TRENDS IN BORRELIA SPP. PREVALENCE IN IXODES SPP. TICKS FROM THE SOUTHEASTERN COASTAL UNITED STATES

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I am submitting herewith a thesis written by Lauren Paul Maestas entitled "TRENDS IN BORRELIA SPP. PREVALENCE IN IXODES SPP. TICKS FROM THE SOUTHEASTERN COASTAL UNITED STATES." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Wildlife and Fisheries Science.

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FROM THE SOUTHEASTERN COASTAL UNITED STATES

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**ABSTRACT**

The Lyme borreliosis (LB) cycle, involving *Ixodes scapularis* and *Borrelia burgdorferi sensu stricto* (*Bbss*), is well documented in the northeastern US, where LB is becoming increasingly prevalent. In coastal North Carolina, *I. affinis* has been shown to have a higher incidence of *Bbss* than *I. scapularis*. My objectives were, to assess changes in prevalence of *Bbss* in *Ixodes* spp. along a transect from Virginia to Florida, and to assess the value of dogs and mesomammals as sentinels for spread of *Borrelia burgdorferi* sensu lato.

*Ixodes* spp. were collected at sites from 37.4° N to 30.0° N in 2011-12. Veterinary clinics from Virginia, to Florida were asked to collect ticks from dogs, and mesomammals were livetrapped in South Carolina and checked for ticks. Positive samples were identified to species by nested PCR and sequenced using the 16S-23S rRNA intergenic spacer genes. A subset of ticks was subjected to 12/16S rRNA genetic procedures.

Overall, 46 ticks were positive for *Bbsl*. Species identify of 22 *Borrelia* DNA samples sequenced was 54% *Bbss*, 32% *B. bissettii*, and 13% *B. miyamotoi*. All *Bbsl*-positive *I. scapularis* for which 12S analysis was completed belonged to the ‘American’ lineage of ticks. Genetic clustering (*F_{ST} values*) was evident among *I. scapularis* positive ticks, indicating an association between infection and haplotype. A total of 370 *Ixodes* spp. ticks were collected from dogs and mammals. Only four were *Borrelia*-positive (one *I. affinis* from a white-tailed deer in Virginia and two *I. affinis* from dogs in North and South Carolina respectively). No *I. scapularis* or *I. affinis* ticks were obtained from mesomammals.

The pronounced prevalence gradient, whereby *Bbsl* was not found in ticks on vegetation south of 35.2°N cannot be explained by an absence of vectors. *Ixodes affinis* was collected in low numbers yet comprised half of the infected ticks identified, highlighting the importance of this species for sylvatic cycles of *Bbsl*. Our furthest-south record of a *Borrelia*-infected tick was from
a dog, demonstrating that dogs can indeed be useful as sentinels for LB. Findings indicate that the risk of locally-acquired *Bbss* infection in the coastal southeast is at present low.
TABLE OF CONTENTS

CHAPTER 1 — INTRODUCTION

1.1 INTRODUCTION ........................................................................................................2

1.2 BACKGROUND: THE ECOLOGY OF LYME BORRELIOSIS IN THE EASTERN UNITED STATES ......................................................................................................................5

1.2.1 Borrelia species diversity ..................................................................................5
1.2.2 Vector tick diversity ............................................................................................6
1.2.3 Etiology of the bacteria in ticks and humans .....................................................8
1.2.4 Genetic variation in Ixodes populations .............................................................9
1.2.5 Vertebrate host diversity ..................................................................................10
1.2.6 Regional variation in the LB cycle ....................................................................13

1.3 OBJECTIVES AND HYPOTHESES ..................................................................16

1.4 LITERATURE CITED .............................................................................................18

CHAPTER 2 - BORRELIA PREVALENCE IN SOUTHEASTERN UNITED STATES

IXODES TICKS CHANGES ALONG A LATITUDINAL GRADIENT

2.1 ABSTRACT ..............................................................................................................33

2.2 INTRODUCTION ....................................................................................................34

2.3 METHODS ............................................................................................................38

2.3.1 Study sites ........................................................................................................38
2.3.2 Collection of ticks from vegetation .................................................................38
2.3.3 Morphometric identification of ticks ...............................................................39
CHAPTER 3 - COMPARISON OF BORRELIA PREVALENCE AND IXODES SPP. TICK INFESTATION OF DOMESTIC CANINES AND WILD MESOMAMMALS IN THE SOUTHEASTERN UNITED STATES

3.1 ABSTRACT...........................................................................................................68
3.2 INTRODUCTION..................................................................................................70
3.3 METHODS.........................................................................................................73
   3.3.1 Sampling sites and protocol........................................................................73
   3.3.2 Laboratory methods.....................................................................................75
   3.3.3 Borrelia detection.........................................................................................75
   3.3.4 Statistics.....................................................................................................77
3.4 RESULTS.............................................................................................................77
   3.4.1 Ticks from companion animals.................................................................77
   3.4.2 Borrelia spp. in ticks from companion animals.........................................78
   3.4.3 Ticks from mesomammals in South Carolina............................................78
3.5 DISCUSSION.....................................................................................................79
CHAPTER 4 - OVERALL CONCLUSIONS

4.1 LYME DISEASE RISK IN COASTAL SOUTHEASTERN UNITED STATES…88
4.2 FUTURE RESEARCH DIRECTIONS………………………………………...90
4.3 LITERATURE CITED…………………………………………………..91

APPENDIX 1. Primers specific to 12S and 16S region of Ixodid ticks…………………94
APPENDIX 2. 12S and 16S gene region conditions for PCR amplification…………………94
APPENDIX 3. Q-PCR Probes and Primers for the 23S segment of Borrelia burgdorferi….95
APPENDIX 4. Borrelia specific nested PCR primers targeting the 16S-23S IGS locus……96
APPENDIX 5. Amplification conditions for Nested IGS PCR…………………………..96
APPENDIX 6. NCBI GenBank submissions for 38 Ixodes haplotypes…………………..98

VITA………………………………………………………………………………….100
LIST OF TABLES

Table 2.1. Names and coordinates of sites where *Ixodes* spp. were collected in 2011-2013
(ordered by decreasing latitude)……………………………………………………….59

Table 2.2. Number of *Ixodes* spp. ticks dragged and flagged per person-hour during
Spring/Summer (March – August) and Fall/Winter (November-January) collections,
2011-2013………………………………………………………………………………60

Table 2.3. Number of *I. scapularis* and *I. affinis* tested for the presence of *Borrelia* spp. by 23S
Q-PCR, number of positive tests, and percent prevalence (P) of infection for both
tick species…………………………………………………………………………….61

Table 2.4. List of 12S taxa, collection sites and GenBank accession numbers. NA = collection
site not known……………………………………………………………………..62

Table 3.1. Locations of veterinary clinics participating in the study (ordered from north to
south)…………………………………………………………………………………85

Table 3.2. Ticks collected by host and state…………………………………………86

Table 3.3 Counts and pathogen prevalence of *I. scapularis* and *I. affinis* removed from domestic
canines…………………………………………………………………………………87

Table 3.4 Counts and pathogen prevalence of *I. scapularis* and *I. affinis* removed from domestic
cats…………………………………………………………………………………..88
LIST OF FIGURES

Figure 1.1. Trends in Lyme borreliosis annual incidence in humans, and *B. burgdorferi* exposure prevalence in domestic canines, for selected eastern coastal states (ordered from north to south)………………………………………………………………………………………………………………...15

Figure 2.1. *F*_\textsubscript{ST} tree showing genetic differences of *Ixodes scapularis* carrying *Bbsl*……..64

Figure 2.2. Trends in haplotype diversity by latitude, using two genes………………………….65

Figure 2.3. Phylogenetic relationship of *I. scapularis* based on12S gene fragments from the southeastern coastal US, representing two distinct lineages…………………..66
Chapter 1.

INTRODUCTION
CHAPTER 1 - INTRODUCTION

1.1 INTRODUCTION

Vector-borne zoonotic pathogens are often maintained in complex transmission cycles involving numerous arthropod vectors and their wild vertebrate hosts (Brown, 1992). Consequently, they are of theoretical interest to disease ecologists, and of practical concern to epidemiologists and public health officials. Comprehensive studies of the importance of potential reservoir hosts and their associated vectors can provide insights into the underlying mechanisms responsible for maintenance of vector-borne pathogens, and as such may provide the necessary knowledge to understand, avoid and control human disease (Brown 1992).

Lyme borreliosis (LB) is the most common arthropod borne disease in the United States (Barbour, 1993, Bacon, 2008). In the US and internationally, the involvement of multiple *Ixodes* spp. and *Borrelia* spp. leads to complex LB transmission cycles, making it important to incorporate an understanding of vector and pathogen species diversity when determining the disease risk posed by these organisms (Oliver, 1996). LB or related disorders have been reported on every continent excluding Antarctica (Mullen and Durden, 2002).

One of the most high-profile vector borne disease systems of northern temperate ecosystems is Lyme borreliosis (commonly termed “Lyme disease”) caused by spirochetes of the *Borrelia* genus and transmitted by ticks of the *Ixodes* genus (Burgdorfer et al. 1982; Steere et al. 1983; Benach et al. 1983; Johnson et al. 1984; Steere et al. 2004). Lyme disease in the northeastern US is a well-researched phenomenon. The ‘classic’ Lyme borreliosis (LB) cycle involves the vector *Ixodes scapularis* (Say, 1821) and the etiological agent *Borrelia burgdorferi sensu stricto* (*Bbss*), *I. scapularis* is the main vector of LB in the northeast (Piesman and Spielman, 1979; Oliver et al. 2003; Steere et al. 2004). This species generally has a 2 year life
cycle and is vector competent for *Bbsl* (Piesman and Spielman, 1979; Yuval and Spielman, 1990; Jacobs et al. 2003; Bowman and Nuttall, 2009). One of the most important aspects to the transmission of *Bbss* to humans is the reverse phenological life cycle of this tick with nymphs feeding before larvae (Spielman et al. 1985). The importance of reverse phenology relates to perpetuation of infection through continual emergence of infected nymphs prior to peak activity of uninfected larvae. *I. scapularis* is the model species for LB understanding in the northeastern US, where this disease is becoming increasingly prevalent in humans and canines (Oliver et al. 2003; Hillyer, 2005; Bacon, et al. 2008).

In other regions of the US *Borrelia*-host cycles occur, but the pathogen species and host species involved and the resulting seasonality and prevalence of infection can be quite different from what is seen in the Northeast. In the Midwest, *I. scapularis* were first documented as being present in northeastern Wisconsin in the late 1960’s (Jackson et al. 1970); since then its distribution has expanded south and westward as has LB in people (Agger et al. 1991; Hamer et al. 2010). Tick densities and prevalence of infection are similar between the Northeast and Midwest (Brisson et al. 2010; Gatewood et al. 2009; Brisson and Dykhuizen, 2004; Caporale, Johnson and Millard, 2005; Duik-Wasser et al. 2006). Similar prevalence of the pathogen would indicate similar infections of humans, yet in the Midwest there is a two-fold decrease in the number of reported cases of Lyme disease from the Northeast. An important point to note is that the phenology of *I. scapularis* in the Midwest differs in length of activity period and higher peak densities in the spring than in the fall (Guerra et al. 2002), with synchronous activity of immature ticks, rather than the reverse phenology typical in the northeast (Hamer et al. 2010).

In the western US, the LB cycle involves two tick species: *Ixodes spinipalpis* (Hadwen and Nuttall, 1916) (critical for sylvatic cycles of the pathogen) and *I. pacificus* (the primary bridge vector to humans) [Brown and Lane1992]. The biology of *I. pacificus* differs from that of *I.scapularis* in the northeast, and circumstantial evidence has shown that *I. pacificus* has
transmitted *B. burgdorferi* to people in fewer than the 24-48 hours commonly required by *I. scapularis* for transmission (Oliver et al. 1993; Lane and Lavois, 1988; Piesman et al. 1987).

Maintenance of the western enzootic cycle differs from the northeast *I. scapularis* white footed mouse (*Peromyscus maniculatus*) model (Brown and Lane, 1992). Rather in western North America the cycle involves the Dusky footed wood rat (*Neotoma fuscipes*) and the tick vectors *I. spinipalus* (previously *I. neotomae* [Norris et al. 1996]) as the primary vector in enzootic cycles and *I. pacificus* as the bridging vector to humans (Brown and Lane, 1992). The western cycle is further complicated by presence of the western fence lizard (*Sceloporus occidentalis*) [a primary host to sub adult *I. pacificus*] which has an immune response that clears infection of ticks or has a zooprophylactic effect on the LB cycle in the west (Lane and Quistad, 1998).

In the Southeast, several closely related *Borrelia* species (termed *B. burgdorferi sensu lato*; *Bbsl*) are maintained by sylvatic cycles broadly similar to those seen in the west (Harrison, 2010; Maggi, 2010). Several authors have suggested that in areas where ticks, people, and canines interact, *I. scapularis* may act as both reservoir and bridging vector for canine- and human-infectious *Bbsl*, with various species potentially playing multiple roles (Clark, 2001; Oliver et al. 2003). Maggie et al (2010) recently challenged this ‘bridge vector’ hypothesis by demonstrating that *I. scapularis* in North Carolina were not infected with *Bbsl* at locations where the prevalence of *Bbsl* in *I. affinis* was prodigious (33.5%) in comparison, and indicating the presence of the pathogen in the southeast (Maggie et al. 2010). This finding may be an indication of the coming quandary, although *I. affinis* is not known to be a human-biting tick (Oliver et al. 1996).

A shift from an *I. scapularis*-dominated LB cycle in the northeast to an *I. affinis*-dominated cycle in the coastal southeast may help explain the low prevalence of human LB cases in southern coastal states. The goal of this study is therefore to map and analyze the emerging LB risk to humans and canines in coastal Virginia (southern), North Carolina, South Carolina, Georgia, and Florida (northern) and explain latitudinal variations in prevalence of *Bbsl*.
1.2 BACKGROUND: THE ECOLOGY OF LYME DISEASE IN THE EASTERN UNITED STATES

Vector-borne disease risk to humans arises from the complex interactions of pathogens with their wildlife reservoirs and associated invertebrate vectors. The diversity of pathogens, ticks and wildlife hosts potentially involved in the maintenance and transmission of Lyme disease in the southeastern U.S. are briefly reviewed below.

1.2.1 Borrelia species diversity

The taxa of *B. burgdorferi* have undergone several revisions leading to the current classification of *Bbss* and *Bbsl* (Farlow et al. 2002). *Bbsl* is found throughout Eurasia, Japan, and North America (Farlow et al. 2002) and consists of at least twenty genospecies worldwide (Schneider et al. 2000; Rudenko et al. 2009; Clark et al. 2013; Margos et al. 2011; Niu et al. 2011; Stanek and Reiter, 2011; Clark et al. 2013), only seven of which are found in the U.S. (*Bbss*, *B. americana*, *B. andersoni*, *B. bissetti*, *B. kurtenbachii*, *B. californiensis*, and *B. carolinensis*); (Clark, 2013, Rudenko et al. 2009; Schneider et al. 2000; Margos et al. 2010). In Europe *B. garinii*, *B. afzelii*, and *B. spielmanii* are also known to be pathogenic (Farlow et al. 2002; Niu et al. 2011; Tsao, 2009). In North America, *Bbss* is the only member of this complex currently known to be associated with human cases of Lyme disease (Mathiesen et al. 1997; Wormser et al. 2008), but “*B. bissetti* –like” DNA was identified in California LB patients (Giard, et al. 2010), as well as the addition of *B. kurtenbachii* which is of interest with regard to human disease (Ogden et al. 2011). Finally, recent publication by Clark et al. (2013) has reported *B. andersoni* and *B. americana* to be associated with human cases of Lyme disease, although the results are somewhat contradictory as testing of the patients was subsequently positive for *Bbss* nearly three years later.
1.2.2 Vector tick diversity

**Ixodes scapularis biology:** *Ixodes scapularis* (Say, 1821) is a nearctic tick species, ranging from South-eastern Canada through Northern Mexico and reaching as far west as Texas (Spielman et al. 1985; Rand et al. 2003; Mechai, et al. 2013; Hamer et al. 2010; Dennis et al. 1998; Guerra et al. 2002; Bishopp and Trembley, 1945). The three-host tick is considered a generalist ectoparsite as hosts include rodents and lizards for immature stages to medium and large size mammals as adults. *I. scapularis* is the principal vector of *Bb* in the Eastern US, as well as various other pathogens (Bowman and Nuttall, 2008). However this species is limited by coastal proximity/humidity levels and elevation (Anderson and Magnarelli, 1980; Rand et al. 2003). Seasonality of this species slightly varies with geographic location, however the commonly accepted seasonality has nymphs emerging first in late spring/early summer and larvae emerging later in the summer; adults of this species are active from fall through early spring (Bowman and Nuttal, 2008; Piesman and Spielman, 1979; Oliver, 1996). Classification of this species has changed through time, and what was once two species is now one. This single species maintains genetic distinctions that have led to classification based on clade. The two main clades include a northern biotype (mostly north of 37°latitude) formerly *I. dammini* which now extends throughout the range of *I. scapularis*, and a southern biotype (below 37° latitude) formerly *I. scapularis* (Stromdahl and Hickling, 2012; Trout, et al. 2009; Steere and Malawista, 1979; Norris et al. 1996). Nymphs of these various clades express different traits such as proclivity for humans (Goddard and Piesman, 2006). One such trait is questing behavior and influence on host selection; Goddard and Piesman (2006) confirmed the difficulty in collecting immature *I. scapularis* in the southern United States.

**Ixodes affinis biology:** There remains little information regarding *I. affinis* and its role in the ecology of LB. The known range of *I. affinis* is South/Central America, Florida, Georgia,
North/South Carolina, and Virginia in coastal areas (Kohls and Rogers, 1953; Oliver, 1987; Guzman-Cornejo, 2007; Harrison, 2010; Nadolny, et al. 2011; Stromdahl and Hickling, 2012). The known hosts in North America include ungulates, carnivores, rodents, lagomorphs, procyonids and avies (Kohls and Rogers, 1953; Oliver, 1987). The seasonality of *I. affinis* includes active adults from March-July (Harrison, 2010), active larvae from fall through spring (Oliver, 1987), and active nymphs from winter to early summer (Oliver, 1987). Consequently, the seasonality of *I. affinis* differs from that of *I. scapularis*. *Ixodes affinis* is thought to go through one lifecycle per year in nature, although adults are thought to be able to overwinter (Oliver, 1987). The variation in phenology between species influenced our collection efforts, and could be of importance if disease emergence in the south follows a different temporal pattern than in the northeast.

**Ixodes angustus biology:** *Ixodes angustus* (Neumann, 1899) occurs in moist, cool, montane habitats throughout North America (Durden and Kierans, 1996), and Eastern Europe. Hosts typically include rodents and their predators with occasional human and domestic dog associations (Peavy et al. 2000; Robins and Kierans, 1992; Hopkins, 1980). *Ixodes angustus* have been shown to be competent vectors for *Bbss* (Peavy et al. 2000), and are the first species of tick outside of the *I. ricinus* complex to be associated with a human case of Lyme disease (Damrow et al. 1989).

**Ixodes minor biology:** *Ixodes minor* (Neumann, 1902), ranges from Central and South America through Florida, Georgia, and South Carolina (Durden and Kierans, 1996; Kierans and Clifford, 1978). Rodents, rabbits, birds, and their predators appear to be the common host for this species (Durden and Kierans, 1996). *Bbsl* has been isolated from *I. minor* in South Carolina (Rudenko, 2009). Relatively little is known about this species in the US.
1.2.3 **Etiology of the bacteria in ticks and humans:** *Bbss* typically develops extracellularly in the midgut of *Ixodes* ticks, accumulating near the microvillar brush and interstitial spaces between epithelial cells; however in some cases it may penetrate the gut wall leading to increases in density of spirochetes in the central ganglia, malphigian tubules, salivary gland excretion and ovaries (Burgdorfer et al. 1989; Piesman, 2001; Mullen and Durden, 2002). While dormant in flat ticks, *Bbsl* is primarily expressing Osp A [Osp are outer surface proteins] (Schwan and Piesman, 2000). Upon commencement of feeding, spirochetes in the midgut replicate, Osp A is down regulated, and Osp C is expressed (Schwan and Piesman 2000; Bowman and Nuttall, 2009). The down regulation of Osp A (midgut binding properties) is thought to allow the spirochete to leave the midgut and enter the salivary gland (Pal et al. 2000; Piesman, et al 2001; Bowman and Nuttall, 2009). The expression of Osp C, coupled with the SALP 15 salivary protein in saliva-assisted transmission, allow the spirochete to then travel through the salivary gland and into the host (Ramamoorthi et al. 2005; Bowman and Nuttall, 2009). These findings have led to the premise of infection with the pathogen typically requiring up to forty eight hours (with a feeding tick) to reach adequate bacterial loads to support transmission of enough bacteria to produce bacteremia (Piesman et al. 1987; Sood et al. 1997). When injected into mammals, the spirochetes migrate and reproduce in the skin (Mullen and Durden, 2002), and may cause a persistent infection if not treated (Steere et al. 2004). They gradually invade the blood stream and spread throughout the body localizing in the bursae of large joints, the nervous system and the heart (Mullen and Durden, 2002). Infections with LB are however variable with some people only experiencing acute infection of the skin, while others may experience chronic symptoms such as heart arrhythmia and arthritis (Steere et al. 2004, Bowman and Nuttall, 2009). *Bbss* can be maintained in tick populations by transstadial propagation; however transovarial transmission would be an inefficient route of maintenance (Mullen and Durden, 2002; Burgdorfer, 1984; Levine, et al. 1985).
1.2.4 Genetic variation in *Ixodes* populations: One hypothesis for geographic variation in the LB cycle in the eastern US is that it may be influenced by underlying genetic variation within *Ixodes* spp. populations; which in turn affects traits of epidemiological importance. Various studies have identified clade structures which are paramount in the transmission of LB (Black and Piesman, 1994; Rich et al. 1995; Norris et al. 1996; Qiu et al. 2002; Fryxell et al. 2012). The northern lineage of *I. scapularis* or the American clade is more likely to transmit LB than the Southern lineages (Qiu et al. 2002). There are differences in behavior between various clades -- for example pronounced North versus South differences in collection methods (such as ‘drag-ability’ of *I. scapularis* nymphs) that may contribute to low rates of human parasitism by *I. scapularis* in the southern US (Stromdahl and Hickling, 2012). Techniques for such studies are rapidly evolving from the use of 12S and 16S markers to the use of cytochrome C (Mechai et al. 2013; Norris et al. 1996), and microsatellites (Chan, 2012). In this study, mitochondrial DNA (MTDNA) analysis was used to compare tick haplotype with the genetic identification and presence of *Bbsl*, where present. Genetic analysis can reveal selective pressures, past events, and evolutionary history- such as the spread of ticks, gene flow, and population structure - that can generate inferences and predictions regarding pathogen transmission (Trout et al. 2009; Black et al. 2001). Hillburn and Satler (1986) identify population size and migration as important factors in the heterozygosity of ticks, as well as the degree to which a species inbreeds. They suggest that genetic variation can best be explained using host abundance, mobility, and host specificity (Hillburn and Satler, 1986; Trout et al. 2009). In chapter two, we test the hypothesis that genetic variation of *I. scapularis* can help explain the relative incidences of Lyme disease in the eastern United States, such that prevalence rates and genetic variation change along a latitudinal gradient.
1.2.5 Vertebrate host diversity

The vertebrate host diversity available to ticks is remarkable; every class of terrestrial vertebrates is known to be parasitized (Sonenshine, 1991). The role of vertebrate hosts in maintenance of the *Borrelia* cycle is two pronged: 1) they serve as reservoirs for the pathogen, and 2) as hosts to maintain tick populations (Sonenshine and Mather, 1994). *Ixodes ricinus* complex ticks have a wide variety of wildlife hosts (Spielman, 1994; Lane et al. 1991). The white-tailed deer is a precarious cog in the Lyme disease machine, necessary for the establishment of tick populations (Anderson et al. 1987; Wilson, et al. 1985). While it does act as the primary host to *I. scapularis* adults, white-tailed deer may also clear *Bbssl* infection (Piesman and Spielman, 1979; Spielman et al. 1985). Medium-sized mammals (mesomammals) are commonly parasitized by *I. scapularis*; though typically the proportion of ticks from these animals is not adequate to support tick populations (Fish and Dowler 1989). Small mammals alternatively serve as the primary hosts of immature *I. scapularis* and as a reservoir in the northeast and midwest (LoGuidace et al. 2003). Reservoir competency among wildlife species varies. Mammals such as the white footed mouse (*P. leucopus*), chipmunks (*Tamias spp.*), and shrews (*Sorex spp.*) are competent reservoirs, and are readily available to ticks (Sonenshine and Mather, 1994). Other species such as white-tailed deer and western fence lizards help to amplify tick populations without passing the bacteria, providing a potential dilution of the bacteria in the environment (commonly referred to as the dilution effect) (Kuo, et al. 2000; Lane and Quistad, 1998; Lane and Loye 1989). Fish and Daniels (1990) used xenodiagnostic techniques to evaluate the role of mesomammals as reservoirs for *Bbss*; showing transmission of the bacteria in skunks (*Mephitis mephitis*) and raccoons (*Procyon lotor*), although less efficient than in mice. Birds also play a role, as several studies have shown potential for certain bird species, such as the American robin (*Turdus migratorius*), to act as reservoirs (Anderson et al. 1990; Richter et al. 2000), while
ticks from other bird species are usually uninfected (Mather et al. 1989). Birds have been further implicated in the movement of ticks and aid in the colonization of new areas (Spielman, 1988). Domestic animals likewise serve as hosts to ticks and commonly acquire tick-borne disease (Bowman et al. 2008). Livestock are commonly parasitized by ticks and infected with \textit{Bbsl} many animals may seroconvert, however not all manifest disease (Bushmich, 1994). Humans serve as incidental hosts to generalist feeding ticks and are commonly infected with tick-borne diseases, including LB (Bacon et al. 2008).

**Borreliosis in domestic canines:** Lyme borreliosis is a disease of canines as well as of humans, and at a broad spatial scale the geographic distribution of \textit{Bbsl} antibody-positive canines (Lindenmayer et al. 1991) corresponds closely to the distribution of human LB cases. In the northeast, rates of LB in humans and canines are positively correlated, with several authors proposing the use of canines as sentinels for this disease in humans (Little et al 2010, Lindenmayer et al. 1991, and Olsen et al. 2000). However, since \textit{I. affinis} infests canines more readily than humans, a southern shift to a more \textit{I. affinis}-dominated sylvatic cycle could produce a corresponding skew towards proportionally more LB in canines than in humans in southern coastal states. Not only is LB a disease of people, and canines, but cats, cattle, horses, and possibly sheep can also become infected with the etiologic agent of Lyme disease (Bushmich, 1994; Mullen and Durden 2002).

Testing of canines for LB has recently shifted away from the classical immunofluorescent assay (IFA), Western Blot, enzyme linked immunosorbent assay (ELISA) and immunoglobulin G (cells vital to the body’s immune response [IgG]) test to the more sensitive SNAP 4Dx\textsuperscript{®} (IDEXX) test. Hamer (2009) showed that tick chemoprophylaxis may reduce sero-survey sensitivity in dogs indicating that caution must be employed when utilizing antibody tests for canines, and rather that ticks off canines should be tested.
There is anecdotal evidence of a recent increase in canine LB cases in South Carolina, without the corresponding increase in human cases (A. Causey, personal communication). One possibility is that dogs are acting as sentinels that are providing early warning of a southward shift of the *I. scapularis-Bbsl* system from the northeast. Alternatively, it is possible that dogs are bitten by a wider range of *Ixodes* species than are humans, particularly *I. affinis*, and thus are being infected via sylvatic cycles of LB discussed above. Consequently, in chapter three with the help of cooperating veterinary clinics in the southeastern US we determined the species composition and pathogen status of ticks found attached to companion animals, including domestic dogs (*Canis familiaris*) and cats (*Felis catus*).

**Borreliosis in wild mesocarnivores:** In chapter 3, we chose to include mesomammals as they are commonly used as sentinels due to large home ranges, ample opportunity for exposure to pathogens, and relative availability (Aguire et al. 2009). Mesomammals are common hosts of immature *I. scapularis* (Carey et al. 1980; Magnarelli et al. 1984; Fish and Dowler, 1989) and can support large numbers of potentially infected ticks (Fish and Daniels, 1990) thereby playing a role in the ecology of LB, especially when used in conjunction with other data. *Bbsl* has been documented in mesocarnivores from the southeast US by numerous sources, including Tackett (2009) and Magnarelli et al (1991) indicating potential promise as a sentinel. As with canine samples, tick data from mesocarnivores may be helpful in monitoring and mapping the spread of LB.

**1.2.6 Regional variation in the Borrelia cycle**

Comprehensive studies of the relative importance of potential reservoir hosts and their associated vectors can provide insights into the underlying mechanisms responsible for
maintenance of a pathogen, and as such may provide the necessary knowledge to understand, avoid and control human disease (Brown and Lane, 1992). Comprehensive studies of tick-borne diseases can be difficult; in many cases studies address only a limited number of components. These components may then be assembled to form all-encompassing conclusions, such as linkages in regional variation of LB.

Lyme disease in the northeastern US is a well-researched phenomenon with a general understanding of the system commonly accepted among professionals. The main vector of LB in this region, *I. scapularis*, and (Piesman and Spielman, 1979; Oliver et al. 2003) generally has a 2 year life cycle and is vector competent for *Bbsl* (Jacobs et al. 2003). One of the most important aspects in the transmission of *Bbss* to humans is the reverse phenological life cycle of this tick, with infected nymphs feeding before uninfected larvae and assisting in the maintenance of enzootic cycles (Spielman et al. 1985). *I. scapularis* is the model species for understanding LB in the eastern US. In the western US, the system has been explained and generally accepted due to much work by Dr. Robert Lane and constituents from the University of California, Berkeley. The western US *Borrelia* cycle involves *I. pacificus* (Cooley and Kohls, 1945) as the vector and human biting tick, and *I. spinipalpis* (previously *I. neotomae*) as the key species that maintains sylvatic cycles but is not known to feed on humans (Brown and Lane, 1992; Norris et al. 1997; Oliver et al. 2003).

While the northeastern and western regions are largely understood, the southeastern US represents a gap in knowledge of LB; owing to the amount of attention drawn to high endemicity areas, and a comparative lack of LB in people from the southeastern US. In the Southeast, several *Bbsl* species are maintained by sylvatic cycles that involve multiple enzootic vectors, including *I. affinis, I. minor,* and *I. angustus* (Oliver et al. 2003; Maggi et al. 2010; Clark et al. 2002; and Damrow et al. 1989). Since *I. scapularis* is widespread in the Southeast (see Dennis et
al. 1998) several authors have suggested that it can serve as a bridge vector for human-infectious 
*Bbsl* species found in the sylvatic cycles described above (Oliver et al. 2003; Hamer et al. 2011; 
and Maggi et al. 2010), since it is a generalist with regard to host selection (Bishopp and 
Trembley, 1945).  A recent study in North Carolina has challenged this ‘bridge vector’ hypothesis 
by demonstrating that *I. scapularis* were not infected with *Bbsl* at several locations where the 
prevalence of Bbsl in *I. affinis* was high (Maggi et al 2010).  This finding may help to explain 
why *Bbss* transmission to humans is uncommon in the southeastern coastal states, despite the 
presence of *Bbsl* in ticks and their wildlife hosts.  For example, the CDC (2011) documented only 
0.4 confirmed cases per 100,000 people in South Carolina in 2010, two orders of magnitude 
lower than the 37.8 cases per 100,000 reported for New Jersey.

With regard to human Lyme disease, North Carolina represents a transition zone between 
the hyper-endemic Northeast, and the non-endemic Southeast (Fig 1).  Furthermore, in North 
Carolina there have been recent reports of new *I. affinis* populations, which appear to be 
expanding its distribution northwards (Maggi et al. 2010, Harrison et al. 2010).  The recent 
discovery of significantly higher prevalence of *Bbss* in *I. affinis* (33.5%) than in *I. scapularis* 
(0%) (Maggi et al. 2010) suggests that more research is needed to understand the role of *I. affinis* 
in endemicity of *B. burgdorferi* in the southeastern US.
Figure 1.1. Trends in Lyme borreliosis annual incidence in humans, and *B. burgdorferi* exposure prevalence in domestic canines, for selected eastern coastal states (ordered from north to south). Bars are means for the two most recent years of data available (2010-2011 for humans, http://www.cdc.gov/lyme/stats/; 2011-2012 for canines, http://www.capcvet.org/parasite-prevalence-maps/; both accessed July 1, 2013). Canine exposure was assessed using immunological tests that may detect exposure from previous years as well as during the reporting year; Bowman et al. 2009).
1.3 OBJECTIVES AND HYPOTHESES

The overall goal of this study was to map the change in prevalence of *B. burgdorferi* in *Ixodes* tick species along a multi-state gradient for LB in the southeastern coastal US. Observed changes in pathogen prevalence are discussed in relation to regional variation in human and canine risk of LB.

My specific objectives were:

1. To compare the prevalence and genetic identity of *B. burgdorferi* and *Ixodes* spp. ticks collected from vegetation, and from domestic dogs, in coastal areas of southern VA, NC, SC, GA, and FL.
2. To test for a relationship between the mitochondrial genotypes of *Ixodes* spp. with the prevalence of *B. burgdorferi* species, and latitude.
3. In South Carolina, to compare the prevalence of *B. burgdorferi* and Ixodid tick species collected from domestic dogs with those from a) vegetation, and b) wild mesomammals.

The hypotheses I tested were:

1) *I. scapularis* will have the highest *B. burgdorferi* prevalence in southern VA, whereas *I. affinis* will have the highest *B. burgdorferi* prevalence in NC and SC.

2) *B. burgdorferi* positive ticks will be less genetically different from one another than to *B. burgdorferi* negative ticks.
3) Tick fauna and *Bbsl* prevalence from domestic dogs will be similar to that of ticks collected from wild mesomammals in South Carolina. Ticks collected from vegetation will not have similar prevalence to those collected from mammals.

Objectives 1 and 2, and Hypotheses 1 and 2, are addressed in Chapter 2. Objective 3 and Hypothesis 3 are addressed in Chapter 3. Both chapters are formatted as scientific manuscripts, to facilitate subsequent submission for publication. Chapter 4 presents the overall conclusions of the study and discusses directions for future research.
1.4 LITERATURE CITED


HILBURN, L.R., AND P.W. SATTLER. 1986. Are tick populations really less variable and should they be? Heredity. 57:113-117.


OLIVER J.H., F.W. CHANDLER, M.P. LUTTRELL, A.M. JAMES, D.E. STALLKNECHT, B.S.


STEERE, A.C., R.L. GRODZICKI, A.N. KORNBLATT, J.E. CRAFT, A.G. BARBOUR, W.
BURGDORFER, G.P. SCHMID, E. JOHNSON, S.E. MALAWISTA. 1983. The spirochetal
STEERE, A.C., J. COBURN; AND L. GLICKSTEIN. 2004. The emergence of Lyme disease. The
diseases with emphasis on the South-Eastern United States. Zoonoses and Public Health.
59:48-64.
TACKETT, K.A. 2009. The Lyme Disease spirochete, Borrelia burgdorferi, in tick species
collected from raccoons (Procyon lotor) and opposums (Didelphis virginiana) trapped in
Warren and Barren counties of south central Kentucky.M.S. Thesis, Department of
Biology, Western Kentucky University, Bowling Green, Kentucky, USA, 32-40 pp.
Phylogeography of Ixodes scapularis from Canines and Deer in Arkansas. Southwestern
Entomologist. 34:273-287.
TROUT FRYXELL, R.T., C.D. STEELMAN, A.L. SZALANSKI, K.L. KVAMME, P.M. BILLINGSLEY,
AND P.C. WILLIAMSON. 2012. Survey of Borreliae in ticks, canines, and white-tailed deer
from Arkansas, U.S.A. Parasites and Vectors. 5:139-147.
decreases transmission of Borrelia burgdorferi spirochetes from infected Peromyscus
leucopus mice to larval Ixodes scapularis ticks. Vector Borne and Zoonotic Diseases.
1:65-74.
TSAO, J.I. 2009. Reviewing molecular adaptations of Lyme Borreliosis spirochetes in the context
of reproductive fitness in natural transmission cycles. Veterinary Research. 40:36.


Chapter 2.

*Borrelia* Prevalence in *Ixodes* Ticks

Changes Along a Latitudinal Gradient
CHAPTER 2 – BORRELIA PREVALENCE IN IXODES TICKS CHANGES ALONG A LATITUDINAL GRADIENT

2.1 ABSTRACT

Introduction: The classic Lyme borreliosis (LB) cycle, involving the vector *Ixodes scapularis* and the etiological agent *Borrelia burgdorferi* sensu stricto (*Bbss*), is well documented in the northeastern US, where LB is becoming increasingly prevalent in humans and canines. Recently, in coastal North Carolina, *I. affinis* has been shown to have a higher incidence of *B. burgdorferi* sensu lato (*Bbsl*) than *I. scapularis*. The objective of this study was to assess changes in prevalence of *Bbss* in *Ixodes* spp. ticks along a north-south gradient.

Methods: Ticks were collected from vegetation at 19 coastal sites from 37.4° latitude (southeastern Virginia) to 30.0° latitude (northeastern Florida) over spring/summer and fall/winter 2011-12. All ticks were identified to species and life stage. *Ixodes* spp. ticks were screened for *Borrelia* spp. using a q-PCR to amplify the 23S gene (Courtney et al. 2004). *Borrelia* positives were identified to species by nested PCR and sequencing of the 16S-23S rRNA intergenic spacer (Bunnikis et al. 2004). A subset of ticks was then subjected to 12S and 16S tick mitochondrial rRNA genetic procedures (Norris et al. 1996).

Results: A total of 1,721 *I. scapularis* and 77 *I. affinis* were collected; 99.0% were adult. The number of ticks dragged per hour increased with decreasing latitude, particularly in winter, whereas the prevalence of *Bbsl* detected decreased with decreasing latitude. Overall, 46 ticks were positive for *Bbsl* (23 *I. affinis* and 23 *I. scapularis*) and these *Bbsl* species included 22 sequenced samples 12 *B. burgdorferi* sensu stricto (54%), 7 *B. bissettii* (32%), and 3 *B. miyamotoi* (13%); 24 specimens remain to be confirmed. No *Borrelia*-positive ticks were detected south of 35N (North Carolina). Genetic clustering based on F<sub>ST</sub> values was evident.
between *I. scapularis* positive ticks, indicating that in addition to the overall result that ticks are genetically clustered by geography some association may also exist between infection and haplotypes. All 23 *Bbsl* positive *I. scapularis* ticks belonged to the American lineage of ticks based on 16S rRNA.

**Conclusions:** Findings indicate that the risk to humans, of locally-acquired LB infection in the coastal southeast is at present low. A clear gradient exists whereby *Bbsl* was not detected in ticks south of 35.2˚N, this cannot be explained by an absence of the vector tick, as *I. scapularis* was abundant throughout our survey area. There was greater genetic similarity in ticks infected with *Bbsl* than those not infected based on the mitochondrial DNA ribosomal RNA 12S gene region. *Ixodes affinis* were collected in very low numbers, yet contributed 50% of the infected ticks we identified highlighting the importance of this species in sylvatic cycles of *Bbsl*. The genetic composition of ticks above and below 35.2˚N was not significantly different.

**Keywords:** *Ixodes scapularis, Ixodes affinis, Borrelia burgdorferi, Borrelia bissettii, Borrelia miyamotoi*, Lyme disease

### 2.2 INTRODUCTION

Lyme borreliosis (LB, commonly termed “Lyme disease”) is caused by infection of *Borrelia* spirochetes transmitted by ticks of the genus *Ixodes* (Burgdorfer et al. 1982; Steere et al. 1983; Benach et al.1983; Johnson et al. 1984; Steere et al. 2004). In the United States, *Borrelia burgdorferi* sensu stricto (*Bbss*) infected tick populations have expanded both in distribution and numbers to create LB “endemic” [endemism is defined by the CDC as a county with established populations of *B. burgdorferi* infected *I. scapularis* (Dennis et al. 1998)] areas in the Northeast and Upper Midwest (Bacon et al. 2008, Tsao, 2009). In the core of these regions, prevalence of
Bbss infection can exceed 50% in adult I. scapularis ticks, and 90% in reservoir-competent host populations (Tsao 2009).

In contrast to the well-described Bbss transmission cycles in northern endemic areas, LB in the southeastern US is topic of great controversy (Stromdahl and Hickling 2012, Clark et al. 2013). CDC maps of confirmed and probable LB case rates indicate a dramatic decline in Bbss human prevalence with decreasing latitude (CDC, 2012). Stromdahl and Hickling (2012) have documented strong latitudinal trends in the species composition of ticks biting military personal, where the further south the collections, the fewer I. scapularis were recovered. Duik-Wasser et al. (2012) have developed models for human risk in the northeast and midwestern US that predict low risk for Lyme disease throughout the southeastern US. Several studies have documented low to undetectable levels of Bbss infections in wildlife and ticks in the southeast; the prevalence of Borrelia spp. infection as reported from I. scapularis in North Carolina and Alabama was quite low; 0.4% (2/531; Magnarelli et al. 1986) and 0% (0/351; Piesman and Sinksy 1988) respectively. Nevertheless, a range of Borrelia species (term B. burgdorferi sensu lato; Bbss) have been identified in southeastern wildlife hosts and non-human-biting ticks (Luckhart et al. 1991; Oliver et al. 1996, Rudenko et al. 2009) and perhaps in humans and human-biting ticks (Clark et al. 2013, but see Stromdahl and Hickling 2012). The extent to which these Bbss species may cause human disease remains uncertain.

Ixodes scapularis (Say, 1821) populations exhibit strong regional and local divergence in population structure (Qui et al. 2002; Humphrey et al. 2010; Mechai et al. 2013). Tick populations in the south eastern US are genetically different with many haplotypes and may result from a historic isolation from northern populations (Avise, 2000; Humphrey et al. 2010). This has led to considerable debate regarding the appropriate taxonomic classification of northern and southern I. scapularis populations (Sonenshine 1993; Telford, 1998). Relationships between
genetic differences and behavioral and epidemiological traits have become a common and informative means of exploration (Black and Piesman, 1994; Norris et al. 1996; Ogden et al. 2011). Genetic analysis will be included to help explain the difference in prevalence of LB in the southeast relative to the northeastern US. Several hypotheses exist with regard to the cause of reduced incidence of LB in the southeastern US (Duik Wasser et al. 2012; Stromdahl and Hickling, 2012); one of those hypotheses to be addressed is tick genetics and effects upon disease risk in the southeastern US.

Divergence in *Ixodes* species is thought to stem from the evolutionary history of nearctic populations of *I. ricinus* complex ticks; *I. pacificus* and *I. scapularis* diverged within this complex in ancient times, as is evident by evolutionary relationships; however divergence of *I. scapularis* populations in the eastern US is more recent (Rich et al. 1995). The commonly accepted theory is that eastern *I. scapularis* populations were separated into relict populations, and these populations spread with glacial retreat leading to differing clades (Rich et al. 1995); this is further supported with genetic evidence indicating a population bottleneck at approximately the same time as the Wisconsin glaciation (Rich et al. 1995; Qui et al. 2001).

Morphological similarities between species in the *I. ricinus* complex have resulted in numerous taxonomic changes to *Ixodes* spp. through time (Camicas et al. 1998; Chan, 2012). Most recently, what were two separate species -- *I. scapularis*, and *I. dammini* -- were combined into the single species *I. scapularis* (Oliver et al. 1993; Kierans et al. 1996). The former was established in the southern US, while the later was found primarily in the northern US (Qui et al. 2001; Chan 2012). Two separate lineages termed “clades” have resulted from this pairing. The northern clade (American clade) is found mainly in the northern US (Qui et al. 2001; Norris et al. 1996), while the southern clade is exclusive to the Southern US (Norris et al. 1996). These clades have further been examined by haplotype; Qui et al. (2001) and Humphrey et al. (2010) have
shown that eleven haplotypes make up the southern clade; seven of which are found exclusively in the southern clade, whilst four are shared between the two clades. The American clade includes ten haplotypes (synonymous with haplotypes of Humphrey et al. 2010), six of which are exclusive to the northeastern and midwestern US.

Evolution and taxonomy of the Lyme disease etiological agent is similarly complex and in flux. Taxa of *B. burgdorferi* have undergone several revisions leading to the current classification of *Bbss* and *Bbsl* (Farlow et al. 2002). *Bbsl* is found throughout Eurasia, Japan, and North America (Farlow et al. 2002) and consists of at least 20 genospecies (Schneider et al. 2000; Rudenko et al. 2009; Niu et al. 2011; Clark, et al 2013) of which only seven of these genospecies have been identified in the US: *Bbss, B. andersoni, B. bissetti, B. kurtenbachii, B. californiensis, B.carolinensis* and *B. americana* (Schneider et al. 2000; Rudenko et al. 2009). In North America, *Bbss* is the only *Bbsl* complex member currently associated with Lyme disease; whereas, in Europe *B. garinii, B. afzelii, and B. spielmanii* are zoonotic pathogens (Farlow et al. 2002; Niu et al. 2011; Tsao, 2009). Recently, Giard et al. (2010) identified “*B. bissetti* –like” DNA in California LB patients, and *B. kurtenbachii* is currently ‘of interest’ with regard to human disease (Ogden et al. 2011). Most recently, Clark et al. (2013) has implicated *B. americana* and *B. andersoni* in human infection, and posited that these infections may be going undetected with current human testing techniques, however these results have yet to be replicated and subsequent testing of the patients over nearly three years were positive for *Bbss*.

The primary objective of this study was to map the change in prevalence of *Bbsl* in Ixodid tick species along a multi-state gradient for LB in the southeastern coastal US. A second aim was to test for a bio-geographical relationship between *Ixodes* spp. genotype, and the observed latitudinal change in *Bbsl* prevalence. Analysis will allow for epidemiological aspects to
be compared with population structure of ticks, collected using various methods, and finally for regional distribution of populations and pathogens.

2.3 METHODS

2.3.1 Study sites

To evaluate the latitudinal gradient, this study focused on the southeastern coastal United States of Virginia, North Carolina, South Carolina, Georgia, and Florida. Some states were subdivided (e.g. northern and southern NC) to increase the spatial resolution of the analysis. Field sites extended from York River State Park, Virginia in the north (37.4° N) to Guana River, Florida in the south (30.0° N); all sampling sites with coordinates are listed with in Table 2.1.

2.3.2 Collection of ticks from vegetation

To assess trends in prevalence along a north-south gradient, we set out to collect 50 adult *I. scapularis* and 50 adult *I. affinis* from north, central, and southern regions of each state’s coastal lowlands. Dates of collection were chosen based on seasonal phenology of target species (M. Toliver, personal communication), such that we targeted *I. affinis* during the spring/summer period (March-August) and *I. scapularis* during the fall/winter period (November-January). At each site, ticks were collected by flagging or dragging a 1m² flannel cloth (Falco and Fish, 1992) through forest, or along roadsides and trails. Our protocol was to drag/flag for a minimum of one up to eight hours per sampling session (we continued sampling until the target sample size of 50 ticks was reached). Location, environmental conditions, dates and habitat descriptions were recorded for each collection. All collected ticks were preserved in vials containing 70% ethanol.
2.3.3 Morphometric identification of ticks

Ticks were identified to species using keys from Kierans and Litwak (1989), Cooley and Kohls (1944), Cooley and Kohls (1945), B. Harrison (unpublished), Clifford et al. (1978), Kierans and Durden (1998), and Durden and Kierens (1996). All ticks were viewed at 100X magnification under a compound light microscope (Nikon SMZ 1500, Nikon Instruments Inc, Japan) and were identified to species and life stage. Identity of a subsample of ticks was corroborated by Dr. Lorenza Beati Ziegler, curator of the U.S. National Tick Collection at Georgia Southern University, as well as being confirmed with sequencing results. All ticks were dabbed dry prior to identification, and returned to 70% ethanol following identification.

2.3.4 Molecular Techniques

**DNA Extraction:** DNA from tick samples were extracted using a DNEasy tissue kit (Qiagen incorporated, Chatsworth, CA) using methods of Beati and Kierans (2001) with slight modification. Samples were removed from ethanol, dried on a paper towel and moved to previously labeled vials. Working inside a laminar flow hood, the distal third of each tick’s idiosoma was cut with a sterile scalpel. A volume of 40 µL defrosted proteinase K (PRO-K) (Fisher bioreagent, Hampton, NH) was added to each flat, unengorged tick. Samples were vortexed and placed in a heated shaker overnight at 56°C for a minimum of 12 hours. 220 µL of buffer AL was added to each sample, which was then incubated at 72°C for ten minutes. 250 µL of 100% ETOH was added and samples were mixed. All liquid content of tick vials (about 700 µL) was transferred to vials containing spin columns, ethanol (70%) was then added to vials containing the tick idiosoma, and vials were stored for future reference. The remaining extraction steps were performed according to manufacturer’s instructions except for the final elution step, which included subsequent addition of 100 µL of hot (72°C) molecular water, which was allowed
to sit for five minutes prior to final centrifugation. Final eluates were then stored at -20°C. This extraction process yielded total genomic DNA. Aliquots were used for haplotype determination and *Borrelia* identification.

**Haplotype determination:** A subsample of *I. scapularis* (up to 49 per site) including both positive (known tick DNA) and negative controls (Molecular water) were tested using 12S and 16S mitochondrial rDNA. This number was determined based upon the number of *Borrelia*-positive ticks in our highest prevalence site, plus doubling the number of PCR negative ticks. Conventional PCR targeting the 12S and 16S genes was used, and the product was sequenced with Ixodidae specific mitochondrial primers (16S+1/16S-2, 12S+1/12S-1), previously published by Norris et al. (1996) [Appendix 1]. Primers were used to amplify a ≈420 base pair region of the 12S gene at a working concentration of 5µM. The protocol of Black and Piesman (1994) was modified for use of PCR beads and changes are as follows. Supermix was prepared using 5µL Tick 12s FWD (12S+1) primer, 5µL Tick 12s Rev (12S-1) primer and 12.5 µL of sterile molecular grade water per sample. Supermix was stored at either -20°C or 4°C depending upon time until use, with the latter not freezing master mix. To each tube, containing one PCR bead (Pure Taq™ Ready To-Go™ PCR beads, GE Healthcare, Little Chalfont Buckinghamshire, UK) was added 22.5 µL of supermix and 2.5 µL of tick DNA. The content of each tube was mixed by gentle flicks of the finger, gently vortexed and centrifuged for a 30 seconds to pool contents. Reactions were placed into a cold block until ready for cycling. The thermal cycler used was a T1000 Thermal Cycler (Biorad), Hercules, CA, USA. Amplification conditions were modified from Black and Piesman (1994) and were as follows. Initial denaturation was at 94°C for 3 minutes followed by five touchdown cycles 94°C for 20-s (denaturation), 65°C for 30-s (annealing), and 72°C for 30-s (elongation) with temperature decreasing by 2°C with each passing cycle. Amplification included 25 cycles at 88°C for 20-s (denaturation), 55°C for 400-s
(annealing), and 68°C for 30-s (elongation). Final elongation was 72°C for 180-s (See Appendix 2). The same protocol was followed for the amplification of a ≈462 base pair segment of the 16S gene, using Tick 16s Fwd (16S+1)/Tick 16s Rev (16S-2) primers.

**Borrelia detection**

**Realtime 23S PCR:** Initial screening of the extracted DNA for *Borrelia* species was accomplished using real time PCR with 23S rRNA Lyme primers (Courtney et al. 2004) [see Appendix 3]. The probe and primers were designed by Dr. Steve Kania and Rupal Brahmhbatt of the University Of Tennessee Department Of Comparative Medicine using Integrated DNA technologies PrimeTime software.

Master mix (MM) was made in a laminar flow hood, using 0.4µL of Roxidine loading buffer, 1 µL of assay mix (primers and probes from applied biosystems) 6.6µL of molecular grade water, and 10µL of MM, which was then pipetted to mix. For each well (specimen), 18 µL of MM was added along with 2 µL of DNA extract for a total 20ul reaction. For each plate, two extraction controls (molecular water run through the DNA extraction protocol), and a positive control (positive control per University of Tennessee School of Veterinary Medicine) was included in each run. Testing was completed using a StepOne Real-Time PCR system from Applied Biosystems (Catalog number: 4376357, Life Technologies, Carlsbad, CA).

**Nested IGS PCR:** *Borrelia*-positive ticks were then subjected to a *Borrelia* specific nested IGS PCR process to replicate DNA for verification and further comparative genetic analysis. We chose to conduct a nested PCR for the purpose of increasing yield and specificity; each nested step was performed in a separate lab to prevent contamination. A nested PCR targeting the 16S-23S intergenic spacer (IGS) loci was used to amplify *Borrelia*-genus specific
DNA using the primers (rrs-rrlA IGS/R) and protocol (slightly modified) of Bunnikis et al. (2004) [see Appendix 4]. Primers were used at a working concentration of 5 µM, and a size of 1100 base pairs. Master mix was prepared by adding 5 µL (of 5 µM IGS Forward) primer, 5 µL (of 5 µM IGS Reverse) primer and 11 µL of sterile molecular grade water per sample. 21 µL of master mix was added to each tube containing one PCR bead (Pure Taq™ Ready To-Go™ PCR beads, GE Healthcare, Little Chalfont Buckinghamshire, UK). 4 µL of total genomic DNA (and controls) were then added to each tube, and contents mixed by gentle flicks of the finger. Reactions were placed into a cold block until ready for cycling. The thermal cycler used was a T1000 Thermal Cycler (Biorad), Hercules, CA, USA. Initial denaturation of each sample was carried out at 94°C for 3 minutes, followed by 5 touchdown cycles 94°C for 20-s (denaturation), 67°C for 30-s (annealing) and 72°C for 60- s, decreasing the temperature by 2°C per cycle. Amplification (See Appendix 4) included 25 cycles of denaturation at 88°C for 20 seconds, 58°C for 400-s (annealing), and 72°C for 30-s (elongation). The final elongation was at 72°C for 180 seconds. The process above was then repeated (nested) using primers FN/ RN. Amplification conditions were as follows. Initial denaturation of each sample was carried out at 94°C for 3 minutes, followed by 5 touchdown cycles 94°C for 20-s (denaturation), 67°C for 30-s (annealing) and 72°C for 60 seconds, decreasing the temperature by 1°C per cycle. Amplification included 25 cycles of denaturation at 88°C for 20- s, 62°C for 400-s (annealing), and 72°C for 30-s (elongation). The final elongation was at 72°C for 180-s.

**Gel Protocol:** PCR products were analyzed by gel electrophoresis, using a 1% agarose gel which was stained with ethidium bromide (1 µL / 10 mL agarose solution). In each gel, we included a molecular ladder (GeneRuler™, 1 KB DNA Ladder, Thermo Scientific) and both a positive and negative control for verification (molecular water run through the DNA extraction
and B31 strain of *Bbss*, respectively). Addition of 1/6 of sample 6x DNA loading dye (Thermo Scientific) was added to each sample for visualization (only to 6 µL of 12S and 16S PCR product). We loaded all PCR products from nested IGS into the gel, whereas only 6µL of 16S and 12S PCR product was loaded and the remainder saved for ExoSAP-IT procedure. PCR product with loading dye was transferred into their respective wells, and ran at 80 Volts for a minimum of one hour. Once a gel had been run, we used a UV light and razor blade to cut out individual bands of Nested IGS DNA for subsequent sequencing.

**Gel recovery and sequencing for Nested IGS:** DNA recovery from nested IGS gels was completed using a Zymoclean™ Gel DNA recovery kit (Epigenetics, Irvine, CA), gel weight was determined and 3 volumes of ADB buffer was added for each volume of gel. Samples then were incubated at 55°C for 5-10 min. Melted agarose solution was placed into a zymo-spin column in a collection tube. Samples were centrifuged for 30-s, and 200µL of wash buffer added to each sample, followed by centrifugation for 30-s; this step was then repeated. Zymo-spin columns were transferred to new tubes, and 30µL of hot molecular water was added directly to the column matrix and allowed to sit for 20 min, samples then were centrifuged and the column vials discarded. Gel DNA extract was then sent to the University of Tennessee’s Knoxville Molecular Biology Resource Facility for sequencing.

**ExoSap-IT DNA clean-up for 16S and 12S PCR Product:**

The remaining dye free PCR product (12S and 16S only) was hydrolyzed, by the addition of ExoSAP-IT® (USB Corporation, Cleveland, OH)) according to the manufacturer’s instructions. The extract was then sent to the University of Tennessee’s Knoxville Molecular Biology Resource Facility for sequencing.
**Alignment and Phylogenetic analysis**

Sequences were edited and assembled using Sequencher 4.10.1 (Gene codes corporation, Ann Arbor, MI); bidirectional sequences were assembled (for a small subset where only unidirectional sequencing worked, we used unidirectional sequences) into consensus sequences. Contigs were then imported into Bioedit v7.2.0 (Ibis Biosciences, Carlesbad, CA), where they were aligned using ClustalW (Thompson, et al. 1994) and nucleotide polymorphisms confirmed. A BLAST (Altschul et al. 1990) search was performed on the aligned consensus sequences to determine tick and pathogen identities through comparison with sequences in GenBank (National Center for Biotechnology Information, Bethesda, MD).

Bayesian Evolutionary Analysis Sampling Trees were created using the MCMC model (BEAST version 1.7.4 software, Drummond and Rambaut 2003) as described by Szalinski et al. (2008). The consensus tree used four Markov chains run for $10^6$ generations and burn-in was left at default value. Viewing of the consensus tree was accomplished using Fig Tree version 1.3.1 software (a component of the Beast package) Representatives of each haplotype were submitted to GenBank (See Appendix 2).

**2.3.5 Statistical analysis**

Statistical analysis was carried out using R studio, version 0.97.320©2009-2012 Rstudio, Inc, Statistix 8 analytic software version 8.0 ©1985-2003 and Microsoft Excel 2010. Linear regressions were used to assess relationships between ticks per sampling hour and latitude. We ran Chi-square tests of association to assess variation in *Borrelia* prevalence among states. Genetic analysis, including Fst and NM values, Tajima’s and Fu and Li’s F and D statistics were calculated using DnaSp 5.10.01 ©2010 Universitat de Barcelona.
2.4 RESULTS

2.4.1 Latitudinal trends in *Ixodes* tick activity

Overall totals of 1,721 *I. scapularis* (17 nymphs and 1704 adults) and 77 *I. affinis* (1 nymph and 76 adults) were collected from the southeastern coastal region of the United States. One adult *I. angustus* was collected at Myrtle Beach State Park.

During fall/winter, we collected an average of 25.1 (±3.13 SEM; standard error of mean) *Ixodes* ticks per hour, with collection success increasing progressively as we moved south (Table 2.2; $R^2 = 0.44, P = 0.0012$). Florida provided the highest densities of *I. scapularis* adults encountered during November 2012 (112 collected per hour at Big Talbot Island State Park). Our collection success in Spring/Summer was much lower--averaging only 7.9 (±1.29 SEM) *Ixodes* ticks per hour--and was not significantly related to latitude ($R^2 = 0.16$, NS).

We collected very few *Ixodes* spp. nymphs (N = 18; 1.0% of all *Ixodes* spp. collected), and those collected were infrequent and in various habitat types during spring/summer. A majority of the *I. scapularis* nymphs came from dragging Victoria’s Bluff Heritage Preserve in South Carolina, 1.42 (±0.34 SEM) nymphs per hour. We collected nymphs in Florida, South Carolina, North Carolina, and Virginia, but never in appreciable numbers. The distribution and seasonality of *I. affinis* at our sites was irregular and distinctly different from that of *I. scapularis* with regard to seasonality, although there was noticeable overlap in the activity of adults of the two species in the early part of spring. Personal observations were that we seemed to find *I. affinis* primarily in swampy areas, with dense understory, such as ferns, whereas *I. scapularis* seemed to be more widely distributed. The only state in which *I. affinis* comprised
>10% of the *Ixodes* ticks collected was North Carolina, where it accounted for 11.3% of the collection.

### 2.4.2 Latitudinal trends in *Borrelia* spp. prevalence

*Borrelia*-infected *I. affinis* and *I. scapularis* were collected from vegetation only in Virginia and North Carolina north of 35.24°N (Table 2.2). In Virginia, 30 of 187 ticks (16%) were positive for *Bbsl*, whereas in North Carolina 16 of 447 ticks (4%) were positive for *Bbsl*. No positive ticks were collected from vegetation in South Carolina, Georgia or Florida. This difference in prevalence among states was highly statistically significant for both tick species (Chi-square test of association; \( X^2 = 111, 4\text{df}, P < 0.0001 \) for *I. scapularis* and \( X^2 = 15.6, 4\text{df}, P = 0.0005 \) for *I. affinis*). In the case of *I. scapularis*, the prevalence of infected ticks was much lower in North Carolina (<1%) than in Virginia (15%; \( X^2 = 52.3, 1\text{df}, P < 0.0001 \)). To date, 9 of the 30 Virginia *Bbsl* positives have been assessed using nested IGS; 55% of those were identified as *Bbss* and were >95% homologous with *Borrelia burgdorferi* (GenBank AF467866), and the remaining 4 were >95% homologous to *B. bissettii* (GenBank JF791763). From North Carolina, 13 of 15 *Bbsl* positives have been identified by IGS with 54% of the samples being >95% homologous with *Borrelia burgdorferi* (GenBank AF467866), 23% of the samples being >95% homologous with *Borrelia bissettii* (GenBank JF791763), and 23% of samples being >98% homologous with *B. miyamatoi* (GenBank AY531879).

*Ixodes affinis* comprised only 7% of all *Ixodes* ticks we collected, yet comprised half of the 46 *Bbsl*-infected ticks that we identified. Overall, *Borrelia*-positives were 12.4 times as frequent in *I. affinis* as in *I. scapularis* (\( X^2 = 140, 1\text{df}, P < 0.0001 \)), with the difference in prevalence between the two species being most pronounced in North Carolina. The prevalence of positive *I. affinis* similarly trended downwards from Virginia (60%) through northern North
Carolina (44%) into southern North Carolina (33%) (Table 2.4), although the number of *I. affinis* available for testing was relatively low and this trend was not statistically significant ($\chi^2 = 1.78$, 1df, $P < 0.41$). One interesting finding was that the sole *I. affinis* nymph collected was also positive for *B. miyamatoi*.

### 2.4.3 Latitudinal trends in *Ixodes* population genetics

#### Analysis of Mitochondrial Haplotypes

We chose to test similar sample sizes from each state for comparison which resulted in a total of 188 12S *I. scapularis* sequences, and 191 16S *I. scapularis* sequences for analysis. This included ticks from various regions and of differing phenotype [*Bbsl* (+) and *Bbsl* (-)]. For the purpose of this thesis, 16S data was only used to confirm American and Southern lineages of ticks.

**12S:** 12SrRNA gene amplification resulted in an amplicon of approximately 350 base pairs. An alignment of 327-bp segment was used for genetic comparisons. From the 188 sequences used a total of 32 haplotypes were identified for the 12S gene (gaps were excluded). Variants 4 and 17 were the most common haplotypes, accounting for 29% and 21% of the haplotypes, respectively, variant 4 representing the Southern lineage, while variant 17 represents the American lineage. Twenty of the haplotypes were unique (i.e., occurring only once). Of the 327 nucleotide characters used for analysis, there were 25 parsimony informative sites of which 22 were parsimony informative with 2 variants, and 3 were informative for 3 variants. There were 263 monomorphic sites, and 36 polymorphic sites. There were 11 singleton variable sites, all of which had 2 variants. Thirty nine sites represented mutations. Tajimas D statistic was not significant ($P$
> 0.10; Tajima’s D= 0.12308), which does not allow predictions on the expansion or decline of populations to be made, however a Tajima’s value >0 indicates that a population may have suffered a recent bottleneck (Holsinger, 2006-2012). Fu and Li’s D and Fu and Li’s F test statistics were not significant ($P > 0.05; D = -2.01; F = -1.453$); however, low values for the test statistic supports the hypothesis of expanding populations rather than a current genetic bottleneck.

Finally analysis on geneflow and migration was conducted. For this task, we utilized Wright’s $F_{ST}$ statistic which revealed high levels of gene flow between all populations for each gene (12S: $F_{ST} = 0.298$, NM= 0.59) suggesting limiting differentiation (Table 2.5). Gene flow and migration rates along a latitudinal gradient demonstrate the general trend, that tick populations are most closely related based on geographic region.

### 2.5 DISCUSSION

This study documents that adult *I. scapularis* are commonly found questing in the southeastern coastal region of the US; indeed we collected more *Ixodes* spp. ticks per hour in the south (137 ± 79 SEM) than the north (69± 40 SEM). Consequently, the hypothesis that the lack of LB in the coastal Southeast results from a scarcity of *I. scapularis* in this region can be rejected.

Despite finding larger numbers of questing ticks in the south, infections with *Bbsl* were not found at latitudes below 35.24˚N. Consequently, the large populations of *I. scapularis* in coastal SC, GA and FL do not presently pose a significant *Bbss* transmission risk to humans. However this risk should continue to be monitored, as tick distributions are presently changing in ways that could lead to increasing human risk in coming years (Stromdahl and Hickling 2012). Results from this study indicate much gene flow in current populations, with the majority of gene flow evident between adjacent states. An overall trend in the degree of genetic relatedness by
geographic region was found where each adjacent state was more genetically similar to adjacent populations than to more distant populations. Within state populations, we found evidence of distinct clustering using the 12S gene, suggesting that genetics may play a significant role in the transmission of \( Bbsl \). Clustering is based upon evolutionary relationships due to genetic distance, with the most genetically similar individuals being the closest together (clustered), indicating that clustered populations share a close genetic relationship. In this case, the \( Bbsl \) positive phenotypes are more closely related to one another, than they are to the \( Bbsl \) negative phenotypes within the same locality (Table 2.5; Figure 2.1). The genetic composition of ticks was evaluated using haplotype diversity; no significant trends in haplotype diversity were demonstrated along a north-south gradient. Average haplotype diversity starting at 84% in the north and ended at 78.5% in the Florida (Figure 2.2) suggesting that there may be coming shifts in the genetic distribution of ticks in the southeast. All 16S \( Bbsl \) positive ticks belonged to the American lineage of ticks; these geographical regions did have representatives of both the Southern and American lineages, further implicating an association between population genetics and risk of LB. Qiu et al. (2002) showed geographical structure among \( I. scapularis \) in the eastern US, supporting Norris et al. (1996) and the finding of both a northern and southern lineage of ticks. While \( I. scapularis \) has been determined to be a single species rather than the northern \( I. dammini \) and southern \( I. scapularis \) (Oliver et al. 1993), these two mitochondrial lineages indicate a noticeable degree of genetic variation between the lineages, and indicate different evolutionary and demographic histories (Qiu et al. 2002). Future studies including larger sample sizes with more \( Bbsl \) positive ticks are needed to strengthen these arguments. The absence of the pathogen in Southern lineage ticks could be due to a number of factors including behavioral differences of the vector, preventing establishment of the pathogen, or possibly the re-evaluation of genetic incompatibility.
The possibility also exists that the reason for not detecting Bbsl is that the pathogen is yet to reach southern populations of ticks, and could change in the future.

Nymphal tick collectability was extremely low compared with the northern US, where nymphs are more readily flagged and dragged than adults (Falco and Fish, 1992). In the Northeast and Midwest, nymphs are the most important life stage in transmission of LB to humans owing to their small size which assists in avoiding detection (Ginsburg et al. 1994). This difference in nymphal questing behavior almost certainly contributes to the low risk of LB south of North Carolina. Previous authors have suggested that these behavioral differences relate to the use of lizards rather than small mammals as primary hosts by immature *I. scapularis* in the South (Oliver et al. 1996; Goddard and Piesman, 2006). Tick adaptation with regard to specific niches and the acquisition of hosts living close to ground-level dictates that ticks should quest in the leaf litter near these animals. Questing height would then be different between southern-adapted ticks and northern-adapted ticks. This indicates that behavior may be as important, or more so than abundance. It would be very helpful to identify genetic markers that would help us map the geographic distribution of these northern-adapted ticks. Both 16S and 12S rDNA seem a promising avenue for addressing this objective, although some more informative markers such as SNP’s and microsatellites are available (Beebe and Rowe, 2008).

While the bulk of this chapter has focused on *I. scapularis*, it is important that we communicate the significance of *Bbsl* infection in *I. affinis*. While this species only constituted 7% of our collection, 30% of these ticks were *Bbsl* positive. In the event that *Bbsl* were to become established in the south at detectable levels, *I. affinis* would be an exemplary amplifying host. While it may not infest humans, it can infest pets, and thereby other human-biting ticks. Recent establishment of *I. affinis* in the southeastern US has been documented (Oliver et al. 1996, Harrison et al. 2010), and based upon prevalence rates in Virginia and North Carolina, this
species may be a secondary method of surveillance for sylvatic \textit{B. b. latus}. \textit{Ixodes affinis} is morphologically similar to \textit{I. scapularis} and as such extra care should be taken in the identification of \textit{Ixodes} ticks in the South. Collection of this species is also quite difficult, and more study of the biology of this tick and its epidemiological potential are warranted.

Maggie et al. (2010) found \textit{B. b. s.} in \textit{I. affinis} in areas of North Carolina where they found no infection in \textit{I. scapularis}. Our results were similar to Maggie et al. (2010) with regard to higher prevalence of \textit{B. b. latus} in \textit{I. affinis} than in \textit{I. scapularis}. Both \textit{I. scapularis} and \textit{I. affinis} are generalist species known to feed on many of the same species, including deer and rodents; however, the difference in detectability of \textit{B. b. latus} would lead you to believe that little to no overlap of feeding upon reservoir competent hosts occurs between these two species. One potentially important host group that \textit{I. affinis} is not known to feed on is the lizards, which have been associated with dilution effects (Oliver, 1996). It is possible that \textit{I. scapularis} at present are not part of the southern LB cycle. This leads to the speculation that there may be a difference in host selection, or abundance, with more samples, primarily \textit{B. b. latus} positives; haplotype diversity should be revisited to analyze the extent of genetic similarity between \textit{B. b. latus} positive ticks, and those without infection.

Tajima’s D value was significant and a negative value was found, which corroborates previous literature (Qui et al. 2002; Van Zee et al. 2013) indicating a low frequency of polymorphism, or an expanding population possibly following a genetic bottleneck. Following these latitudinal changes, there is slight variation in the values of Fu and Li, and Tajima which could indicate population expansion in a southerly, and northerly direction, where it seems that much population expansion is occurring. Expansion between the various clades could lead to more ticks available in the \textit{B. b. latus} cluster and to a change in the dynamics of LB ecology in the
with either the introduction of the pathogen at higher levels, or the addition of a species that is better adapted to the transmission of *Bbss*. 
2.6 LITERATURE CITED

ALTSCHUL, S.F., GISH, W., MILLER, W., MYERS, E.W. & LIPMAN, D.J. 1990. BLAST

Press, Cambridge, USA.

BACON, R.M., K.J. KUGELER, P.S. MEAD. 2008. Surveillance for Lyme disease---United States,

BEATI, L. AND KEIRANS, J.E. 2001. Analysis of the systematic relationships among ticks of
the genera *Rhipicephalus* and *Boophilus* (Acari: Ixodidae) based on mitochondrial 12S

BENACH, J.L., E.M. BOSLER, J.P. HANRAHAN, J.L. COLEMAN, G.S. HABICH, T.F. BAST, D.J.
CAMERON, J.L. ZIEGLER, A.G. BARBOUR, W. BURGDORFER, R. EDELMAN, AND R.A.
KASLOW.1983. Spirochetes isolated from blood of two patients with Lyme disease. New

are different niches. Genetics 168:713-722.

BRISSON, D., M.F. VANDERMAUSE, J.K. MEECE, K.D. REED, D.E. DYKHIUZEN. 2010. Shared and
vicariant evolutionary histories of Northeastern and Midwestern *Borrelia burgdorferi*
populations. Emerging Infectious Diseases 16:911-917.


CHAN, C.T. 2012. Comparative analysis of microsatellite and mitochondrial genetic variation in *Ixodes scapularis*. Master’s Thesis, Georgia Southern University, Statesboro GA.


HOLSINGER, K.E. 2010. Tajima’s D, Fu’s T’s Fay and Wu’s H, and Zeng et al.’s E.


MARGOS, G., A. HOJGAARD A, R.S. LANE R.S., M. CORNET, V. FINGERLE, N. RUDENKO, N.


APPENDICES
### TABLE 2.1. Names and coordinates of sites where *Ixodes* spp. were collected in 2011-2013 (ordered by decreasing latitude).

<table>
<thead>
<tr>
<th>SITE NAME</th>
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<th>LON.</th>
</tr>
</thead>
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Table 2.2 Number of *Ixodes* spp. ticks dragged and flagged per person-hour during Spring/Summer (March – August) and Fall/Winter (November-January) collections, 2011-2013. Most of the collected ticks were adults (see text for details). Collection sites are ordered by decreasing latitude. SP = State Park; NWR = National Wildlife Refuge; WMA = Wildlife Management Area.

<table>
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<th>Fall/Winter</th>
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Table 2.3 Number of *I. scapularis* and *I. affinis* tested for the presence of *Borrelia* spp. by 23S q-PCR, number of positive tests, and percent prevalence (P) of infection for both tick species. Collection sites are ordered by decreasing latitude.

<table>
<thead>
<tr>
<th>State</th>
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<th><em>I. affinis</em></th>
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<td></td>
<td></td>
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<td>N (+)ve</td>
<td>P</td>
<td>N (+)ve</td>
<td>P</td>
</tr>
<tr>
<td>VA</td>
<td>York River SP</td>
<td>37.41</td>
<td>48</td>
<td>10</td>
<td>21%</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Kiptopeke SP</td>
<td>37.17</td>
<td>5</td>
<td>2</td>
<td>40%</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Chippoke’s Plantation SP</td>
<td>37.14</td>
<td>39</td>
<td>3</td>
<td>8%</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Dismal Swamp NWR</td>
<td>36.72</td>
<td>50</td>
<td>6</td>
<td>12%</td>
<td>9</td>
</tr>
<tr>
<td>All Virginia</td>
<td></td>
<td></td>
<td>142</td>
<td>21</td>
<td>15%</td>
<td>15</td>
</tr>
<tr>
<td>Northern</td>
<td>Dismal Swamp SP</td>
<td>36.51</td>
<td>50</td>
<td>1</td>
<td>2%</td>
<td>21</td>
</tr>
<tr>
<td>NC</td>
<td>Merchant’s MP SP</td>
<td>36.44</td>
<td>48</td>
<td>0</td>
<td>0%</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Mattamuskeet NWR</td>
<td>35.48</td>
<td>52</td>
<td>0</td>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Goose Creek SP</td>
<td>35.46</td>
<td>47</td>
<td>0</td>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td>All Northern NC</td>
<td></td>
<td></td>
<td>197</td>
<td>1</td>
<td>1%</td>
<td>25</td>
</tr>
<tr>
<td>Southern</td>
<td>Cliffs of the Neuse SP</td>
<td>35.24</td>
<td>43</td>
<td>1</td>
<td>2%</td>
<td>4</td>
</tr>
<tr>
<td>NC</td>
<td>Croatoan National Forest</td>
<td>34.86</td>
<td>50</td>
<td>0</td>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Cedar Point Rec. Area</td>
<td>34.69</td>
<td>56</td>
<td>0</td>
<td>0%</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Holly Shelter Game Refuge</td>
<td>34.42</td>
<td>51</td>
<td>0</td>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td>All Southern NC</td>
<td></td>
<td></td>
<td>200</td>
<td>1</td>
<td>1%</td>
<td>9</td>
</tr>
<tr>
<td>SC</td>
<td>Little Pee Dee River Landing</td>
<td>33.93</td>
<td>54</td>
<td>0</td>
<td>0%</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Myrtle Beach SP 2</td>
<td>33.65</td>
<td>56</td>
<td>0</td>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Frances Marion NF</td>
<td>33.18</td>
<td>50</td>
<td>0</td>
<td>0%</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Edisto Beach SP</td>
<td>32.52</td>
<td>50</td>
<td>0</td>
<td>0%</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Victoria's Bluff HP</td>
<td>32.27</td>
<td>57</td>
<td>0</td>
<td>0%</td>
<td>7</td>
</tr>
<tr>
<td>All South Carolina</td>
<td></td>
<td></td>
<td>267</td>
<td>0</td>
<td>0%</td>
<td>16</td>
</tr>
<tr>
<td>GA</td>
<td>Sapelo Island Reserve</td>
<td>31.45</td>
<td>24</td>
<td>0</td>
<td>0%</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Jekyll Island SP</td>
<td>31.11</td>
<td>36</td>
<td>0</td>
<td>0%</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Crooked River SP</td>
<td>30.83</td>
<td>5</td>
<td>0</td>
<td>0%</td>
<td>4</td>
</tr>
<tr>
<td>All Georgia</td>
<td></td>
<td></td>
<td>65</td>
<td>0</td>
<td>0%</td>
<td>7</td>
</tr>
<tr>
<td>FL</td>
<td>Big Talbot Island SP</td>
<td>30.50</td>
<td>58</td>
<td>0</td>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Guana River WMA</td>
<td>30.02</td>
<td>42</td>
<td>0</td>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td>All Florida</td>
<td></td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0%</td>
<td>0</td>
</tr>
</tbody>
</table>

1. One *Ixodes* adult could not be identified to species level; it tested (-)ve for *Borrelia* spp.
2. One *I. angustus* collected; it tested (-)ve for *Borrelia* spp.
3. Three positive ticks from NC were 99% homologous to *B. miyamatoi*. 
**Table 2.4.** List of 12S taxa used in phylogenetic tree Figure 2.3, includes collection sites GenBank accession numbers, and publisher. NA = collection site not known.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Collection Site</th>
<th>GenBank No.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>I. scapularis</em></td>
<td>Collier Co., FL</td>
<td>L43901</td>
<td>Norris et al. (1996)</td>
</tr>
<tr>
<td><em>I. scapularis</em></td>
<td>Collier Co., FL</td>
<td>L43900</td>
<td>Norris et al. (1996)</td>
</tr>
<tr>
<td><em>I. scapularis</em></td>
<td>Liberty Co., GA</td>
<td>L43899</td>
<td>Norris et al. (1996)</td>
</tr>
<tr>
<td><em>I. scapularis</em></td>
<td>Orangeburg, SC</td>
<td>L43898</td>
<td>Norris et al. (1996)</td>
</tr>
<tr>
<td><em>I. scapularis</em></td>
<td>Adams Co., MS</td>
<td>L43897</td>
<td>Norris et al. (1996)</td>
</tr>
<tr>
<td><em>I. scapularis</em></td>
<td>Morrison Co., MN</td>
<td>L43896</td>
<td>Norris et al. (1996)</td>
</tr>
<tr>
<td><em>I. scapularis</em></td>
<td>Jackson Co., AL</td>
<td>L43895</td>
<td>Norris et al. (1996)</td>
</tr>
<tr>
<td><em>I. scapularis</em></td>
<td>Payne laboratory, OK</td>
<td>L43893</td>
<td>Norris et al. (1996)</td>
</tr>
<tr>
<td><em>I. scapularis</em></td>
<td>Bulloch Co., GA</td>
<td>L43892</td>
<td>Norris et al. (1996)</td>
</tr>
<tr>
<td><em>I. scapularis</em></td>
<td>Payne laboratory, OK</td>
<td>L43891</td>
<td>Norris et al. (1996)</td>
</tr>
<tr>
<td><em>I. scapularis</em></td>
<td>McIntosh Co., GA</td>
<td>L43890</td>
<td>Norris et al. (1996)</td>
</tr>
<tr>
<td><em>I. scapularis</em></td>
<td>Payne laboratory, OK</td>
<td>L43889</td>
<td>Norris et al. (1996)</td>
</tr>
<tr>
<td><em>I. scapularis</em></td>
<td>Currituck laboratory, NC</td>
<td>L43888</td>
<td>Norris et al. (1996)</td>
</tr>
<tr>
<td><em>I. scapularis</em></td>
<td>Currituck laboratory, NC</td>
<td>L43887</td>
<td>Norris et al. (1996)</td>
</tr>
<tr>
<td><em>I. scapularis</em></td>
<td>Payne laboratory, OK</td>
<td>L43886</td>
<td>Norris et al. (1996)</td>
</tr>
<tr>
<td><em>I. scapularis</em></td>
<td>Barnstable Co., MA</td>
<td>L43885</td>
<td>Norris et al. (1996)</td>
</tr>
<tr>
<td><em>I. scapularis</em></td>
<td>Jackson Co., AL</td>
<td>L43884</td>
<td>Norris et al. (1996)</td>
</tr>
<tr>
<td><em>I. scapularis</em></td>
<td>Jackson Co., AL</td>
<td>L43883</td>
<td>Norris et al. (1996)</td>
</tr>
<tr>
<td><em>I. scapularis</em></td>
<td>Bulloch Co., GA</td>
<td>L43882</td>
<td>Norris et al. (1996)</td>
</tr>
<tr>
<td><em>I. scapularis</em></td>
<td>Bulloch Co., GA</td>
<td>L43881</td>
<td>Norris et al. (1996)</td>
</tr>
<tr>
<td><em>I. scapularis</em></td>
<td>McIntosh Co., GA</td>
<td>L43879</td>
<td>Norris et al. (1996)</td>
</tr>
<tr>
<td><em>I. scapularis</em></td>
<td>Liberty Co., GA</td>
<td>L43878</td>
<td>Norris et al. (1996)</td>
</tr>
<tr>
<td><em>I. ricinus</em></td>
<td>NA</td>
<td>L43903</td>
<td>Norris et al. (1996)</td>
</tr>
<tr>
<td><em>I. pacificus</em></td>
<td>California</td>
<td>L43902</td>
<td>Norris et al. (1996)</td>
</tr>
</tbody>
</table>
Table 2.5. Fst (Nm Values) for 12S mtDNA comparing genetic variation within and between *Ixodes scapularis* ticks based on State of collection and presence or absence of *Borrelia* spp. This table presents the values that Figure 2.1 is based upon.

<table>
<thead>
<tr>
<th>Analysis by state</th>
<th>Virginia (+)</th>
<th>Virginia (-)</th>
<th>North Carolina (+)</th>
<th>North Carolina (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virginia (+)</td>
<td>0.01628</td>
<td></td>
<td>0.15476</td>
<td>0.04701 (10.136034)</td>
</tr>
<tr>
<td>Virginia (-)</td>
<td></td>
<td>0.08844 (5.153550)</td>
<td>0.04701 (10.136034)</td>
<td>-0.02201</td>
</tr>
<tr>
<td>North Carolina (+)</td>
<td>0.15476</td>
<td>0.08844</td>
<td>0.19472 (2.0677896)</td>
<td></td>
</tr>
<tr>
<td>North Carolina (-)</td>
<td>0.04701 -0.02201</td>
<td>0.19472</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2.1. Fst tree showing genetic relatedness of *Ixodes scapularis* populations (defined by region of collection) using 12S rDNA. The tree indicates that phenotype (i.e., infection with *BbsI*) is important within a geographical area, with infected populations being less genetically different than uninfected populations. This tree is based on values from Table 2.5.
Figure 2.2. Trends in haplotype diversity, or percentage of ticks from each region of a particular haplotype, using 12S haplotypes and moving north to south. This figure demonstrates that the diversity of haplotypes between ticks in different geographic regions follows a general trend with slight peaks in North Carolina and Georgia, yet generally decreasing with latitude. Diversity of haplotypes is indicated on the bars, and ordinate.
Figure 2.3. Phylogenetic relationship of the *Ixodes scapularis* 12S rDNA (338 bp) amplified from ticks collected throughout the southeastern coastal United States. GenBank references in the format I_scap_accession number. My samples are shown by Variant (Var_ID). This tree clearly shows two distinct clades of *I. scapularis* the Southern lineage on top and American lineage below confirmed with phylogenetic analysis of 16S data (not included).
CHAPTER 3

COMPARISON OF BORRELIA PREVALENCE AND IXODES SPP. TICK
INFESTATION OF DOMESTIC CANINES AND WILD MESOMAMMALS IN THE
SOUTHEASTERN UNITED STATES
CHAPTER 3 - COMPARISON OF BORRELIA PREVALENCE AND IXODES SPP. TICK INFESTATION OF DOMESTIC CANINES AND WILD MESOMAMMALS IN THE SOUTHEASTERN UNITED STATES

3.1 ABSTRACT

Introduction: Lyme Borreliosis (LB) is a tick-transmitted disease to both canines and humans. At a broad spatial scale, the geographic distribution of Borrelia burgdorferi sensu lato (Bbsl) antibody-positive canines corresponds closely to the distribution of human LB cases. The purpose of this project was to test the hypothesis that domestic canines and wild mesomammals can be used as sentinels for possible southward expansion of Bbsl infection in Ixodes populations.

Methods: Twenty-four veterinarians from Virginia, North Carolina, South Carolina, and Florida were asked to collect ticks from companion animals brought to their clinics. Ticks were also collected from vegetation at 5 coastal sites from 39.3°N latitude (northeastern South Carolina) to 32.7°N latitude (southeastern South Carolina) over spring/summer and fall/winter 2011-12. Collected ticks were morphologically identified to species and life stage. All Ixodes ticks were then PCR screened for the presence of Bbsl by amplifying the 12S-23S intergenic spacer (IGS). Bbsl PCR positive samples were confirmed with bidirectional sequencing. Tick diversity from mesomammals and companion animals from South Carolina were compared, and comparisons were made between states for companion animals.

Results: In total, 328 animals were checked for ticks, of which canines (n = 263), felines (n = 48), raccoons (n = 9), Virginia opossums (n = 6), deer (n = 1), and a feral hog (n = 1). A total of 892 ticks were collected from 311 companion animals; this total comprised 302 I. scapularis (34%), 232 A. americanum (26%), 157 Rhipicephalus sanguineous (18%), 140 Dermacentor variabilis (16%), 39 Amblyomma maculatum (4%), 22 I. affinis (2%). Of the 324 Ixodes ticks
collected, only 4 were Borrelia-PCR positive. These four ticks were all I. affinis and were collected from a deer in Virginia, a canine in North Carolina, and two from a canine in South Carolina. The bacteria from the North Carolina I. affinis from a canine were 96% homologous to Borrelia bissetti (GenBank JF791763) the remaining 3 samples are yet to be sequenced.

Conclusions: Tick fauna and Bbsl prevalence in companion animals and mesomammals was similarly low. The absence of I. ricinus complex ticks from mesomammals, and the level of diversity of other tick species, was in agreement with previous studies in the southeast (e.g., Kollars et al. 2009). All positive ticks were I. affinis, which is a tick not known to bite people; this could account for relatively higher levels of LB in canines than in humans in the Southeast. Our furthest-south record of a Borrelia-infected tick was from a dog, demonstrating that companion animals can indeed be useful as sentinels for LB emergence. The paucity of Ixodes ticks on the mesomammals suggests that these species would not be cost-effective sentinels (unless perhaps they are being collected for another reason).

Keywords: Borrelia, ticks, surveillance, mesomammals, companion animals, canines
3.2 INTRODUCTION

Infection with vector-borne pathogens is innately variable in space and time as transmission cycles depend on intricate relationships between pathogens, vectors, and hosts (Randolph, 2010). Many of these interactions are responsive to changing environmental conditions, exposure, and time spent in high risk areas (Randolph, 2010; Cutler, et al. 2010). Assessing risk in areas of emerging disease or low risk areas can be very challenging due to veiled cycles and confounding variables. The use of companion animals as sentinels has been suggested to assist in demarcating these areas (Falco et al. 1993; Hamer et al. 2009 Little et al. 2010). The use of sentinels as a means of disease detection has long been accepted, and in the case of canines has been evaluated (Duncan et al. 2004). Hamer, et al. (2009) demonstrated the importance of testing ticks for Borrelia rather than using antibody titers in areas with inherently low prevalence of LB. Serosurveillance in canines is more acceptable than in people as onset of illness is typically weeks to months after infection, allowing the immune system time to produce antibody (Little et al. 2010). However, with serological tests one must be able to differentiate active infection from past exposure which is difficult. One means of avoiding difficulty is the potential to screen ticks from the animal host (Magnarelli et al. 1987; Straubinger et al. 1997). Companion animals are typically in close proximity to their owners and at higher risk of coming into contact with ectoparasite vectors (Hamer et al. 2009; Little et al. 2010). Past testing for LB was completed using a two tier approach (immunofluorescent assay followed by a western blot), and has been phased out due to high cost, inefficiency (Duncan et al. 2004) and inability to detect active infection rather than prior exposure. Testing of ticks is one way to circumvent such issues and as such was our method of choice. Idexx Laboratories has also solved this problem to continue the use of serology by using C6 antibodies, and the SNAP®4DX test (Duncan et al. 2004).
Canines are six times more likely to be seropositive for *B. burgdorferi* than humans (Eng et al 1988), although many animals may not exhibit manifestation of clinical signs (Mullen and Durden, 2002). It logically follows that pet owners with infected pets would then be at a higher risk of contracting *B. burgdorferi*. Rand et al. (1991) demonstrated that seropositive canines were associated with high-risk areas prior to onset of human cases of disease. In North Carolina, an area of the southeast in which the prevalence of LB is low, canines has been employed for LB surveillance (Duncan et al. 2004). Bowman et al. (2009) used canines as sentinels on a broader, multistate scale. A recurring issue with serological tests is cross reactivity of antibodies to multiple antigens in canines and humans (Hamer et al. 2009). Serology also presents drawbacks with regard to insensitivity of serology to early infection, and detection of late manifestations of the disease which are no longer pathognomonic (Wormser, 2006; Littman, 2006).

Medium sized mammals (hereafter mesomammals) have been implicated of being wildlife reservoirs for various tick-borne zoonotics, including LB (Fish and Daniels, 1990). Aguirre et al. (2009) proposed that mesomammals could serve as sentinels because they typically inhabit large home ranges, and incur heightened opportunity to encounter infected vectors. However, mesomammals may not play a significant role in sylvatic cycles of LB due to comparatively low levels of infection in nature (Fish and Daniels, 1990). Even though prevalence may be low in these species their size and ability to harbor large numbers of ticks could contribute to a portion of the infected tick population (Fish and Daniels, 1990). Alone, mesomammals may not be a great model for LB surveillance due to low infection rates, but jointly mesomammals and canines may prove useful in surveillance.

Bowman et al. (2009) brought to light an area in Florida where the prevalence of LB in canines was significantly higher than the rest of the state as a whole. In neighboring North Carolina *I. affinis* a known vector competent tick for LB (Oliver et al. 1987) has been
documented with 33% prevalence of Bbsl, whereas I. scapularis in the area were uninfected (Maggi et al. 2010). Ixodes affinis is not known to be a human biting tick, and has been recovered from companion animals (Oliver et al. 1987). If this species is present and feeding on companion animals, this could be one explanation for case rates of LB in canines being higher than in people.

Surveillance programs that identify areas with both the presence of the pathogen and the vector may identify the risk of a disease and its spread earlier and more accurately than those that focus on only a single method (Daniels et al. 1993). Various studies have employed multiple methods, evaluating canine exposure and vector prevalence as a warning system for encroaching LB (Rand et al 1991, and Daniels et al. 1993); other studies have used antibody prevalence rates from medium-sized mammals and deer to evaluate human risk (Magnarelli et al. 1991). We aim to similarly evaluate mesomammals in conjunction with companion animals.

The southeastern US represents a gap in knowledge of LB, while some areas have been explored, many more are in the wait. Multiple studies have been conducted in Virginia, North Carolina, Georgia, and Florida, however little has been done or can be found in the South Carolina literature. Comparing the ticks collected from mesomammals, and canines in the state may be useful in evaluating sylvatic cycles of LB in South Carolina, and will augment what literature exists. In this study we aimed to evaluate and compare various surveillance methods throughout our geographical study area using ticks from mesomammals, and companion animals. The objectives of this study were to (1) compare the prevalence of LB in ticks from companion animals along a latitudinal gradient and (2) to compare the prevalence of LB in ticks from companion animals and ticks and tissue from wild mesomammals in South Carolina.
3.3 MATERIALS AND METHODS

3.3.1 Sampling sites and protocol

Site Establishment: Sites were chosen for proximity to the coast, and geographic distribution through the state (i.e., we wanted representative samples throughout the state). For these reasons we chose to collect ticks from mesomammals and target veterinarians in the vicinities of Myrtle Beach State Park, Little Pee Dee River Landing, Francis Marion National Forest, Edisto Beach State Park, and Victoria’s Bluff Heritage Preserve, veterinarian locations are listed by latitude in (Table 3.3). Grid locations (approximately 100m$^2$) for trapping mesomammals were established as directed by local authorities, as many of these sites were in areas of recreational use, and safety of the animals and the general public were of the utmost importance. Edisto Beach was not trapped, as vegetation sampling there began late in the study.

Collection of ticks from companion animals: In May 2012, we contacted twenty-four veterinarians from the southeastern USA, with a goal of achieving voluntary participation from at least four veterinary practices from Virginia, North Carolina, South Carolina, and Florida. We were successfully able to recruit a total of 24 clinics; nine from Virginia, six from North Carolina, six from South Carolina, and three from Florida (See Table 3.1). Collection kits consisting of instructions and empty labeled vials were assembled and mailed to each clinic. Clinics were contacted regularly to prompt them to collect, to check on progress, and to determine if more materials were needed. Tick samples were requested to be removed using forceps and stored in 70-80 percent ethanol. In addition information on county, breed, date, and preventatives were requested.
Collection of ticks and tissue from wild mesomammals: Mesomammal collections were limited to South Carolina for purposes of time and budget constraints. Four to five tomahawk live traps (81cmx25cmx31cm Model 108 Tomahawk Live trap company, Tomahawk, Wisconsin, USA) were set for mesocarnivores at the corner of each grid (approximately 100m apart), and one trap in the center when available, totaling up to five tomahawk traps per grid. Sardines in water were used for bait. Virginia opossum (Didelphis virginiana) were handled without the use of anesthetics; all other mesocarnivores were anesthetized with a Ketamine-Xylazine cocktail with Atipamezole reversal following protocols set forth by the Lyme disease gradient project (IACUC 1849). All visible ticks were taken from each host and stored in 70% ethanol. Ear biopsies were also taken, to supplement data from ticks.

Tick identification: Ticks were identified to species using keys from Kierans and Litwak (1989), Cooley and Kohls (1944), Cooley and Kohls (1945), Harrison (unpublished), Clifford et al. (1978), Kierans and Durden (1998), and Durden and Kierens (1996). All ticks were viewed at 100X magnification under a compound light microscope Nikon SMZ 1500 (Nikon Instruments Inc, Japan) and identified to species and life-stage. Confirmation of identity for a subsample of ticks was cross-checked by consultation with Dr. Lorenza Beati Ziegler of the US National Tick Collection. Voucher specimens are maintained in the University of Tennessee Center for Wildlife Health.

3.3.2 Laboratory methods

DNA Extraction: Host tissue samples were DNA extracted using a DNEasy tissue kit (Qiagen incorporated, Chatsworth, CA) following manufacturer’s directions for animal tissue.
Ticks were extracted using methods of Beati and Kierans (2001) with slight modification. Samples were removed from ethanol, dried on a paper towel and moved to previously labeled vials. Working inside a laminar flow hood, the distal third of each tick’s idiosoma was cut with a sterile scalpel. A volume of 40 µL defrosted proteinase K (PRO-K) (Fisher bioreagent, Hampton, NH) was added to each flat, unengorged tick (for engorged ticks we doubled the volumes of ETOH, buffer AL, and buffer ATL). Samples were vortexed and placed in a heated shaker overnight at 56°C for a minimum of 12 hours. 220 µL of buffer AL was added to each sample, which was then incubated at 72°C for ten minutes. 250 µL of 100% ETOH was added and samples were mixed well. All liquid content of tick vials (about 700 µL) was transferred to vials containing spin columns, ethanol (70%) was then added to vials containing the tick idiosoma, and vials were stored for future reference. The remaining extraction steps were performed according to manufacturer’s instructions except for the final elution step, which included subsequent addition of 100 µL of hot (72°C) molecular water, which was allowed to sit for five minutes prior to final centrifugation. Final eluates were then stored at -20°C. This extraction process yielded total genomic DNA, including tick DNA, potential bacterial DNA (Borrelia species) and any potential host DNA. Aliquots were used for screening, and Borrelia identification.

3.3.3 Borrelia detection

Real-time 23S PCR: Initial Borrelia screening used real-time PCR to amplify the 23s rRNA region of Borrelia using the previously published Lyme primers (Courtney et al. 2004) which were designed by Dr. Steve Kania and Rupal Brahmbhatt of the University of Tennessee Department of Comparative Medicine using Integrated DNA Technologies PrimeTime software (primers are listed in appendix 3). Master mix (MM) was made in a laminar flow hood, using 0.4 µL of Roxidine loading buffer, 1 µL of assay mix (primers and probes from applied
biosystems) 6.6 µL of molecular grade water, and 10 µL of MM, which was then pipetted up and down to mix. Eighteen µL of MM was added to each well (AB48 well optical reaction plate), followed by two µL of DNA extract (tick or host). Two extraction controls (molecular water run through the DNA extraction protocol), and a positive control (positive control per University of Tennessee School of Veterinary Medicine) were included in each run. PCR reactions were carried out by a StepOne Real-Time PCR system from Applied Biosystems. Ticks testing positive were further screened by means of a nested IGS process.

**Nested IGS PCR:** All samples which tested Bbsl positive by means of real-time PCR were subjected to a nested PCR for differentiation of *Bbss* from *Bbsl*. The nested protocol amplified the intergenic spacer region (IGS, ~1100 bp) of *Borrelia* using the primers and protocol (Appendix 4) reported by Bunnikis et al. (2004).

Primers were diluted to a working concentration of 5 µM, and master mix was prepared using 5 µL of 5 µM IGS Forward primer, 5 µL of 5 µM IGS Reverse primer and 11 µL of sterile molecular grade water per sample. One PCR bead (Pure Taq™ Ready To-Go™ PCR beads (GE Healthcare, Little Chalfont Buckinghamshire, UK) was added to each tube, followed by the addition of 21 µL of master mix to each tube. 4 µL of tick DNA was then added to each tube. PCR reactions were carried out in a T1000 Thermal Cycler (Biorad), Hercules, CA, USA. This process was then repeated using primer IGSFN and IGSRN, and amplification conditions (Appendix 4) from Bunnikis et al. (2004).

**Gel Protocol:** PCR products were analyzed using gel electrophoresis and using a 1% agarose gel (Tris base, acetic acid and EDTA and agarose) to visualize the IGS amplicon. Additionally, 1 µL of ethidium bromide was added for each 10 mL of agarose solution. To each sample 5 µL of 6x
DNA loading dye (Thermo Scientific) was added. 15 μL of GeneRuler™ (1 KB DNA Ladder, .5 mg/mL) was added to the first well in the gel, and then addition of the entire sample with loading dye was added to the gel. Each gel was run at 80 Volts for a minimum of one hour, and visualized with UV light. Successful amplicons were individually cut out from the gel and purified using a Zymoclean™ Gel DNA recovery kit (Epigenetics, Irvine, CA) following the manufacturers directions. Our sole amendment was the use of molecular grade water rather than elution buffer and allowing heated molecular grade water to sit on the Zymo-spin columns for 20 minutes prior to final centrifugation. Purified DNA from gel extractions were then sent to the University of Tennessee’s Knoxville Molecular Biology Resource Facility for bi-directional sequencing. Resulting sequence reads were compared to NCBI GenBank samples to confirm and identify the pathogen.

3.3.4 Statistics

Chi square tests were conducted using Statistix 8 analytic software version 8.0 ©1985-2003. Chi square tests of association were used for comparison of pathogen prevalence between species from north to south.

3.4 RESULTS

3.4.1 Veterinary collected ticks from all species

Veterinary clinicians collected a total of 1050 nymphal and adult ticks from 313 different hosts (263 canines, 48 felines, 1 while-tailed deer, and a feral hog). The collection contained six species of ticks (Table 3.2), of which 302 (29%) were *I. scapularis* and 29 (3%) were *I. affinis*. These *Ixodes* ticks were all adults; however one unknown *Ixodes sp.* larva was collected from a domestic canine in North Carolina. Comparison of the proportion of *I. scapularis* and *I. affinis*
parasitizing canines and felines among states were significant such that *I. scapularis* was significantly higher on both species ($\chi^2 = 49.25$, 5df, $P < 0.0001$) (Table 3.3). The mean number of *I. affinis* per infested animal was 0.09 (±0.86 SEM) *I. affinis*, whereas the mean number of *I. scapularis* per animal was 0.95 (± 0.86 SEM). No clear trends in species composition across latitudinal gradients were observed, other than low levels of parasitization by *I. affinis*. There were no *I. affinis* collected in Florida, but they were collected from all other sampled states. No data were available on how many inspected animals were tick free, nor did we compare tick infestation among seasons (because collection date was not reported by several clinicians).

### 3.4.2 *Borrelia* spp. in ticks from companion animals

Of the 143 *Ixodes* spp. ticks from hosts that were screened for *Borrelia*, only four adult *I. affinis* were PCR positive for the IGS *Bbsl* gene (Table 3.3). One *I. affinis* was collected from a white-tailed deer from Virginia, one from a dog in North Carolina, and two from a dog in South Carolina. The only sample sequenced to date (the specimen from the North Carolina canine) was 96% homologous to a previously amplified *B. bissetti* (GenBank JF791763). There was no significant difference in *Borrelia* spp. prevalence among host-collected *Ixodes* spp. by state ($\chi^2 = 0.77$; df = 3; $P = 0.85$), nor among *I. affinis* by state ($\chi^2 = 2.08$; df = 2; $P = 0.35$).

### 3.4.3 Ticks from mesomammals in South Carolina

Fifteen wild mesomammals were captured and inspected for ticks; these comprised nine raccoons (*Procyon lotor*) and six Virginia opossums (*Didelphis virginiana*). The majority (10 of 15) of the animals were tick-free upon visual inspection. Of the four hosts with ticks, a total of 12 ticks were collected consisting of nine *I. texanus* recovered from two different raccoons, and three *D. variabilis* recovered from two opossums. One additional raccoon with ticks was captured and
released without processing. None of the ticks (n = 12), and none of ear biopsies (n = 15), tested positive for \textit{Bbsl}, each animal with ticks was parasitized by only a single tick species, one raccoon had multiple ticks, while all other captured mesomammals had only a single tick found on them.

\section*{3.5 DISCUSSION}

Neither the species composition of ticks from companion animals, nor the prevalence of \textit{Bbsl} within their ticks, exhibited significant variation by latitude in this study. Interestingly, the four \textit{Borrelia}-positive ticks from hosts were \textit{I. affinis}. Future studies should target the collection of \textit{I. affinis} further investigate this finding. Evaluation of the rate of parasitization of \textit{I. affinis} on companion animals and potential association with pathogens may help address the skew in infections between people and pets in the southeast. Although this study did not demonstrate statistical significance, personal observation indicates that \textit{I. affinis} may more commonly parasitize felines than canines. The presence of infected \textit{I. affinis} in the southeast could either act as an amplifier and cause higher rates of infection among human biting ticks, or maintain sylvatic cycles in \textit{I. affinis} while rarely crossing over to human biting ticks such as \textit{I. scapularis}. Ticks in the \textit{I. ricinus} complex can be morphologically difficult to distinguish, especially immature stages. That being said the possibility of misidentification of immature \textit{I. affinis} as \textit{I. scapularis} is a valid concern.

Intriguingly neither \textit{I. scapularis} nor \textit{I. affinis} were collected from mesomammals, yet other studies have found each species on mesomammals in southern states (Oliver et al. 1987; Pung et al. 1994; Tackett, 2009). We were trapping at times when both tick species were active, as we collected questing ticks from vegetation in areas near our trap sites. Other studies in the
southeast have documented similar findings with little to no parasitization by *I. scapularis* in Missouri and Tennessee (Kollars et al. 1993; Kollars et al. 1999).

Even though *Bbss* prevalence in the Southeast is markedly lower than in the Northeast, it is still important to check oneself and ones pets for ticks (Oliver, 1996). In the Northeast, the nymphal stage of *I. scapularis* is the most important lifestage in transmission of *B. burgdorferi* to humans, while adults are the primary vector to canines (Little et al. 2010). LB is a concern not only for humans, but also for companion animals; infection with *B. burgdorferi* has been shown to cause arthritis and althralgia in pets, and can occasionally lead to death (Little et al. 2010).
3.6 LITERATURE CITED


from Southeastern and Mid-Atlantic States. Vector Borne and Zoonotic Diseases. 4:221-229.


TACKETT, K.A. 2009. The Lyme Disease spirochete, Borrelia burgdorferi, in tick species collected from raccoons (Procyon lotor) and opposums (Didelphis virginiana) trapped in Warren and Barren counties of south central Kentucky. M.S. Thesis, Department of Biology, Western Kentucky University, Bowling Green, Kentucky, USA, 32-40 pp.

TABLE 3.1. Locations of veterinary clinics participating in the study (ordered from north to south).

<table>
<thead>
<tr>
<th>Clinic name</th>
<th>Location</th>
<th>Latitude</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Virginia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anderson’s Corner Animal Hospital</td>
<td>Toano</td>
<td>N37.3797</td>
</tr>
<tr>
<td>Toano Animal Clinic</td>
<td>Toano</td>
<td>N37.3797</td>
</tr>
<tr>
<td>Jolly Pond Veterinary Hospital</td>
<td>Williamsburg</td>
<td>N37.2749</td>
</tr>
<tr>
<td>Colonial Veterinary Clinic</td>
<td>Williamsburg</td>
<td>N37.2749</td>
</tr>
<tr>
<td>VCA Boulevard Animal Hospital</td>
<td>Newport News</td>
<td>N37.0710</td>
</tr>
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<td>The Oaks Veterinary Clinic</td>
<td>Smithfield</td>
<td>N36.9822</td>
</tr>
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<td>Virginia Beach</td>
<td>N36.8345</td>
</tr>
<tr>
<td>Beach Pet Hospital</td>
<td>Virginia Beach</td>
<td>N36.8345</td>
</tr>
<tr>
<td>Harbour Vet Office</td>
<td>Suffolk</td>
<td>N36.7413</td>
</tr>
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<td></td>
</tr>
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<td>Sunbury</td>
<td>N36.4214</td>
</tr>
<tr>
<td>Lannon’s Animal Hospital</td>
<td>Elizabeth City</td>
<td>N36.2944</td>
</tr>
<tr>
<td>Pasquotank Animal Hospital</td>
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Table 3.2. Counts of tick species, by life stage, removed from domestic animal hosts by collaborating veterinarians in Virginia, North Carolina, South Carolina and Florida. The collection period was approximately March, 2012 to May, 2013. Additional ticks were collected from live-trapped mesocarnivores (mesoc.) in South Carolina, and from a hunter-killed feral hog (F.hog) in Florida.

<table>
<thead>
<tr>
<th>State</th>
<th>Host species</th>
<th>No. of hosts with ticks</th>
<th>Ixodes scapularis</th>
<th>Ixodes affinis</th>
<th>Ixodes texanus</th>
<th>Amblyomma maculatum</th>
<th>Amblyomma americanum</th>
<th>Dermacentor variabilis</th>
<th>Rhipicephalus sanguineous</th>
<th>Total ticks</th>
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<td>0</td>
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<td>0</td>
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<td>3</td>
<td>5</td>
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<td>0</td>
<td>0</td>
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<td>10</td>
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<td>61</td>
<td>29</td>
<td>69</td>
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<td>0</td>
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<td>6</td>
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<td>302</td>
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<td>9</td>
<td>39</td>
<td>96</td>
<td>102</td>
<td>153</td>
<td>143</td>
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Table 3.3  Counts and pathogen prevalence of *I. scapularis* and *I. affinis* removed from domestic dogs and tested for the presence of *B. burgdorferi*. Ticks were collected by collaborating veterinarians in Virginia, North Carolina, South Carolina and Florida over the period of March, 2012 to May, 2013. Clinics are ordered by decreasing latitude.

<table>
<thead>
<tr>
<th>State</th>
<th>Clinic</th>
<th>Latitude</th>
<th>Dogs with <em>Ixodes</em> spp. ticks</th>
<th><em>I. scapularis</em></th>
<th><em>I. affinis</em></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
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<td>Tested (+ve)</td>
<td>Prev (%)</td>
<td>Tested (+ve)</td>
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<tr>
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<td>The Oaks</td>
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<td>8</td>
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<td>All Virginia</td>
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<td></td>
<td>13</td>
<td>15</td>
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</tr>
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<td>Goose Creek</td>
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<td>-</td>
</tr>
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<td>19</td>
<td>0</td>
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<td>Dillon</td>
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<tr>
<td>All Florida</td>
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<td>18</td>
<td>18</td>
<td>0</td>
</tr>
</tbody>
</table>

¹ Information on the number of tick-free dogs was not available.
² Anderson’s Corner Veterinary Hospital also collected three *I. affinis* from a white-tailed deer -- one was *B. burgdorferi*-positive.
³ Includes one *B. burgdorferi*-negative *Ixodes* tick that could not be identified to species.
Table 3.4 Counts and pathogen prevalence of *I. scapularis* and *I. affinis* removed from domestic cats and tested for the presence of *B. burgdoferi*. Ticks were collected by collaborating veterinarians in Virginia, North Carolina, South Carolina and Florida over the period March, 2012 to May, 2013.

<table>
<thead>
<tr>
<th>State</th>
<th>Cats with <em>Ixodes</em> spp. ticks</th>
<th><em>I. scapularis</em></th>
<th><em>I. affinis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tested</td>
<td>(+)ve Prev (%)</td>
<td>Tested (+)ve Prev (%)</td>
</tr>
<tr>
<td>VA</td>
<td>10</td>
<td>3 0 0</td>
<td>2 0 0</td>
</tr>
<tr>
<td>NC</td>
<td>19(^2)</td>
<td>6 0 0</td>
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</tr>
<tr>
<td>SC</td>
<td>6</td>
<td>6 0 0</td>
<td>3 2 66</td>
</tr>
<tr>
<td>FL</td>
<td>3</td>
<td>3 0 0</td>
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</tr>
</tbody>
</table>

1 Information on the number of tick-free cats was not available.

2 Includes one *B. burgdorferi*-negative *Ixodes* tick that could not be identified to species.
CHAPTER 4 - OVERALL CONCLUSIONS

4.1 LYME DISEASE RISK IN THE COASTAL SOUTHEASTERN UNITED STATES

The risk of Lyme disease in the southeastern US can at present best be described as very low with undetectable levels of *B. burgdorferi* sensu stricto in *I. scapularis* below the 35th latitude in North Carolina. We report prevalence rates for *Bbsl* in *I. scapularis* collected from vegetation along the following north-south gradient: Virginia (15%), North Carolina (0.5%), South Carolina (0%), Georgia (0%), and Florida (0%). No positive *I. scapularis* were recovered from canines or mesomammals in the southeast. We detected no *Bbss* in *I. scapularis* south of North Carolina which is important because *I. scapularis* is the main vector of Lyme disease in the eastern US (Sonenshine et al. 1993). Diversity of *Ixodes* spp ticks recovered by dragging/flagging included three species which are vector competent for *Bbsl* (Oliver et al. 2003; Damrow et al. 1989); they are listed in order of greatest abundance (1721 *I. scapularis*, 77 *I. affinis*, and 1 *I. angustus*). In South Carolina, comparison between *Bbsl* (+) and (-) ticks was not possible as no *I. scapularis* were infected.

Stromdahl and Hickling (2012) assert that the density of *I. scapularis* in the Southeast is an order of magnitude lower than densities in the Northeast. In this study, *I. scapularis* were collected in abundance from vegetation at our southern sites, disproving one hypothesis for the differential in Lyme disease in the northeastern and southeastern US being due to low numbers of ticks. A second hypothesis regarding this differential relates to nymphal parasitization due to behavioral differences in *I. scapularis* nymphs (Piesman, 2002; Goddard and Piesman, 2006) and host choice. Our findings support variance in nymphal collectability which could relate to host preference (Goddard and Piesman, 2006). A third hypothesis implicates tick genetics (Northern vs Southern ticks) (Stromdahl and Hickling, 2012; Trout Fryxell et al. 2012)
as a reason for differential prevalences of Lyme disease among geographic regions. We compared mitochondrial genotypes of *I. scapularis* ticks from Virginia through Florida to address this question and our findings, while not significant implicate genetic differentiation as a factor in the risk of *Bbsl* infection of ticks. We observed both the American and Southern genetic lineages in our *I. scapularis* populations, and noticed all *Bbsl* positive ticks were also associated with the 16S American clade ticks, as proposed by Qui et al. (2002). Those ticks that were PCR positive with *Bbsl* were genetically similar among themselves compared to than were infection-free ticks in the same population. Given these genetic differences, it seems possible that there is some inherent difference between the northern and southern clade of *I. scapularis* that alters their ability to become infected, or transmit *Bbsl*. If so, this could help explain why there is such a difference in LB case rates in the northeastern and southeastern US.

*Ixodes affinis* were not collectected in appreciable numbers, but from those collected it is evident that they follow the same trends with regard to prevalence as *I. scapularis*. Wherever *I. scapularis* positives were detected (State and site-wide), so too were *I. affinis* found *Bbsl* positive (although almost always at a higher prevalence). Virginia had a 60% prevalence of *Bbsl* in *I. affinis* whereas North Carolina had a 41% prevalence, and similar to *I. scapularis* no infection was evident south of 35°N. No *I. affinis* were collected in Florida, or from mesomammals. At the southern limit of our *Bbsl* detection (33.9°N) were two positive *I. affinis* off the same dog from a vet in South Carolina suggesting by extension that LB risk to canines extends further south than it does for humans, and that risk maps based on canine serology data (e.g., Bowman et al. 2009), need to be interpreted cautiously in southeastern states. Analysis of bacterial strains is still to be completed, but three *Borrelia* genospecies have so far been identified in our tick collection. These are *B. burgdorferi* sensu stricto, the causative agent of Lyme disease (Oliver et al. 1993), *B. bissetti* which is of interest due to the recent association of “*B. bissetti*-like
DNA” being associated with Lyme disease in California (Giard et al. 2011). The third detected genospecies is *B. miyamatoi*, which is a relapsing fever group bacterium that is distinctly separate from the *Bbsl* complex (Fukanga et al. 1995). *B. miyamatoi* has recently been recognized to cause a zoonotic disease that shares several similarities with Lyme disease (Chowdri et al. 2013). Due to differences in the nature of *Bbsl* and *B. miyamatoi* detection of *B. miyamatoi* in people can be missed, or it can be misdiagnosed Gugliotta et al (2013).

### 4.2 FUTURE RESEARCH DIRECTIONS

Several aspects of this study that could have been better accomplished are acquisitions of larger sample sizes of mesomammals, companion animals, *I. affinis*, and genetic samples. Genetic markers for *I. affinis* and ecological studies of this species may shed some light on which *Bbsl* is circulating in the southern US. The use of additional genetic markers such as nuclear markers or microsatellites would also contribute greatly to the results. Inclusion of ticks from small mammals in the southeast would add an additional dimension whereby cryptic cycles may be revealed, and overall detectability of LB may increase. We are uncertain of the ability of our primers to pick up *B. americana*, and with the putative association of this species with human disease (Clark et al. 2013), retesting some tick collections with an assay that is more sensitive assays to the presence of this pathogen may be useful. Overall the inclusion of genetic analysis is vital to the understanding of ecosytems and helps in explaining the unseen goings on of systems that would otherwise be missed. A particular strength of this study is that is provides considerable information on the status of *Bbsl* and *Ixodes* spp. ticks down the eastern seaboard in 2012-13, such that is will serve as an invaluable baseline for comparison with the result of other surveys of emerging tickborne diseases that we foresee being undertaken in future decades.
4.3 LITERATURE CITED


transmission of the Lyme disease spirochete from the southeastern United States.

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APPENDICES

Appendix 1. Primers specific to 12S regions of ticks in the family Ixodidae (Norris et al. 1994).

Primer 1: Tick 12s Fwd 5’--TAC TAT GTT ACG ACT TA -3’
Primer 2: Tick 12s Rev 5’--AAA CTA GGA TTA GAT ACC C-3’
Primer 3: Tick 16s Fwd 5’--CCG GTC TGA ACT CAG ATC AAG T-3’
Primer 4: Tick 16s Rev 5’--TWR RGA CAA GAA GAC CCT AWG A-3’

Appendix 2. 12S and 16S gene region conditions for PCR amplification of Ixodes ticks.

Amplification conditions:

- Denaturation 3 min @ 94C
- Denaturation 20 sec @ 94C 5 touchdown cycles, decreasing temperature -2/cycle
- Annealing 30 sec @ 65C
- Elongation 100 sec @ 72C
- Denaturation 20 sec @ 88C 25 cycles
- Annealing 400 sec @ 55C
- Elongation 30 sec @ 68C
- Final elongation 3 min @ 72C
Appendix 3. Real-Time Probes and Primers specific for the 23S gene segment of *Borrelia burgdorferi*

Probe:
PrimeTime Probe--/56-FAM/AG ATG TGG T/ZEN/A GAC CCG AAG CCG AGT G/3IABkFQ/

Primer 1:
PrimeTime Primer 1 5’--CGA GTC TTA AAA GGG CGA TTT AGT-3’

Primer 2:
PrimeTime Primer 2 5’--GCT TCA GCC TGG CCA TAA ATA G- 3’
**Appendix 4.** *Borrelia*-genus specific nested PCR primers targeting the 16S-23S IGS locus.

Outer IGS: PCR 1

Primer 1: IGS Forward 5’--GTA TGT TTA GTG AGG GGG GTG-3’
Primer 2: IGS Reverse 5’--GGA TCA TAG CTC AGG TGG TTA G- 3’

Inner IGS: PCR 2

Primer 1: IGS FN 5’--AGG GGG TGA AGT CGT AAC AAG- 3’
Primer 2: IGS RN 5’--GTC TGA TAA ACC TGA GGT CGG A- 3’

**Amplification conditions: PCR 1**

Denaturation 3 min @ 94C

\[
\begin{align*}
\text{Denaturation} & : 20 \text{ sec} @ 94C \\
\text{Annealing} & : 30 \text{ sec} @ 67C \\
\text{Elongation} & : 100 \text{ sec} @ 72C \\
\text{Denaturation} & : 20 \text{ sec} @ 88C \\
\text{Annealing} & : 400 \text{ sec} @ 58C \\
\text{Elongation} & : 30 \text{ sec} @ 72C \\
\text{Final elongation} & : 3 \text{ min} @ 72C
\end{align*}
\]

5 touchdown cycles, decreasing temperature 2/cycle

25 cycles
Amplification conditions: PCR 2

5 touchdown cycles, decreasing temperature -1/cycle

Denaturation 3 min @ 94C

Denaturation 20 sec @ 94C

Annealing 30 sec @ 67C

Elongation 100 sec @ 72C

Denaturation 20 sec @ 88C

Annealing 400 sec @ 62C

Elongation 30 sec @ 72C

Final elongation 3 min @ 72C

Appendix 5. NCBI GenBank submissions for 32 *Ixodes* 12S haplotypes.
Contact Information:

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Release Date Information:

- Release immediately after it is processed.

Reference information:

- Sequence authors: Lauren P. Maestas, Rebecca T. Trout Fryxell, and Graham J. Hickling.
- Publication information: Unpublished,

**32 Ixodes 12S haplotypes**

1. Bankit ID: *Ixodes scapularis*
   a. Definition: *Ixodes scapularis* 12S Mitochondrial rDNA
   b. Source: *Mitochondrion Ixodes scapularis* (Black -legged tick)
   c. Organism: *Ixodes scapularis*
   d. Reference: Bases 1 to 438
   e. Authors: Maestas, L.P., Trout Fryxell, R.T., Miller, D.L., and Hickling, G.J.
   f. Title: *Borrelia* prevalence in *Ixodes* ticks changes along a latitudinal gradient
   g. Haplotype: Variant X
   h. Specimen Collection Location: Each individual
   i. Sequence:
   j. Comments:

**32 Ixodes 12S haplotypes**

1. Bankit ID: *Ixodes scapularis*
   a. Definition:
   b. Source:
   c. Organism:
   d. Reference:
   e. Authors:
   f. Title:
   g. Haplotype:
   h. Specimen Collection Location:
   i. Sequence:
   j. Comments:
Z. *Borrelia* gene genotypes.

1. Bankit ID:
   a. Definition:
   b. Source:
   c. Organism:
   d. Reference:
   e. Authors:
   f. Title:
   g. Haplotype:
   h. Specimen Collection Location:
   i. Sequence:
   j. Comments:
VITA

Lauren Maestas was born to Herbert Maestas and Dolly Gallegos in Holman, New Mexico. He attained his BS in Wildlife conservation biology from Humboldt State University and followed that with a MS in wildlife health from the University of Tennessee.