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Ecology and Disease Potential of the Black-Legged Deer Tick, Ixodes Scapularis Say, in Mississippi

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By

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To assess the seasonality and disease potential of *Ixodes scapularis* Say in north Mississippi, ixodid ticks were collected by drag cloth method at two sites in north Mississippi weekly from August 1, 2010 through July 31, 2011 and tested for molecular evidence of disease agents via polymerase chain reaction (PCR) assay. In addition, environmental data were observed and recorded for each collection date. *I. scapularis* nymphs (n=6) were collected in August, September, March, and May, perhaps reflecting a seasonally bimodal distribution, while adults (n=256) were found October through May with a peak in March. No statistically significant relationship between environmental data and number of *I. scapularis* was found. No *I. scapularis* were PCR positive for *Borrelia burgdorferi* or *Anaplasma phagocytophilum*, and four were positive for *Babesia odocoilei*, a cervid babesiosis.

DEDICATION

To my parents, Barbara and Burt, who have continually supported me throughout my endeavours, both academic and personal. Without your love and guidance, I wouldn't be the person I am today. Thank you.

To my human partners and my feline companions: you make this world a better place, and encourage compassion and kindness. I am grateful for you.

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CHAPTER I

HISTORY, TAXONOMY, BIOLOGY AND DISEASE ECOLOGY OF *IXODES SCAPULARIS* SAY

Background

Ticks are obligate blood-sucking parasites that may cause nuisance effects by their bites as well as transmit disease organisms. The black-legged deer tick, *Ixodes scapularis* Say, is a well-known tick species, belonging to the acarine family Ixodidae, the hard-bodied ticks. Ixodid ticks can transmit a wide range of pathogens across a breadth of vertebrate hosts, including humans. In the United States, *I. scapularis* is a known vector of *Borrelia burgdorferi*, *Babesia microti*, and *Anaplasma phagocytophilum*, the causative agents of Lyme disease, babesiosis, and human granulocytic anaplasmosis, respectively (Wallis et al. 1978, Burgdorfer et al. 1982, Dumler 2011, Telford et al. 2011). As a result of its particularly high medical significance, *I. scapularis* was the first chelicerate arthropod to be chosen for large-scale genomic analysis (Ghosh et al. 2007) via the *Ixodes scapularis* Genome Project, which sequenced the Wikel strain of laboratory-reared *I. scapularis*.

Taxonomy

Ixodes scapularis (Figure 1.1, 1.2) was first identified by Thomas Say in 1821 (Cooley and Kohls 1945). No type specimen exists, but a lectotype was designated by

Spielman et al. in 1979 (Spielman et al. 1979). However, according to the rules for zoological nomenclature under the International Code of Zoological Nomenclature (ICZN), this specimen designated by Spielman is actually a neotype (Keirans et al. 1996). It was identified while Spielman et al. were in the process of describing the "new" species, *I. dammini*. Primarily based on the morphology of nymphs and the geographical distribution of all life stages, they separated southern populations of *I. scapularis* and northern populations of *I. dammini* (Spielman et al. 1979, Keirans et al. 1996, Piesman 2002). Subsequently, mating experiments demonstrated that adults of *I. scapularis* and *I. dammini* can produce viable offspring, thus indicating that *I. dammini* is not a valid species (Oliver et al. 1993b), therefore the name *I. dammini* has been assigned as a junior synonym.

Biology

There are 21 records of *I. scapularis* in the Cooley and Kohls 1945 review of *Ixodes* ticks in North America, showing a concentration in the southern U.S., as well as reports from the upper Midwest and eastern U.S. (Hooker et al. 1912, Cooley and Kohls 1945) in habitats providing high humidity, which it requires to avoid desiccation. The tick is generally associated with deciduous forest and habitats containing abundant leaf litter (Piesman 2002) to provide adequate humidity. Ixodid ticks lose water primarily through their cuticle, and Lees noted that *Ixodes* ticks were observed to lose water at rates which were 10-15 times higher than *Dermacentor andersoni*, the Rocky Mountain wood tick (Lees 1946). All life stages are susceptible to desiccation, with larvae losing water more rapidly than adults or nymphs (Yoder and Spielman 1992). As with other ixodid ticks, *I. scapularis* is most vulnerable to water imbalance or desiccation when engaging

in questing behavior, which involves the tick leaving the sheltered leaf litter for areas with higher temperatures and lower humidity. As with other ixodid ticks, *I. scapularis* is most vulnerable to water imbalance or desiccation when engaging in host-seeking behavior, whereby the tick leaves the sheltered leaf litter for areas with higher temperatures and lower humidity. Ticks utilize two types of host-seeking behavior: active (hunter), in which ticks actively search out a host, and passive (ambush or questing), in which they sit and wait for contact by a host. Ticks which utilize active host-seeking behavior can respond to host stimuli from great distances, as is the case with *Amblyomma americanum*, the lone star tick, which can respond to a carbon dioxide source up to 21 meters away (Waladde and Rice 1982). Questing behavior, which is utilized by most ticks, including *I. scapularis*, consists of ticks climbing vegetation and raising the first pair of legs to attach to passing hosts. The legs bear sensilla that act as chemoreceptors and mechanoreceptors (Waladde and Rice 1982, Sonenshine 2008) which enable ticks to respond to stimuli created by a potential host. Both kairomones (chemicals released by one species or organism which benefit individuals of another species without providing benefit to the emitting species) and tactile stimulation are important cues involved in tick questing (Waladde and Rice 1982). Detection of carbon dioxide and ammonia is accomplished by an organ on the dorsal surface of the front pair of tarsi, Haller's organ (Sonenshine 2008), and distal sensilla on the tarsi assist ticks in sensing movement of a host (Waladde and Rice 1982).

Life Cycle

The developmental stages of *I. scapularis* consist of egg, larva, nymph, and adult (Oliver 1989). The life cycle has been heavily studied in the northern U.S. and takes 2-3

years to complete (Eisen and Lane 2002). In the summer, eggs deposited in the early spring by females hatch, and larvae quest for a blood meal at that time. Larval *I. scapularis* are reported to disperse only 2-3 meters from their egg mass (Stafford 1992). If they find a host, feed, and engorge before September, they will drop off the host, molt into nymphs, and overwinter in the leaf litter, and if they feed after September, they will overwinter as engorged larvae (Yuval and Spielman 1990). Larvae which do not feed do not molt(?) to the next life stage. Engorged larvae that are ready to molt or unfed nymphs become active in the spring and quest for hosts throughout the summer. Once they feed on a host and engorge, they drop from the host and molt into adults which are then active in the fall through early spring. In the spring, females who have fed and mated will oviposit and then die. Eggs oviposited by these females will eclose in summer, at which time the larvae will emerge to quest, thus beginning the cycle again.

Adult mating habits vary, and there are reports of fed adults *I. scapularis* mating both on and off of the host (Rogers 1953, Harris 1959, Yuval and Spielman 1990) as well as unfed males and females mating (Harris 1959). Like other Ixodid ticks, *I. scapularis* females oviposit one large egg batch, which range in number from 850 to 3,000 (Wilson et al. 1990, Oliver et al. 1993b).

Host Associations

Ixodes scapularis is known as a three-host tick, meaning the mobile stages, larvae, nymphs, and adults all feed on a different host. Larvae and nymphs have an extremely broad host range, and will feed on at least 31 mammalian species and 49 avian species in the Great Lakes and northeastern U.S. regions (Anderson 1988, 1989). In addition, immature *I. scapularis* have been recorded feeding on common five-lined

skinks (*Plestiodon fasciatus*) and fence lizards (*Scleroporus undulatus*) (Hixson 1941, Apperson et al. 1993, Oliver et al. 1993a, Durden et al. 2002). Interestingly, Hixson observed that the majority of immature ticks feeding on *S. undulatus* were dead, and the others showed signs of decreased fitness (Hixson 1941). While the larvae and nymph's host range is broad, passerine birds and reptiles are very important for maintaining *I. scapularis* populations in the South (Oliver et al. 1993a). In the North and East, the primary hosts for *I. scapularis* larvae and nymphs are white-footed mice, *Peromyscus leucopus*, Eastern chipmunks, *Tamias striatus* (Anderson 1988), and gray squirrels, *Sciurus carolinensis* (Lane et al. 1991). Adults have been recorded on at least 12 species of mammals (Anderson 1988), but they do not appear to feed on birds (Bishopp and Trembly 1945). They typically feed on white-tailed deer, *Odocoileus virginianus* (Main et al. 1981) and other large mammals, including dogs, cats, and humans (Harris 1959) (Piesman 2002).

Seasonality

The seasonal activity of questing adults is fairly well known, consisting of peak populations during October through December (Rogers 1953), with another peak in February (Goddard 1986, Mackay and Foil 2005) and March (Harris 1959). Adults do not actively quest for a host during the summer months (Rogers 1953) (Kollars et al. 1999). Little is known about the seasonality of immature *I. scapularis* in the South. Goddard and Piesman (2006) collected larvae in mid-June and nymphs in April and May, while a study in South Carolina with a non-standardized collecting protocol collected larvae from rodents from April through August, and four nymphs in June, August, and

September (Clark et al. 1998). Upon examination of lizards in Georgia, larvae were found March through October and nymphs in May through September

The larvae and nymphs of *I. scapularis* are very difficult to collect with drag cloths and flags (Goddard and Piesman 2006), and most collecting attempts in the South have yielded very low numbers of larvae and nymphs (Mackay and Foil 2005, Goddard and Piesman 2006, Diuk-Wasser et al. 2010). One explanation for low collection numbers of *I. scapularis* larvae and nymphs is that these immature ticks are highly susceptible to desiccation, and are at an increased risk of desiccation while questing out of the humid, sheltering leaf litter (Needham and Teel 1986) (Yoder and Spielman 1992). Rogers (1953) noted that *I. scapularis* larvae in Florida tended to stay close to the leaf litter rather than climbing up grass or foliage as is common among other Ixodid ticks. Although it was performed in the northern US, data from a study in which removing sheltering leaf litter in the environment resulted in a 72-100% population reduction of *I. scapularis* (Schulze et al. 2009) lends support to the idea that desiccation could be a deterring factor to *I. scapularis* questing high on vegetation in the South. It can be argued that the humidity is higher in the southern U.S. than the north, however, and more work is warranted to determine if removing leaf litter in the South would impact *I. scapularis* populations as it does in the North. Secondly, in the southern U.S., *I. scapularis* larvae and nymphs frequently feed on ground-dwelling species of lizards which move freely through the leaf litter (Oliver et al. 1993a) (Apperson et al. 1993), (Durden et al. 2002) thus allowing them to remain below the leaf litter. If the immature ticks are not actively questing above the leaf litter, collection with a drag cloth is very difficult.

Disease Ecology

Ixodes scapularis was originally thought to vector no human pathogens (Hooker et al. 1912) (Rogers 1953), yet, in just the last 40 or so years has emerged as the most important tick-borne disease vector in the United States, being a competent vector of *Borrelia burgdorferi* (Burgdorfer 1984), *Babesia microti* (Homer 2000), and *Anaplasma phagocytophilum* (Hodzic et al. 1998). *Ixodes scapularis* was brought to the spotlight when it was first identified as a vector for *B. microti* in Massachusetts in 1976 (Spielman 1976), and interest in the tick rose rapidly when, two years later in 1978, it was identified as a vector for Lyme disease in the northeast, specifically Connecticut (Wallis et al. 1978). *Anaplasma phagocytophilum* (formerly *Ehrlichia phagocytophila* (Dumler et al. 2001)) was identified from patients in Wisconsin and Minnesota in 1994 (Chen et al. 1994) and, since the closely-related *I. ricinus* is a vector of *E. phagocytophila* among European sheep, cattle, and goats, transmission of *E. phagocytophila* by *I. scapularis* was correctly hypothesized at that time. Since being identified as a vector for these pathogens, extensive work has been done to evaluate the disease potential of *I. scapularis* in the northern and central U.S. in relation to Lyme disease (Diuk-Wasser et al. 2012) (Ostfeld et al. 1995) (Piesman and Sinsky 1988) (Piesman 2002), babesiosis (Spielman et al. 1981), and to a lesser extent, *A. phagocytophilum* (Adelson et al. 2004).

A combination of the tick's expanding range and human modification of the landscape has contributed greatly to increased human-tick contact and potential for disease transmission (Ostfeld et al. 1995). Additionally, human land use over the past 60 years has transformed the Northeastern United States from a primarily agricultural area to one which is heavily focused on residential development. There are now forests in

varying stages of growth near and on residential lots, which provide ideal habitats for *I. scapularis* and its host animals while increasing human-tick interaction (Lane et al. 1991). Additionally, in the Midwest and Northeast, human-tick contact is maximized because increased summer human outdoor activity coincides with the peak of nymphal questing times.

Lyme Disease

Ixodes scapularis is primarily known for being one of the main vectors of the etiological agent of Lyme disease, *Borrelia burgdorferi*, the most common and frequently reported vector-borne disease in the United States (CDC 2002), with 30,158 reported cases and 22,561 confirmed cases in 2010 (CDC 2012a) (Figure 1.3). Although disputed (Herman-Giddens 2012), the southern United States is considered a low-risk area for Lyme disease (Diuk-Wasser et al. 2006) (Diuk-Wasser et al. 2012), from 2006 to 2010, four cases of Lyme disease were reported to the Mississippi Department of Health, supporting the low risk of Lyme disease in the state.

The genome of *Borrelia burgdorferi* was sequenced in 1997 (Fraser et al. 1997). These bacteria are motile spirochetes which are neither gram-negative nor gram-positive (Barbour and Hayes 1986) (Shapiro and Gerber 2000). In order for the life cycle of *B. burgdorferi* to be completed, a tick vector and two or more wild host species (small vertebrates for the larvae and nymphs and a large mammal for adults) are required (Schauber and Ostfeld 2002). Although adult *I. scapularis* may transmit *B. burgdorferi,* nymphal ticks are the primary life stage with a significant role as a vector for *B. burgdorferi* transmission to humans (Piesman et al. 1987, Stafford et al. 1998, Piesman 2002). Infected female ticks can transovarially transmit *B. burgdorferi*, but is not

significant in the life cycle (Piesman et al. 1986, Lane and Burgdorfer 1987, Magnarelli et al. 1987, Schoeler and Lane 1993) Larval ticks primarily acquire the spirochete while feeding on a small infected vertebrate, rodent such as the white-footed mouse, *Peromyscus leucopus* (Anderson et al. 1983) or the cotton mouse, *Peromyscus gossypinus* (Galbe and Oliver 1992). In the South, larvae and nymphs often feed on lizards, and some species such as southeastern five-lined skink (*Eumeces inexpectatus*) and the green anole (*Anolis carolinesis*), may act as competent reservoirs for *B. burgdorferi* (Levin et al. 1996).

In approximately 70% of cases, early Lyme disease presents clinically with erythema migrans (EM) lesions typically around the tick bite area, as well as systemic symptoms such as fatigue, fever, headache, stiff neck, arthralgias, myalgias, and regional adenopathy (Spach et al. 1993). Lyme carditis, characterized by atrioventricular heart block and/or myopericarditis may develop and usually requires hospitalization (Wormser et al. 2006). For early Lyme disease, doxycycline is the antibiotic of choice, except in cases where it is contraindicated, such as pregnancy. Lyme disease has a multisystemic nature, affecting multiple organ systems. Long-term sequelae may occur in late stage Lyme disease and include Lyme arthritis and neuroborreliosis, which are treated with the antibiotic ceftriaxone, among others (Wormser et al. 2006).

EM lesions are characteristic “bull's eye” patterned rashes which were once uniquely associated with *Ixodes* ticks and Lyme disease, but have now been identified on patients following bites from *Amblyomma americanum*, the Lone Star tick (Masters et al. 1998). When associated with *A. americanum* bites, EM rashes are referred to as Masters’ disease, STARI (southern tick associated rash illness) (Telford and Goethert 2008), or

southern Lyme disease (James et al. 2001). Due to the presence of EM lesions in other tick-associated disease syndromes, they should never be used as a single diagnostic tool, especially when patients live in an area where both *I. scapularis* and *A. americanum* are present.

Human Granulocytic Anaplasmosis

Human granulocytic anaplasmosis (HGA), is an ehrlichiosis-like disease caused by *Anaplasma phagocytophilum*. Closely related to the genera *Rickettsia* and *Ehrlichia*, *A. phagocytophilum* was formerly classified as an ehrlichiosis, *Ehrlichia phagocytophila,* until Dumler et al proposed that it be included in the genus *Anaplasma* (Dumler et al. 2001). First identified as an *I. ricinus*-vectored disease in European cattle, sheep, and goats, *E. phagocytophila* was identified from human patients in Wisconsin and Minnesota in 1994 (Chen et al. 1994). The tick vector was correctly hypothesized as *I. scapularis* based on the fact that *I. scapularis* and *I. ricinus* are in the same complex, and may transmit the same types of disease agents.

HGA has been reported in the United States, Europe, and Asia. In the United States, it is primarily found in New England and north central and Pacific states, though it has been reported in the South (McQuiston et al. 1999) (Dumler et al. 2001). In 2010, there were 1,761 reported cases of *A. phagocytophilum* in the United States (CDC 2012a). There has been at least one adult *I. scapularis* found infected in Mississippi with *Anaplasma phagocytophilum* (Goddard, J., Varela-Stokes, A.S., unpublished data).

Anaplasma phagocytophilum are obligate intracellular bacteria which colonize neutrophils, the most abundant type of white blood cells. *Ixodes scapularis* ticks acquire the bacteria while feeding on an infected host, and the bacteria are passed transstadially

from one life stage of the tick to the next, however, they are not transovarially transmitted. This means that unfed *I. scapularis* larvae are incapable of transmitting the diseases to other hosts (Dumler et al. 2005) (Dumler 2011). While the primary mammalian reservoir for *A. phagocytophilum* in the United States is the white-footed mouse, *P. leucopus*, raccoons, *Procyon lotor*, and gray squirrels, *Sciurus carolinensis* have been demonstrated as competent reservoir hosts in a laboratory setting (Levin et al. 2002).

HGA is characterized by headache, fever, myalgia, progressive leukopenia, thrombocytopenia, and anemia (Eng et al. 1990), and symptoms typically present after a 5-10 day incubation period. In addition to a patient's symptoms and history of a tick bite, Wright-stained blood smears, PCR, and serologic testing are used to diagnose cases of HGA (Dumler et al. 2007). Treatment of choice is doxycycline.

Babesiosis

Babesiosis is a malaria-like disease caused by blood parasites in the order Piroplasmida, family Babesiidae. Members of the family Babesiidae are one of the most ubiquitous and widespread blood parasites in the world based on numbers and distribution of species in animals, and occur on every continent except Antarctica (Homer et al. 2000) (Senanayake et al. 2012). The first case of human babesiosis was reported in Yugoslavia in 1957 (Skrabalo and Deanovic 1957) (Gorenflot et al. 1998). In the United States, human babesiosis is primarily caused by *Babesia microti* (Franca 1910) , a rodent parasite. An organism which has a >99% sequence similarity to *Babesia divergens* (M'Fadyean & Stockman 1911), a primarily-European zoonotic cattle *Babesia*, has been detected in a patient in the U.S. (Beattie et al. 2002), (Telford and Goethert 2008). In

addition, three other nearly-indistinguishable strains, WA_1 in Washington (Quick et al. 1993), (Herwaldt et al. 2004), CA_1 in California (Persing et al. 1995), and MO_1 in Missouri (Herwaldt et al. 1996) (Gorenflot et al. 1998) (Telford and Goethert 2008) have been described in the United States. Babesiosis was not a nationally notifiable disease until 2011, and the CDC reports 632 cases in 2011 (CDC 2012b).

Babesia are obligate intraerythrocytic parasites which infect a massive variety of vertebrate hosts such as rodents, birds, cervids, and humans (Homer et al. 2000). Larval ticks feeding on *P. leucopus* is important for the maintenance of *B. microti* in sylvatic and enzoonotic systems in the United States (Homer et al. 2000), as other larger hosts on which adults feed, such as white-tailed deer, are incompetent reservoirs for *B. microti* (Telford et al. 2011).

Clinical symptoms of infection are caused by the asexual reproductive stage of the organism in the erythrocytes of the host and the subsequent lysis of host cells, and symptoms are similar to malaria in humans (Homer et al. 2000). Lower levels of parasitemia in the blood can present with headache, fever, chills, nausea, vomiting, myalgia, altered mental status, disseminated intravascular coagulation, anemia with dyserythropoiesis, hypotension, respiratory distress and renal insufficiency, while higher levels are associated with nausea, emesis, night sweats, weight loss and hematuria (Homer et al. 2000). Infections with *B. microti* are often subclinical and self-limiting in healthy patients, requiring no treatment, however they can be life-threatening in individuals who are splenectomized, immunocompromised, or receiving immunosuppressive therapy (Krause et al. 1996, Gorenflot et al. 1998, Telford and Goethert 2004). In the case of coinfection with *Borrelia burgdorferi*, infections with *B.*

microti may elicit an immune response that results in establishment of higher numbers of *Borrelia* spirochetes in the host, causing the patient to experience more symptoms than a singular infection (Krause et al. 1996). Treatment is most frequently a combination of quinine and clindamycin (Telford et al. 2011).

Objectives

Since there is a paucity of information concerning the seasonal questing activity of *I. scapularis,* the first objective of this study was to analyze the questing activity of larval, nymphal, and adult *I. scapularis* in north Mississippi. The second objective was to screen collected *I. scapularis* for the presence of: *Borrelia burgdorferi*, *Babesia*, and *Anaplasma phagocytophilum* in order to evaluate the risk of exposure to these three human disease agents from *I. scapularis* in Mississippi.

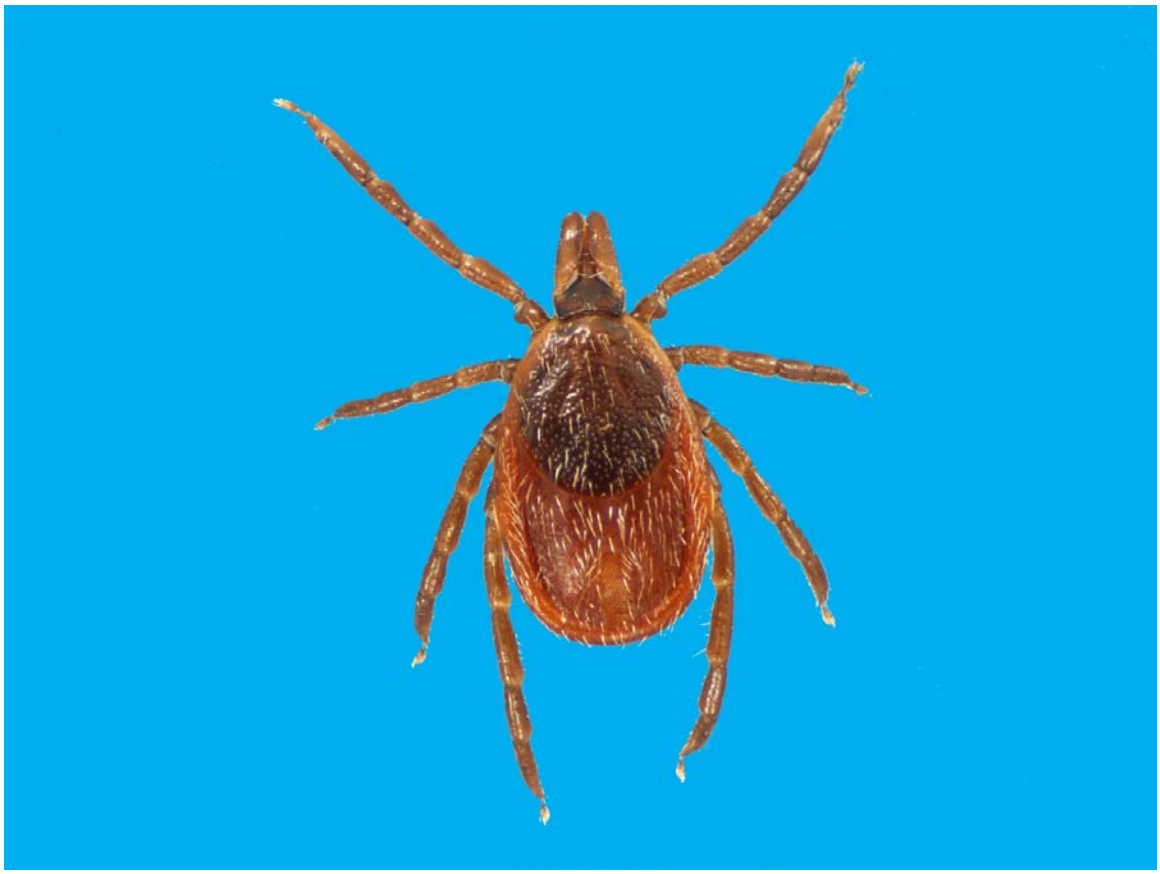

Figure 1.1 Female *Ixodes scapularis* (Photo courtesy Dr. Blake Layton, Mississippi State University Extension Service)

Figure 1.2 *Ixodes scapularis* male (Photo courtesy Dr. Blake Layton, Mississippi State University Extension Service)

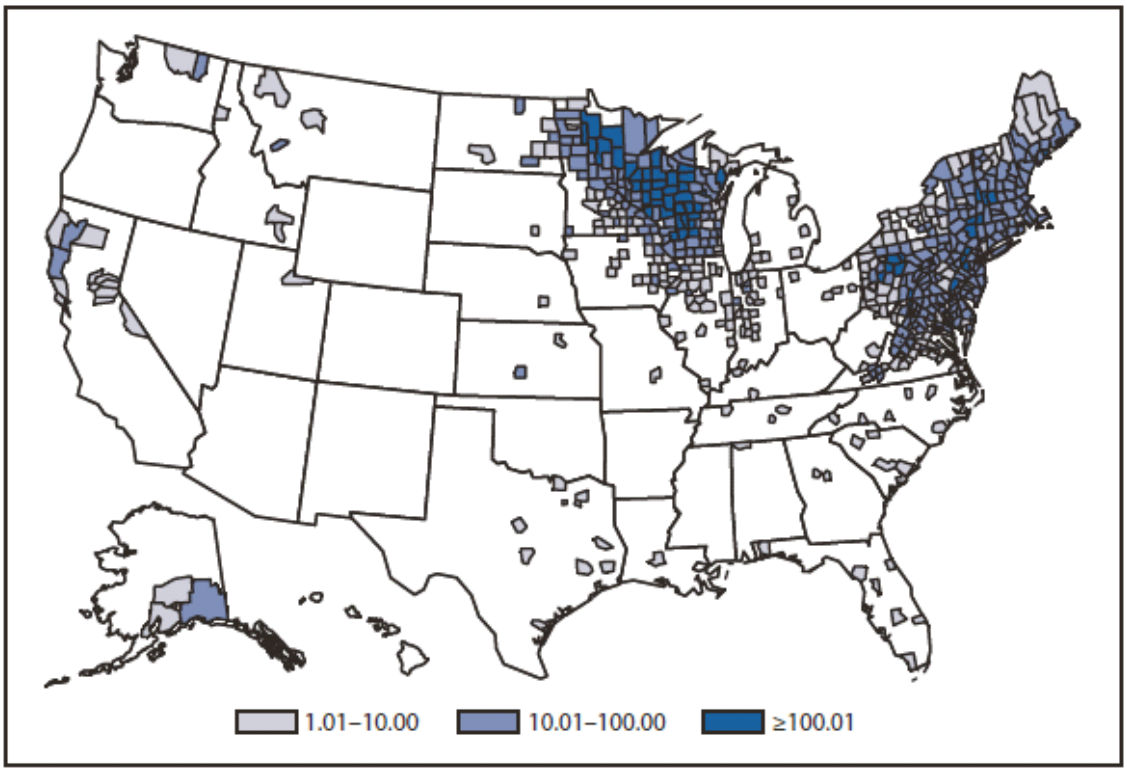

Figure 1.3 Lyme Disease Incidence Per 100,000 Population of Reported Confirmed Cases, by County – United States, 2010 (Image courtesy of CDC)

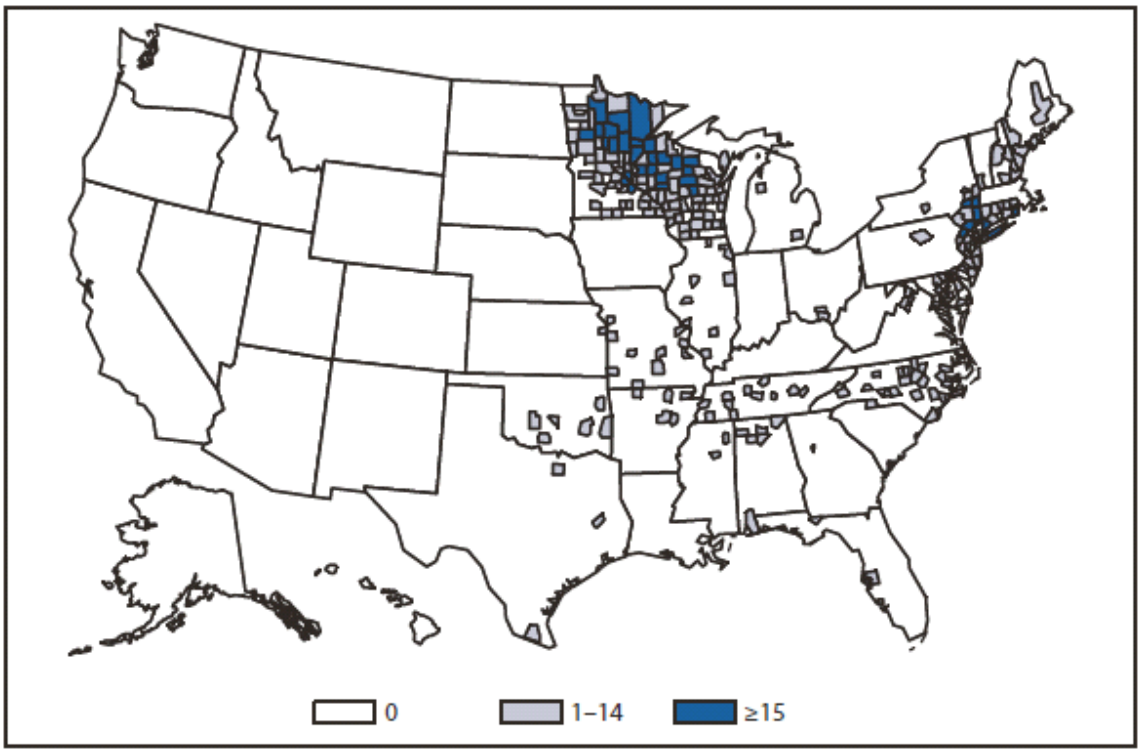

Figure 1.4 Human Granulocytic Anaplasmosis Reported Cases, by County – United States, 2010 (Image courtesy of CDC)

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CHAPTER II

SEASONALITY OF *IXODES SCAPULARIS* IN NORTH MISSISSIPPI

Introduction

The black-legged tick, *Ixodes scapularis* Say , is a North American hematophagous ectoparasite capable of vectoring several pathogens of medical significance across a breadth of vertebrate hosts, including humans. In the United States, *I. scapularis* is a known vector of *Borrelia burgdorferi*, *Babesia microti,* and *Anaplasma phagocytophilum*, the causative agents of Lyme disease, babesiosis, and human granulocytic anaplasmosis, respectively (Wallis et al. 1978) (Anderson 1989) (Telford et al. 2011) (Dumler 2011). These pathogens are maintained in a sylvatic cycle and humans acquire them via the bite of an infected *I. scapularis*, so to minimize human-tick interaction and the instances of disease, it is very important to understand the relative abundance and seasonality of all life stages of *I. scapularis* (Mackay and Foil 2005). As a direct result of human disease transmission involving biting *I. scapularis*, the seasonality and life cycle of *I. scapularis* has been heavily studied in the central and northeastern U.S., however, less is known about the biology of this tick in the South (Mackay and Foil 2005), (Goddard 1992), (Goddard and Piesman 2006).

In a ten-year survey of human-biting ticks in Mississippi, *I. scapularis* comprised 9.2% of human tick bites reported, making it the fourth most common tick found on humans in Mississippi (Goddard 2002), and a field study in northwest Florida determined

I. scapularis to be the most common tick collected utilizing a drag cloth (Cilek and Olson 2000). There has been limited work describing questing activity of adult *I. scapularis* in Mississippi (Goddard 1986) and immatures (Goddard and Piesman 2006) (Diuk-Wasser et al. 2010) (Diuk-Wasser et al. 2006) as well as population estimates of adult *I. scapularis* (Goddard and Goddard 2008) and relative risk of acquiring those ticks while outdoors (Goddard et al. 2011). The objective of this study was to analyze the questing activity of larval, nymphal, and adult *I. scapularis* in north Mississippi.

Collection Sites

Two ½ hectare sites in north Mississippi were selected for dragging, one located at Wall Doxey State Park near Holly Springs (Marshall Co.) and the other at Noxubee National Wildlife Refuge near Starkville (Oktibbeha Co.) (Figure 2.1). These locations were chosen because they were wooded with a medium-dense canopy and contained leaf litter and suitable host animals for *I. scapularis*. The terrain at Noxubee was lowland, mostly flat with a large, sloping slough covering at least ¼ of the site. For several days following rain, the slough would fill with water and prevent complete sampling of that area. The site was located in an area which is periodically subjected to flooding, in a forest management practice known as green-timber reservoir management, or GTR. Trees on both sites were primarily a mix of *Pinus* species, mainly *Pinus taeda*, the loblolly pine, and *Pinus palustris*, the longleaf pine. At Wall Doxey, the pines were mixed with upland mixed oak-hickory terrain, including deep ravines and steep slopes. The underbrush of both sites was comprised of thorny *Smilax* and *Vaccinium* species, while the ground had thick leaf litter cover and fallen logs. Each site had small clearings with sparse canopy and clumps of *Panicum* and other grasses.

Mammalian host species visually observed in both sites included white-tailed deer, *Odocoileus virginianus*, and gray squirrels, *Sciurus carolinensis.* Reptilian hosts observed in abundance were five-sined skinks, *Eumeces fasciatus*, broad-headed skinks, *Eumeces laticeps,* and eastern fence lizards, *Scleroporus undulatus.* The most frequently encountered passerine bird species hosts were house wrens, *Troglodytes aedon,* house sparrows, *Passer domesticus*, northern mockingbirds, *Mimus polyglottos,* and thrushes in the family Turdidae. Non-passerine bird species such as wild turkeys, *Meleagris gallopavo*, were also observed on both of the sites.

Collection Methods

During the one-year period from August 1, 2010 through July 31, 2011, ticks were collected by dragging a 1-m^2 corduroy cloth in two ½ hectare sites located in north Mississippi. Both sites were visited once per 7-day period for one year, for a total of 104 site visits. Temperature, sky condition, and relative humidity were recorded for each collecting date, and collecting attempts were made each visit regardless of weather conditions. In order to collect from the plots in an efficient manner, each site was visually divided into 25 lanes and collecting was performed by transecting the sites in these predetermined lanes. The drag cloth was checked for ticks every 10 meters, and all ticks found attached to the cloth were removed and placed in vials containing 95% ethanol. Before each site visit, a random lane was chosen for slower, more intensive dragging. Vegetation and leaf litter was agitated more thoroughly in the selected lane, and the cloth was checked every five meters. Due to low numbers of all life stages of *I. scapularis* and collecting discrepancies, it was not possible to statistically analyze the random lanes for increased numbers of *I. scapularis*. However, as other studies have noted, *I. scapularis* is

notoriously difficult to collect in the South (Mackay and Foil 2005) (Goddard and Piesman 2006), and based on the limited data obtained from this study, no conclusions can be drawn as to whether intensive flagging is a more effective collecting method than dragging for *I. scapularis* in the South.

In the lab, adult *I. scapularis* were counted then identified to species and sex utilizing Pictorial Key to the Adults of Hard Ticks, Family Ixodidae (Ixodida: Ixodidae), East of the Mississippi River (Keirans and Litwak 1989). All adult *I. scapularis* collected in April and May were checked to ensure they were not *I. affinis*, a closely-related species which is active in summer and whose range overlaps with *I. scapularis.* All *Ixodes* nymphs collected were sent to Dr. Richard Robbins (Armed Forces Pest Management Board, Washington DC) for identification to species. One *I. scapularis* nymph and one adult male and female pair have been deposited as voucher specimens in the Mississippi Entomological Museum, Mississippi State University. The remaining *I. scapularis* were stored for future PCR analysis, and other species collected were stored for future studies.

Statistical Analyses

The data analysis for this study was generated using SAS software, Version 9.2 of the SAS System for Windows. Copyright © 2008 SAS Institute Inc. Collection data for adult ticks collected at both sites were analyzed to determine whether the number of *I. scapularis* adults collected was a function of temperature or vapor pressure deficit (Diuk-Wasser et al. 2010). Collection data for nymphs was not analyzed due to low collection numbers (n = 3 at each site). Data from both sites were pooled for weeks 11-45 and since PROC UNIVARIATE indicated an unacceptable level of skewness (1.7173), data were

transformed using log. This brought the skewness to 0.4630, which is within the acceptable range of (-1 to 1). A General Mixed Model Analysis using the PROC GLIMMIX procedure of SAS was then performed on log of (count +1) ticks. This model was chosen because it is robust.

Results

Over the course of one year, 104 collection attempts were made and a total of 256 adult and 6 nymphal *I. scapularis* were collected from the two collection sites (see Appendix A for raw collection data). From Wall Doxey State Park, 233 adults were collected, and 23 were collected from Noxubee National Wildlife Refuge. Three nymphal *I. scapularis* were collected from each collecting site, and no larval *I. scapularis* were collected at either site. (Table 2.1)

The first adult *I. scapularis* was collected during the 12th week of collecting on October 19, 2010 at Wall Doxey, and the second adult *I. scapularis* was collected the same week on October 21, 2010 at Noxubee (Figure 2.2). The last adults collected were found on April 3, 2011 at Noxubee during week 36 of collecting, and on May 25, 2011 at Wall Doxey during week 42 of collecting. The most adults were caught in March (97 adults, 37.9% of all *I. scapularis* collected), and no adults were collected from June through mid-October.

The first *I. scapularis* nymph was collected on August 2, 2010 at Wall Doxey. Two more nymphs were collected at Noxubee on September 3, 2010 and September 16, 2010. The next nymph was collected on March 14, 2011 at Noxubee, then two more on May 17, 2011 at Wall Doxey. (Figure 2.3). Although data were very limited, it appears

that the nymphs may have been questing in a bimodal fashion, with peaks in fall and spring.

Results of the Analysis of Variance are provided in Table 2.2. No significant correlation was found between VPD and number of ticks collected, or temperature and VPD combined and number of ticks collected. The equation used was: log (count +1) = -1.511 + 0.2175 week – 0.0039 week^2. $R^2 = 0.43$.

Discussion

Results of this study are consistent with previous studies in which attempts to collect *I. scapularis* larvae and nymphs in the South by dragging have yielded low numbers of adults and extremely low numbers of larvae and nymphs (Goddard 1986) (Mackay and Foil 2005) (Piesman 2002) (Diuk-Wasser et al. 2012). During 29 hours of dragging, Goddard and Piesman managed to collect only 12 *I. scapularis* larvae in mid-June (Goddard and Piesman 2006), but even those collection numbers are rare in the South. A two-year, carefully-executed dragging study in southwest Mississippi yielded zero *I. scapularis* larvae and nymphs (Goddard et al. 2003).

In this present study, other *Ixodes* nymphs were collected (18 *I. brunneus* and 6 *I. dentatus*), so we feel confident that the collecting method did not exclude nymphal *I. scapularis*. *Ixodes scapularis* is not a highly-mobile tick, with hatching larvae actively dispersing approximately 2-3 meters from the egg mass (Stafford 1992), so collecting 256 adult *I. scapularis* between the two sites is indicative that a corresponding number of larvae and nymphs must also be present somewhere in the environment. Possible reasons for their absence during drag cloth sampling in the South are: 1) collecting attempts made during times when they are not active, 2) immature *I. scapularis* are somehow not able to

attach or remain on drag cloths, 3) other vegetation blocks or prevents contact with the cloth, and 4) immature *I. scapularis* do not quest high enough in vegetation to make contact with the cloth.

Concerning the first possibility, historically, many studies involving *I. scapularis* have been limited by short periods of collecting. In 2005, a large research project began collecting *I. scapularis* nymphs from 304 locations east of the 100th meridian, restricting collection dates within this year to 19 May through 27 August (Diuk-Wasser et al. 2010). Collecting in the 16 states south of the 39th parallel yielded so few immature *I. scapularis* (n = 21, and zero in Mississippi) that the sites in that area were only sampled for the first year out of the total three year study. Considering that four of the six *I. scapularis* collected in my study were collected outside of the date range used in the Diuk-Wasser study, it is reasonable to conclude that the Diuk-Wasser study could have missed questing *I. scapularis* nymphs by not sampling over a longer time period. However, the present study was conducted year-round to avoid this problem. The second point, that immature *I. scapularis* cannot attach or remain on the drag cloth, has been refuted by studies in the northeast, midwestern, and northern U.S. (Stafford 1993, Guerra et al. 2002, Diuk-Wasser et al. 2006, Diuk-Wasser et al. 2010) where high numbers of immature *I. scapularis* were collected with a drag cloth. Other vegetation blocking the tick from making contact with the cloth also was not of great concern in this study, as other studies have been conducted using a drag cloth in areas with similar thick, thorny ground cover and still retrieved immature *I. scapularis* (Stafford 1993, Falco et al. 1999, Diuk-Wasser et al. 2006, Diuk-Wasser et al. 2010, Diuk-Wasser et al. 2012). I hypothesize that immature *I. scapularis* are difficult to collect with a drag cloth due to the

fact that they spend most of this time deep in the leaf litter. This could be tested in the future by utilizing a Berlese funnel to sample leaf litter, although it would be labor-intensive to adequately sample an area. Rogers (1953) noted that *I. scapularis* larvae in Florida tended to stay close to the leaf litter rather than climbing up grass or foliage as is common among other Ixodid ticks. If immature *I. scapularis* are relying on lizards as hosts which move freely through leaf litter as their primary food sources, remaining in leaf litter would allow for more contact with the hosts.

Considering the documented presence of *I. scapularis* adults in north Mississippi and the noncorresponding low numbers of *I. scapularis* larvae and nymphs collected in this study, more research is warranted to clarify questing behavior of immature *I. scapularis* in the South.

Table 2.1 *Ixodes scapularis* collected between August 1, 2010 and July 31, 2011

Site	Adults	Nymphs
Wall Doxey	233	3
Noxubee	23	3
Totals	256	6

Table 2.2 Results of statistical analysis of questing behavior in relation to vapor pressure deficit

Source of Variation	Num DF	Den DF	F Value	Pr > F
Week	1	52	0.000	0.9480
VPD	1	52	1.87	0.1772
Week*VPD	1	52	0.39	0.5336
Week*Week	1	52	8.08	0.0064
Week*Week*VPD	1	52	2.86	0.0968

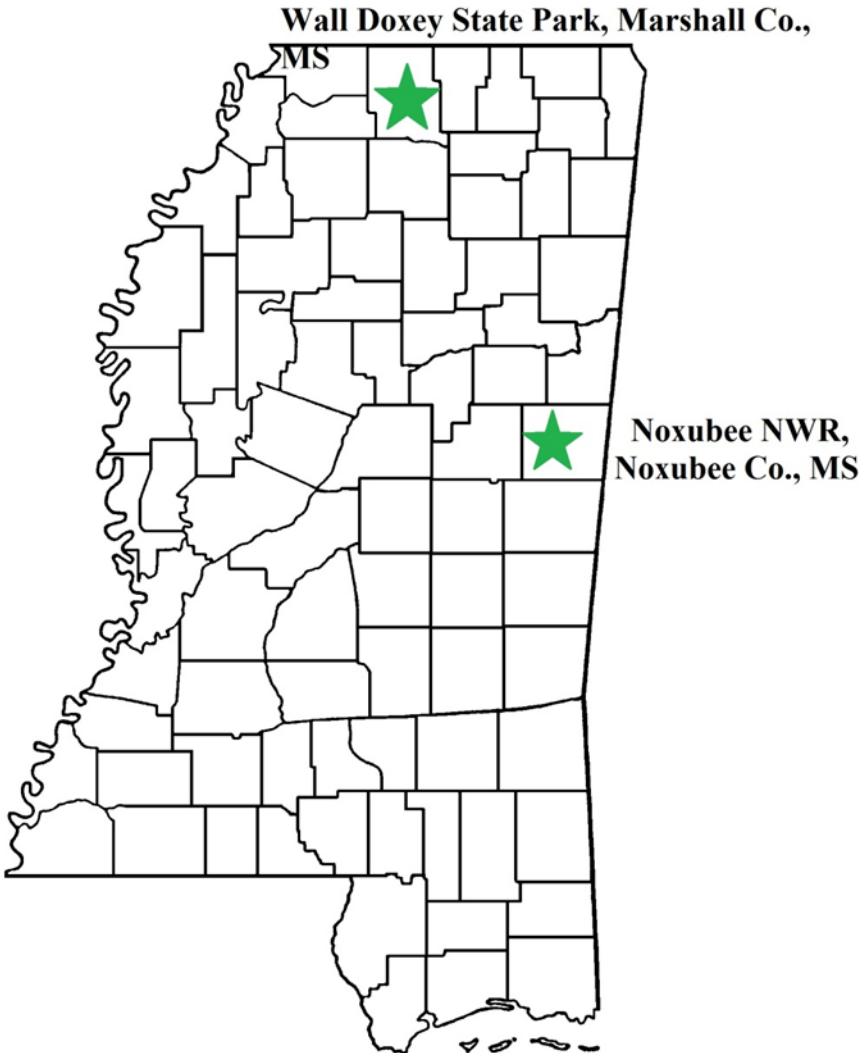

Figure 2.1 Collection sites in Mississippi (Photo courtesy of Joe MacGown, Mississippi State)

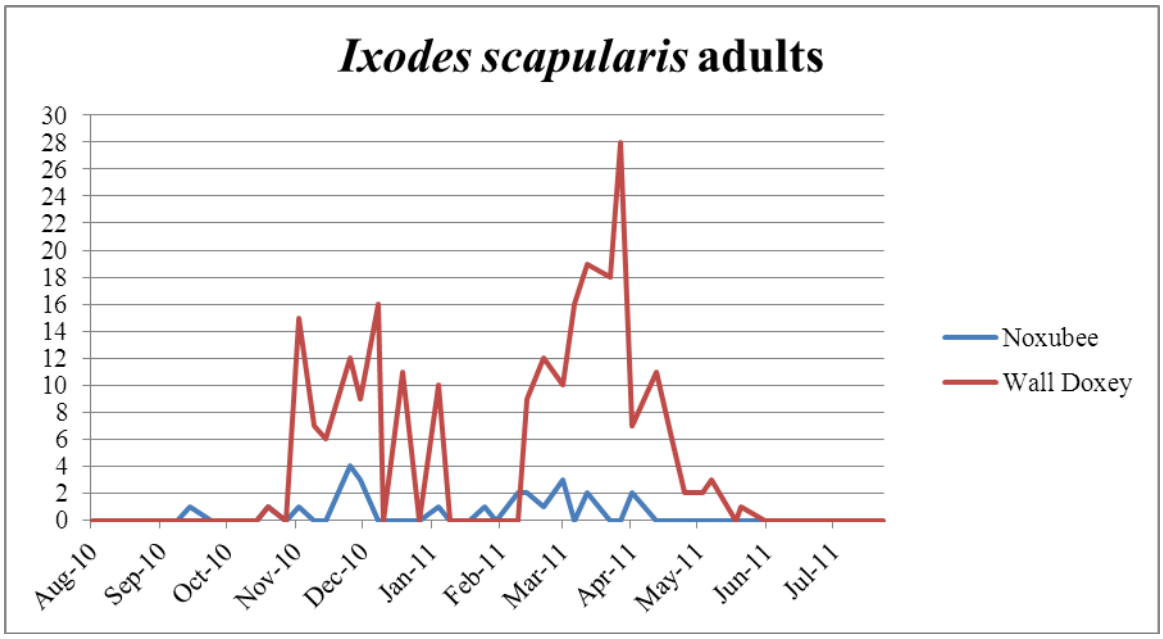

Figure 2.2 Number of adult *Ixodes scapularis* collected from Wall Doxey State Park and Noxubee NWR weekly between August 1, 2010 and July 31, 2011.

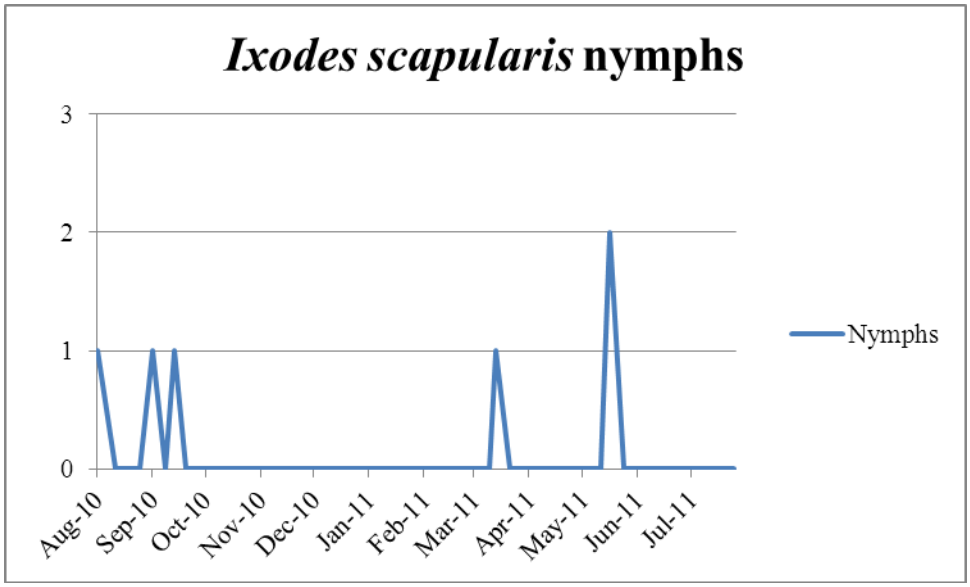

Figure 2.3 Number of nymphal *Ixodes scapularis* nymphs collected from Wall Doxey State Park and Noxubee NWR weekly between August 1, 2010 and July 31, 2011.

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CHAPTER III

DISEASE POTENTIAL OF *IXODES SCAPULARIS* IN NORTH MISSISSIPPI

Introduction

Ixodes scapularis, the black-legged tick, is a North American hard tick species capable of transmitting several pathogens of medical significance across a breadth of vertebrate hosts, including humans. Originally described by Say in 1821 and once thought to not serve as a disease vector (Hooker et al. 1912, Rogers 1953), *I. scapularis* is now considered the most medically important tick in the United States. *Ixodes scapularis* is a known vector of *Borrelia burgdorferi*, *Babesia microti*, and *Anaplasma phagocytophilum*, the causative agents of Lyme disease, babesiosis, and human granulocytic anaplasmosis, respectively (Burgdorfer et al. 1982, Anderson 1989, Lane et al. 1991, Dumler 2011, Telford et al. 2011). *Ixodes scapularis* was first recognized as a disease vector when it was found to be a transmit *B. microti* in Massachusetts in 1976 (Spielman 1976). Interest in the tick rose rapidly when, two years later in 1978, it was identified as a vector for Lyme disease in the Northeast, specifically Connecticut (Burgdorfer et al. 1982, Steere et al. 1983). *Anaplasma phagocytophilum*, formerly *Ehrlichia phagocytophila* (Dumler et al. 2001), was identified from patients in Wisconsin and Minnesota in 1994 (Chen et al. 1994). Since the closely-related *I. ricinus* is a vector of *E. phagocytophila* among European sheep, cattle, and goats, transmission of *E. phagocytophila* by *I. scapularis* was correctly suspected at that time.

From the time it was first identified as a disease vector, additional studies have been done to evaluate the disease potential of *I. scapularis* in the northern and central U.S. in relation to Lyme disease (Diuk-Wasser et al. 2012) (Ostfeld et al. 1995) (Piesman and Sinsky 1988) (Piesman 2002), babesiosis (Spielman et al. 1981), and to a lesser extent, *A. phagocytophilum* (Adelson et al. 2004), however, little is known of the disease potential of *I. scapularis* in the southern U.S. A limited study by Goddard et al surveyed ticks in Mississippi for a variety of disease agents and found *I. scapularis* infected with an unidentified spotted fever group rickettsial species and *Ehrlichia* species, but none infected with *Borrelia* organisms (Goddard et al. 2003). There has been at least one adult *I. scapularis* found infected in Mississippi with *Anaplasma phagocytophilum* (Goddard, J., Varela-Stokes, A.S., unpublished data), and to our knowledge nobody has surveyed ticks in Mississippi for *Babesia* organisms. The objective of this study was to survey adult *I. scapularis* collected in north Mississippi for molecular evidence of three different genera of disease agents: *Borrelia, Babesia,* and *Anaplasma* and to assess the disease risk of this tick in north Mississippi.

Materials and Methods

Tick Collection Methods

Two ½ hectare sites in north Mississippi were selected for dragging, one located at Wall Doxey State Park near Holly Springs in Marshall County and the other at Noxubee National Wildlife Refuge near Starkville in Oktibbeha County (figure, map). These locations were chosen because they were wooded with a medium-dense canopy and contained leaf litter, and suitable host animals for *I. scapularis*. During a one-year period from August 1, 2010 through July 31, 2011, each site was visited once per 7-day

week, totaling 104 site visits. In order to collect from the plots in an efficient manner, each site was visually divided into 25 lanes and collecting was performed by transecting the sites in these predetermined lanes. The drag cloth was checked for ticks every 10 meters, and all ticks found attached to the cloth were removed and placed in vials containing 95% ethanol. Once in the lab, ticks were identified to species and life stage, and adults were sexed.

PCR Analyses

Tick DNA was extracted using the illustra triplePrep Kit (GE Healthcare, Piscataway, NJ) and a modified manufacturer's protocol which eliminated the first wash in PBS. Ticks were removed from ethanol, dried, placed in individual labeled microcentrifuge tubes where they were chopped using a new, sterile #11 scalpel blade. Chopped ticks were vortexed with 50ul lysis buffer and 10ul Proteinase K. After incubation for one hour at 56ºC, DNA was extracted according to manufacturer's protocol.

Water controls were included with set of samples extracted and every set of samples subjected to PCR assay. To ensure successful extraction, all samples were tested by PCR amplification of a portion of the tick mitochondrial 16S rRNA gene (Black and Piesman 1994). For *A. phagocytophilum*, the major surface protein gene, *msp2*, was amplified with msp2-3f and msp2-3r primers (Massung and Slater 2003). For detection of *Babesia* spp., testing was performed targeting 18S rRNA using primers KIM18SF and KIMREV2 (M. Yabsley, unpublished data). Samples that demonstrated a product with initial 18S rRNA primers were subsequently re-tested using a nested PCR protocol to amplify another region of the 18S rRNA gene of apixomplexans using primers 5.1 and

3.1 in the primary amplification and RLBH-F and RLBH-R in the secondary amplification (Allen et al. 2008). To test for *Borrelia* spp., we used a nested PCR targeting the *flaB* gene using the primers FLALL and FLARL for primary amplification and FLARS and FLALS for secondary amplification (Barbour et al. 1996). Samples that produced amplicons in the initial *flaB* PCR assay were then subjected to a hemi-nested PCR targeting the 16S rRNA gene. The hemi-nested PCR used primers 8F and 16RNAR in the primary amplification and primers 16RNAR and 16RNAL in the secondary amplification (Varela et al. 2004) (Table 3.1). All PCR assays were performed in Bio-Rad DNA Engine Thermal Cyclers. For PCR conditions, see Appendix B, All products were visualized by electrophoresis in 2% agarose gels containing ethidium bromide.

Positive controls were included in all PCR experiments. The positive control for tick mitochondrial 16S rRNA PCR assays was genomic DNA from a male Gulf Coast tick, *Amblyomma maculatum*. The positive control for *A. phagocytophilum* PCR assays was genomic DNA from cultured *A. phagocytophilum*. For *Babesia* PCR assays, the positive control was genomic DNA from a clinical sample of infected whole dog blood obtained from Mississippi State College of Veterinary Medicine. The positive control for *B. burgdorferi* PCR testing was a 1:100 dilution of stock sample *B. burgdorferi* Sh-2-82 strain, originally isolated from an *I. scapularis* from Shelter Island, NY.

Results and Discussion

A total of 244 adult *I. scapularis* were examined for the presence of disease agents. . Amplicons from amplification of the tick mitochondrial 16S rRNA gene were detected in 100% of tick samples, indicating that all DNA extractions were successful. No tick samples were positive for DNA of *Anaplasma phagocytophilum* by PCR of the

msp2 gene. Initially, 82 samples were positive by nested PCR for *Borrelia* sp. when targeting the *flaB* gene, but upon subsequent PCR using the 16sRNA gene, they were all negative, indicating that the initial results were likely false positives due to laboratory contamination with *flaB* amplicons. Twelve PCR products were amplified using the 18S rRNA gene for *Babesia.* Upon sequencing, four of the ticks matched *Babesia odocoilei,* a non-zoonotic cervid babesiosis which is transmitted by *I. scapularis* (Waldrup et al. 1989a) and is prevalent through the southern United States, from eastern Texas (Waldrup et al. 1989b) through Florida (Telford and Forrester 1991), and southern Virginia (Perry et al. 1985).

Five *I. scapularis* nymphs collected from this study were to be pooled and tested for the presence of disease agents by the Centers for Disease Control in Fort Collins, CO. However, the technician performing the extraction was not successful, and unfortunately DNA from these ticks was lost.

Based on the relatively low number of *I. scapularis* collected in our study as well as the negative PCR results for human pathogens, *I. scapularis* appears to represent a very low disease risk to humans in north Mississippi. This is supported by the previous survey of tick diseases in Mississippi (Goddard et al. 2003). Lyme disease is primarily transmitted by nymphal *I. scapularis* (Lane et al. 1991), and the low numbers of nymphs collected in this study is consistent with the work of Diuk-Wasser et al. (Diuk-Wasser et al. 2012) and as discussed in Goddard et al. (Goddard 2002) supports the idea that there is little evidence that *I. scapularis* is involved in Lyme disease transmission in Mississippi, as discussed by Goddard (Goddard 2002).

Table 3.1 Gene targets used for *I. scapularis* disease agent screening.

Product	**Target Gene**
Tick DNA	16S rRNA
Anaplasma phagocytophilum	*msp2*
Babesia spp.	18S rRNA
Borrelia burgdorferi	*flaB*
Borrelia burgdorferi	16S rRNA

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APPENDIX A

IXODES SCAPULARIS RAW COLLECTION DATA

Date Collected	Stage	Sex	Ambient Temp	Humidity	Sky Condition
8/2/2010	N	N/A	89	77	Clear
10/19/10	A	M	74	49	Clear
11/05/10	A	M	47	47	Clear
11/05/10	A	M	47	47	Clear
11/05/10	A	M	47	47	Clear
11/05/10	A	M	47	47	Clear
11/05/10	A	M	47	47	Clear
11/05/10	A	M	47	47	Clear
11/05/10	A	M	47	47	Clear
11/05/10	A	M	47	47	Clear
11/05/10	A	M	47	47	Clear
11/05/10	A	M	47	47	Clear
11/05/10	A	M	47	47	Clear
11/05/10	A	M	47	47	Clear
11/05/10	A	F	47	47	Clear
11/05/10	A	F	47	47	Clear
11/05/10	A	F	47	47	Clear
11/05/10	A	F	47	47	Clear
11/05/10	A	F	47	47	Clear
11/09/10	A	M	64	45	Clear
11/09/10	A	M	64	45	Clear
11/09/10	A	M	64	45	Clear
11/09/10	A	M	64	45	Clear

11/09/10	A	F	64	45	Clear
11/09/10	A	F	64	45	Clear
11/09/10	A	F	64	45	Clear
11/18/10	A	F	48	98	Overcast
11/18/10	A	F	48	98	Overcast
11/18/10	A	F	48	98	Overcast
11/18/10	A	F	48	98	Overcast
11/18/10	A	M	48	98	Overcast
11/18/10	A	M	48	98	Overcast
11/24/10	A	F	68	81	Cloudy, rain
11/24/10	A	F	68	81	Cloudy, rain
11/24/10	A	F	68	81	Cloudy, rain
11/24/10	A	F	68	81	Cloudy, rain
11/24/10	A	F	68	81	Cloudy, rain
11/24/10	A	F	68	81	Cloudy, rain
11/24/10	A	F	68	81	Cloudy, rain
11/24/10	A	M	68	81	Cloudy, rain
11/24/10	A	M	68	81	Cloudy, rain
11/24/10	A	M	68	81	Cloudy, rain
11/24/10	A	M	68	81	Cloudy, rain
11/24/10	A	M	68	81	Cloudy, rain
12/04/10	A	F	57	84	Cloudy

12/04/10	A	F	57	84	Cloudy
12/04/10	A	F	57	84	Cloudy
12/04/10	A	F	57	84	Cloudy
12/04/10	A	M	57	84	Cloudy
12/04/10	A	M	57	84	Cloudy
12/04/10	A	M	57	84	Cloudy
12/04/10	A	M	57	84	Cloudy
12/04/10	A	M	57	84	Cloudy
12/09/10	A	F	38	42	Cloudy
12/09/10	A	F	38	42	Cloudy
12/09/10	A	F	38	42	Cloudy
12/09/10	A	F	38	42	Cloudy
12/09/10	A	F	38	42	Cloudy
12/09/10	A	F	38	42	Cloudy
12/09/10	A	F	38	42	Cloudy
12/09/10	A	F	38	42	Cloudy
12/09/10	A	F	38	42	Cloudy
12/09/10	A	M	38	42	Cloudy
12/09/10	A	M	38	42	Cloudy
12/09/10	A	M	38	42	Cloudy
12/09/10	A	M	38	42	Cloudy
12/09/10	A	M	38	42	Cloudy

12/09/10	A	M	38	42	Cloudy
12/09/10	A	M	38	42	Cloudy
12/29/10	A	F	60	44	Cloudy
12/29/10	A	F	60	44	Cloudy
12/29/10	A	F	60	44	Cloudy
12/29/10	A	F	60	44	Cloudy
12/29/10	A	F	60	44	Cloudy
12/29/10	A	F	60	44	Cloudy
12/29/10	A	F	60	44	Cloudy
12/29/10	A	M	60	44	Cloudy
12/29/10	A	M	60	44	Cloudy
12/29/10	A	M	60	44	Cloudy
12/29/10	A	M	60	44	Cloudy
12/29/10	A	M	60	44	Cloudy
01/04/11	A	F	63	30	Clear
01/04/11	A	F	63	30	Clear
01/04/11	A	F	63	30	Clear
01/04/11	A	F	63	30	Clear
01/04/11	A	F	63	30	Clear
01/04/11	A	F	63	30	Clear
01/04/11	A	F	63	30	Clear
01/04/11	A	M	63	30	Clear
01/04/11	A	M	63	30	Clear

01/04/11	A	M	63	30	Clear
02/17/11	A	F	61	72	Clear
02/17/11	A	F	61	72	Clear
02/17/11	A	F	61	72	Clear
02/17/11	A	F	61	72	Clear
02/17/11	A	M	61	72	Clear
02/17/11	A	M	61	72	Clear
02/17/11	A	M	61	72	Clear
02/17/11	A	M	61	72	Clear
02/17/11	A	M	61	72	Clear
02/24/11	A	F	68.3	78	Cloudy
02/24/11	A	F	68.3	78	Cloudy
02/24/11	A	F	68.3	78	Cloudy
02/24/11	A	F	68.3	78	Cloudy
02/24/11	A	F	68.3	78	Cloudy
02/24/11	A	F	68.3	78	Cloudy
02/24/11	A	F	68.3	78	Cloudy
02/24/11	A	F	68.3	78	Cloudy
02/24/11	A	M	68.3	78	Cloudy
02/24/11	A	M	68.3	78	Cloudy
02/24/11	A	M	68.3	78	Cloudy
02/24/11	A	M	68.3	78	Cloudy

03/01/11	A	F	48.3	52	Cloudy
03/01/11	A	F	48.3	52	Cloudy
03/01/11	A	F	48.3	52	Cloudy
03/01/11	A	F	48.3	52	Cloudy
03/01/11	A	F	48.3	52	Cloudy
03/01/11	A	F	48.3	52	Cloudy
03/01/11	A	M	48.3	52	Cloudy
03/01/11	A	M	48.3	52	Cloudy
03/01/11	A	M	48.3	52	Cloudy
03/01/11	A	F	48.3	52	Cloudy
03/10/11	A	F	41.2	85	Clear
03/10/11	A	F	41.2	85	Clear
03/10/11	A	F	41.2	85	Clear
03/10/11	A	F	41.2	85	Clear
03/10/11	A	F	41.2	85	Clear
03/10/11	A	F	41.2	85	Clear
03/10/11	A	F	41.2	85	Clear
03/10/11	A	F	41.2	85	Clear
03/10/11	A	F	41.2	85	Clear
03/10/11	A	F	41.2	85	Clear
03/10/11	A	M	41.2	85	Clear
03/10/11	A	M	41.2	85	Clear

03/10/11	A	M	41.2	85	Clear
03/10/11	A	M	41.2	85	Clear
03/10/11	A	M	41.2	85	Clear
03/10/11	A	M	41.2	85	Clear
03/17/11	A	F	61	57	Clear
03/17/11	A	F	61	57	Clear
03/17/11	A	F	61	57	Clear
03/17/11	A	F	61	57	Clear
03/17/11	A	F	61	57	Clear
03/17/11	A	M	61	57	Clear
03/17/11	A	M	61	57	Clear
03/17/11	A	M	61	57	Clear
03/17/11	A	M	61	57	Clear
03/17/11	A	M	61	57	Clear
03/17/11	A	M	61	57	Clear
03/17/11	A	M	61	57	Clear
03/17/11	A	M	61	57	Clear
03/17/11	A	M	61	57	Clear
03/17/11	A	M	61	57	Clear
03/17/11	A	M	61	57	Clear
03/17/11	A	M	61	57	Clear

03/17/11	A	M	61	57	Clear
03/22/11	A	F	66	72	Overcast
03/22/11	A	F	66	72	Overcast
03/22/11	A	F	66	72	Overcast
03/22/11	A	F	66	72	Overcast
03/22/11	A	F	66	72	Overcast
03/22/11	A	F	66	72	Overcast
03/22/11	A	F	66	72	Overcast
03/22/11	A	F	66	72	Overcast
03/22/11	A	F	66	72	Overcast
03/22/11	A	F	66	72	Overcast
03/22/11	A	F	66	72	Overcast
03/22/11	A	M	66	72	Overcast
03/22/11	A	M	66	72	Overcast
03/22/11	A	M	66	72	Overcast
03/22/11	A	M	66	72	Overcast
03/22/11	A	M	66	72	Overcast
03/22/11	A	M	66	72	Overcast
03/22/11	A	M	66	72	Overcast
03/31/11	A	F	45.8	64	Cloudy
03/31/11	A	F	45.8	64	Cloudy
03/31/11	A	F	45.8	64	Cloudy

03/31/11	A	F	45.8	64	Cloudy
03/31/11	A	F	45.8	64	Cloudy
03/31/11	A	F	45.8	64	Cloudy
03/31/11	A	F	45.8	64	Cloudy
03/31/11	A	F	45.8	64	Cloudy
03/31/11	A	F	45.8	64	Cloudy
03/31/11	A	F	45.8	64	Cloudy
03/31/11	A	F	45.8	64	Cloudy
03/31/11	A	F	45.8	64	Cloudy
03/31/11	A	F	45.8	64	Cloudy
03/31/11	A	F	45.8	64	Cloudy
03/31/11	A	F	45.8	64	Cloudy
03/31/11	A	F	45.8	64	Cloudy
03/31/11	A	F	45.8	64	Cloudy
03/31/11	A	M	45.8	64	Cloudy
03/31/11	A	M	45.8	64	Cloudy
03/31/11	A	M	45.8	64	Cloudy
03/31/11	A	M	45.8	64	Cloudy
03/31/11	A	M	45.8	64	Cloudy
03/31/11	A	M	45.8	64	Cloudy
03/31/11	A	M	45.8	64	Cloudy
03/31/11	A	M	45.8	64	Cloudy

03/31/11	A	M	45.8	64	Cloudy
03/31/11	A	M	45.8	64	Cloudy
03/31/11	A	F	45.8	64	Cloudy
04/07/11	A	F	65	63	Partially cloudy
04/07/11	A	M	65	63	Partially cloudy
04/07/11	A	M	65	63	Partially cloudy
04/07/11	A	M	65	63	Partially cloudy
04/07/11	A	M	65	63	Partially cloudy
04/07/11	A	M	65	63	Partially cloudy
04/07/11	A	M	65	63	Partially cloudy
04/12/11	A	F	77	30	Clear
04/12/11	A	F	77	30	Clear
04/12/11	A	F	77	30	Clear
04/12/11	A	F	77	30	Clear
04/12/11	A	F	77	30	Clear
04/12/11	A	F	77	30	Clear
04/12/11	A	M	77	30	Clear
04/12/11	A	M	77	30	Clear
04/12/11	A	M	77	30	Clear
04/12/11	A	M	77	30	Clear
04/12/11	A	M	77	30	Clear
04/22/11	A	F	73.5	87	Cloudy

04/22/11	A	M	73.5	87	Cloudy
04/22/11	A	M	73.5	87	Cloudy
04/22/11	A	M	73.5	87	Cloudy
04/22/11	A	M	73.5	87	Cloudy
04/22/11	A	M	73.5	87	Cloudy
04/29/11	A	M	71.4	56	Clear
04/29/11	A	M	71.4	56	Clear
05/04/11	A	F	55.6	54	Clear
05/04/11	A	M	55.6	54	Clear
05/12/11	A	F	75.3	76	Cloudy
05/12/11	A	F	75.3	76	Cloudy
05/12/11	A	M	75.3	76	Cloudy
05/17/11	N	N/A	62.6	46	Clear
05/17/11	N	N/A	62.6	46	Clear
05/17/11	A	M	62.6	46	Clear
05/17/11	A	M	62.6	46	Clear
5/25/11	A	F	82	78	Overcast

APPENDIX B

PCR CONDITIONS

Primer	PCR Conditions
16S	92 ºC 1 min 11 cycles of 92 ºC 1 min 48 ºC 1 min 72 ºC 1 min 30 sec followed by 33 cycles of 92 ºC 1 min 54 ºC 35 sec 72 ºC 1 min
MSP2F, MSP2R	95 ºC 2 min 41 cycles of 94 ºC 30 sec 55 ºC 30 sec 72 ºC 1 min followed by 72 ºC 5 min
FLAB 1º	95 ºC 3 min 40 cycles of 95 ºC 1 min 55 ºC 1 min 75 ºC 1 min
FLAB 2º	95 ºC 3 min 40 cycles of 95 ºC 1 min 57 ºC 1 min 75 ºC 1 min
KIMF, KIMR	95 ºC 5 min 40 cycles of 95 ºC 30 sec 45 ºC 45 sec 72 ºC 45 sec followed by 72 ºC 5 min
16s Borr 1º	95 ºC 3 min 40 cycles of 95 ºC 30 sec 55 ºC 30 sec 72 ºC 2 min
16s Borr 2º	95 ºC 3 min 40 cycles of 95 ºC 1 min 50 ºC 1 min 75 ºC 1 min 30 sec

	followed by 95 ºC 1 min 75 ºC 7 min
Hepat 1º	94 ºC 3 min 30 cycles of 94 ºC 1 min 55 ºC 1 min 72 ºC 1 min 30 sec followed by 72 ºC 5 min
Hepat 2º	94 ºC 1 min 40 cycles of 94 ºC 1 min 50 ºC 1 min 72 ºC 1 min 30 sec