; Montane Red-Cedar hardwood
	Tuttle Educational State Forest	Dusty Ridge Park	
Lower North Carolina (LowerNC)	Lake James State Park, Paddy's creek	Lake Norman State Park; Love Valley	Mixed hardwood, pine, hemlock; Oak-hickory, sweet gum, pine

Tick Sampling

Two commonly used approaches for sampling ticks, particularly *I, scapularis,* are flagging and dragging (Rulison et al., 2013). Flagging involves sweeping a flannel or cotton cloth that is attached like a flag to a hand-held pole or dowel (Fig. 9) through leaf litter or vegetation (Rulison et al., 2013). Dragging, on the other hand, involves pulling the equivalent material (i.e., flannel or cotton) behind the investigator, typically by a rope attached to a basal pole, with the pole horizontal and perpendicular to the direction of movement (Rulison et al., 2013). The idea behind the use of a flannel cloth for flagging is that the flannel's furry nature mimics the fur of any passing mammal within the landscape, causing the questing ticks to latch onto the cloth. Flagging was used because it was more appropriate for this study due to the nature of the topography of the landscape which was not flat throughout and had lots of shrubs and rocky outcrops which would make dragging difficult and less efficient. Flags were made of white flannel cloth of 1x1 m attached to a 1.5 m long pole (Fig. 9). Each transect flagged was 100-m long. After every 10-20 m distance of flagging, the flags were carefully inspected on both sides for any attached tick. At least 7 transects were established for flagging at each site (Table 2). Along each transect, the flag was swept over the top of bushes, leaf litter, rocks, or low vegetation. The furriness of the flannel also provides the ticks with tiny structures to hold on to, allowing easy capture. The white color of the cloth makes it easier to detect attached ticks. Each transect was georeferenced using a hand-held GPS (Garmin GPSMAP 64sc). The relative humidity and temperature at each site were recorded using a mini-temperature humidity meter (UNI-T, UT333). Tick flagging was done on rain-free days in the late mornings (when the morning dew had dried out and the weather is not too hot to prevent questing) or late afternoons to avoid the hottest and least humid times of the day (Schulze et al., 1998). Ticks that had latched

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onto the flannel cloth were collected using pointy tweezers and placed in labeled 1.5 mL

Eppendorf tubes filled with 95% ethanol, one tube per transect. Ninety-five percent ethanol kills

and preserves the ticks. In the lab, the ticks were temporarily stored in a -20°C freezer. The ticks

were later identified with the aid of a dissecting microscope and tick identification guides (e.g.,

Coley, 2015; Durden & Keirans, 1996; Keirans & Litwak, 1989). Non-blood-fed ticks were

placed in 1.5 mL capped vials and shipped to CDC for infection screening.

Figure 9. A typical flag composed of a flannel cloth attached to a pole (left) and a demonstration of how flagging was done in one of the field sites (right).

Testing Procedure of *Ixodes scapularis* for *Borrelia burgdorferi* Infection Done by Centers for Disease Control and Prevention (CDC) Personnel.

Confirmed *I. scapularis* nymph and adult tick samples (non-blood fed) were sent to the

CDC for testing. This was made possible due to an ongoing collaboration between the

Communicable Disease Branch of the NC Division of Public Health and the CDC's Division of

Vector-Borne Diseases. The DNA of each tick sent to the CDC was extracted using a modified

version of the protocol for DNA extraction from field-collected ticks (Graham et al., 2016,

2018). The DNA extraction process involved first, homogenizing the ticks using 545 mg 2.0 mm

yttria-stabilized zirconium oxide beads in a Qiagen 470 µL lysis mix comprised of buffer ATL, 20 µl proteinase K, and 0.5% DX anti-foaming reagent (Graham et al., 2018). The tick sample was then disrupted for 2 minutes using a Biospec Mini-Beadbeater-96 before incubating for approximately 10-12 minutes at 56ºC (Graham et al., 2018). After incubation, samples were centrifuged for 30 seconds at 1000 x g, and then 200 µl of each sample was processed using the Qiagen (QIAcube HT) automated nucleic acid isolation system as well as the Qiagen-Cador Pathogen 96 kit (Graham et al., 2018). Samples were then combined with Qiagen VXL mixture and binding buffer ACB to 650 µl and subjected to 3-minute vacuuming at 35 kPa (Graham et al., 2018). After vacuuming was complete, the column was washed using 600 µl of an AW1 buffer and vacuumed for 2 minutes at 35 kPa29. DNA was finally eluted by the addition of 100 µl AVE buffer to the column, incubated for 2 minutes, then vacuumed for 6 minutes at 55 kPa (Graham et al., 2018). Each extract was then screened for *Borrelia burgdorferi*, *B. mayonii*, *B. miyamotoi*, *Borrelia* sp., *Anaplasma phagocytophilum*, and *Babesia microti* using a pair of multiplex real-time PCR assays (Hojgaard et al., 2014). Modifications included the use of a pan-*Borrelia* 16S target in place of the *B. burgdorferi* "gB31" target (Parola et al., 2011). Samples that tested positive for *Borrelia* underwent further testing to detect and distinguish *B. miyamotoi*, *B. burgdorferi* s.s., and *B. mayonii* using a duplex real-time PCR assay targeting the oppA2 gene in *B. burgdorferi* s.s. and *B. mayonii* (Pritt et al., 2016).

Permits and Collaborations

I obtained a Virginia Scientific Collection, Research, and Survey Permit (#062250) from the Virginia Department of Game and Inland Fisheries to conduct surveys in Bedford, Pulaski, Carroll, and Patrick Counties. For sampling in North Carolina, a permit (19-SC01252, 20-SC01252, and 21-SC01252) was obtained from the North Carolina Wildlife Resources

Commission as well as the NC Division of State Parks to sample ticks by flagging on state-owned lands throughout the sampling period. Approval to trap rodents was obtained from University of North Carolina Institutional Animal Care and Use Committee (IACUC; #18-002, and 2-1006).

Table 2. Flagged sites and sampling effort during the Summer and Winter seasons.

Site	Summer transects			Winter transects	
	Year 1	Year 2	Year 3	Year 1	Year 2
Camp Raven Knob/Sparta	30	9	-	12	-
Claytor Lake	12	-	-	-	-
New River Trail State Park at Hiwassee	14	-	-	12	-
Cumberland Knob	12	8	-	-	-
New River Trail State Park at Byllesby	16		-	12	-
New River Wagoner Access	14	10	-	12	-
New River State Park, 221 Access	12	8	-	12	-
New River State Park, Alleghany Access	16	8	-	12	-
Rocky Knob Park	13	-	-	-	-
Clawson-Burnley Park	17	12	10	12	-
Rocky Knob Recreational Area	14	-	-	-	-
Fairy Stone State Park	10	-	-	12	-
Smith Mountain Lake state Park	12	-	-	12	-
Smithey's Creek Wildlife Recreational Area	17	12	-	18	-
Rendezvous State Forest	12	-	-	-	12
Stone Mountain State Park	17	11	10	-	11
Lake James State Park	-	12	12	-	12
Lake Norman State Park	12	7	12	-	12
Mortimer	-	-	12	-	12
Tuttle Educational State Forest	-	-	12	-	12
Rocky Face Mountain State Park	-	-	12	-	12
Dusty Ridge Park	-	-	12	-	12

Data Analysis

Bar graphs were used as describe the data, showing the differences in *I. scapularis* tick densities and infection rates between the New River and corresponding western Piedmont region for each of the seven banding locations along the north-south gradient. The "ggplot2", "tidyverse" and "dplyr" packages in the R software were used to generate the plots. The Mann-Whitney U (Wilcoxon) test, which is a non-parametric test was used to determine significant differences between two samples, was used to compare the differences between tick densities and infection rates for each pair (i.e., a New River and a western Piedmont site) in a tier. Tick density was calculated as the number of ticks for an area divided by the total number of transects for that area or site. Infection prevalence is the number of infected ticks divided by the number of ticks tested. Density of infected nymphs was determined by calculating the product of the infection prevalence and the tick density.

Generalized linear models (GLM) under the negative binomial family, which is used for over-dispersed (i.e., where the conditional variance is greater than the conditional mean) count data, were used to address the relationship between the different life stages of *I. scapularis* (dependent variable) and the following independent variables: Region (as New River or Piedmont) and Gradient (as north-to-south gradient). The sites were grouped as belonging to one of the following based on the geographic tiers: upper Virginia (UpperVA), Lower Virginia (LowerVA), Border of North Carolina (BorderNC), Upper North Carolina (UpperNC), Mid North Carolina (MidNC), Low North Carolina (LowNC), and Lower North Carolina (LowerNC). The tiers were numerated from 0 to 6 (i.e., UpperVA to LowerNC, respectively). Weighted logistic regression, which is used for binary responses but also for fractional or proportional data, was used to analyze *B. burgdorferi* infection rate data in *I. scapularis* against the covariates

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Gradient and 'Region'. When there was significant interaction term, I ran the independent models by 'Region' using zero intercept since biologically, 'Region' and 'Gradient' cannot be meaningful when there exist no ticks. Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) were used to select the best model for each life stage regression model. The candidate models included whether a second-order polynomial and/or an interaction between any of these parameters was best in predicting the observed outcome. The model with the least AIC and BIC that had meaningful biological interpretation was selected as the best model. The number of transects and number of tested ticks were used as weights to standardize differences in the numbers of transects and number of tested nymphs used in the GLM and the logistic model, respectively. The "MASS" package in R software was used to perform the regression models. The Z-value is the statistic generated in Generalized Linear Models (GLM). Positive Z-values result from values that are above the mean, and negative Z-values are from values below the mean.

RESULTS

General

The three-year data collection, from June 2018 to July 2021, involved a total of 660 transects of 100-m each and yielded a total of 3,137 ixodid ticks. About 96% of these ticks (n = 3,019) were *Ixodes scapularis* (Table 3). The remaining ticks comprised 24 (8 males:16 females) American dog ticks (*Dermacentor variabilis*), 50 (7 larvae; 39 nymphs; 1 male; 3 females) Lone star ticks (*Amblyomma americanum*), 10 (1 larva, 1 nymph, 6 males and 2 femlaes) Brown dog ticks (*Rhipicephalus sanguineus*), and 34 (3 larvae, 8 nymphs, and 23 adults) Asian long-horned ticks (*Haemaphysalis longicornis*) (Table 3).

Out of the 3,019 *I. scapularis* ticks, 2,200 (70.13%) were larvae, 768 (25.44%) were nymphs and the remainder (5.33%) were adults (Table 2.4). For all the three active life stages sampled, the New River region recorded more *I. scapularis* than western Piedmont, comprising 86.65% (2,616) of the total *I. scapularis* ticks collected (Table 4). Overall, the New River had about 5 times higher *I. scapularis* nymph density (i.e., 2.86 ± 0.47 [SE] nymphs/100 m) compared to western Piedmont (i.e., 0.56 ± 0.09[SE] nymphs/100 m) The UpperVA tier, which was the northernmost tier in Virginia, had the highest number of *I. scapularis* larvae and adults (Table 4). The North Carolina counties that share a border with Virginia to the north (i.e., Alleghany – BorderNC, and Ashe – UpperNC) recorded the highest abundance of *I. scapularis* nymphs both in the New River and western Piedmont regions (Table 4). No adult *I. scapularis* were collected in the southernmost sites

Table 3. Relative abundances and relative percentages (in parenthesis) of the distribution of the different tick species that were collected throughout the study.

Species	Common name	Abundance (%)
Amblyomma americanum	Lone star tick	50 (1.59)
Dermacentor variabilis	American dog tick	24 (0.77)
Haemaphysalis longicornis	Asian long-horned tick	34 (1.08)
Ixodes scapularis	Blacklegged tick	3019 (96.24)
Rhipicephalus sanguineus	Brown dog tick	10 (0.32)

Table 4. Summary of total numbers of Ixodes scapularis ticks sampled from north (UpperVA) to south (LowerNC) in the New River and western Piedmont regions, by life stage.

Tier	Larva	Nymph	Adult	Larva		Nymph		Adult	
				NR	PD	NR	PD	NR	PD
UpperVA	900	89	73	900	0	88	1	73	0
LowerVA	166	37	14	55	111	19	18	10	4
BorderNC	211	419	24	150	61	378	41	24	-
UpperNC	85	166	40	73	12	121	45	39	1
MidNC	693	38	11	601	92	28	10	6	5
LowNC	1	11	3	0	1	11	0	2	1
LowerNC	36	2	0	36	0	2	0	0	0
Total	2200	768	161	1815	277	647	115	154	11

Ixodes scapularis Nymph Abundance Model

The best model for the *I. scapularis* nymphs indicated significant effects of 'Region' (i.e., New River/western Piedmont), a second order polynomial effect of 'Gradient' (i.e., north-to-south), and a significant 'Region' by 'Gradient' interaction (Table 5), indicating that the distribution *I. scapularis* nymph density from UpperVA to LowerNC was hump-shaped (Table 6; Figure 2.6). With reference to the New River region, *I. scapularis* nymph density at the western Piedmont region decreased significantly by 2.8 ($p < 0.0001$; Table 6) without the effect of gradient. The 'Gradient' effect indicated a significant increase of 1.6 *I. scapularis* nymphs from the north (UpperVA) up to a point along the 'Gradient' and then a significant decline by

0.52 nymphs towards the southmost tier (p < 0.0001; Table 6), without the effect of the 'Region'. The significant interaction term indicated that the slopes of the hump in the two 'Regions' with respect to the 'Gradient' were significantly different (Table 6). To understand the interaction better, models for the two regions were run separately. The rate of increase in the number of *I. scapularis* nymphs from UpperVA towards the crest of the hump was higher for the New River region (2.3) compared to the western Piedmont [0.9] (Tables 7 & 8). The slope of decline in *I. scapularis* nymphs from Upper VA to LowerNC was steeper for the New River region (0.6) compared to the western Piedmont (0.3) (Tables 7 and 8). The graph and heat map suggests that the peak of the hump is occurring around MidNC (Figure 10).

The spread of *I. scapularis* nymphs extended further south along the New River than at the western Piedmont region (Figure 10). The farthest extent in the New River region at which *I. scapularis* nymphs were found was in LowNC (i.e., Caldwell County) whereas that of the western Piedmont region ended at MidNC (i.e., Wilkes County) [Figure 10].

Table 5. Illustrative list of tested models for nymph density estimates AIC and BIC, and the R^2 (in percentage). The model used in prediction is indicated in boldface.

Regression models for density estimates	AIC	BIC	R^2 (%)
Region + Gradient	1176.63	1193.02	36.29
Region + Gradient + (Site:Gradient)	1176.82	1197.31	36.29
Region + Gradient + Gradient^2	1078.69	1099.17	54.48
Region + Gradient + (Site:Gradient)+ Gradient^2	**1072.67**	**1097.24**	**55.76**
Region + Gradient+(SiteGradient) + Gradient^2 + (SiteGradient^2)	1073.68	1102.35	55.90

Table 6. Negative binomial regression of the best model that predicted the effect of the explanatory variables on *I. scapularis* nymphal density. The northernmost site, UpperVA, and New River were the references used in the model to represent the variables Gradient and Region, respectively.

glm.nb (formula = Nymph ~ Gradient + Region + (Region:Gradient) + Gradient^2, weights = Transect number.)				
Parameter	**Estimate**	**Std. Error**	**z value**	**Pr (>|z|)**
(Intercept)	1.032	0.245	4.216	< 0.0001
Piedmont	-2.777	0.440	-6.304	< 0.0001
North-South gradient	1.623	0.237	6.859	< 0.0001
(North-South gradient)^2	-0.525	0.056	-9.363	< 0.0001
Region: N_S gradient	0.461	0.160	2.876	0.00403
AIC = 55.76			D^2 (Pseudo R^2) = 55.76%	

Figure 10. Distribution of *I. scapularis* nymph density (± standard error) (left) and heat map (right) at New River and corresponding western Piedmont regions from north to south. Numbers on the bars indicate the number of transects. Each transect = 100-m. Asterisk(s) indicate statistically significant differences.

Table 7. Negative binomial model of *I. scapularis* nymphs in relation to the north-to-south gradient in the western Piedmont Region alone. Intercept was assumed to be 0 because there is no Gradient without ticks.

glm.nb (formula = Nymph ~ 0 + Gradient + Gradient^2, weights = Transect number.)				
Parameter	**Estimate**	**Std. Error**	**z value**	**Pr (>|z|)**
North-South gradient	0.713	0.244	2.9419	0.00351
(North-South gradient)^2	-0.314	0.069	-4.563	<0.0001

Table 8. Negative binomial model of *I. scapularis* nymphs in relation to the north-to-south gradient in the New River Region alone. Intercept was assumed to be 0 because there is no Gradient without ticks.

glm.nb (formula = Nymph ~ 0 + Gradient + Gradient^2, weights = Transect number.)				
Parameter	**Estimate**	**Std. Error**	**z value**	**Pr (>|z|)**
North-South gradient	1.696	0.202	8.399	< 0.0001
(North-South gradient)^2	-0.466	0.057	-8.32	< 0.0001

Logistic Model of *Ixodes scapularis* Nymphal Infection

The best model from Table 9 was selected to investigate the effect of 'Region' and 'Gradient' on *I. scapularis* nymphal infection prevalence. The second order polynomial without interaction was the best model predicting the effect of 'Region' and 'Gradient' on nymphal infection prevalence. The distribution of the nymphal infection prevalence was hump shaped although the pattern was not as evident as that of the nymphal density. The increasing portion of the hump showed marginal significance whereas that of the decreasing end was insignificant (Table 10). I found marginal significance in terms of the 'Gradient' alone (Table 10). From UpperVA towards the peak, the odds of finding infected nymphs increases by 1.89 times up to the peak of the hump without the effect of 'Gradient' (Table 10). By 'Region', nymphal infection prevalence along the New River was highly significant compared to the western Piedmont (Table 10). The odds of finding an infected nymph in the New River region was 98% more than the odds of finding an infected nymph in the western Piedmont region.

Borrelia burgdorferi infection rates in *I. scapularis* nymphs along the New River region extended further south along the north-to-south gradient compared to that of the western Piedmont which ended in Wilkes County (UpperNC) (Figure 11a). The heat map shows nymphal infection highest at the MidNC and UpperNC tiers of the New River region. However, the BorderNC and UpperNC tiers at the New River region also showed quite high infection prevalence (Figure 11b). The western Piedmont region had an area of highlighted nymphal infection prevalence at the LowerVA tier in Patrick County (Figure 11b).

Table 9. Illustrative list of competing models assessed for nymph density estimates AIC and BIC, and the R2. The best is boldened.

Regression models for density estimates	AIC	BIC	R^2(%)
Region + Gradient	59.80	61.92	53.85
Region + Gradient + (Region:Gradient)	60.20	63.03	57.14
Region + Gradient + Gradient^2	**58.72**	**61.56**	**60.18**
Region + Gradient + (Region:Gradient) + Gradient^2	59.61	63.15	62.46
Region + Gradient +(Region:Gradient) + Gradient^2 + (Region:Gradient^2)	61.57	65.82	62.55

Table 10. Weighted logistic regression on the effect of the explanatory variables on *I. scapularis* nymphal infection prevalence. UpperVA and New River (NR) were used as reference levels in the model to represent the variables Gradient and Site, respectively. The model was weighted using the number of nymphs tested for *B. burgdorferi.*

glm (formula = (Nymphal Prevalence) ~ Gradient + Region + Gradient², family = "binomial")				
Parameter	**Estimate**	**Std. Error**	**z value**	**Pr (>|z|)**
(Intercept)	-1.544	0.303	-5.095	< 0.0001
Piedmont	-2.324	0.605	-3.842	< 0.001
Gradient	0.638	0.3332	1.919	0.055
(Gradient)²	-0.152	0.089	-1.700	0.0892
AIC = 58.72			D² (Pseudo R²) = 60.18%	

Table 11. Logistic model of *I. scapularis* in relation to the north-to-south gradient in the western Piedmont region alone.

glm (formula = Nymph prevalence ~ 0 + Gradient + Gradient², weights = Tested nymphs.)				
Parameter	**Estimate**	**Std. Error**	**z value**	**Pr (>|z|)**
(North-South gradient)	-2.241	1.741	-1.228	0.198
(North-South gradient)²	0.363	0.392	0.924	0.356

The second order polynomial model for the western Piedmont showed an initial decrease in 'Gradient' from north to south and then an increase (Table 11). However, these changes were

not significant. The peak in the nymphal infection prevalence in the western Piedmont region was at LowerVA (Figure 11). In the case of the New River region, a humped shape was observed. The nymphal infection prevalence increased significantly by 2.63 folds from UpperVA up to the peak of the hump (Table 12). Afterward, it decreased significantly by 25% towards the southmost end where nymphal infection prevalence was detected.

Table 12. Logistic model of *I. scapularis* in relation to the north-to-south gradient in the New River region alone.

glm (formula = Nymph prevalence ~ Gradient + Gradient^2, weights = Tested nymphs.)				
Parameter	**Estimate**	**Std. Error**	**z value**	**Pr (>|z|)**
(North-South gradient)	0.968	0.149	-9.800	< 0.0001
(North-South gradient)^2	-0.282	0.051	-5.545	0.0001

Figure 11. Distribution of *I. scapularis* nymph infection prevalence (left) and heat map (right) at New River and corresponding western Piedmont regions from north to south. Numbers on bars indicate number of nymphs tested. Locations without numbers indicate the absence of nymphs. Asterisk(s) indicate a statistically significant difference between the two sites under comparison.
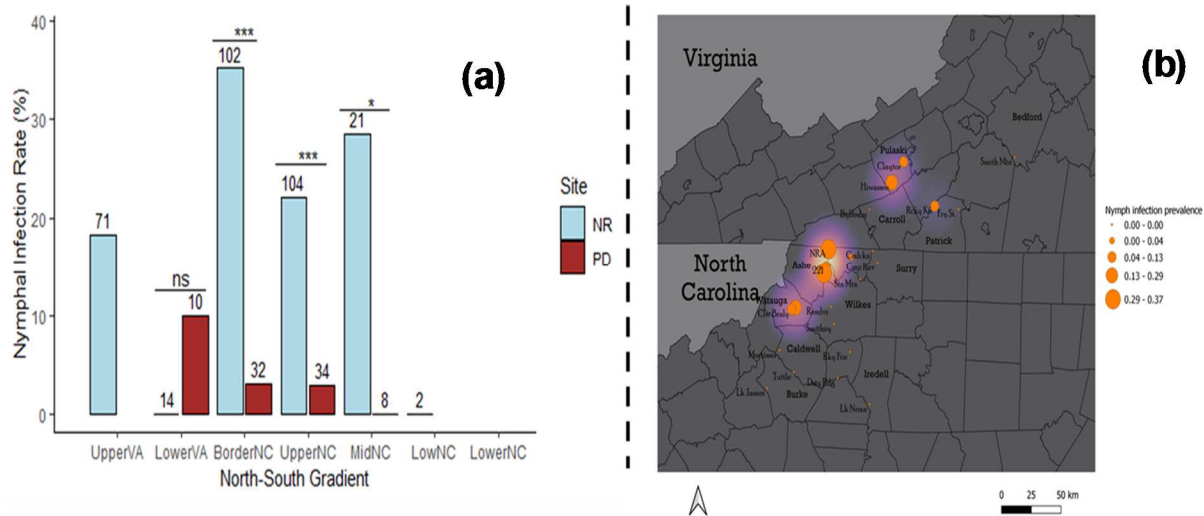
Relationship between *Ixodes scapularis* Nymphal Density and Infection Prevalence in Them

The data suggested a positive relationship between *Ixodes scapularis* nymph density and *Borrelia burgdorferi* prevalence. The relationship, to about a nymphal density of three, showed marked increase in *B. burgdorferi* prevalence. The results predicted that about one in five ticks would be infected with *B. burgdorferi* (Figure 12). The relationship weakened as the nymph density increased (Figure 2.8). Infection rates in New River sites in BorderNC (i.e., Alleghany County), UpperNC (i.e., Ashe County), and MidNC (i.e., Watauga County) were above the expected (Figure 12). At the MidNC site in Watauga County, one *I. scapularis* nymph/100 m was likely to show a 30% rate of *B. burgdorferi* infection (Figure 12).

Density of infected nymphs is determined by multiplying the nymph density by their respective infection rates in each site. Indeed, in the New River sites, the density of infected *Ixodes scapularis* nymphs was highest at the BorderNC site in Alleghany County followed by UpperVA (Pulaski County) and UpperNC (Ashe County) sites (Figure 13). The southernmostsite with density of infected nymphs in the New River region was MidNC (in Watauga County) [Figure 13].

In the New River sites, the density of infected *I. scapularis* nymphs was highest at the BorderNC site in Alleghany County followed by UpperVA (Pulaski County) and UpperNC (Ashe County) (Figure 13). In the western Piedmont region, the density of infected nymphs was low and extended as far south as UpperNC (Wilkes County) as against Watauga for the New River region (Figure 13).

Figure 12. Relationship between Prevalence and *Ixodes scapularis* density. Watauga, Ashe, and Alleghany had B. burgdorferi infection rates above the predicted blue line. Caldwell and Wilkes also had *B. burgdorferi* infection rates below the predicted line.
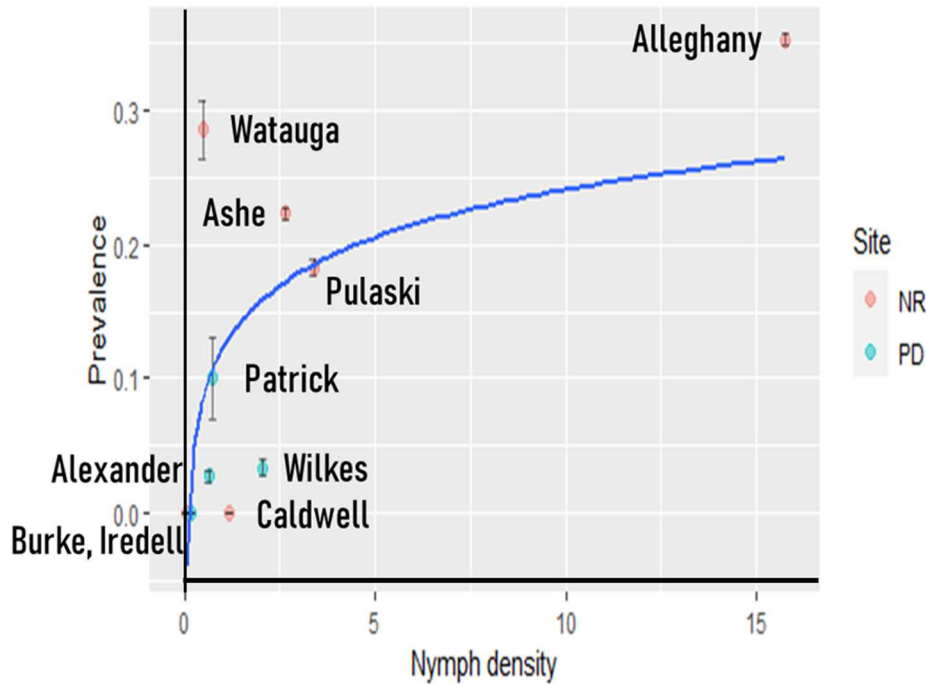
Table 13. A generalized linear model of *Borrelia burgdorferi* prevalence against *I. scapularis* nymphal density for the two regions combined and each region separately. The family and the weights used were Gaussian and tested nymphs, respectively. Log (Nymph prevalence) ~ log (Nymph density).

Predictor	Estimate	Std. error	t-value	Pr(>|t|)
log (Nymph density (Piedmont + New River)	0.113	0.012	9.136	< 0.0001
log (Nymph density) (Piedmont alone)	0.687	1.177	2.364	0.036
log (Nymph density) (New River alone)	3.022	0.275	5.726	0.00277

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Figure 13. Density of infected *Ixodes scapularis* nymphs in the New River and western Piedmont regions from the north (UpperVA) to the south (LowerNC). Values on bars represent the density of nymphs.
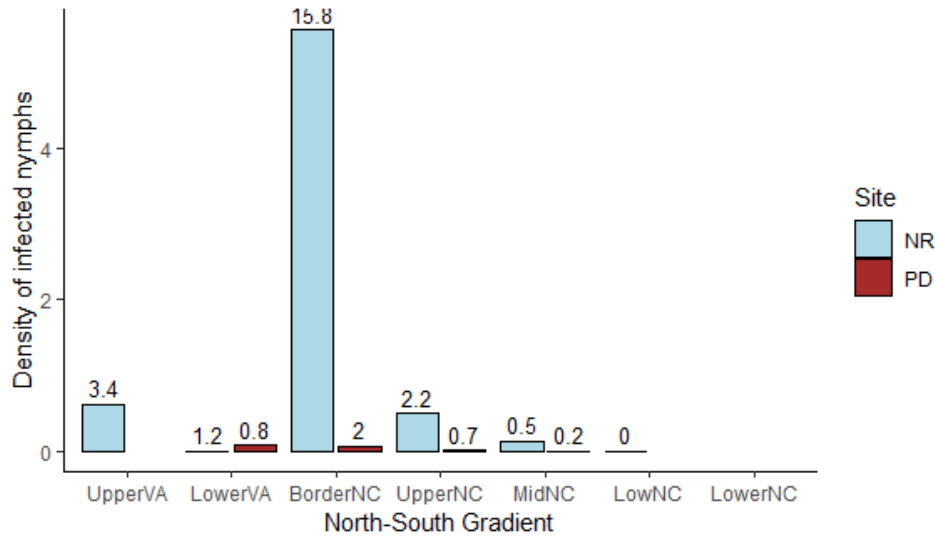
Adult *Ixodes scapularis* Regression Model

The best competing model was a second-order polynomial with effects of 'Region', 'Gradient', and an interaction term (Table 14). The second-order polynomial indicated a humped shape pattern in the adult density. The insignificant 'Gradient' effect made it unclear whether the hump was inverted or not. The initial increase or decrease (depending on whether the hump was inverted or not) in adult *I. scapularis* density from UpperVA was 0.053 adult ticks/100 m. However, that increase, or decrease was not significant (Table 15). The other part of the hump either increased or decreased significantly by 0.15 adults/100 m (Table 15). There were more adult *I. scapularis* in the New River than the western Piedmont. The density of adult *I. scapularis* decreased by 4 ticks/ 100m in the western Piedmont compared to the New River, without the 'effect of 'Gradient' (Table 15). The significant interaction term indicated that there

was an effect of 'Region' on the 'Gradient'. The difference in slope of the 'Gradient' between the New River and western Piedmont region was 0.63 (Table 15).

The independent regression models by 'Region' showed that the hump was inverted. in both the New River and western Piedmont region. The decrease and increase in adult *I. scapularis* at the western Piedmont region were not significant (Table 16). However, the New River region showed a significant decrease by 3.7 adult ticks/ 100 m up to the trough and then a significant rise by 0.7 (Table 17). The southernmost point of adult *I. scapularis* detection was the same for the New River and western Piedmont (that was at LowNC) (Figure 14). The heat map showed that the initial decrease was from UpperVA to BorderNC where it began to increase (Figure 14).

Table 14. Illustrative list of tested models for nymph density estimates AIC and BIC. The model in bold was the best model that was used in making the prediction.

Regression models for density estimates	AIC	BIC	R^2(%)
Region + Gradient	364.23	377.28	50.00
Region + Gradient + Region: Gradient	360.36	377.27	52.37
Region + Gradient + Gradient^2	359.54	376.34	52.68
Region + Gradient + Region: Gradient + Gradient^2	**354.24**	**374.05**	**55.47**
Region+Gradient+Region:Gradient+Gradient^2+(RegionGradient^2)	355.82	379.83	55.74
Region + Gradient + Gradient^2 + (Site: Gradient^2)	355.78	376.36	54.96

Table 15. Negative binomial regression of the best fit model that predicts the effect of the explanatory variables on *I. scapularis* adult density. UpperVA and New River were the references used in the model to represent the variables Gradient and Site, respectively.

glm.nb (formula = Adult ~ Gradient + Site + Site: Gradient + Gradient^2, weights = Transect no.)				
Parameter	**Estimate**	**Std. Error**	**z value**	**Pr (>|z|)**
(Intercept)	1.401	0.353	3.968	< 0.0001
Piedmont	-3.946	0.752	-5.249	< 0.0001
Gradient	0.053	0.299	0.176	0.8604
Gradient^2	-0.153	0.060	-2.532	0.0114
Piedmont: Gradient	0.630	0.241	2.619	0.0088
AIC = 354.24		D^2 (Pseudo R^2) = 56.7%		

Table 16. Negative binomial model of *I. scapularis* adult in relation to the second order polynomial of the north-to-south gradient in the western Piedmont Region alone. Intercept was taken as 0.

glm.nb (formula = Adult ~ 0 + Gradient + Gradient^2, weights = Transect number.)				
Parameter	**Estimate**	**Std. Error**	**z value**	**Pr (>|z|)**
(North-South gradient)	-0.697	0.5117	-1.362	0.173
(North-South gradient)^2	0.017	0.111	0.149	0.882

Table 17. Negative binomial model of *I. scapularis* adult in relation to the second order polynomial of the north-to-south gradient in the New River Region alone. Intercept was taken as 0.

glm.nb (formula = Adult ~ 0 + Gradient + Gradient^2, weights = Transect number.)				
Parameter	**Estimate**	**Std. Error**	**z value**	**Pr (>|z|)**
(North-South gradient)	-3.686	1.138	-3.238	0.0012
(North-South gradient)^2	0.726	0.248	2.926	0.00344

Figure 14. Distribution of *I. scapularis* adult density (± standard error) (left) and heat map (right) at New River and corresponding western Piedmont regions from north to south. Numbers on the bars indicate the number of transects. Each transect = 100-m. Asterisk(s) indicate statistically significant differences.
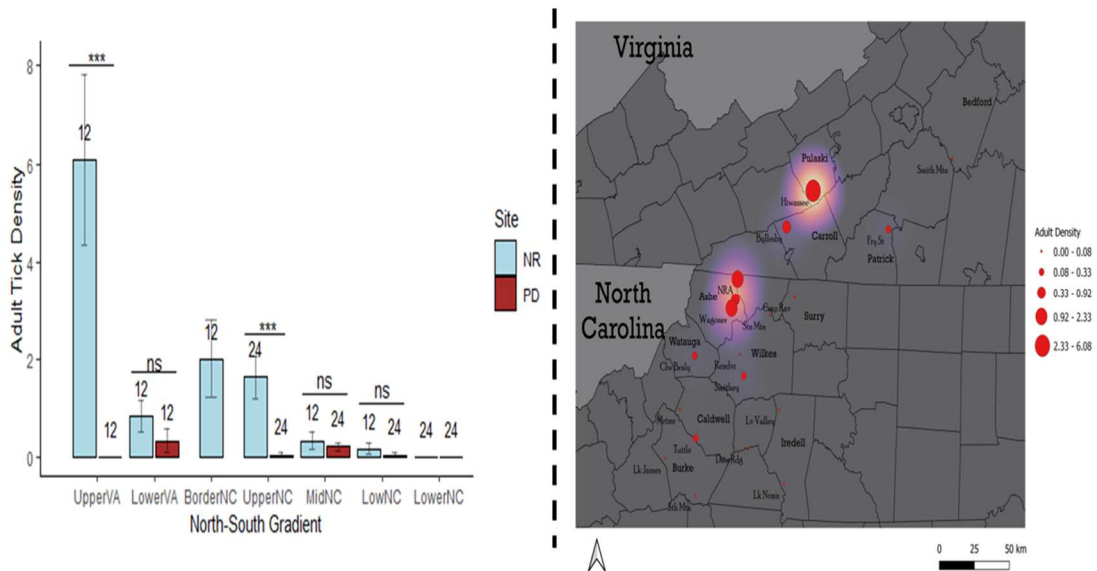
Adult *Ixodes scapularis* Infection Rate

The best model was a second-order polynomial with effects of 'Gradient' and 'Region' without an interaction effect. The distribution of the adult *I. scapularis* infection was humped shape. The odds of adult *I. scapularis* being infected increased 2.72 times from North to the peak of hump and then decreased significantly by 25% (Table 18). There was no significant difference in terms of adult tick infection in the New River compared to the western Piedmont (Table 18). The humped shape in the adult *I. scapularis* infection prevalence was not clear as was proven by the independent models by 'Region' showing no significant differences (Tables 20 & 21). This was proven when both the Gradient and second order polynomial of the gradient showed no statistical significance when ran separately (Tables 20 & 21). The humped shape was marginally significant for the western Piedmont (Table 20).

Along the 'Gradient' and 'Region', the infection prevalence in adult *I. scapularis* did not seem to follow a particular pattern (Figure 15). The infection prevalence along the western Piedmont showed up in LowerVA and MidNC, skipping tiers between these two areas (Figure 15). Along the New River, the infection prevalence was highest at UpperNC but was not much different from UpperVA and MidNC tiers (Figure 15).

Table 18. Weighted logistic regression of competing models on the effect of Region and Gradient on adult *I. scapularis* infection rates. The New River region and the UpperVA tier were used as references. The best competing model is in bold print.

Regression models for density estimates	AIC	BIC	R^2(%)
Region + Gradient	49.21	50.41	2.88
Region + Gradient + Region: Gradient	49.32	50.92	9.6
Region + Gradient+ Gradient^2	**45.05**	**46.64**	**24.70**
Region + Gradient + Region: Gradient + Gradient^2	46.86	48.85	25.36
Region +Gradient +Region:Gradient +Gradient^2+Region: Gradient^2	44.73	47.12	40.00
Region + Gradient +Gradient^2 + Site: Gradient^2	46.45	48.44	26.80

Table 19. Weighted logistic regression of the effect of Region and Gradient on adult *I. scapularis* infection prevalence. UpperVA and New River were used as reference levels in the model to represent the variables Gradient and Region, respectively. The model was weighted using the number of nymphs tested for *B. burgdorferi*.

glm (formula = (Adult Prevalence) ~ Gradient + Region+ Gradient^2, family = "binomial")				
Parameter	**Estimate**	**Std. Error**	**z value**	**Pr (>|z|)**
(Intercept)	-0.283	0.255	1.110	0.2672
Piedmont	-0.137	0.719	-0.191	0.8488
Gradient	1.002	0.428	2.341	0.0192
Gradient^2	-0.285	0.124	2.296	0.0217
AIC = 45.05			D^2 (Pseudo R^2) = 17.6%	

Table 20. Logistic model of adult *I. scapularis* infection rate in relation to the north-to-south gradient in the western Piedmont region alone.

glm (formula = Adult prevalence ~ 0 + Gradient + Gradient^2, weights = Tested adults.)				
Parameter	**Estimate**	**Std. Error**	**z value**	**Pr (>|z|)**
(North-South gradient)	2.523	1.501	1.682	0.0927
(North-South gradient)^2	-0.735	0.400	-1.839	0.0660

Table 21. Logistic model of adult *I. scapularis* infection rate in relation to the north-to-south gradient in the New River region alone.

glm (formula = Adult prevalence ~ 0 + Gradient + Gradient^2, weights = Tested adults.)				
Parameter	**Estimate**	**Std. Error**	**z value**	**Pr (>|z|)**
(North-South gradient)	0.328	0.224	1.344	0.179
(North-South gradient)^2	-0.039	0.084	-0.456	0.649

Figure 15. Distribution of *I. scapularis* adult infection prevalence (left) and heat map (right) at New River and corresponding western Piedmont regions from north to south. Numbers on bars represent number of adults tested. Locations without numbers indicate the absence of adults. Asterisk(s) indicate statistically significant differences.
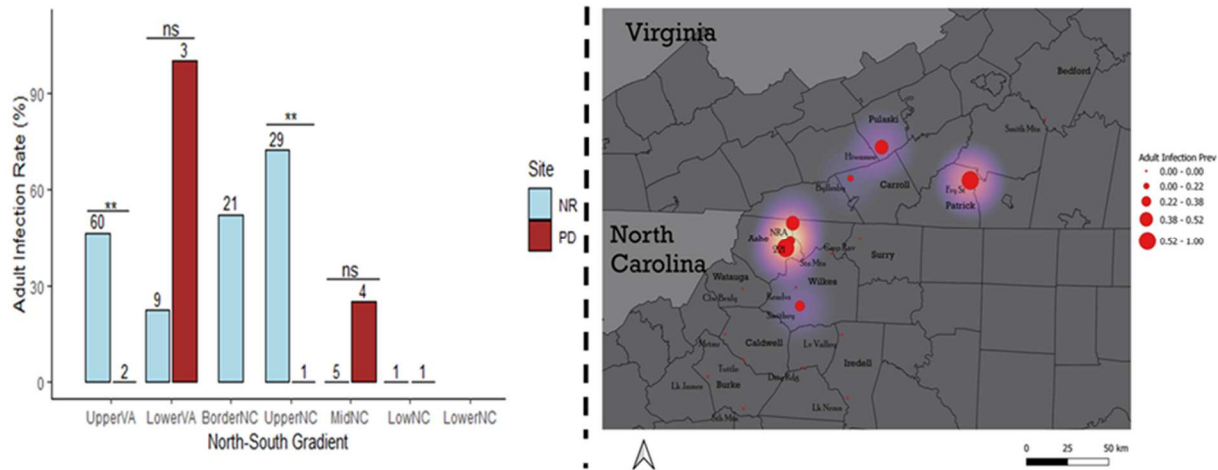
Distribution and Density of *Ixodes scapularis* Larvae

The best competing model for the *I. scapularis* larva indicated a humped shape distribution of larval density (Table 22). Based on that model, there is 1.8 times more *I. scapularis* larvae in the New River region compared to the western Piedmont region (Table 23). This is evident in the bar graph which showed that for every tier, the *I. scapularis* larval density is higher in the New River region than the western Piedmont region (Figure 16). In terms of the gradient, *I. scapularis* density increased significantly by one *I. scapularis* larva from the north up to a point and then begin to decrease significantly by 0.3 southward (Table 23). The individual models for the regions indicated a difference in the slopes between the two 'Regions'. The increase in *I. scapularis* larvae in the New River region, approaching the hump, was higher than

that of the western Piedmont (Tables 24 & 25). Similarly, the decrease in *I. scapularis* tick

density was also higher in the New River than the western Piedmont.

Table 22. Negative binomial regression of the best fit model that predicts the effect of the

explanatory variables on *I. scapularis* larval density. UpperVA and New River were the

references used in the model to represent the variables Gradient and Site, respectively.

Regression models for density estimates	AIC	BIC	R^2 (%)
Region + Gradient	991.97	1007.32	15.00
Region + Gradient + Site:Gradient	988.36	1016.84	19.11
Region + Gradient + Gradient^2	**986.82**	**1007.16**	**17.97**
Region + Gradient + Site:Gradient + Gradient^2	986.70	1011.11	18.96
Region + Gradient + Site:Gradient + Gradient^2 + Site:Gradient^2	988.36	1016.84	19.11
Region + Gradient + Gradient^2 + Site: Gradient^2	987.14	1011.55	18.75

Table 23. Negative binomial regression of the best fit model that predicts the effect of the

explanatory variables on *I. scapularis* larval density. UpperVA and New River (NR) were the

references used in the model to represent the variables Gradient and Site, respectively.

Glm.nb (formula = (Larval Density) ~ Site + Gradient + Gradient^2)				
Parameter	**Estimate**	**Std. Error**	**z value**	**Pr (>|z|)**
(Intercept)	2.678	0.605	4.424	< 0.0001
Piedmont	-1.815	0.424	-4.279	< 0.0001
Gradient	0.813	0.444	1.832	0.067
Gradient^2	-0.286	0.078	-3.662	0.0002
AIC = 17.97			D^2 (Pseudo R^2) = 18.0 (%)	

Table 24. Negative binomial model of *I. scapularis* larvae in relation to the second order polynomial of the north-to-south gradient in the western Piedmont region alone. Intercept was taken as 0.

glm.nb (formula = Larva ~ 0 + Gradient + Gradient², weights = Transect number.)				
Parameter	**Estimate**	**Std. Error**	**z value**	**Pr (>|z|)**
(North-South gradient)	1.549	0.474	3.269	0.001
(North-South gradient)²	-0.415	0.115	-3.621	0.0003

Table 25. Negative binomial model of *I. scapularis* larvae in relation to the second order polynomial of the north-to-south gradient in the New River Region alone. Intercept was taken as 0.

glm.nb (formula = Larva ~ 0 + Gradient + Gradient², weights = Transect number.)				
Parameter	**Estimate**	**Std. Error**	**z value**	**Pr (>|z|)**
(North-South gradient)	2.081	0.462	4.501	<0.0001
(North-South gradient)²	-0.434	0.108	-4.019	< 0.0001

Figure 16. Larval density of *I. scapularis* ticks (± standard error) at New River sites and corresponding western Piedmont reference sites for each gradient point from north to south. Numbers on the bars indicate the number of transects. Each transect = 100-m. Asterisk(s) indicate statistically significant differences.
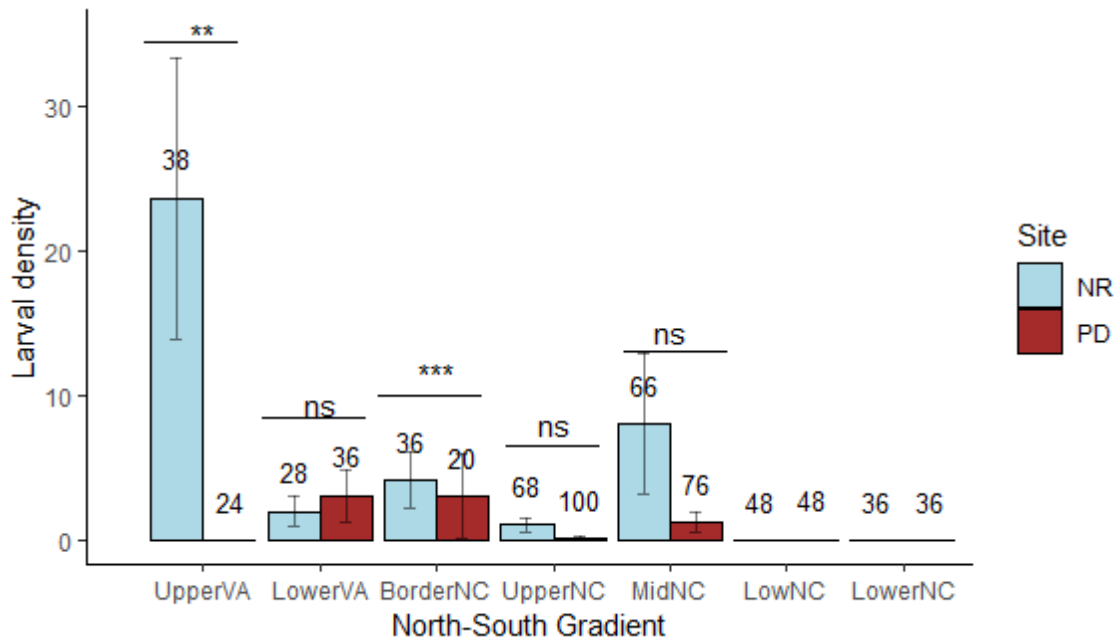
DISCUSSION

Distribution of *Ixodes scapularis* Nymphs

The nymphal distribution realized did not support my predicted north-to-south gradient. However, it is not rare for species to exhibit humped shape distribution pattern across geographic space (e.g., Holt et al., 1997; McGill & Collins, 2005). Animals are known to exhibit a humped shape when moving from one area to another as was shown in a griffon vulture study in Israel (Spiegel et al., 2013). Cortinas & Kitron, (2006) described a similar event when they recorded significantly higher annual *I. scapularis* prevalence in several Illinois River counties than in Ogle County which had previously recorded the highest *I. scapularis* tick activity in the state. As it

stands, the current wavefront of uninfected and infected *I. scapularis* invasion appear to be concentrated in Alleghany and Ashe Counties which share a border with Virginia. In terms of the mechanism of invasion, the initial introduction of non-infected *I. scapularis* ticks further south than infected ticks supports the tick-first invasion hypothesis. The scenario occurs when there is an initial invasion of uninfected ticks into a new area by their vertebrate hosts before the gradual secondary invasion of the spirochete by infected ticks and/or rodents (Hamer et al., 2010). The tick-first hypothesis reported in this study is consistent with the observations by Teague (2018) who conducted a study in the western Piedmont region of North Carolina. He flagged for ticks and collected them off hunter harvested deer to investigate the entomological risk of Lyme disease in the study area and to determine whether there was a north-to-south gradient in tick and pathogen distribution. He found that there was a decreasing gradient in *I. scapularis* density from the north to the south. The same was true for the *B. burgdorferi* infection they carried. However, the non-infected tick's distribution had spread further south than that of the infected ticks, indicating the tick-first scenario. A related study, which forms the second chapter of my dissertation further supported the tick-first hypothesis through the absence of cryptic vectors on rodents and *B. burgdorferi* in rodents within areas where *I. scapularis* was not found. This implies that the spirochetes are likely not to be present in areas where the *I. scapularis* is absent, refuting the spirochete-first hypothesis.

The recorded higher *I. scapularis* ticks in the New River region compared to the western Piedmont region is consistent with the hypothesis that the New River is facilitating the spread of *I. scapularis* ticks. Multiple studies have mentioned higher tick abundances along river corridors although none of them studied the role of river corridors on tick spread directly (e.g., (Cortinas & Kitron, 2006; Rand et al., 2007). Coastal areas are more likely to show high abundances of *I.*

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scapularis because such areas natural provide the moderate temperature and humidity required for the survival of ticks. Brownstein et al. (2003) used temperature and vapor pressure to predict the spatial occurrence of *I. scapularis* across the United States. Their model indicated the occurrence of *I. scapularis* along the coastal states of the eastern United States and the Mid-west. Although their data showed the occurrence of ticks, it did not differentiate between the northern and the southern tick populations which may indicate why we observe differences in Lyme disease risk when comparing the north to the south.

The association of *I. scapularis* with rivers in noncoastal sites has previously been modeled by Kitron et al. (1992). It is worth noting that the ticks themselves do not move much (Buczek et al., 2020), hence, their association with riparian habitats is dependent on their hosts. For instance, Carroll & Schmidtmann (1996) in a field experiment to establish how far *I. scapularis* nymphs and adults may disperse reported that *I. scapularis* nymphs and adults cover only 2-3 meters and 5 meters, respectively. Therefore, any feature that enhances the dispersal of *I. scapularis* hosts is what causes the spread of Lyme disease.

River corridors are reported to facilitate and direct the movement of organisms, materials, and resources (Bennett, 1999). Likewise, riparian zones are reported to be preferred movement habitats for many terrestrial animals such as mammals (e.g., (Clements et al., 2011), and birds (e.g., (Lees & Peres, 2008). Walter, et al. (2011) studied the space use of sympatric deer in a riparian ecosystem and found that white-tailed deer moved along forested, riparian areas.

Distribution of *Borrelia burgdorferi* Infection in *Ixodes scapularis* Nymphs

The discovery of a humped-shaped distribution of the *B. burgdorferi* infection rates among the different life stages of *I. scapularis* were also indicative of an invasion phenomenon. The increased *B. burgdorferi* infection rate along the New River compared to the Piedmont

reference region may also be attributed to the association of *Peromyscus leucopus* with riparian habitats. Arvidson (2009) investigated whether riparian corridors supported rodent species as much as xeric (i.e., dry) habitats do by sampling rodents along the Hillsborough River in Hillsborough County located on Big Pine Island in Florida. His results showed that *Peromyscus gossypinus* (cotton mouse)*, a closely related sibling species to *P. leucopus*, was well adapted to and had higher abundance in riparian corridors compared to other rodent species - *Sigmodon hispidus* (hispid cotton rat) and *Ochrotomys nuttalli* (golden mouse). Arvidson's results suggested that riparian corridors may provide dispersal routes for some species such as *Peromyscus* spp., but not all species of rodents. This means that the New River may be enhancing the relative abundance of *P. leucopus*, the primary reservoir host of the Lyme disease spirochete.

Relationship between *Borrelia burgdorferi* Prevalence and *Ixodes scapularis* Nymphal Density

The density of nymphs within an area was positively correlated with *B. burgdorferi* prevalence in *I. scapularis* ticks. *Ixodes scapularis* larvae are born without the pathogen. Larvae pick up the pathogen through an infective bite when they acquire their first blood meals. Adults on the other hand feed more frequently on deer which are non-competent reservoir hosts. The nymphs have already taken their first blood meals and feed on reservoir hosts. Hence, nymphs are the main life stage that maintains the enzootic transmission cycle of *B. burgdorferi. Ixodes scapularis* nymphs emerge in spring before the emergence of larvae in mid-summer. This permits nymphs that had already fed once and likely to be infected to transmit the spirochete to newly born competent and non-infected reservoir hosts before the emergence of the larvae, thereby increasing the chances of larvae feeding on infected hosts.

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There was a non-linear positive relationship between nymphal prevalence and density. MidNC (Watauga County), a New River site, showed an unexpectedly high *B. burgdorferi* prevalence at a low nymphal density. The site (Clawson-Burnley Park) was the only one that was in the midst of a town. It is a highly visited park and the forest seemed more fragmented than that of the state parks. Fragmented landscapes support *P. leucopus* abundance and density (Allan et al., 2003). Other less competent reservoir hosts such as chipmunks, shrews, and squirrels, were all found in the park during rodent trapping sessions for another study. The high human density makes Watauga County an area of high Lyme disease risk. Watauga is also more mountainous compared to the other sites. According to Lantos et al. (2021), the spread of Lyme disease southward is influenced by the mountains and mentioned that climate change may have made higher elevations more suitable for the survival and development of *I. scapularis* and their hosts, similar to what was observed with *I. ricinus* in Europe (Gern, 2008).

Distribution of *Ixodes scapularis* Adult and Larva, and *Borrelia burgdorferi* Infection Rates in Adults

Although the model for the adult *I. scapularis* density depicted a humped shape distribution, the individual models did not show any significance in the humped distribution. This was because the intercept of the best model was negative, and it does not make biological sense for ticks to behave in a certain pattern when they are absent (negative means below zero which is impractical in real life. It may be that the sample size obtained for the adult *I. scapularis* ticks was small. In areas where western Piedmont recorded higher *B. burgdorferi* infection rates, the number of *I. scapularis* ticks under consideration was too few to make a valid conclusion. However, the larval distribution was humped shape. The detection of non-infected adult *I.*

scapularis further south than *B. burgdorferi* strengthens the evidence supporting the tick-first hypothesis.

The New River and Lyme Disease in Western North Carolina

This study suggests that the New River is providing a suitable conduit for at least one of the components needed to create a Lyme disease niche condition. The past reports of increasing Lyme disease cases in the New River Valley areas of Virginia (Rife, 2017) and North Carolina (e.g., Lantos et al., 2015; NC Division of Public Health, 2019; Neupane et al., 2021) support my findings.

It is unclear whether it is the northern or southern clade of *I. scapularis*, or even an interbreed of these two clades that are occurring in western North Carolina. It is known that the southern clade usually parasitizes lizards and rarely bites humans (Apperson et al., 1993; Dennis et al., 1998; Durden et al., 2002; Levine et al., 1997). A pilot study by (Arsnoe et al., 2015) showed that nymphs collected from a northern state (Wisconsin) were 12 times more likely than nymphs collected from southern states of North and South Carolina to quest above the leaf litter. Questing above the leaf litter is predictive of human LD risk because it is more likely for a tick above ground to locate a passing human than a tick on the ground covered by leaf litter. This points the evidence of rising Lyme disease cases in northwestern North Carolina to either the northern clade or a hybrid of the two clades. The interbreeding of these two clades is supported by taxonomic studies that showed that the two clades are reproductively compatible and genetically similar (Oliver et al., 1993; Wesson et al., 1993; Xu et al., 2020).

The Way Forward

Lantos et al. (2015), showed that Lyme disease was at the verge of reaching North Carolina following the Appalachian Mountains route. In 2019, data on human Lyme disease

cases in North Carolina showed that the disease cases clustered around the proposed line of invasion is ongoing. This study provides entomological risk data on the disease in northwestern North Carolina. Nymphal and adult tick density as well *B. burgdorferi* infection showed that the southward spread of the disease is happening faster along the New River valley than corresponding western Piedmont region. The current hotspot of the vector and pathogen is concentrated around Alleghany and Ashe Counties. The southernmost extent of *Borrelia burgdorferi* infection prevalence appears to be in Watauga and surrounding counties. Patrick County may in the future become a focus from which *I. scapularis* and *B. burgdorferi* will be hitting the Piedmont region. Public health efforts should be focused around these areas.

CHAPTER III: EVALUATE IF THE SMALL MAMMAL COMMUNITY IN
NORTHWESTERN NORTH CAROLINA IS PERMISSIVE FOR THE ESTABLISHMENT OF
BORRELIA BURGDORFERI ENZOOTIC CYCLE

ABSTRACT

Rodents are carriers of myriad of pathogens that may cause infectious diseases. *Peromyscus leucopus* is regarded as the principal reservoir host of *Borrelia burgdorferi. Ixodes scapularis* and *B. burgdorferi* are thought to be invading the northwestern North Carolina area. It is unknown whether the vector and pathogen can become established once introduced. This study was conducted to determine whether the rodent community in northwestern North Carolina is permissive for successful establishment of the tick and the pathogen following introduction. A total of 3,881 trapnights yielded 174 small mammals belonging to nine species: star-nosed mole (*Condylura cristata*), pine vole (*Microtus pinetorum*), woodland jumping mouse (*Napaeozapus insignis*), golden mouse (*Ochrotomys nuttalli*), White-footed mouse (*Peromyscus leucopus*), eastern gray squirrel (*Sciurus carolinensis*), hispid cotton rat (*Sigmodon hispidus*), shrew (*Sorex* sp.), and eastern chipmunk (*Tamias striatus*). *Peromyscus leucopus*, the principal reservoir host of *B. burgdorferi*, constituted 88.51% of the total number of individuals caught and dominated both along the New River and western Piedmont. *Peromyscus leucopus* was also the only rodent species with attached ticks. All the ticks collected off *P. leucopus* were *I. scapularis.* Per-capita tick infestation rate of *P. leucopus* in the New River was 14-times higher compared to western Piedmont; a result that is consistent with higher infection prevalence of *P. leucopus* in the New River region. Apart from *P. leucopus,* one *Microtus pinetorum,* one *Tamias striatus*, and two *Sciurus carolinensis*, also tested positive for *B. burgdorferi.* Watauga County was the southernmost site along the New River where *B. burgdorferi* was detected while Stone Mountain

State Park (between Alleghany and Wilkes Counties) was the southernmost along the western Piedmont. The results showed that *I. scapularis* and *B. burgdorgdorferi* can become established after invasion.

INTRODUCTION

Rodents as Effective Pathogen Reservoirs and Spreaders

Rodents (Order Rodentia) are the largest order of extant mammals and constitute about 42% of the global mammal biodiversity (Capizzi et al., 2014). Rodents play important roles in ecosystems by serving as food source for other organisms (Meckstroth et al., 2007), recycling nutrient via their burrowing activities (Roy, 2022), and shaping plant communities through selective seed predation (Jansen & Forget, 2001; Weighill et al., 2017). Nevertheless, rodents can also be agents of pathogen spread, posing serious public health threats. Traits possessed by rodents that make them effective pathogen reservoirs and spreaders include, being highly opportunistic (i.e., being able to adapt to changes in the environmental conditions and colonize new and often disturbed habitats), high reproductive rate, and rapid turnovers [i.e. short life spans] (Mills, 2006). In addition, rodents (e.g., rats and mice) are cosmopolitan (found almost everywhere except Antarctica and some islands) which makes the infectious agents they carry also widespread. Based on the pace-of-life hypothesis, animals with short life span such as small rodents invest little in long-term immune defenses (i.e., adaptive immunity). Rather, they channel resources for the development and maintenance of adaptive immune responses into life processes such as reproduction. (e.g., Ardia et al., 2003; Previtali et al., 2012). The little investments put into the development of immune defenses by animals with short life cycles such as *P. leucopus* makes them competent hosts (i.e., having the ability to maintain a pathogen in its body over extended periods of time that permits the circulation of the pathogen in the system]

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(Gern et al., 1998). In some cases, a single individual rodent may harbor more than one pathogen species (termed, coinfection) and be able to transmit them both/all simultaneously (Dahmana et al., 2020; Kim et al., 2006; Rabiee et al., 2018). For example, the white-footed mouse, which is the primary reservoir host of the pathogen that causes Lyme disease (LD), *Borrelia burgdorferi*, can also maintain *Borrelia miyamotoi* in its system simultaneously and effectively transmit both to another host through the vector, *Ixodes scapularis*. It is therefore not surprising that rodents host more than 60-80 zoonotic diseases (Han et al., 2016; Luis et al., 2013; Meerburg et al., 2009), and play a critical role in the transmission and spread of many pathogens around the world (Meerburg et al., 2009).

The Role of Rodents within the Enzootic Cycle of *Borrelia burgdorferi*

Some small rodents are vital for the maintenance of *B. burgdorferi* by acting as reservoir hosts. The transmission of *B. burgdorferi* from one reservoir host to another requires the vector, *I. scapularis*. *Ixodes scapularis* has a three-host life cycle. The tick feeds once as a larva, then as a nymph, and finally (mostly the females) as adults. When *I. scapularis* feeds on an infected reservoir host at any of the active immature active life stages, it picks up the *B. burgdorferi* pathogen during the blood-feeding process. Once the pathogen is picked up, it remains with the tick throughout the remainder of its life cycle (i.e., transstadial transmission). The infected tick also transmits the pathogen to another host when it next feeds, and the cycle continues. For a host to be a competent reservoir of *B. burgdorferi*, it must (1) take up a critical dose of the *B. burgdorferi* pathogen during a blood-meal, and (2) allow the pathogen to multiply and survive in parts of its body that are accessible to the ticks (Stanek et al., 2002). Depending on how rodents differ in their effectiveness at meeting these criteria, their level of competence as reservoir hosts will vary.

In the northeastern United States, the white-footed mouse (*Peromyscus leucopus*) is considered the principal reservoir host of *B. burgdorferi* and the preferred host by larval and nymphal *I. scapularis* ticks (Ostfeld et al., 2001). A study on the reservoir competence of white-footed mouse and chipmunks which was calculated as the mean proportion of newly molted nymphs infected by an individual rodent was found to be 0.94 and 0.69 for white-footed mouse and chipmunk, respectively (Schmidt & Ostfeld, 2001). This meant that *P. leucopus* infected over 90% of *I. scapularis* that blood-fed on them compared to eastern chipmunks (*Tamias striatus*) that infected about 70% (Schmidt & Ostfeld, 2001). Aside from *P. leucopus* being a preferred host by *I. scapularis*, it is also highly susceptible to *B. burgdorferi* (Mather et al., 1989).

A study that compared the relative potential of rodents as reservoirs of *B. burgdorferi* found that only three species were consistently infested with immature *I. scapularis* [(i.e., white-footed mice:16.4±1.9, chipmunks: 3.2 ± 2.1, and voles: 3.1 ± 1.1)] (Mather et al., 1989). In the study, white-footed mouse was the most dominant comprising 68.56% (i.e., 1,424 out of 2,077) of the rodents captured. *Ixodes scapularis* larvae were found over five times more on *P. leucopus* (16.4 ± 1.9) than the rest (Mather et al., 1989).The authors infected the three rodents and allowed 100 larvae to blood feed from these rodents. A sample size of 15 to 20 of the newly molted nymphs that had detached from these rodents were tested for the presence of *B. burgdorferi* to determine the relative potential of the three rodent species. Their results showed 90% infectivity in *P. leucopus*, 70% in *T. striatus*, and 5.5% in *M. pennsylvanicus* (Mather et al., 1989). Using epidemiologic models, they estimated that one *P. leucopus* can infect as many nymphal ticks as 12 chipmunks or about 221 meadow voles (Mather et al., 1989). Brisson et al. (2008) used a technique called signature matching that identified the vertebrate species from with an infected

nymph had taken its blood meal as a larva by investigating the outer surface protein C (ospC) genotypes to determine the reservoir competence of a series of small vertebrates. They found that 25.5% had fed on masked shrew, at least 23.5% from white-footed mouse, 20% from short-tailed shrew, 4.4% from chipmunks, and less than 3% from squirrels.

Data from a study in the southeastern states of Georgia, South Carolina, and Florida showed that *P. gossypinus* (cotton mouse), *Sigmodon hispidus* (Hispid cotton rat), and *Neotoma floridana* (eastern woodrat) were readily infected with *B. burgdorferi* and remained infected and infective to competent tick vectors for extended periods, often for life (Oliver et al., 2003). Certain birds can also act as reservoir hosts of *B. burgdorferi* where they may be responsible for the large-scale spread of the pathogen. However, a study in the northeastern United States that used field and published data showed that although birds can be responsible for the long-range dispersal of *B. burgdorferi*, rodents tend to be more important reservoir hosts of *B. burgdorferi* maintaining a stable enzootic cycle of transmission and persistence (Giardina et al., 2000).

The Dilution Effect Hypothesis

The above studies showed that not all species within an area are reservoir hosts and that even among the reservoir hosts, the level of reservoir competence varies. This premise reinforces the concept of biodiversity and disease risk. A biodiverse rich area has a higher diversity of species, with some being highly competent while other are less or not at all. Within such a community, the chances of transmitting a pathogen from one reservoir host to another by a vector are reduced. This is because the chances of a vector picking up the pathogen from a reservoir host are reduced, and even when the vector does locate a reservoir host and gets infected, the chances of the vector transmitting the pathogen to another reservoir host are reduced since there are many other species with varying reservoir competence in such a

biodiverse area (e.g., Schmidt & Ostfeld, 2001). This phenomenon, whereby increased biodiversity reduces disease risk, is termed the "dilution effect". In certain cases where a biodiverse habitat consists primarily of the dominant species, or most of the species within the area are competent hosts then biodiversity may increase disease risk (i.e., amplification effect) (Randolph & Dobson, 2012).

In the context of Lyme disease ecology, a forest habitat that has high rodent biodiversity (e.g., often, large forest patches) results in reduced probability of the *I. scapularis* tick encountering a *P. leucopus*, because its abundance is "diluted" by the presence of other less competent small mammals, thereby reducing transmission rate from and to the ticks. This is supported by a study on the effect of forest patch size on Lyme disease risk in Dutchess County, southeastern New York, which revealed that large forest patches (> 5 ha.) had a relatively low density of infected nymphs (DIN) compared to small forest patches (< 1-ha.) (Allan et al., 2003a). The small forest patches in their experiment on average had three times as many *I. scapularis* ticks and seven times more infected ticks as did larger forests (>5 ha.) (Allan et al., 2003).

Lyme Disease Risk in the Southeast

The rodent community structure can determine *B. burgdorferi* transmission risk since not all rodents are competent in transmitting the *B. burgdorferi* pathogen. For example, Apperson et al. (1993) in their study on the relative utilization of reptiles and rodents as hosts by juvenile *I. scapularis* in the coastal plains of North Carolina, trapped rodents using Sherman traps and reptiles by hand, or pitfall and funnel traps with drift fence. The authors found *I. scapularis* larvae on only 3.74% (i.e., 4 out of 107) of *Peromyscus* mice and none on other rodent species captured. For the reptiles, no *I. scapularis* was found on the captured snakes but 36.73% of the

lizards examined (give numbers of infested/total lizards) were infested with 188 *I. scapularis* larvae and 47 *I. scapularis* nymphs (Apperson et al., 1993). Lizards, typically, considered incompetent reservoir hosts of *B. burgdorferi.* Therefore, the high frequency of *I. scapularis* infesting lizards in the Southeast is one possible explanation for why there is a low Lyme disease risk in the Southeast compared to the Northeast.

Before the 1990s, the vector that was responsible for transmitting *B. burgdorferi* in the Northeast (then called *I. dammini*) was thought to be of a different species from that of the Southeast (*I. scapularis*). The two groups have now been proven to be the same species (Oliver et al., 1993; Wesson et al., 1993), but are classified into different clades (Xu et al., 2020). The two clades are the American and Southern Clades. The difference between the two clades is thought to be related to the preference of the northern populations to feed on rodents compared to southern populations (Goddard et al., 2015). The infection rate of *B. burgdorferi* was estimated to be 2-8% in southern *I. scapularis* adults (Diuk-Wasser et al., 2010; Oliver, 1996), compared to average infection rates of 50% in adult ticks in New England (Barbour & Fish, 1993).

It is not clear if the differences between the two clades is caused by genetic or environmental factors. Arsnoe et al. (2019) conducted a common garden experiment where they collected female *I. scapularis* ticks from deer in 15 different states, ranging from 'high risk' areas through 'transition' areas to 'low risk' areas. The gravid females were allowed to oviposit, and the eggs and larvae were reared in the lab. Newly molted nymphs from the same mother were put in two geographically different environments – one in Michigan and the other in Tennessee. Forty to 59 of nymphs from same mother were put in both Michigan and Tennessee environments. The authors then observed their questing behaviors. They found that although the nymphs, regardless of origin, spent more time under leaf litter, those from high Lyme disease

risk areas quested higher than those from transition and Lyme disease areas, irrespective of the environmental condition. Their findings suggested that differences in the numbers of Lyme disease cases across states in the eastern United States were linked to the questing behavior of the ticks. Ticks that quested high on dowels and on top of leaves where associated with high Lyme disease areas (Arsnoe et al., 2019). Their findings showed that the differences in the questing abilities of the *I. scapularis* nymphs were more related to behavior (genetics) than to the physical environment. Questing probabilities of nymphs from Virginia was 11.5% compared to 1.7% for North Carolina (Arsnoe et al., 2019).

Relationship between Rodents and the Invasion of *Ixodes scapularis* and *Borrelia burgdorferi into* Northwestern North Carolina

Putting the disease niche concept in the light of Lyme disease, the invasion of an area by infected and uninfected ticks does not mean that these components alone can main the disease niche. The transmission cycle must involve reservoir hosts that will cycle the *B. burgdorferi* among rodent communities in the environment. Rodents are also needed to serve as possible blood meal hosts for the *I. scapularis* ticks. According to Oliver et al. (2003), for any geographic area, it is critical to identify the main reservoir host species of *B. burgdorferi* by studying the rodent community structure. The distribution and abundance of rodents within an area can determine whether *I. scapularis* and *B. burgdorferi* can become established after being introduced to a new area. Therefore, evaluating the relative abundance of *P. leucopus* and other rodent species in northwestern North Carolina will shed light on the potential for establishing a persistent enzootic transmission cycle in this part of the state.

Aim

In this chapter, the aim was to characterize the local and regional rodent community of the New River valley and western Piedmont and its role in *B. burgdorferi* spread and persistence. Should the vector and pathogen successfully invade, the rodent community species composition and relative abundance of most competent hosts may determine whether they can become established or not.

Hypothesis

Hypothesis 1: Permissive or Non-permissive Environment

There are two possibilities, that is, either the environment is dominated by *P. leucopus*, making it suitable for the establishment of an effective enzootic transmission cycle, or the small mammal species within the environment are evenly distributed, making the area less permissive for the establishment of *B. burgdorferi* transmission cycle. The permissive environment increases the chances of *I. scapularis* encountering the reservoir host, *P. leucopus*, hence increasing the efficiency of pathogen transmission. The non-permissive environment would decrease the chances of *I. scapularis* encountering a *P. leucopus* because the species are more evenly distributed in such an environment.

Hypothesis 2: New River versus Piedmont

Should the permissiveness in the two 'Regions' be similar, *B. burgdorferi* infection rates in *P. leucopus* and attached ticks found in the New River region would be higher than those in the western Piedmont region. The infection rate would also extend further south in the New River region compared to the western Piedmont. My previous chapter showed that the New River is serving as a conduit that is facilitating the spread of *I. scapularis* and the pathogens they carry. For this reason, many *I. scapularis* would infect rodents in the New River than the western

Piedmont. Many of the engorged ticks collected off *P. leucopus* in the New River region would

be infected than those collected off *P. leucopus* in the western Piedmont.

Prediction

I predicted that for a permissive environment, the rates of *B. burgdorferi* infection in *P.*

leucopus would be higher than if the environment is not permissive. Overall infection rate in *P.*

leucopus in the New River region would be higher than the western Piedmont. The tick burden

on small mammals in the New River region would also be higher than that of the western

Piedmont.

METHODS

Sampling Sites

I established two regions, the New River and western Piedmont regions. Along each

region, I established five sampling sites. Each site on the New River had a corresponding parallel

site in the western Piedmont region. The sites were restricted to North Carolina because I was

interested in what happens in North Carolina after the *I. scapularis* and *B. burgdorferi* invasion.

The sites were selected along the same flagging transects used in the previous chapter. The sites

were selected to include areas where *I. scapularis* had been previously collected in relatively

high densities and other southern sites where no *I. scapularis* was observed through flagging

from the previous study. This would provide an indication of the current point of *I. scapularis*

and *B. burgdorferi* establishment and further test the cryptic enzootic cycle consistent with the

"spirochete-first" hypothesis. The sampling sites were North Carolina state parks or forests

located along the New River or western Piedmont except for Clawson Burnley Park (Figure 3.1)

which was a county recreational green space. There were selected sites along the New River and

corresponding western Piedmont sites (Figure 17). All the sites were mixed hardwood forest

habitats comprising oak, hickory, maple, hemlock, pines, shrubs, and coves in variable

proportions. Some parks such as Cumberland Knob, New River State Parks, and Clawson-

Burnley Park have patches of open fields of lawn. The edge habitats included meadows, forbs,

and herbs. All sites supported recreational activities such as hiking, biking, swimming, kayaking,

camping, and picnicking. Only Rendezvous State Forest permits regulated hunting.

Figure 17. Map of counties where small rodents were sampled, indicating the specific sites within the New River (blue) and western Piedmont (red) (indicated by green circular dots). (NRA –New River State Park, Alleghany; CMBK – Cumberland Knob; NR221 – New River State Park at Highway 221; CRK – Camp Raven Knob; NRW – New River State Park, Wagoner Access; SMSP – Stone Mountain State Park, CLBP – Clawson-Burnley Park; RSF – Rendezvous State Forest; LJSP –Lake James State Park; LNSP – Lake Norman State Park). The inserted map shows the location of sampling sites within North Carolina.
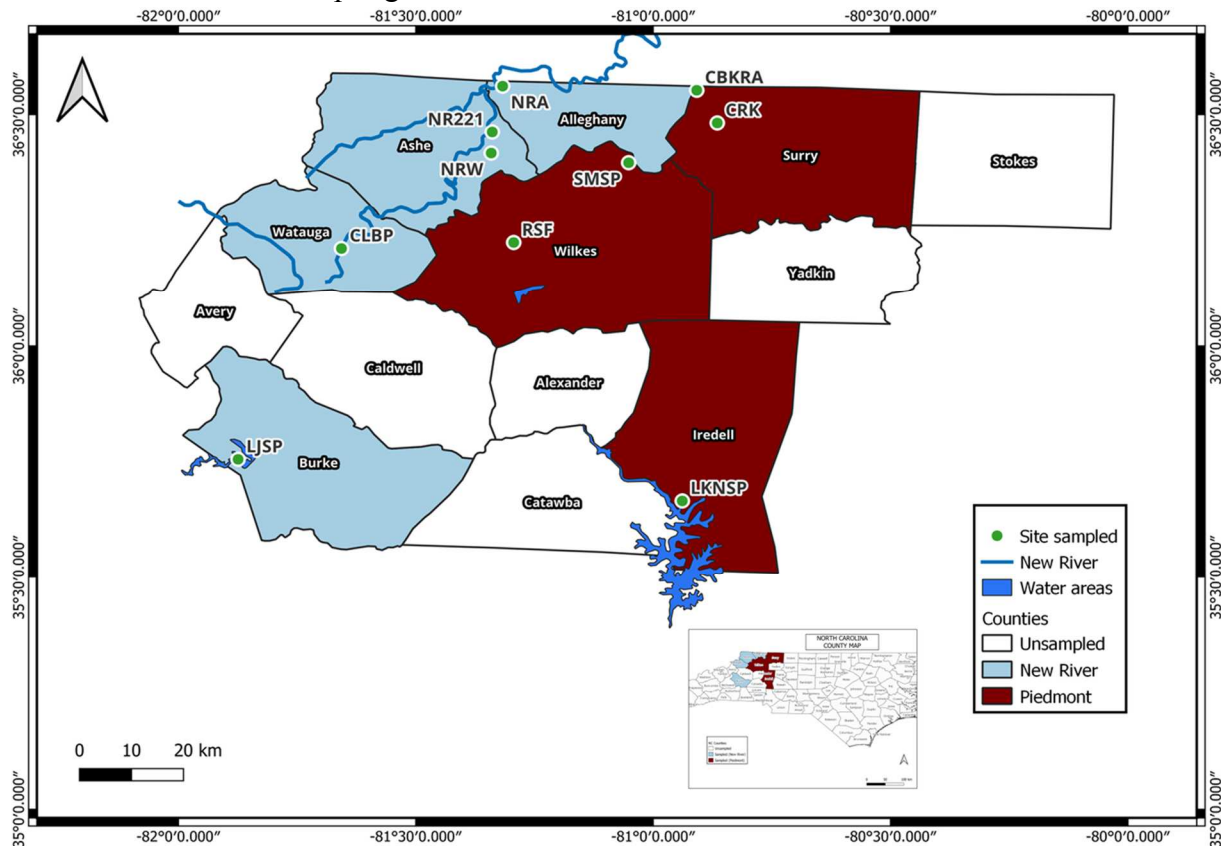
Methodology

    Small Mammal Trapping

Small mammals were live-trapped in the spring-summer time of each year between 2019-2021 (2019: April - July; 2020: July - August; 2021: April, - June) to coincide with the period of nymph and larva emergence. The selected trapping sites were within areas that had been previously flagged for ticks. Duration of each rodent trapping session was: two nights per site in 2019 and then increased to three consecutive nights in subsequent years to increase trapping success. All sites were visited once a year. Rendezvous State Forest was the only site that was visited once throughout the entire trapping period. Sherman collapsible traps (HB Sherman Traps Inc., Tallahassee, FL, USA) and Longworth traps (Anglian Lepidopterist Supplies, Norfolk, England) were used. The use of these two different trap types simultaneously helps reduce the bias estimate of species composition and improve capture efficiency (Anthony et al., 2005). Each site had four 100-m transects each comprising 10 pairs of traps, one Sherman and one Longworth at every trap station. Approximately, 10-m was reserved between adjacent trap stations. Every trap was labeled based on the transect line (as either A1 to A10, B1 to B10, C1 to C10, or D1 to D10, depending on the transect name, A to D) and the trap type (either Sherman or Longworth). Flagging tapes were used to mark every trap station for verification purposes and ease of trap location during trap inspection and re-setting. Each trap station's flagging tape had the same label as indicated on each trap. In addition, each transect line was georeferenced. Mixed seeds, comprising sunflower seeds, millet seeds, and peanuts were used as preferred bait in 2019. The bait was changed to peanut butter (Great Value Creamy Peanut Butter, Walmart Stores Inc., Bentonville, AR, USA) mixed with fresh oats (Great Value Old Fashioned Oats, Walmart Stores Inc., Bentonville, AR, USA) in subsequent years for improved trapping success. The traps were

set at sunset and inspected at sunrise each day. Protocols for trapping and handling rodents were by the American Society of Mammologists guidelines (Sikes & Gannon, 2011).

For every terrestrial small mammal trapped, the following standard morphometric measurements were taken: Total Length (TOTL): total body and tail length; from tip of the nose to end of the tail; Tail Length (TL): from the base of tail at a right angle to the body to the tip of tail; Head-and-Body Length (HBL) (i.e., TOTL – TL); Hind Foot Length (HFL): from heel to the nail of the longest toe; Ear Length (EL): from the basal notch to the distal tip of the pinna; Weight (WT) in grams. Each animal was identified using Reid's (2006) field guide and examined for reproductive condition (non-reproductive, abdominal, or scrotal testes in males, and lactating, pregnant, and non-reproductive [i.e., not pregnant or lactating] in females). *Peromyscus leucopus* and *P. maniculatus* are morphologically similar. The differentiation between these two species was based on the tail length, extent of bicoloration and hindfoot length (e.g., Bruseo et al., 1999). The sex (using anogenital distance, which is longer in males) and age (assigned into three broad class ranges: juvenile, subadult, and adult based on weight) were also determined. Before being released at the point of capture, ear tissue samples were collected from both ears of each small mammal, using a 2-mm ear-punch (Miltex Instruments, York, PA), and then color marked on the belly using a permanent marker. Each punched ear was sanitized using 70% ethanol swabs before and after punching. The 70% ethanol swabs were also used to sterilize each punch kit before and after each use. Tissue samples and collected ectoparasites were separately stored in 95% ethanol for future testing for the presence of *B. burgdorferi*. Recaptures within a trapping session were only rechecked for ectoparasites, reweighed, and released at the site of capture (Hamer et al., 2010). The tripping mechanism for each trap was carefully checked anytime the traps were being set to maintain a relatively constant degree of sensitivity. The trap success rate

was calculated by taking the number of empty and/or tripped traps into account. Trapping success was calculated as the quotient of the number of successful captures and the number of traps set deducted from the product of half the number of tripped traps (i.e., no. of successful traps / (no. of traps set - 0.5*number of tripped traps). The assumption was that on average, tripped traps were open for half the night (Hamer et al., 2010). The University of North Carolina at Greensboro Institutional Animal Care and Use Committee (IACUC) training modules and permits (# 18-002 for 2018-2020 and 21-006 for 2021) for trapping and handling rodents and other small mammals were used. Licenses to trap in the selected sites were obtained from the North Carolina Wildlife Resources Commission under the collection license # SC01252.

Ectoparasite Sampling

Prior to ear punches, all individual small mammals were inspected for attached ectoparasites and the tick burden (i.e., the number of ticks per animal) was recorded. The pelage of every small mammal caught was systematically examined for attached ticks, paying particular attention to the ears, groin, foot, and main body where ticks are not easily dislodged (Barnett & Dutton, 1995; Durden et al., 1991). Any tick observed was collected with pointed forceps. The collected ectoparasites were put in labeled 1.5 mm Eppendorf tubes containing 95% ethanol. In the laboratory, the ticks were kept at -20°C for future identification. The number of ticks collected off one rodent represented the rodent's tick burden.

DNA Extraction from Small Mammal Ear Tissue Samples

To avoid contamination of samples with external DNA, extraction of total DNA from ear tissue and tick, and qPCR analysis were conducted in different rooms. The protocol for DNA extraction of ear samples followed the outlined procedure from QIAGEN's DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany). One ear tissue sample from each rodent trapped from

the field was transferred into sterile microcentrifuge tubes then 180 µL of buffer ATL, and 20 µL of proteinase K were added. The solutions and tissue sample were mixed by vortexing for 30 s and then incubated at 56°C for 1 hour for cell lysis. Then 200 µL of Buffer AL was added, vortexed for 30 s, and incubated at 56ºC for another hour for cell lysis. After incubation, 200 µL of 100% ethanol was added and vortexed for 30 s. The 600 µL of samples were then transferred into a DNeasy Mini spin column placed in a 2 mL collection tube. The mixture was centrifuged at 8,000 rpm for 1 minute. Then the flow through and collection tube were discarded, and the spin column was put into a new 2 mL microcentrifuge tube. A 500 µL volume of wash buffer AW1 was added and centrifuged at 8,000 rpm for another 1 minute, after which the flow through and collection tube was discarded. The spin column was transferred to a new 2 mL collection tube, and then 500 µL of buffer AW2 was added and centrifuged at 14,000 rpm for 3 minutes. The spin column was put into a 2 mL microcentrifuge tube after discarding the flow through and collection tube. The DNA in the spin column was eluted by adding 50 µL of nuclease-free sterile water to the center of the spin column membrane and centrifuged at 8,000 rpm for 1 minute. This procedure was followed for each small mammal ear tissue sample. The DNA extract was stored in a -20º C freezer until needed.

DNA Extraction from Ticks Collected Off Small Mammals

Due to the hardiness of tick tissues, extra initial steps are involved in DNA extraction from ticks compared to DNA extraction from small mammal ear tissue. Each 2 mL microcentrifuge tube (Fisher Scientific, Hampton, NH), containing approximately 12 sterilized 3 mm diameter glass beads (#11-312A, Fisher Scientific, Hampton, NH) with 180- µL ATL buffer and 20-µL of proteinase K was prepared beforehand. Then, each tick was placed on the microtube's inside walls, then cut into very tiny pieces using sterile steel surgical scalpel blades

(Royaltek stainless, Fisher Scientific). The samples were homogenized in a FastPrep™ FP120 Cell Disruptor (Thermo Electron Corporation, Carlsbad, CA) for 2 cycles of 40 seconds each, then incubated at around 56°C for 1 hour and then vortexed for about 15 seconds before proceeding to the next step. The steps that followed (from adding AL buffer to eluting the DNA using PCR water) were the same as described above.

Detection of *Borrelia burgdorferi* and *Borrelia miyamotoi* from Extracted DNA Samples

The presence of *B. burgdorferi* sensu lato and *B. miyamotoi* DNA was determined using multiplex quantitative real-time PCR primers, probe, and amplification conditions of the 16s rRNA gene described by Tsao et al., 2004. The 20-µL qPCR mixture consists of 10 µL of 2 x TaqMan Universal Mastermix, 1 µL each of 16S forward primer, 16S reverse primer, *B. burgdorferi* probe, and *B. miyamotoi* probe, and 4 µL PCR water. DNA from *B. burgdorferi* strain B31 and sterile water were positive and negative PCR controls, respectively. *Borrelia burgdorferi* DNA (10^1/spirochetes/µl) was obtained from Dr. Jean Tsao, Michigan State University. After amplification and real-time data acquisition, analysis was performed with a BIO-RAD CFX Manager Software. DNA extraction and qPCR analysis was done in Loganathan Ponnusamy's laboratory at the Dearstyne Entomology Building, North Carolina State University, Raleigh, North Carolina under his guidance and supervision.

Data Analysis

Trapnights referred to the number of traps set for a night. For disturbed traps, they were assumed to have been opened for half the time throughout the night (Hamer et al., 2010). I calculated the relative abundances of each species by habitat. Comparisons were made between New River and western Piedmont. Relative abundance or relative frequency of capture of a

rodent species was determined by calculating the total number of individuals of that species for a site divided by the total number of individuals of all species for that site. Tick burden referred to the number of ticks on a rodent. To determine the mean number of ticks per rodent species per site, the total number of ticks collected for that species for a given site was divided by the number of rodents belonging to that species. *Borrelia burgdorferi* was counted as present in a rodent when the PCR cycle number (Cq value) was between 20 and 40. PCR numbers below 20 were regarded as background noise, while those above 40 were considered as having too few non-specific amplicons to be considered a positive detection. These values were determined based on the concentration of *B. burgdorferi* in the positive control which was 10 spirochetes/1 µL. Standard errors for proportional data were determined as the square root of the product of the proportion of positives (p), minus its inverse divided by the sample size, n (i.e., $\sqrt{[(p*(1-0))/n]}$). The standard error in tick burden was calculated as the square root of the standard deviation. The Chi-square test was used to determine p-values for proportional data using Microsoft Excel (2010 version). The statistical significance in tick burdens between the western Piedmont and New River regions was determined using the generalized linear model under the negative binomial family (used the R software). Bar graphs were used to analyze the relative abundances, and variations in *P. leucopus* proportions as related to the number of attached ticks and *B. burgdorferi* prevalence.

RESULTS

Rodent Distribution and Abundance and the *Borrelia burgdorferi* Infection Rates

A total of 3,881 trapnights (i.e., after accounting for disturbed traps) yielded 174 small mammals belonging to nine species: one star-nosed mole (*Condylura cristata*), one pine vole (*Microtus pinetorum*), one woodland jumping mouse (*Napaeozapus insignis*), six golden mice

(*Ochrotomys nuttalli*), 154 white-footed mice *(Peromyscus leucopus)*, five eastern gray squirrels (*Sciurus carolinensis*), three hispid cotton rat (*Sigmodon hispidus*), two shrews (*Sorex* sp.), and one eastern chipmunk (*Tamias striatus*) (Table 26). *Peromyscus leucopus, Ochrotomys nuttalli,* and *Sorex* sp. were common to both the New River and western Piedmont (Table 26). *Peromyscus leucopus* dominated the overall rodent community with a relative frequency of 88.51% (Table 26). Its dominance was recorded in every site, both at the New River and western Piedmont sites, except for Lake Norman where *P. leucopus* was one less than *S. hispidus* (Table 26). Comparatively, there were 57.14% more *P. leucopus* in western Piedmont than the New River. However, their relative abundances were not significantly different (Figure 18).

Out of the 174 small mammals captured,170 were screened for *B. burgdorferi* of which 21.76% were found to be infected (numbers of non-*Peromyscus leucopus* that were tested were very few due to their low relative abundances [Table 26]). All the rodents that tested positive for *B. burgdorferi* were *P. leucopus* except for four individuals of three species: two *S. carolinensis* (one from New River, Ashe, and the other from New River, Alleghany), one *M. pinetorum* (from New River, Alleghany), and one *T. striatus* (from New River, Ashe). *Peromyscus leucopus* infection in the New River region was significantly higher (p=0.00072) than that of western Piedmont (Figure 18). Infected rodents were found as far south as Watauga County for the New River region while at western Piedmont, *B. burgdorferi* infection in rodents was not detected after (north of Wilkes County) (Figure 20). Approximately 53.83% of the *P. leucopus* in Watauga County were infected with *B. burgdorferi*.

Out of the 37 infected rodents, 64.86% were in the New River area (i.e., 24 against 13). Among the 13 infected rodents in western Piedmont, five were in Cumberland Knob (BorderNC), one in Camp Raven Knob (UpperNC), and the rest were from Stone Mountain State

Park (MidNC), which is at the border between Alleghany and Wilkes County (Figure 18). Stone Mountain contributed 56.12% to the entire *P. leucopus* caught in western Piedmont. No *B. burgdorferi* infection was detected in rodents from the southernmost sites, LowerNC (refer to Figure 20).

Tick Burden on Rodents

In all, 98 ticks comprising 79 larvae and 19 nymphs were collected on 37 rodents. All the ticks were *Ixodes scapularis* ticks and were all found on *P. leucopus*. Out of the 98 *I. scapularis* ticks, 11.22% were found in western Piedmont and the rest in the New River region. Of the larvae infesting rodents, 26.58% were infected with *B. burgdorferi* while 42. 11% of the nymphs were infected. Three *B. burgdorferi*-infected larvae were from non-infected *P. leucopus*. All the rest of infected larvae (n=18) came from infected *P. leucopus* mice. All the eight *I. scapularis* nymphs were from the New River area (Figure 19). Two of these nymphs were collected off an infected rodent. The remaining six were collected off five *P. leucopus* individuals. All the infected nymphs were from the Alleghany and Ashe County sites. Out of the 21 infected larvae, 20 (95.24%) were from the New River region. Only one was found infected in the western Piedmont region (Figure 18). Only eight of the ticks were collected off rodents in the western Piedmont. The remaining 98 immature ticks were collected off *I. scapularis* in the New River region. In terms of per capita tick burden, I recorded 1.4 ticks per *P. leucopus* in the new River compared to 0.11 tick per *P. leucopus* in the western Piedmont. (Figure 18).

Table 26. Rodent species caught in the New River and western Piedmont region in a north-to-south gradient, beginning from the sites at the North Carolina-Virginia border to the southernmost sites in Burke and Iredell Counties (refer to Figure 17).

Numbers in parentheses refer to the tick burden per individual of that species. The parenthesis at the totals refers to the tick burden for all the rodents caught in a given site throughout the study.

Scientific name	NC-VA Border		UppperNC		MidNC		LowNC		LowerNC		Total	
	New River	Piedmont	New River	Piedmont	New River	Piedmont	New River	Piedmont	New River	Piedmont	New River	Piedmont
Condylura cristata	-	-	1 (0.0)	-	-	-	-	-	-	-	1	
Microtus pinetorum	1 (0.0)	-	-	-	-	-	-	-	-	-	1	
Napaeozapus insignis	-	-	-	1 (0.0)	-	-	-	-	-	-		1
Ochrotomys nuttalli	1 (0.0)	-	-	2 (0.0)	-	2 (0.0)	-	-	1 (0.0)	-	2	4
Peromyscus leucopus	2 (1.5)	33 (0.03)	28 (1.17)	4 (0.0)	6 (1.00)	55 (0.13)	13 (4.15)	4 (0.75)	7 (0.0)	2 (0.0)	56	98
Sciurus carolinensis	-	-	4 (0.0)	-	-	-	1	-	-	-	5	
Sigmodon hispidus	-	-	-	-	-	-	-	-	-	3 (0.0)		3
Sorex sp.	-	-	-	-	-	1 (0.0)	1	-	-	-	1	1
Tamias striatus	-	-	-	-	-	-	1	-	--	-	1	
Total rodents/site	**4 (0.75)**	**33 (0.03)**	**33 (0.99)**	**7 (0.00)**	**6 (1.00)**	**58 (0.12)**	**16 (3.38)**	**4 (0.75)**	**8 (0.0)**	**5 (0.0)**	**67**	**107**

Figure 18. Relative frequency of *P. leucopus* in mammal communities of the New River and western Piedmont (far left bars). Numbers on the bars represent the number of *P. leucopus* in the two areas. Prevalence of *B. burgdorferi* in *P. leucopus* in the New River and Western Piedmont (middle bars). Numbers on the bars represent the number of *P. leucopus* that were tested (middle bars). Tick burden on *P. leucopus* in the New River and western Piedmont (far right bars). Numbers on the bars represent the number of *P. leucopus* in the two areas. Error bars are standard error bars.
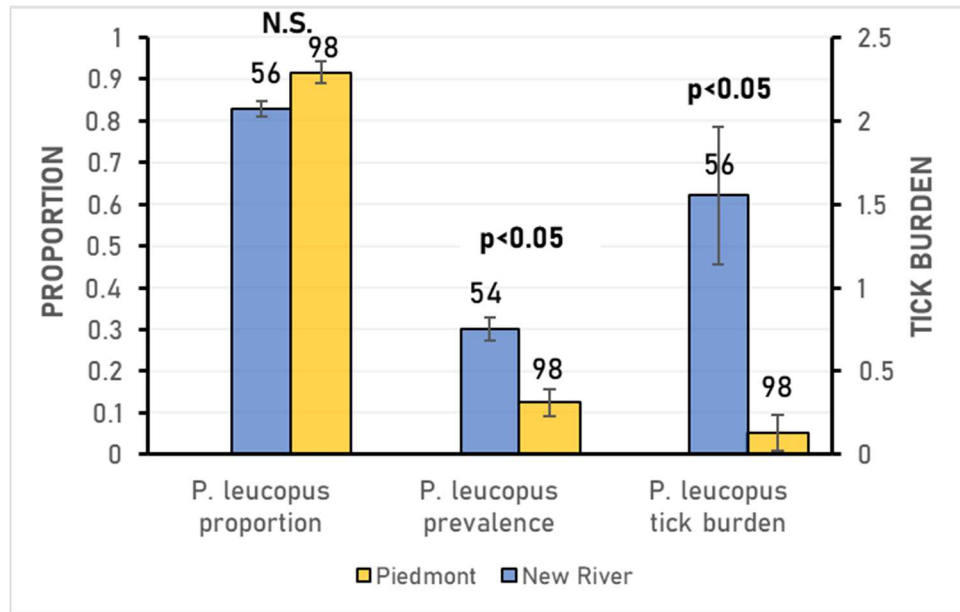
Figure 19. *Borrelia burgdorferi* **prevalence along a north-to-south gradient in the New River and western Piedmont.** Error bars represent standard error bars. Numbers on the bars represent the number of small mammal ear tissues that were tested.
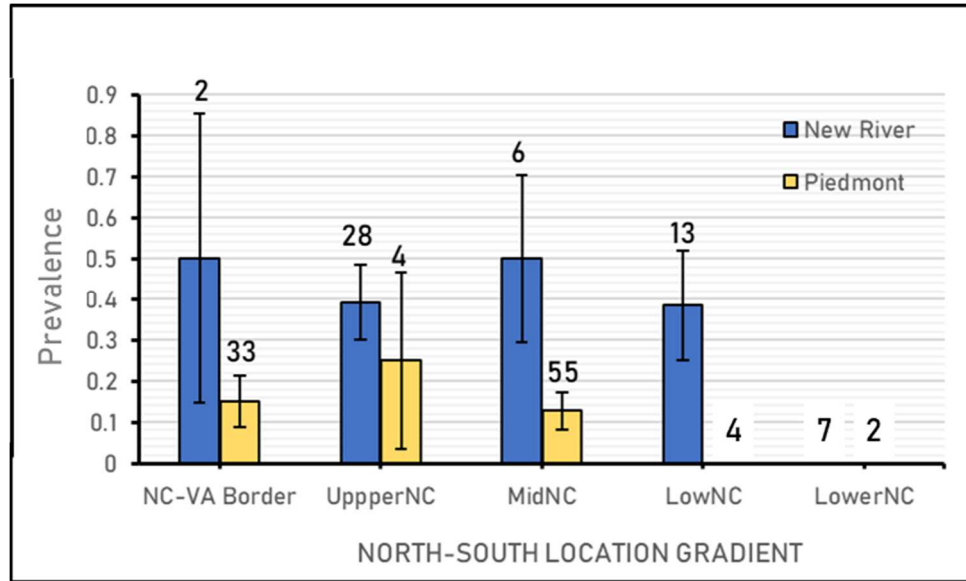
Figure 20. Infection rate in *I. scapularis* **larvae and nymphs collected off** *P. leucopus* **in the New River and western Piedmont.** Error bars represent standard error bars. Numbers on the bars represent the number of ticks tested.
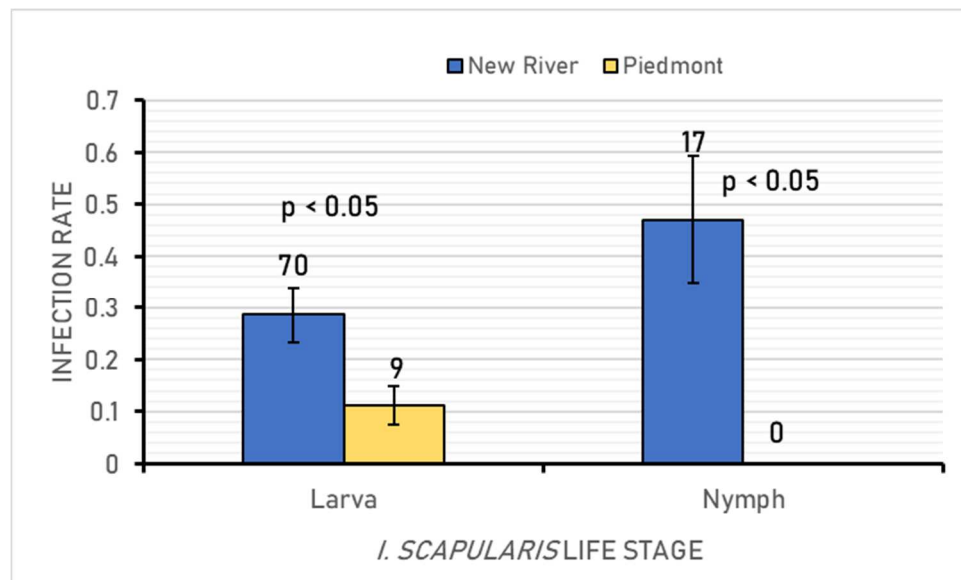
DISCUSSION

Distribution and Relative Abundance of *Ixodes scapularis*

This study is the first of its kind to be conducted in northwestern North Carolina and provides additional knowledge on the spread of *I. scapularis* and *B. burgdorferi* from the northern state of Virginia into North Carolina along the Appalachian Mountains and Piedmont foothills. *Peromyscus leucopus*, the principal reservoir host of *B. burgdorferi* dominated the rodent community from north to south except for the southernmost county of Iredell. An assessment of the rodent community in the Piedmont of North Carolina by (Kapfer & Muñoz, 2012) likewise found that *P. leucopus* dominated the rodent community. Another study in 1996 on the distribution, diversity, and host specificity of Bartonella in rodents from the Southeastern United States found *P. leucopus* as the dominant species in North Carolina Piedmont (i.e., Morrow Mountain Park in Stanly County and Catawba College Reserve in Rowan County) (Kosoy et al., 1997). A recent thesis by Mellis, (2021) on the spatial variation in mammal and ectoparasite communities in the foothills along the southern Appalachian Mountains also found that *P. leucopus* was the dominant species. A study of the effect of riparian zones on small mammal communities in the southern Appalachians of North Carolina, Macon County did not find any difference in small mammal community structure between the riparian and non-riparian zones and attributed this to the lack of structural and vegetative distinction between these two zones (Laerm et al., 1997).

The permissiveness of the western North Carolina environment for *Borrelia burgdorferi* establishment

All the areas trapped were large woodland forests in State Parks or county parks with little effect from fragmentation. However, the relative proportion of *P. leucopus* in the area

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compared to other rodent species was significantly high. The dominance of *P. leucopus* in the area is consistent with the suitability of the environment for the establishment of an efficient *B. burgdorferi* transmission cycle. The similar relative proportion of *P. leucopus* in both the New River and western Piedmont suggests that *P. leucopus* naturally constitutes most of the small mammals in the area. Even though the relative proportion of *P. leucopus* was similar between the two regions, infection rate and tick burden were significantly higher in the New River than the western Piedmont. This is consistent with the New River hypothesis in the previous chapter which showed that *I. scapularis* density and their infection rates were higher in the New River than the western Piedmont.

Tick Burdens on *Peromyscus leucopus* and Tick *Borrelia burgdorferi* Infections

The significant tick burden in the New River area despite the comparatively lower abundance of *P. leucopus* compared to western Piedmont is also consistent with the use of the New River as a potential route for invasion by *I. scapularis*. The high numbers of *I. scapularis* infesting *P. leucopus* and their corresponding high infection rates suggested that *P. leucopus* was not only preferred by *I. scapularis* but also a competent reservoir host for *B. burgdorferi* as demonstrated by other studies (e.g., Schmidt & Ostfeld, 2001). *Peromyscus leucopus* may not necessarily be the preferred host by immature *I. scapularis*. It may be that *P. leucopus* is more tolerant of *I. scapularis* in terms of poor grooming etc. as suggested by Davidar et al. (1989). Larval infection in *I. scapularis* ticks indicated that the *B. burgdorferi* pathogen is probably already established in the *P. leucopus* population in the study area and circulation enzootically in that system. The detection of *B. burgdorferi* infection in a chipmunk, eastern gray squirrels, and a pine vole is consistent with previous findings which implicated voles, squirrels, and chipmunks as other potential competent hosts (Mather et al., 1989). The high *P. leucopus* infection rate

together with the high *I. scapularis* larval infection (collected from rodents) indicates that

throughout the northern part of northwestern North Carolina, there is an efficient enzootic

transmission cycle of the *B. burgdorferi* with the southern part of that region having a strong

potential for it once the tick and the pathogens are introduced. The fact that I found some

infected larvae from non-infected rodents is of great interest, resulting possibly from partial

feeding on several hosts or possibly transovarial transmission. This, obviously, warrant further

investigation.

The Three Mechanisms of *Ixodes scapularis-Borrelia burgdorferi* Invasion

Hamer et al. (2010) showed that there are three mechanisms of tick invasion: (1) tick-

first, (2) dual-invasion, and (3) spirochete-first. In the tick-first scenario, initial surveillance of

ticks and competent hosts in an area shows no sign of pathogen and ticks' presence. The initial

detection of ticks before the later detection of the pathogen supports the tick-first scenario.

When both competent hosts, their attached ticks and unattached ticks are found to be infected

simultaneously then it is consistent with the dual-invasion scenario. Likewise, when no *I.*

scapularis is detected but rather other ticks and infection in competent hosts as well as some of

the other ticks then it is supportive of the spirochete-first scenario.

The absence of *B. burgdorferi* infection in *P. leucopus* from my southernmost sites where

I. scapularis was absent, and the non-detection of other tick species on *P. leucopus*, indicated

that the mechanism of invasion cannot be the spirochete-first. The tick-first scenario suggests

that the non-infected ticks are carried into new areas by incompetent hosts such as the white-

tailed deer before the invasion by the slower moving reservoir hosts that introduce infected

ticks(Hamer et al., 2010). The detection of *I. scapularis* on *P. leucopus* suggested that it could be

the dual invasion or the tick-first mechanism. However, I found from the previous chapter that non-infected ticks were leading the invasion which is consistent with the tick-first hypothesis.

Concluding Remarks

The dominance of *P. leucopus*, their level of infection, and their high tick burden in northwestern North Carolina indicates that the area is highly permissive for the establishment of an effective *B. burgdorferi* enzootic cycle. Preference of *I. scapularis* for *P. leucopus* or inefficient groom by *P. leucopus* might be the reason for their high tick burdens. The tick-first mechanism of invasion suggests that a non-reservoir host, possibly the white-tailed deer (*Odocoileus virginianus*), may be responsible for the initial spread of *I. scapularis* into the region.

In terms of public health implications, these findings of high environmental enzootic suitability of this region for Lyme disease establishment and spread are consistent with human case distribution (Figure 6) suggest that the reported and probable human cases of Lyme disease in northwestern North Carolina were locally acquired. The suitability of the environment suggests that the western North Carolina region may experience increased number of cases soon. Therefore, emphasis should be given to public education. Specifically, people should be educated on how to minimize tick bites by checking themselves regularly when they go out in the woods for recreation. It is also important that people wear clothes that are (i) bright enough to easily spot a tick on them; (ii) impregnated with chemicals for controlling ticks such as permethrin; and (iii) well tucked into the socks and at the waist to make it difficult to ticks to reach the skin. In addition, it is important to take a bath right after returning from the woods and have clothes washed in hot water and dried with heat. Medical professionals must also be made aware of the current status of Lyme disease in northwestern North Carolina. Long-term

monitoring of the rodent composition and the effect of environmental variables such as temperature, relative humidity, leaf litter size, forest type and plant species can be utilized to determine the spatial eco-epidemiology of Lyme disease within the region to be able to provide effective policies to relevant stakeholders.

CHAPTER IV: DETERMINE THE PHENOLOGY AND PATTERNS OF INFECTION IN THE INVADING IXODES SCAPULARIS TICKS IN THE NORTHWESTERN NORTH CAROLINA CORRIDOR

ABSTRACT

Lyme disease is the most common vector-borne disease in the United States. Each year, over 30,000 cases of the disease are reported by state health departments to the Centers for Disease Control and prevention (CDC). Lyme borreliosis is caused by B*orrelia burgdorferi* and transmitted by the ixodid tick, *Ixodes scapularis* (blacklegged tick). Previous cluster analysis study of Lyme disease cases in endemic Virginia predicted the invasion of Lyme borreliosis into western North Carolina following the Appalachian Mountains. Differences in climate and host distribution and abundance can alter the phenology of the invading ticks. This study sought to investigate the phenology of *I. scapularis* ticks as they invade North Carolina through the northwestern corner. Two sites, one in Alleghany County and the other in Ashe County, which are known from previous study to be current hotspots of *I. scapularis* invasion were selected for the study. The sites were flagged monthly for ticks. Some of the ticks were sent to CDC for screening for *B. burgdorferi* after morphological identification using appropriate keys. The data showed that tick questing activity pattern was similar to the asynchronous phenology pattern that is observed in the Northeast. Nymphal activity pattern spanned from May to October with most activity occurring during late Spring and early Summer. Modeling data showed that altitude, temperature, and relative humidity affect nymph distribution. There were slight variations between the two sites in terms of temporal tick activity patterns, hence, the need for continuous monitoring programs to develop predictive models that can effectively help to manage and inform the public on periods of high Lyme disease risk.

INTRODUCTION

In Chapter 2, I discussed about the spatial dimension of the putative spread of *Ixodes scapularis* and *Borrelia burgdorferi* into northwestern North Carolina, paying attention to geographic features along the route on invasion that may be facilitating their spread. and the mechanism of invasion. In Chapter 3, I discussed the reservoir component of the system to understand if there is a possibility for the establishment of a Lyme disease niche through the establishment of *B. burgdorferi* transmission cycle. In this chapter, I will focus on the temporal dimension of the *I. scapularis-B. burgdorferi* system. Specifically, I will address the seasonal dimension of the life cycle of *I. scapularis* ticks and evaluate its relatedness to the typical two-year *I scapularis* life cycle observed in other hyper endemic regions of Lyme disease.

Phenology and Its Importance in Vector-borne Diseases

Phenology is the timing of a vector's life cycle (US National Phenology Network, 2020). By understanding the phenology of disease vectors, we can predict when certain vector-borne diseases will emerge because the transmission of the pathogen carried by the vector is related to the variations in the vector's seasonal activities (Levi et al., 2015; MacDonald, 2018; US National Phenology Network, 2020). For example, Lyme disease will spread during periods of nymphal *I. scapularis* activity because the nymphs are not easily spotted on the skin due to their small size. Information about the phenology of a disease can direct proper public health attention to periods of increased vector activity in order to minimize the incidence of the disease. For example, cholera, which is caused by the bacteria *Vibro cholerae* (an organism in aquatic environments), and vectored by the common housefly, *Musca domestica*, is common during the wet seasons in endemic areas, therefore, adequate information on good personal and environmental hygiene during this season is a necessary step towards the prevention of a cholera

outbreak. Understanding the environmental conditions suitable for the vector and its life cycle

can provide essential intervention methods by helping to know which factors affect their survival

and when they can be targeted for effective control. Currently, it is unclear whether the invading

I. scapularies ticks would have a phenology similar to that of endemic states like Virginia as

changes in ecological dynamics have influence on the seasonality of vector species (Levi et al.,

2015). According to Hamer et al. (2012), the dynamics and processes affecting tick and pathogen

densities and spread into newly-invaded areas may differ from those in endemic areas, because

of geographic variation in factors such as photoperiod, climate, habitat suitability as well as

varying population dynamics as the organisms become established.

Phenology of Lyme Disease in Endemic Northeastern States

In the northeast and central United States, the life cycle of *I. scapularis* lasts for two

years (Figure 21). It is a three-host life cycle because each active life stage (i.e., larva, nymph,

and adult) must acquire a blood meal from a different host. The life cycle begins with egg laying

by the replete *I. scapularis* female spring. The female dies following egg laying, while the male

dies following mating. Eggs hatch around mid-summer (if earlier, larvae do not blood feed until

mid-summer) (Yuval & Spielman, 1990). New *I. scapularis* larvae quest for blood meal from

mid-summer until late fall of the same year (Yuval & Spielman, 1990). Larvae that blood feed

during the summer months molt into nymphs (soon afterwards) that stay inactive until the

following year (Yuval & Spielman, 1990). Larvae that blood fed in late fall overwinter into the

following year before molting into nymphs during springtime (Yuval & Spielman, 1990).

Nymphs quest (i.e., seek for host) from late spring to mid-summer with their activity peaking

around early summer The few larvae that did not feed the previous year overwinter and seek for

hosts concurrently with nymphs in spring (Lindsay et al., 1998). Nymphs that blood feed early

(i.e., during the spring-summertime) molt into adults that quest during mid-fall of the same year (Ostfeld et al., 2006). However, nymphs that blood feed around mid-summer do not molt into adults until spring of the following year. Most blood fed nymphs that develop into adults the following year die (Yuval & Spielman, 1990). Therefore, feeding before late summer is crucial for the nymphs to develop into adults (Yuval & Spielman, 1990). However, unfed nymphs that emerged late in the first year overwinter and quest the following year so that nymphs are the only life stage with more than one cohort coexisting in nature because they survive two seasons (Yuval & Spielman, 1990). The adults that emerged the following year and those that overwintered without blood feeding the previous year do so during early spring. Adult *I, scapularis* that are unsuccessful in acquiring blood meals die by the beginning of summer.

In the next year, the nymphs emerge first in early spring through early summer. The larvae, on the other hand, re-emerge in early spring together with the nymphs (Daniels et al., 1996; Yuval & Spielman, 1990). Nymphs actively seek for blood meals from spring up until early Summer (Anderson, 1989). Those nymphs that were successful in acquiring blood meals molt into adults that become active in Fall, overwinter, and continue seeking for a suitable host up until early Spring. *Ixodes scapularis* adults that were unsuccessful in acquiring blood meals die by summer of the second year. *Ixodes scapularis* nymphs that do not obtain a blood meal by the end of mid-summer stay inactive until the following year's Spring.

Ixodes scapularis Life Cycle and Lyme Disease Risk

The effect of seasonal pattern on *I. scapularis* activity has been postulated as a crucial factor in determining the efficiency and rate of transmission cycles of pathogens associated with this tick species (Levi et al., 2015; Piesman & Spielman, 1979; Randolph, 1998; Wilson & Spielman, 1985). For the transmission cycle of *B. burgdorferi* to persist, in the absence of

efficient transovarial transmission (i.e., transmission from mother to eggs), larvae and nymphs

must feed on the same infective hosts within a timeframe that spans the duration of

infectiousness of the host (Levi et al., 2015).

Figure 21. The typical two-year and three-host life cycle of I. scapularis. The asynchronous

phenology occurring in this mode of I. scapularis life cycle makes B. burgdorferi transmission

cycle effective. Illustrated by Sankey (2015).
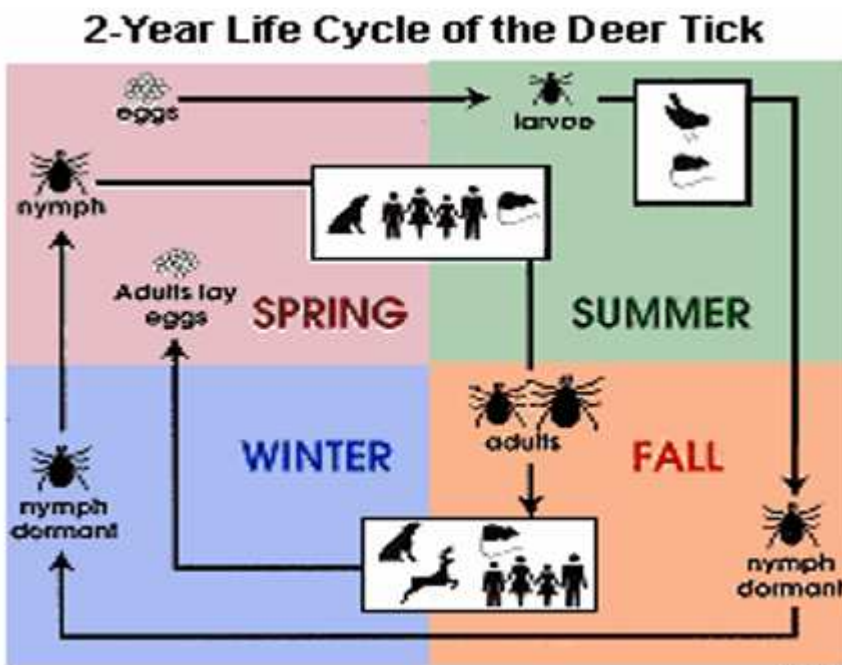
Ixodes scapularis phenology can be either asynchronous or synchronous (Figure 22).

Asynchronous phenology, as commonly observed in the northeastern and central United States,

involves the emergence of the nymphs before the larvae. This ensures that the infective nymphs

transmit the *B. burgdorferi* to new and old uninfected generations of the reservoir host such as

Peromyscus leucopus before the emergence of the uninfected larvae (Levi et al., 2015). This

phenomenon ensures that many reservoir hosts are infected before the larvae begin to quest to

ensure the perpetuation of the enzootic transmission cycle. Asynchronous phenology is effective

in cases where reservoir hosts are able to maintain infectivity from nymphs long enough until larval peak emergence (Levi et al., 2015).

In the case of *I. ricinus* in Europe, and *I. persulcatus* in Asia, all the active life stages can feed synchronously (Kurtenbach et al., 2006), with the degree of synchrony varying geographically and possibly determined by climate (Randolph et al., 2000). Low infection prevalence of *B. burgdorferi* in nymphal ticks in Europe and Asia, compared to eastern North America, is thought to arise in part from synchronous feeding of larvae and nymphs in this Eurasian territory (Kurtenbach et al., 2006). There is a brief incident of synchrony in the life cycle of *I. scapularis* in the northeast. This occurs when the few larvae that did not blood feed the previous year overwinter and feed concurrently with nymphs in summer (Levi et al., 2015). In areas where synchronous phenology if the sole mode of *B. burgdorferi* transmission, the period of infectivity in reservoir hosts need not be long because the nymphs infect rodents which then infect larvae within the same timeframe (Levi et al., 2015).

Periods of peak in *I. scapularis* nymphs is important in influencing human exposure risks. In the northeastern United States, for example, the late spring to early summer timing of peak nymphal activity coincides with human outdoor activity, which is thought to increase risk of human exposure (Barbour & Fish, 1993). Substantial changes in the timing of this peak could influence the coincidence of tick and human activity, affecting epidemiological patterns (Levi et al., 2015). Model simulations have also suggested that along a latitudinal gradient from northeastern to southeastern United States, larvae may be active progressively earlier in the year, resulting in nymphs being more likely to appear and be active in autumn (Ogden et al., 2008). This emergence of larvae prior to nymphal activity has been observed in some studies of southeastern *I. scapularis* populations (e.g., Clark et al., 1998), and may be contributing to the

inefficient *B. burgdorferi* transmission cycle observed in southeastern states (Oliver, 1996). A more recent study by Ogden et al. (2008) on the phenology of *I. scapularis* at the Mattamuskeet National Wildlife Refuge, in the coastal region of North Carolina, showed larval activity peaking in June, well before the nymphal peak in late summer to autumn. This could be related to the low Lyme disease incidence in eastern North Carolina, and possibly other southeastern states (Gatewood et al., 2009; Ogden et al., 2007; Schwartz et al., 2017).

Figure 22. The two possible phenology in *I. scapularis* life cycle. (a) Synchronous phenology, where the second minor larval and nymphal peak occur concurrently. (b) Asynchronous phenology, where the nymphal peak occurs ahead of that of the larvae.
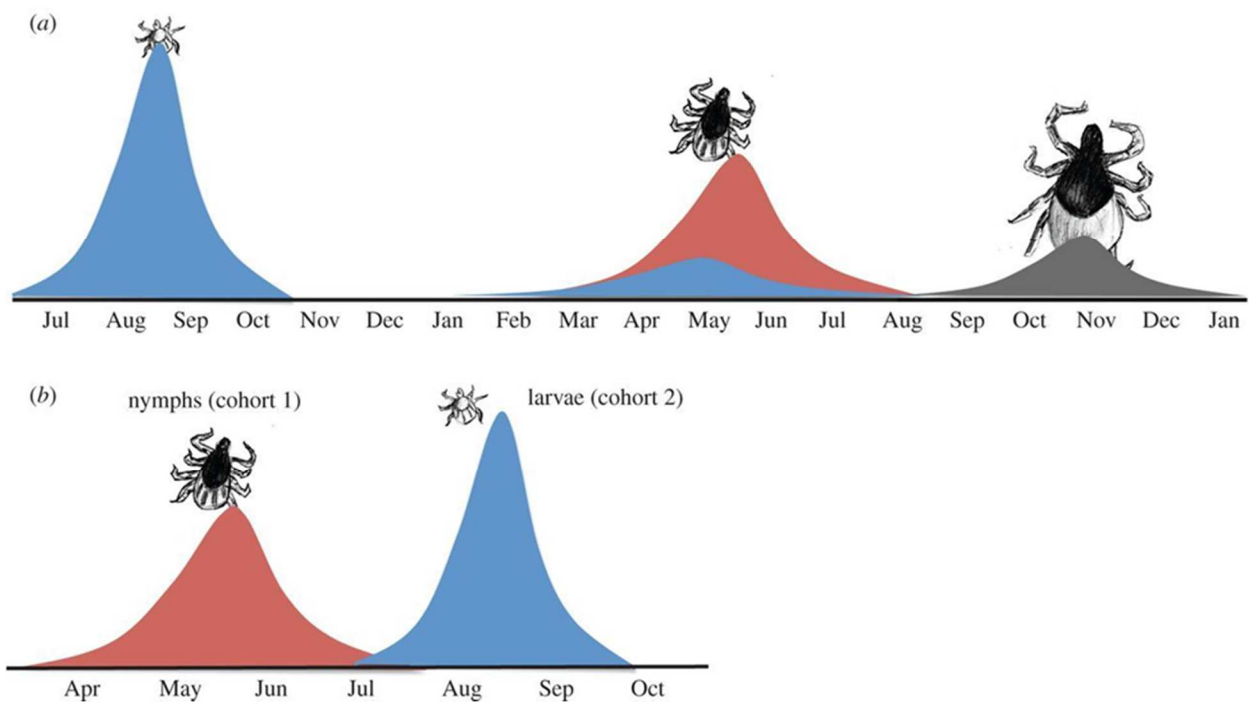
Phenology of *Ixodes scapularis* in Southwestern Virginia and Northwestern North Carolina

There are two clades of *I. scapularis*, the northern and southern clades (Xu et al., 2020). The northern clade (i.e., those in the Northeast that are responsible for majority of Lyme disease cases) has been found to quest above leaf litter, hence more likely to bite humans (Arsnoe et al.,

2019). The southern clade (found in the southern states), on the other hand, quest below the leaf litter which reduces its probability of coming into contact with humans, hence low Lyme disease risk (Arsnoe et al., 2019).

Morris et al. (2022) studied the phenology of *I. scapularis* ticks in southwestern Virginia where reported Lyme disease cases have increased over a short period of time (Lantos et al., 2015). They collected ticks on monthly basis (from February 2018 to January 2019) for a year in southwestern Virginia. In southeastern Virginia, sampling was done during winter months and once every fortnight for the rest of the year. They compared the phenology of these ticks in the Southeast (Montgomery and Roanoke Counties) to that of the Southwest (Princess Anne and Norfolk Counties) to determine if it was the northern population of ticks that were spreading south. The authors collected 1,936 *I. scapularis* (1,496 larvae, 306 nymphs, 134 adults) in southwestern Virginia and 134 *Ixodes affnis* (adults), 116 *I. scapularis* (54 larvae, 13 nymphs, 49 adults) (Morris et al., 2022). In southwestern Virginia, the authors collected *I. scapularis* nymphs from April through October with activity peaking May through July. In southeastern Virginia, nymphs were only active from May through August, with activity peaking in June (Morris et al., 2022). Their results showed differences in *I. scapularis* from the two areas, concluding that the above-ground questing behavior of *I. scapularis* ticks in southwestern Virginia indicated that northern populations of *I. scapularis* are invading southward as reported earlier (e.g., Brinkerhoff et al., 2014; Hickling et al., 2018; Lantos et al., 2015)[Figure 2.2 in Chapter 2].

Study Aim

The aim of this study was to <u>investigate the phenology and patterns of infection in the invading *I. scapularis* ticks in the northwestern North Carolina corridor.</u> This is to further understand whether the seasonal variations in the *I. scapularis* life cycle poses Lyme disease

threats to humans and their companion animals in northwestern North Carolina. Specifically, I aimed to study the phenology of *I. scapularis* as it pertains to northwestern North Carolina to determine the possibility of creating an established system of pathogen cycle as observed in the northeast.

Approach

I sampled the two hotspots of *I. scapularis* which I determined based on previous study. Sites were sampled on the same day each month for a minimum of 12 months. The seasonal variations in *I. scapularis* density for each life stage would provide data on the phenology of the species. Their infection rates would also determine which months are the high-risk months.

Hypothesis

Many previous studies around the northwestern parts of North Carolina indicated that Lyme disease is expanding southward (S. Apperson et al., 1990b; Brinkerhoff et al., 2014; Hickling et al., 2018; Lantos et al., 2015). Based on this premise, I hypothesized that the phenology of *I. scapularis* ticks in the northwest would be comparable to that of the Southeast.

METHODS

Sampling Sites

The New River sites at Alleghany access and Highway 221 access, which had been identified as current hotspots with high *I. scapularis* tick densities and *B. burgdorferi* infection rates were chosen for the study (Figure 23). The two sites are in Alleghany and Ashe counties, respectively. They are both forested habitats characterized by second- or third growth trees. Oak-Hickory hardwood form about half of the forest cover. Other tree types are maples, pines, hemlocks, American beech, black gum, black locust, yellow birch, and yellow poplar (North Carolina Division of Parks and Recreation, 2015). Dogwood, sourwood, sassafras, huckleberries,

alders, and hydrangeas constitute the understory (NC Division of Parks and Recreation, 2015).

The parks are open for recreational purposes such as picnicking, kayaking, hiking, and camping.

Figure 23. Map of the two counties bordering Virginia (shaded gray) where the sites for flagging were located. NRA: New River State Park, Alleghany access (red); NR221: New River State Park, 221 Access (blue).
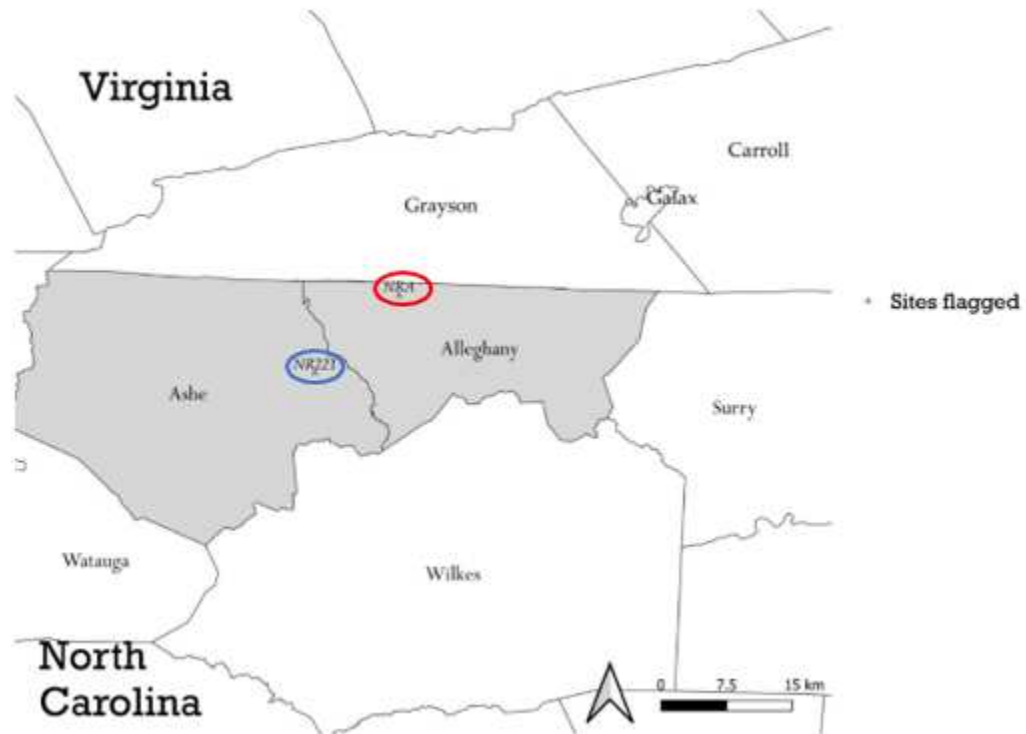
Methodology

Tick Collection

The phenology study focused on sampling *I. scapularis* ticks at these sites over at least a one-year span. Flagging was done once every month from November 2020 (only the Ashe County site was surveyed in November and December 2020) to December 2021. Sampling was done between the middle to the end of the month, depending on when the weather conditions were suitable for flagging. Twelve transects of 100-m each were established for each site and for most of the months, flagging was done on the same day. When tick sampling for both sites were not done on the same day, it was completed no more than two days later. The flag (1-m by 1-m

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cloth attached to a 1.2-m wooden pole) was swept along the forest floor, over rock surfaces and logs in a systematic manner. An effort was made to sample the same transects each month. Flagging transects were georeferenced using a hand-held Geographic Positioning System (GPS) (Garmin GPSMAP 64sc). Flagging transects included mostly the forest edges where ticks are known to inhabit. When flagging each transect, the flag was checked periodically for any attached ticks, every 10-20 meters depending on the terrain. Ticks that had latched onto the flagging cloth were collected using a pointed forceps and put in 1.5-mL Eppendorf tubes containing 95% ethanol and labeled accordingly all ticks collected from one transect were place in the same vial. In the laboratory, the collected ticks were stored in a -20°C freezer until needed for processing and identification. Ticks were morphologically identified using dissecting microscopes and tick identification manuals (Clifford et al., 1961; Durden & Keirans, 1996; Keirans & Litwak, 1989; Sonenshine, 1979). Some of the adults and nymphs were later sent to CDC for pathogen screening.

Borrelia burgdorferi Infection Screening

A total of hundred confirmed unfed *I. scapularis* ticks, 50 nymphs collected during the spring-summer time and 50 adults collected during the fall-winter time were sent to CDC for pathogen testing. The testing was done as part of a collaboration between the Communicable Disease Branch of the North Carolina Division of Public Health and the CDC's Division of Vector-Borne Diseases. The DNA of each tick sent to CDC was extracted using a modified version of the protocol for DNA extraction from field-collected ticks (Graham et al., 2016, 2018). The DNA extraction process involved first, homogenizing the ticks using 545 mg of 2.0 mm sized yttria-stabilized zirconium oxide beads in an Qiagen 470 µL lysis mix comprised of buffer ATL, 20 µL proteinase K, and 0.5% DX anti-foaming reagent (Graham et al., 2018). The

tick sample was then disrupted for 2 minutes using a Biospec Mini-Beadbeater-96 before incubating for approximately 10-12 minutes at 56ºC (Graham et al., 2018). After incubation, samples were centrifuged for 30 seconds at 1000 x g, and then 200 µL of each sample was processed using the Qiagen (QIAcube HT) automated nucleic acid isolation system as well as the Qiagen-Cador Pathogen 96 kit (Graham et al., 2018). Samples were then combined with Qiagen VXL mixture and binding buffer ACB to 650 µL and subjected to 3-minute vacuuming at 35 kPa (Graham et al., 2018). After vacuuming was complete, the column was washed using 600 µL of AW1 buffer and vacuumed for 2 minutes at 35 kPa (Graham et al., 2018). DNA was finally eluted by the addition of 100 µL AVE buffer to the column, incubated for 2 minutes, then vacuumed for 6 minutes at 55 kPa (Graham et al., 2018). Each extract was then screened for *Borrelia burgdorferi*, *B. mayonii*, *B. miyamotoi*, *Anaplasma phagocytophilum*, and *Babesia microti* using a pair of multiplex real-time PCR assays (Hojgaard et al., 2014). Modifications included the use of a pan-*Borrelia* 16S target in place of the *B. burgdorferi* "gB31" target (Parola et al., 2011). Samples that tested positive for *Borrelia* underwent further testing to detect and distinguish *B. miyamotoi*, *B. burgdorferi* s.s., and *B. mayonii* using a duplex real-time PCR assay targeting the oppA2 gene in *B. burgdorferi* s.s. and *B. mayonii* (Pritt, Mead, et al., 2016).

Permits and Collaborations

I obtained a permit from the NC Wildlife Resources Commission (#20-SC01381 and 21-SC01381) to sample ticks by flagging in state-owned lands throughout the sampling period.

Data Analysis

Line graphs of tick questing activity against months and seasons were plotted for each site separately and combined using Windows Excel (Microsoft 365 version). Tick activity was calculated as the quotient of the tick density per square meter for a given life stage for each

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month or season and the total tick density per square meter for the entire sampling period (i.e., either for the sum of the months or for the sum of a given season) (Fish, 1995). These proportional values of determining the tick activity compensates for the plotting of large tick activity values that fit the chart without having to break the scale of the dependent variable (tick activity) to be able to plot very small and large values on the same chart area. A similar strategy was adopted for the density of nymphs and adults infected with *B. burgdorferi* across the months in which nymphs and adults were actively questing. Density of infected ticks is the product of the tick density (i.e., number of ticks/100m^2) and the prevalence (i.e., the mean number of infected ticks per transect).

RESULTS

General

Out of a total of 1,728 *I. scapularis* ticks collected, 55% were collected from the Ashe County site while the remainder were collected from the Alleghany County site. Overall, larvae were the most collected (57.23%), followed by nymphs (28.87%), and then adults (14.0%) (Table 27). There were more individuals of *I. scapularis* per life stage collected at the Ashe County site (i.e., 49 more larvae, 19 more nymphs, and 98 more adults) than the Alleghany County site. The New River site in Alleghany County was the only site that had a month where sampling yielded no tick (i.e., December) (Table 27). In May, all three life stages of *I. scapularis* were recorded at the New River State Park in Ashe County (Table 27).

Table 27. *Ixodes scapularis* **ticks collected per month by life stage at the New River State Park sites in Ashe and Alleghany Counties.** Values in parenthesis indicate tick density (i.e., ticks/100-m).

MONTH	Ashe			Alleghany		
	Larva	Nymph	Adult	Larva	Nymph	Adult
Nov-20	0 (0.0)	0 (0.0)	6 (0.5)	-	-	-
Dec-20	0 (0.0)	0 (0.0)	2 (0.2)	-	-	-
Jan-21	0 (0.0)	0 (0.0)	1 (0.1)	0 (0.0)	0 (0.0)	1 (0.1)
Feb-21	0 (0.0)	0 (0.0)	43 (3.6)	0 (0.0)	0 (0.0)	16 (1.3)
Mar-21	0 (0.0)	1 (0.1)	52 (4.3)	0 (0.0)	1 (0.1)	28 (2.3)
Apr-21	0 (0.0)	0 (0.0)	21 (1.8)	0 (0.0)	0 (0.0)	11 (0.9)
May-21	3 (0.3)	70 (5.8)	1 (0.1)	3 (0.3)	64 (5.3)	0 (0.0)
Jun-21	7 (0.6)	133 (11.1)	0 (0.0)	4 (0.3)	121 (10.1)	0 (0.0)
Jul-21	144 (12.0)	40 (3.3)	0 (0.0)	128 (10.7)	39 (3.3)	0 (0.0)
Aug-21	320 (26.7)	7 (0.6)	0 (0.0)	296 (24.7)	7 (0.6)	0 (0.0)
Sep-21	45 (3.8)	5 (0.4)	0 (0.0)	39 (3.3)	5 (0.4)	0 (0.0)
Oct-21	0 (0.0)	2 (0.2)	34 (2.8)	0 (0.0)	2 (0.2)	11 (0.9)
Nov-21	0 (0.0)	0 (0.0)	3 (0.3)	0 (0.0)	0 (0.0)	5 (0.4)
Dec-21	0 (0.0)	0 (0.0)	7 (0.6)	0 (0.0)	0 (0.0)	0 (0.0)
Tick #	**519 (3.1)**	**258 (1.5)**	**170 (1.0)**	**470 (3.3)**	**239 (1.7)**	**72 (0.5)**

Tick Activity

A bimodal distribution was observed for adult *I. scapularis* in both sites. Adult tick activity peaked in early spring (i.e., February and March) and in early fall (i.e., October) [Figure 24 A & B]. The higher of the two peaks in adult tick activity was in early fall in Alleghany while that of Ashe was of about the same peak as late fall (Figure 24 A & B). By late spring, adult activity had ceased. Nymphal activity started from early-, mid-spring to early fall had only one peak (Figure 24 A & B). Nymphal activity preceded larval activity (Figure 24). Nymphal tick activity in Alleghany County had two peaks, the normal peak from mid-spring to early fall and a little peak in early fall (Figure 24 B). In the Ashe County site, the peak in *I. scapularis* nymph activity was unimodal (Figure 24 B). Larval activity spanned from mid-summer to early fall, peaking in August at both the Alleghany and Ashe County sites. From July to October, both nymphs and larvae were active. All the three life stages of *I. scapularis* were active in September-October in different activity proportions at the Alleghany site (Figures 24 B). When winter conditions are suitable, adult *I. scapularis* quests as seen in Alleghany and Ashe (Figures 24 A, B, C, & D).

Figure 24. Stage specific monthly tick activity fraction (A) and (C), and monthly distribution of *I. scapularis* (B) and (D) by life stage for Alleghany and Ashe, respectively. (A) and (C) is the fraction of each life stage by month to the total recorded throughout the study for that life stage. Monthly distribution of *I. scapularis* by life stage. (B) and (D) is the proportion of each life stage recorded for a given month compared to the total number of ticks collected for that month.
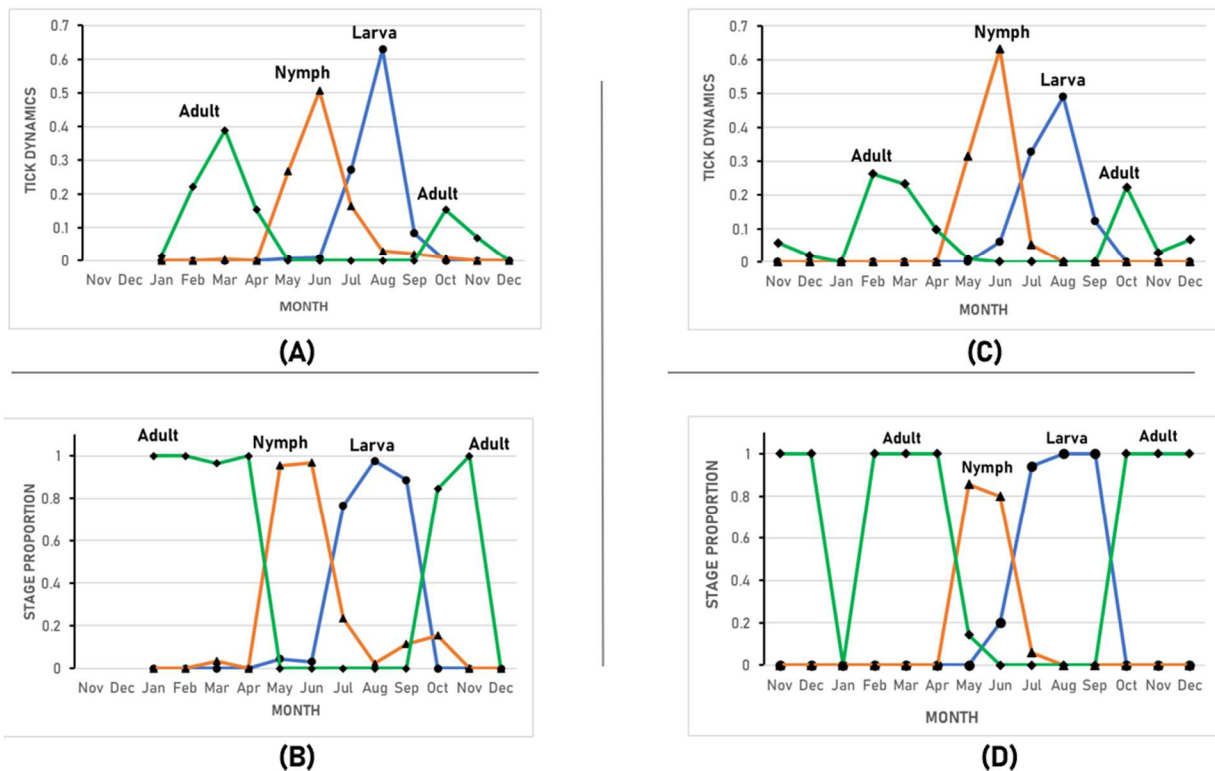
Borrelia burgdorferi Infection in Nymphs and Adults

Ixodes scapularis nymphs were most infective during early spring and early fall (Figure 25). Adults were most infective during early spring and late fall (Figure 25). At any period that nymphs and adults were active, *B. burgdorferi* was detected in the ticks (Figure 25). From late

spring to early fall, nymphs are active and circulating the pathogen among reservoir hosts and *I. scapularis* larvae.

Figure 25. Seasonal variation of *B. burgdorferi* infection in *I. scapularis* nymphs (blue bar) and adults (green bar). Numbers on bars represents the number of tested nymphs.
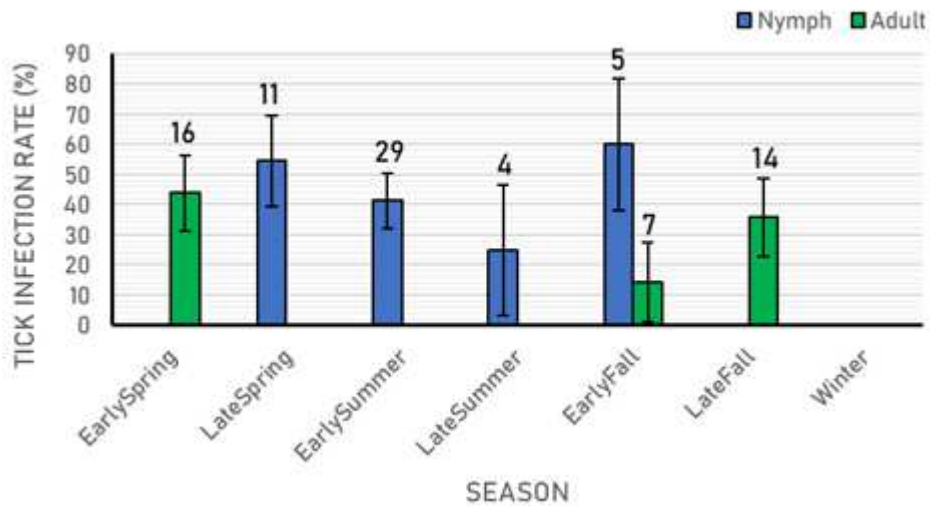
DISCUSSION

Seasonal Variations in the Phenology

The phenology of *I. scapularis* ticks at the two different study sites were similar and comparable to the phenology of *I. scapularis* ticks observed in the northeast (e.g., Fish, 1995). Although the observed tick activity pattern in this study is comparable to the general phenology pattern of *I. scapularis* in the northeast. In western North Carolina, the first and second adult peaks occurred one month earlier with adult tick activity in January and February, unlike in southern New York which was from March to June (Figure 26). Seasonal climatic differences may be causing subtle but significant changes in tick activity as the ticks invade new areas. The seasonal pattern of activity of *I. scapularis* ticks determines the rate and efficiency of transmission cycles of pathogens (Levi et al., 2015; Randolph, 1998) because the seasonal

pattern determines the time it takes for an engorged tick that fed on an infected host to develop and molt into the next life stage. A long-term study on the phenology of *I. scapularis* within the Appalachian and Piedmont regions may provide further insight as to whether the phenomenon may change in the future.

Asynchronous Phenology

Asynchronous phenology occurs when larval and nymphal activities do not occur concurrently. Instead, the nymphs emerge before the larvae. The emergence of *I. scapularis* nymphs before the larvae result in the effective transmission of *B. burgdorferi* from infected nymphs to newly recruited reservoir hosts. In this way, many competent hosts are infected so that when the larvae emerge, the chances of them picking up *B. burgdorferi* is from a host is enhanced (Levi et al., 2015). The phenology of the ticks in northwestern North Carolina showed similar phenology patterns to that of northeastern ticks. This observation suggests that the invading ticks are probably northern populations that are invading southward. The *I. scapularis* in the southwestern Virginia and northwestern North Carolina are likely to be northern clade of *I. scapularis* that are invading south. The phenology of southern populations of *I. scapularis* is different from that of the northern populations (Ogden et al., 2018). In addition, southern populations of *I. scapularis* were reported to be rare in the Appalachian Mountains and Piedmont region of North Carolina (Apperson et al., 1993), and Maryland (Amerasinghe et al., 1992). Recent cluster of human Lyme disease cases were found to be occurring in southwestern Virginia along the Appalachian Mountains (Lantos et al., 2015). Morris et al. (2022) studied the seasonal variation of blacklegged ticks in southwestern Virginia, where Lyme disease cases are reported to be rising, and southeastern Virginia, where Lyme disease cases are reported as low. They surveyed different life stages of *I. scapularis* ticks in the southeast and southwest of

Virginia for 12 months. They found that the phenology of *I. scapularis* populations from the two regions were different. The phenology of the southwestern ticks (adult activity: January to March; nymphal activity: April to October; larval activity: June to October) was consistent with the phenology of northeastern populations. This suggests that the northern populations are invading south, following the Appalachian Mountains, which may be providing suitable psychrophilic environment for the invading ticks. In the southeast, tick activity was much more prolonged (adult activity: January to May, October to December; nymphal activity: May to August; larval activity: June to November). Morris et al. (2022) also collected significantly more *I. scapularis* larvae and nymphs in the southwest than the southeast. Adult tick densities did not differ between the west and the east. Their findings were consistent with the hypothesis suggesting the southward invasion of *I. scapularis* ticks following the Appalachian Mountains. The similarities between the patterns in the northeast and that of northwestern North Carolina (Figure 26), may mean that the *B. burgdorferi* transmission cycle through the reservoir host and vector can be efficiently maintained in northwestern North Carolina.

Figure 26. Comparison between seasonal Ixodes scapularis ticks in southern New York (left; Fish, 1995) and northwestern North Carolina (right – this study).

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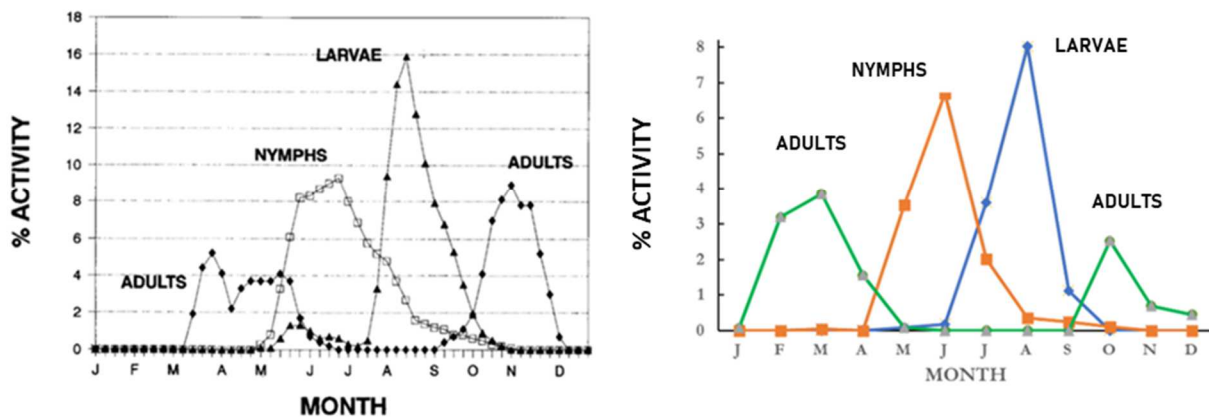
Infection Prevalence in *Ixodes scapularis* Nymphs and Adults

Usually, it is expected that *B. burgdorferi* infection rates in adult ticks should be higher than that of nymphs. This is because adults have already blood fed twice, once as larvae and once as nymphs against nymphs who have fed once. Hence, probability of infection is expected to be higher in adults compared to nymphs. However, I recorded higher *B. burgdorferi* infection in *I. scapularis* nymphs than adults. It is not clear whether this is a cyclical occurrence or a seasonal event. Long-term monitoring of the vertebrate community may provide clues. Early spring to early fall (but mostly late spring to early summer) seems to be the timing for nymphal activities, hence efforts are required to raise Lyme disease risk awareness with the public during those periods on nymphal activity.

Conclusion

The asynchronous phenology suggests that there is an efficient *B. burgdorferi* transmission cycle. The spring-summer period of nymphal emergence in asynchronous phenology coincides with the breeding periods of *P. leucopus* [which starts from spring and ends in fall] (Korytko & Vessey, 1991), ensuring that enough of these mice are infected with *B. burgdorferi* by the questing infectious nymphs before the questing period of the larvae from mid-summer to fall. Periods of increased human outdoor activities coincides with this period of elevated nymphal activity, increasing human Lyme disease risk.

People within the study area must be educated on tick-bite prevention strategies in order to minimize their exposure to *B. burgdorferi* infected ticks. Healthcare professionals also need to be educated on the current status of the pathogen in the region so that they can diagnose flu-like symptoms as possible *B. burgdorferi* infection after further screening and interview with potential patients of the disease.

CHAPTER V: GENERAL DISCUSSION

Some unique geographic features may play a role in infectious disease spread by providing the permissive environment for the spread of the epizootiological components of the disease through the invasion and establishment of the disease niche. The invasion of certain components of the disease niche without the presence of the other components would not lead to establishment of the disease niche. In my dissertation research, I aimed to assess the possibility that *Ixodes scapularis* and *B. burgdorferi* are invading into North Carolina through the northwestern corner of NC and that the New River along the route is facilitating the spread of the ticks and the pathogen they carry. I also aimed to characterize the rodent community and the phenology of *I. scapularis* within the region of invasion to understand the permissiveness of this environment for the establishment of an effective enzootic transmission cycle.

My first aim focused on investigating where *I. scapularis* and *B. burgdorferi* are invading from and how certain topographic features along its path may be facilitating their southward spread. In that aim, I hypothesized that the New River which connects southwestern Virginia to northwestern North Carolina would be facilitating the north-to-south spread of *I. scapularis* and the pathogen they carry. I expected to find a north-to-south decreasing pattern in tick density and their pathogen infection prevalence. However, I observed a humped shape pattern with the peak around Alleghany and Ashe Counties. The humped shape may be indicative of an ongoing invasion. Spiegel et al. (2013) found a similar trend in their study of griffon vulture in Israel. The observation of higher tick densities along the New River compared to the western Piedmont, together with a more southern distribution along the New River than the western Piedmont was consisted with my prediction. The finding suggested that the New River is facilitating the spread of the tick and pathogen. My findings showed that the *I. scapularis and B. burgdorferi* are

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invading into northwestern North Carolina through the southwestern edge of Virginia with the

New River serving as a putative corridor that is facilitating their southward spread. The more

southward spread of *I. scapularis* compared with *B. burgdorferi* suggested that the vector, *I.

scapularis* is leading the invasion wave followed by the pathogen, *B. burgdorferi*. This finding is

consistent with the tick-first mechanism of invasion. Although this study was relatively short

compared to Hamer et al.'s study, previous studies that found *I. scapularis* to be rare in the

southern Appalachian Mountains is an indication that the ticks were previously not present. The

detection of *I. scapularis* and *B. burgdorferi* supports the rising human Lyme disease cases in the

New River Valley areas of southwestern Virginia and northwestern North Carolina. Although the

ticks collected in my study were not genetically analyzed to ascertain which clade they belong to,

the ease in the collection of ticks, especially larvae and nymphs, through flagging was an

indication that they were questing above the leaf litter than underneath it. Northern populations

are known to quest on top of leaf litter compared to the southern populations that spend more

time under leaf litter, making them difficult to collect by flagging/dragging (Arsnoe et al., 2019).

Another study in Virginia that compared seasonal variations in Virginia's southwestern *I.

scapularis* populations to those of the southeast found that the phenology of the two tick

populations were different with the southwestern *I. scapularis* populations being more similar to

the phenology of *I. scapularis* populations in northeastern United States (Morris et al., 2022).

This supports the idea that the ticks in the southwest of Virginia and northeast of North Carolina

are more likely to belong to the northeastern clade. Phylogenetic analysis needs to be done to on

ticks collected from northeastern United States, southwestern Virginia, and northwestern North

Carolina to verify if they truly belong to the same clade.

The support for the role of the New River in facilitating the spread of Lyme disease can be strengthened if another study could compare regions to the west of the New River. This study focused on the western Piedmont region that was to the east of the new River. To show that it is not just the mountains causing the difference in *I. scapularis* densities and their *B. burgdorferi* infection rates, a study considering the region that is west of the New River would be informative.

The second and third aims of my dissertation investigated the suitability of environment in northwestern NC to the establishment of an effective enzootic transmission cycle. Specifically, I looked at the rodent community dimension (Aim 2) and the phonologic (seasonal) dimension of the disease niche. When a vector successfully invades a new area, there must be competent hosts to be able to efficiently circulate the pathogen with the animal population to establish an enzootic transmission cycle. The introduction of the pathogen might not necessarily mean that *B. burgdorferi* will be transmissible among the rodent community. For instance, in the case of asynchronous phenology where the nymphs emerge first before the larvae, the competent hosts must be able to retain the pathogen long enough to coincide with the emergence of the pathogen. In addition, the vector responsible for the transmission, *I. scapularis*, must have a phenology that matches the availability of the host and ensures effective transmission to non-infected ticks. Based on this background, I investigated the permissiveness of the rodent community and the phenology of the *I. scapularis* ticks to determine if they would support the establishment of a disease niche.

In my second aim, I found that *Peromyscus leucopus*, the primary reservoir host for *B. burgdorferi*, was the dominant rodent species in the entire studied areas of northwestern North Carolina. This sets the environment for the establishment of a Lyme disease niche through the

efficient circulation of *B. burgdorferi* among the rodent community. My findings showed that other competent rodents such as voles and chipmunks were also present in the region but at a much lower abundance. Shrews are also competent hosts. To obtain a holistic understanding of the permissiveness of the environment to the establishment of a Lyme disease niche, future studies may involve the use of larger traps, and other vertebrate trapping techniques such as pitfalls and mist netting in order to ascertain the contribution of other vertebrate hosts that were not trapped in this study to the permissiveness of the environment to Lyme disease niche establishment.

This was a short-term study; multi-year continuous study would be needed to further document this pattern of putative tick and *B. burgdorferi* invasion. Furthermore, in areas where the enzootic cycle is established, long term studies can characterize multi-year population dynamics of rodents (and their drivers, e.g., acorn mast years (Ostfeld et al., 2006)) on infection dynamics in the enzootic system as well as in humans. The availability of food resource for the different hosts *I. scapularis* hosts such as *Odocoileus virginianus* and *P. leucopus* can determine the relative risk to Lyme disease in an area over many years. For example, improved acorn production in the northeast was found to boost the abundance of both *P. leucopus* and the white-tailed deer (Ostfeld et al., 2006). The consequent effect was the increased abundance of both I. *scapulari*s larvae (from eggs laid by *I. scapularis* females that blood fed on deer) and *P. leucopus* (due to increased reproductive success as a result of food abundance) during spring-summer of the first year of *I. scapularis* life cycle. The earlier emergence of infected *I. scapularis* nymphs ahead of the new generation's *I. scapularis* larvae caused increased *B. burgdorferi* infection rates among *P. leucopus* individuals. The abundance of *P. leucopus* increased the contact rate between infected nymphs and uninfected *P. leucopus* (both the newly

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recruited and existing uninfected ones). Many of the new generation's *I. scapularis* larvae that quested later became infected because of the higher density of infected *P. leucopus*. The overall effect was increased rate of *B. burgdorferi* infection in *I. scapularis* nymphs in the second year which correlated with heightened human LB risk in the second year (Ostfeld et al., 2006).

In aim 3, I investigated the phenology of the invading *I. scapularis* and to determine if the emergence periods of the different life stages in their life cycle coincides with periods of host abundance to be able to efficiently transmit the pathogen enzootically. The phenology of the *I. scapularis* ticks is equally important since transovarial transmission in *I. scapularis* is negligible, most transmission to ticks is horizontal ((Kardatzke et al., 1992) resulting in newborn larvae being uninfected. Hence, larvae may acquire *B. burgdorferi* infection during their first bloodmeal. Once, the infection is acquired, it is maintained through transstadial transmission (i.e., throughout the remainder of the tick's life cycle). The infected nymph may then transmit the acquired infection to a non-infected host when it blood feeds. Therefore, *B. burgdorferi* transmission to new hosts occurs during the nymph or adult phases. The timing in the emergence of nymphs and larvae determines whether a penology can be described as asynchronous or synchronous. If the nymphs emerge before the larvae, then it is asynchronous. If both larvae and nymphs emerge concurrently then it is synchronous. My study showed that the phenology of the *I. scapularis* ticks in northwestern North Carolina is "asynchronous" similar to that reported from hyperendemic regions of Lyme disease in northeastern United States.

However, we do not know the lasting effect of climate change on the phenology of the invading *I. scapularis* ticks. Further studies need to be done, probably under different climatic conditions to see the effect of climate change on the questing behavior of the assumed northern

population of *I. scapularis* in the southeastern region of the United States. This may provide an idea of the possible southward extent that could be reached by these invading *I. scapularis*.

After establishing that it is the northern clade that is invading the south, we may need to understand the possibility of interbreeding between the northern and southern populations and how that may impact the questing abilities of the hybrid populations. Questing abilities in the northern and southern populations have been shown to be more driven by inherently behavioral differences between the two clades and not just their environment (Arsnoe et al., 2019) (Arsnoe et al., 2019).

Concluding Remarks

Put together, my study showed that there is a perfect storm set for the establishment of *I. scapularis* and *B. burgdorferi* in North Carolina. It is just a matter of time. Certain geographic features along the path of invasion may also create hotspots from where the spread of the ticks and pathogen may occur. The current hotspot for the establishment of Lyme disease seems to be around the New River Valley area in Alleghany and Ashe Counties.

There is the need for state regulated programs that will ensure regular monitoring of the *I. scapularis* populations and the *B. burgdorferi* infection rates in *P. leucopus* and the ticks through active surveillance. This is because it is unknown whether the *I. scapularis* populations may be able to spread and adapt to other less permissible climatic conditions beyond the Appalachian Mountains and how that may influence their questing behavior. Public health officials need to educate the public, especially those living in and around Ashe and Alleghany counties on tick safety protocols such as the wearing of permethrin-treated clothes when out in the woods, especially during the spring-summer time when the nymphs are questing. Healthcare officials especially those in and around Alleghany and Ashe need to be briefed on the status of Lyme

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disease in their area and begin considering flu-like symptoms as possibility of *B. burgdorferi*

infection.

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