Investigation of Ticks and Tick-Borne Pathogens in Deciduous Forests of Eastern Central Alabama

by

Xiaodi Wang

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Approved by

Derrick K. Mathias, Chair, Assistant Professor of Entomology
Alana L. Jacobson, Assistant Professor of Entomology
David W. Held, Associate Professor of Entomology

Abstract

Ticks are obligate hematophagous arthropods, and as vectors of human disease, they are second only to mosquitoes in medical importance. There are many unknowns in Alabama regarding ticks and tick-borne diseases that require further study. In 2015, we sampled ticks across eight sites located in or near Auburn, AL, and investigated tick density, diversity and pathogen prevalence. Seven tick species were collected, but 97.71% of all samples were a single species, the lone star tick, *Amblyomma americanum*, the primary vector of ehrlichiosis. For prevalence studies, a multiplex qPCR assay was used to screen DNA samples from lone star ticks simultaneously for five pathogens. Our results revealed an absence of either *Rickettsii parkeri* or Panola Mountain *Ehrlichia*, but we did identify the presence of three bacterial species: *R. amblyommii* (54.51%), *Ehrlichia chaffeensis* (0.27%) and *Ehrlichia ewingii* (0.45%). Moreover, , we observed that questing ticks were unequally distributed within habitats. Thus, in 2016, we investigated factors that influence spatial variation of questing *A. americanum* within a single forested habitat. The hypothesis was tha questing behavior is driven by a combination of factors associated with microclimate, vegetation, and animal hosts. A stepwise Poisson regression model was used to examine these relationships. Our best-fit model included six explanatory factors: forest-floor gravimetric moisture, forest-floor depth, tree diversity, canopy cover, the number of available hosts, and weekly mean relative humidity.

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List of Abbreviations

CanCo	Canopy Cover
CH	Chewacla State Park
FFD	Forest-floor Depth
GM	Forest-floor Gravimetric Moisture
HD	Hickory Dickory Park
LW	Lake Wilmore
LK	Louise Kreher Forest Ecology Preserve
MaxT	Weekly Maximum Temperature
MeanRH	Weekly Mean Relative Humidity
MeanSD	Weekly Mean Saturation Deficit
TK	Tuskegee National Forest
TK.5	Tuskegee National Forest Compartment 5
TK.15	Tuskegee National Forest Compartment 15
TK.20	Tuskegee National Forest Compartment 20
TotHst	Total Number of Available Hosts
TrDiv	Tree Diversity
WV	Westview Park
#Nymph	Total Number of Questing *Amblyomma americanum* Nymphs

Chapter 1

Overview of Tick Biology and Role in Pathogen Transmission

Ticks transmit a wide variety of pathogens and have become notorious vectors of diseases that have a substantial impact on both humans and domestic animals. Worldwide, tick-borne diseases (TBDs) of both medical and veterinary importance are on the rise (Nicholson et al. 2010). Lyme borreliosis, for example, is now the most commonly reported vector-borne disease in the United States and resulted in over 33,000 confirmed or probable human cases in 2014 (CDC 2015a). Moreover, the CDC recognizes that this is an underestimate of the actual number of cases, and based on commercial laboratory testing and medical insurance claims, the actual number is likely closer to 300,000 (CDC 2015b). Less common diseases associated with tick bites include anaplasmosis, babesiosis, ehrlichiosis, Rocky Mountain spotted fever, and tularemia among others, all of which are of significant importance for public health in the United States (at least regionally). In addition, ticks are capable of spreading agents that cause disease in livestock and companion animals, including bovine anaplasmosis and babesisosis and canine ehrlichiosis. Deleterious effects of tick infestation on animal production has also been reported, which may lead to economic losses (Jongejan and Uilenberg 2004). Moreover, tick bites can cause severe allergic reactions and there is evidence of toxins in the saliva of some tick species that cause paralysis in both humans and animals (Chand et al. 2016, Araujo et al. 2016). Despite the medical and veterinary importance of ticks, our understanding of tick ecology in the United

States is dominated by studies of the black-legged tick, *Ixodes scapularis*, particularly populations of this species occurring in states in the Northeast and around the Great Lakes. In the Southeast, data from states neighboring Alabama suggest that the lone star tick, *Amblyomma americanum*, is the most common human-biting tick in the region and that of the TBDs endemic to the U.S., Lyme disease is less prevalent than other TBDs, such as Rocky Mountain spotted fever (Stromdahl and Hickling 2012). Thus, the focus of this thesis is to begin to improve our knowledge of the distributions of ticks and tick-borne pathogens in Alabama, as well as our understanding of factors that influence host-seeking behavior among ticks, especially *A. americanum*. The objective of this chapter is to review the biology and role in pathogen transmission of the major tick species native to Alabama.

1.1 Tick morphology and systematics

Ticks are obligate hematophagous ectoparasites belonging to the order Ixodida, which falls in the class Arachnida along with mites. Like their insect cousins, archnids belong to the phylum Arthropoda but belong to the subphylum Chelicerata, which differ in the structural components of the mouthparts compared to the mandibulate subphylum Hexapoda. Chelicerates feature structures called chelicerae, which are specialized digits used for cutting host tissues. Over 900 tick species have been described worldwide, which have been grouped into three families: Ixodidae (hard ticks) including more than 700 species, Argasidae (soft ticks) consisting of roughly 200 species, and Nuttalliellidae with only one described species (Guglielmone et al. 2010, Apanaskevich et al. 2011, Horak et al. 2013, Apanaskevich et al. 2013, Dantas-Torres et al.

2012, Nava et al. 2013, Venzal et al. 2012, 2015). It is practicable to differentiate the first two well-established families with the naked eye. Hard ticks have visible anterior mouthparts from the dorsal view with a sclerotized scutum just posterior to the capitulum. The length of the scutum is a sexually dimorphic character that allows one to distinguish between adult males and females. On the contrary, soft ticks lack sexual dimorphism and a hard scutum, and the mouthparts are not visible from the dorsal view (Goddard and Layton 2006).

Of the tick families, Ixodidae is the most thoroughly investigated group of the three in the United States due to its greater medical and economic importance (Belozerov 2008). Ixodidae has been further divided into two major groups, the Prostriata and Metastriata, which can be easily distinguished based on the position of the anal groove. Although these groups have not been assigned an official taxonomic level, it is a useful distinction from a practical perspective, as it is the first feature invariably used by morphological keys for hard ticks. In the Prostriata, the anal groove is anterior to the anus, and this group consists of a single genus, *Ixodes*, which contains the only known vectors of the agents of Lyme disease. In the Metastriata, the anal groove is posterior to the anus; and this group is comprised of 11 genera, including *Amblyomma*, *Dermacentor*, and *Rhipacephalus*, each of which contains species of medical or veterinary significance. Useful morphological features for distinguishing among genera and species include the length of the mouthparts (i.e., hypostome, chelicerae, and palps), the shape of the basis capituli, the presence or absence and length of coxal spurs, and the number and arrangement of dentition of the hypostome (Keirans and Litwak 1989, Durden and Keirans

1996, Keirans and Durden 1998, Coley 2015).

1.2 Tick life cycles

All Ixodid ticks have four life stages: eggs, larvae, nymphs, and adults. Once eggs hatch, larvae and all successive life stages (except adult males of some species) feed exclusively on blood once during that specific stage. The blood is used primarily for development and molting to the next stage by larvae and nymphs and for reproduction by adults. Unfed immature ticks are small, usually less than 2 mm in length, which makes them difficult to identify. However, larvae can be distinguished easily from nymphs and adults by having six legs instead of eight (Kleinjan and Lane 2008). Nymphs are smaller than adults, are sexually immature, and easily distinguished by the lack of a genital pore. Adults are the only stage in which sex can be determined, and several morphological characters are sexually dimorphic. In male hard ticks, for example, the scutum covers the entire dorsum, while in females only the anterior portion of the dorsum is covered allowing the abdomen to expand substantially during blood feeding (Keirans and Litwak 1989).

Hard ticks exhibit one-, two- or three-host life cycles depending on their feeding behavior. For one-host life cycles, engorged larvae and nymphs remain on the same host throughout development. Mating also occurs on this host, so it is only after feeding and reproduction is complete that females drop off to lay eggs. In contrast, for three-host life cycles, each life stage must find a new host to obtain a blood meal. Female adults may mate on or off the host (varies among species) and then lay eggs in the natural environment. In fact, more than 90% of hard

tick species are characterized by a three-host life cycle. It takes larval ticks three to four weeks to hatch from eggs. Upon hatching, each larva must find, attach to and feed on a suitable host (typically a small vertebrate) for about three to six days. After detaching from the host, larvae find a sheltered place, such as leaf litter on the forest floor, and develop into nymphs, a process that takes two to four weeks. Similar to larvae, nymphs can survive from six to nine months without feeding, but typically feed on medium- or large-sized hosts for five to ten days before dropping off. Adequate shelter and at least a two-week period are required for successful development and molting to the adult stage. Adult ticks can survive as long as 18 months without feeding, which makes it possible for the ixodid life cycle to take up to three years in temperate environments with short growing seasons. Both sexes tend to attach to large-sized mammals and usually mate on the host after a short period of feeding, except for species in the genus *Ixodes* that mate off the host. Females continuously feed for about one week and can increase their body mass up to 100 times compared to their prefeeding mass. Because the scutum of males covers the length of the body, there is less room for expansion during blood feeding, so males take much smaller blood meals. Thus, the size of an engorged male is only a fraction of a fully engorged female. After digesting the blood meal, female hard ticks typically oviposit a few thousand eggs in a single clutch, which takes days or even weeks to complete in a suitable environment. Finally, after oviposition is complete, the exhausted females die (Mullen and Durden 2009, p. 500; Sonenshine and Roe 2013, pp. 60-64).

1.3 Tick ecology and behavior

Over their evolutionary history, ticks have adapted to a wide range of habitats and climatic conditions worldwide and are found on every continent except Antarctica. In addition to habitat diversity, ticks also display a range of life styles related to host preferences. Because each life stage must blood feed before developing to the next stage (or reproducing in the case of adults), tick biology is closely tied to host-seeking strategies and behaviors. It can be argued that over the course of a tick lineage's evolutionary history, the degree of host specialization is the most important factor in shaping the biology of present-day taxa. What we now perceive to be important features of tick ecology have been shaped and refined to reflect each species' life style. Although factors that influence all ticks, such as microclimate, limits certain species into a narrow physiological range of humidity and temperature, the majority of ticks collectively display an amazing range in the conditions in which they live. This section briefly reviews variation in tick life styles and some of the major factors that govern tick biology, namely climate and tick-host interactions.

1.3.1 *Nidicolous and non-nidicolous ticks*

The broad ecological range observed among ticks has been shaped by selection imposed by off-host habitats associated with specific host-driven life styles. Ecologically, ticks can be divided into two broad groups, nidicolous and non-nidicolous, which are differentiated by where ticks live when off the host. Nidicolous ticks live in close proximity to the host and are found in nests, burrows, caves or other secluded enclosures used by their preferred host species. In contrast,

non-nidicolous ticks occupy open spaces of forests, brushlands, or grassland habitats. Taxonomically, nidicolous ticks include both ixodid (hard) and argasid (soft) ticks, although argasids are more commonly associated with this life style. The nymphal and adult stages of soft ticks feed more frequently and for shorter durations (~ 30 minutes) than hard ticks, so being in proximity to the host at certain stages of the cycle is important for survival and reproduction. Argasids are also extremely long lived and can survive for longer periods without blood meals than ixodid ticks. Therefore, they are adapted to survive periods between nest or burrow abandonment and re-colonization by a new host (Sonenshine and Roe 2013, pp. 39-45). In contrast, most non-nidicolous ticks belong to family Ixodidae. Due to their "free-living" nature, non-nidicolous ticks have adapted to a broader variety of habitats compared to nidicolous ticks, particularly those that follow the three-host life cycle. However, for each species there is an optimal habitat type that features climatic conditions and hosts for which a given species is best suited. The majority of non-nidicolous species spend more than 90% of their time living off-host (Oliver 1989). Thus, climate tends to more strongly affect non-nidicolous tick survival because the habitat of nidicolous ticks is often more buffered from the macroenvironment. Not only does climate more strongly influence survival of non-nidicolous ticks, but it also strongly influences host-seeking behavior (i.e., questing). Although climate affects tick species differently, questing tends to occur within limited ranges of humidity and temperature in the environment (Randolph 1997, Ogden et al. 2004). Another important difference between these life styles is that non-nidicolous ticks can respond to host stimuli at greater distances compared to nidicolous species (Beelitz and Gothe 1991). Therefore,

compared to nidicoulous ticks, non-nidicolous ticks are better adapted to overcome the challenge of finding hosts when they are not present in their immediate surroundings.

1.3.2 *Effects of climate on ticks*

The majority of three-host ixodid ticks spend their time living off-host; therefore, the inhabiting environment between blood-meals influences numerous aspects of tick biology (Needham and Teel 1991). Habitats that can be colonized by tick populations are roughly delineated by climatic conditions of the macroenvironment, while between blood meals ticks are more directly exposed to and responsive to the range of microenvironments within a habitat. These may vary substantially in terms of suitability for tick survival or host-seeking behavior. Microenvironments are highly influenced by sets of microscale climatic conditions (e.g., relative humidity, temperature) (Sonenshine and Roe 2013, p. 21), which in turn are influenced by numerous factors, such as the structure of the microenvironment, soil type, degree of insolation, etc. Although these factors certainly influence the floral, faunal, and microbial communities present at the microscale, the biological processes in which they engage result in outputs that feed back into and influence their immediate surroundings, shaping the microenvironment in complex ways (Geiger 1965, Chen et al. 1999) Thus, understanding distributions of ticks at different spatial scales, requires an understanding of how abiotic factors influence tick biology.

1.3.2.1 *Humidity and water balance*

For all terrestrial arthropods, the maintenance of body water is critical for survival. Ticks are no exception as water balance is a major factor for their development and longevity. The threshold for maintaining water level at a steady state is called the critical equilibrium humidity (CEH), ranging from approximately 75% to 94% relative humidity (RH). In other words, the threshold represents a counterbalance between water efflux and influx at atmosphere when ticks can maintain body water equilibrium. Therefore, if the humidity falls below this threshold, ticks will continuously lose water (Knülle and Wharton 1964), which may lead to desiccation and death. In fact, most water loss in ticks occurs through integumentary loss because of a high surface-to-volume ratio and limited openings on the body to the atmosphere (Needham and Teel 1991). In addition, when ticks are host seeking, a rise in locomotor activity and exposure to less buffered conditions (i.e., lower humidity, air flow, etc.) increase rates of water loss (Lees 1946, 1947; Yoder et al. 1997; Fielden et al. 1999).

Despite risk of desiccation, ticks may survive for months or years without feeding. This period is longer than most other arthropods, as they possess many features that conserve energy and water (Balashov 1968). Water conservation in unengorged ticks is complex and involves numerous mechanisms. These include those that are (i) physiological: deposition of cuticular wax that enhances water-proofing, spiracular closing devices that prevent excessive water loss, and excretion of nitrogenous waste that is nearly dry (guanine or other related products); (ii) behavioral: aggregating to suppress water-loss (particularly as larvae), seeking out optimal

conditions for water retention and host attachment, and the restriction of host-seeking to specific times or seasons that reduces dehydration (Yoder et al. 1997, 2015; Benoit and Denlinger 2010, Benoit et al. 2006). Other than water retention, some species, such as *Ixodes scapularis,* have the ability of actively uptaking water vapor from the subsaturated atmosphere when off-host (Bowman and Sauer 2004). This characteristic allows ticks to extend the questing period by counteracting water loss during questing. Interestingly, although ticks require water for survival, most ixodids actually avoid contact with liquid water (Krober and Guerin 2000). Exceptions include xerophilic species that possess the ability to imbibe free water (Yoder and Spielman 1992, Yoder et al. 2006).

1.3.2.2 *Temperature*

In addition to humidity, temperature is also fundamentally important to tick survival, and microclimatic temperature has been shown to explain much of the variation in tick behavior and population dynamics within a given habitat (Schulze and Jordan 2003, Rynkiewicz and Clay 2014, Hubalek et al. 2003, Harlan and Foster 1990). The research literature suggests that temperature influences tick biology in complex ways. For example, water balance, active sorption mechanisms and mortality and development rates are all closely related to temperature of the soil, leaf litter or ambient air (Knülle 1966, McEnroe and McEnroe 1973, McEnroe 1979, Hubalek et al. 2003).

As described above, tick biology is strongly influenced by microclimate, which varies substantially on both daily and seasonal time scales. Thus, temperature is a major factor in determining seasonal and spatial variation in tick activity and population dynamics (Estrada-Pena 2003). While the influences of temperature are not yet completely understood, studies have revealed relationships between tick activities and threshold temperatures, below which ticks are not active. Threshold temperatures of specific species and stage have been estimated for tick locomotor activity (3.9-9.8˚C), host-seeking activity (7.2-13.9˚C), and cold temperature survival (-18.5-11.6˚C) (Clark 1995, Vandyk et al. 1996, Schulze et al. 2001, Olson and Patz 2011).

On a much broader scale, temperature and humidity work together to limit the geographic ranges of various species. Ticks of higher heat tolerance tend to function better in drier environments than those with lower heat tolerance (Yoder et al. 2006). However, there exists a critical temperature (the critical transition temperature, or CTT) that denotes the point of abrupt water loss in ticks (Yoder et al. 2005), whereby temperatures above the CTT cause death due to desiccation. On the other hand, cooler and more humid habitats may have deleterious consequences on fitness due to negative impacts on reproduction (Burks et al. 1996). Thus, although extremes of temperature and humidity influence tick populations in different ways, they work in combination to influence species-specific patterns of occurrence and spatial distributions.

1.3.3 *Diapause*

To survive environmental extremes, particularly those associated with low temperatures, some tick species enter diapause (Sonenshine and Roe 2013b, p. 9). Two forms of diapause occur among ticks with the most common being behavioral (host-seeking) diapause. This occurs after hatching or molting when conditions induce a quiescent state. Ticks in behavioral diapause do not quest even when hosts are in close proximity. This state is primarily induced by exposure to short day lengths (Belozerov et al. 2002, Cabrera and Labruna 2009). The second type is called morphogenetic (developmental) diapause and happens after a blood meal (i.e. embryonated eggs, engorged larvae, nymphs and adult females). Morphogenetic diapause is characterized by delayed ecdysis or oviposition (Belozerov 1982) and is most commonly induced by differences between pre- and post- feed conditions of photoperiod and temperature (Randolph 2004).

Diapause in some cases can also enable ticks to synchronize their active periods with climatic conditions that favor questing, as well as activity of potential hosts. This is an important survival-promoting strategy that appears to have evolved in a number of non-nidicolous tick species (Sonenshine 1993, p. 24). In addition, it is noteworthy that exogenous quiescence, is yet another form of dormancy (i.e., non-diapause dormancy) that has been recognized in ticks. To reduce desiccation stress, for example, *Dermacentor variabilis* will be quickly activated by long day conditions to actively host-seek and feed (Yoder et al. 2015). This quick alternation between two states differs remarkably from diapause, as diapausing arthropods cannot readily adjust from a deep inactive syndrome to an active state (Denlinger 2002). However, because

short-day response by ticks appear to vary among species, there is still debate whether tick dormancy is a real diapause syndrome or simple quiescence. Resolving this issue will require further studies (Belozerov 2008).

1.3.4. *Tick-host interactions*

Since ticks are obligate blood feeders, it is imperative that each life stage finds a host. Therefore, in addition to environmental determinants, tick distributions are also limited by host presence and abundance (Klompen et al. 1996, Cumming 2002). Ticks adapted to specific habitats are more likely to encounter hosts that have adapted to the same habitat, and for some species, studies have concluded that population dynamics are driven by seasonal fluctuations in host densities (Schauber and Ostfeld 2002, Wang et al. 2015). In addition to serving as food sources, hosts also influence tick distributions by serving as vehicles for transport among locations, as the dispersal capabilities of ticks is limited (Falco and Fish 1991).

Ticks demonstrate two types of host-selection strategies: (i) host-specific, which is practiced by highly selective tick species, and (ii) opportunistic, which is utilized by generalist species that feed on a wide range of hosts (Sonenshine 1975). It is hypothesized that the two different strategies are the outcome of evolution associated with life style (i.e., nidicolous vs. non-nidicolous), habitat specificity, physiological factors, and the ability of ticks to avoid host rejection (Trager 1939). In fact, more than 85% of ixodid ticks possess relatively strict host specificity, which in turn, may be a driving mechanism of parasite diversity (Mullen and Durden

2009). From a medical and public health perspective, ticks that display the opportunistic strategy are most commonly involved in pathogen transmission.

An aspect of host-seeking behavior closely tied to host-selection strategies is the method used to locate hosts. Host-locating methods are grouped into two categories: passive (ambush) and active (hunter) strategies (Mullen and Durden 2009, p. 502). The former strategy is employed by most non-nidicolous ticks. Ambush ticks acquire hosts by sitting and waiting for hosts to pass by and then quickly grasping onto them when in close proximity. Ambush ticks are said to "quest" because of the posture observed as they extend their forelegs anterolaterally and hold onto vegetation or some other substrate with their remaining legs. Haller's organ, a sensory structure located in each foreleg, contains olfactory sensilla that are likely used to identify potential hosts (Parola and Raoult 2001). Depending on life stage, ticks quest at different heights, which determines, at least in part, the range of hosts that ambush ticks will encounter. Immature ticks tend to quest near the ground and will therefore attach to small-sized animals, such as rodents, lizards, and ground-feeding birds. In contrast, adult ticks usually quest higher and attach to larger animals (Miller et al. 2016). It is unclear which factors drive this variation in questing height, but it is hypothesized to be a combination of factors including host preferences and physiological limitations (e.g., desiccation tolerance). In contrast to ambush ticks, hunter ticks actively seek out hosts and attack them. This strategy is most common in harsh environments where the passive strategy is not feasible (e.g., xeric conditions). In these cases,

hunter ticks emerge from shelters, such as soil, sand or duff, and then run across the ground to initiate contact with and then grasp onto the host (Mullen and Durden 2009, p. 502).

In addition to serving as sources of blood meals, hosts also serve as a means of dispersal for many tick species. There is evidence that times and locations of dropping off the host after ticks feed are non-random. The underlying hypothesis is that "drop-off behavior" has been selected so fed ticks disperse to optimal habitats (e.g., appropriate shelter and microclimate) at times of day conducive to survival, which influences their spatial distributions (Vredevoe et al. 1997). Host-activity patterns, for example, influence nidicolous and non-nidicolous ticks in opposite ways. The former drop from inactive hosts in nests or burrows, while the latter often drop to the ground at the time of maximum host activity. Furthermore, it appears that photoperiod and scotophase (i.e. dark phase of the light:dark cycle) of the environment and the circadian rhythm of the ticks may act as the dominant exogenous and endogenous factors, respectively, influencing the timing of drop-off (George 1971; Sonenshine 1993, p. 41).

1.4 Important tick-borne pathogens, vectors, and associated diseases of the Southeast

More than 25 species of hard ticks are commonly found in the eastern United States (Keirans and Litwak 1989). Only a small portion of these are medically important since many are narrow host specialists that rarely bite humans. The overwhelming majority of tick-borne pathogens are zoonotic, and therefore, commonly cycle between ticks and wild animals. Humans tend to be incidental or dead-end hosts (i.e., hosts that prevent the complete development of parasites

and thus block their transmission to the next host), and contract disease when bitten by ticks that are opportunistic in their biting behavior. Below, the general process of pathogen-transmission by ticks is described, along with the tick species of medical and public health importance in the Southeast, the pathogens they transmit, and the associated illnesses.

1.4.1 *Biology of pathogen transmission by ixodid ticks*

Collectively, ticks transmit a wider variety of infectious organisms than any other group of hematophagous arthropods, including viruses, protozoa, fungi, rickettsiae and other bacteria (Jongejan and Uilenberg 2004). Pathogenic tick-borne viruses are the cause of rare but potentially severe diseases, including Colorado tick fever virus, Powassan virus, tick-borne encephalitis virus, Heartland virus, and Bourbon virus (Mansfield et al. 2009, Ebel 2010, Yendell et al. 2015, Mattar and Gonzalez 2016, Vasconcelos and Calisher 2016). As for pathogenic protozoa transmitted by ticks, the most common human disease is babesiosis, an infection of red blood cells, which became a nationally notifiable disease in 2011. In 2013, the year for which the most recent data are publicly available, 1,762 cases were reported across 22 states (CDC 2015c). However, 95% of the cases occurred in just seven states, five in New England along with Wisconsin and Minnesota. Although the severity of babesiosis ranges from asymptomatic to life threatening, most infections are manageable, particularly if identified early. Comparatively, very few cases of tick-borne illnesses caused by fungi have been reported (Brites-Neto et al. 2015). Of all the pathogens transmitted by ticks, bacterial agents are most important from medical and public health perspectives. In the United States, the most

widespread and significant tick-borne pathogens include *Borrelia burgdorferi*, *Ehrlichia chaffeensis*, and *Rickettsia rickettsii*, which are the primary agents of Lyme disease, ehrlichiosis, and Rocky Mountain spotted fever, respectively. Interestingly, species in the genera *Ehrlichia* and *Rickettsia* are proteobacteria belonging to the order Rickettsiales and are obligate intracellular bacteria (Narasimhan and Fikrig 2015). In other words, these microorganisms are incapable of living outside of host cells and in the human host display tropism, meaning that they preferentially infect specific cell types. The characteristic tropism and pathology of these pathogens will be described further below.

A complete transmission cycle of an arthropod-borne disease depends upon three components: a pathogen, a vertebrate host, and an arthropod-host or vector (Mullen and Durden 2009, p. 20). For the pathogen, this type of life cycle requires remarkable flexibility, as it must be able to infect and multiply in both a vertebrate host and an arthropod vector. For hosts to be involved in transmission, the species must not only be susceptible to the pathogen, but also support a level of infection in the blood that is sufficient to infect a vector. Likewise, for a vector to be competent, it must be susceptible to infection and capable of transmission, most commonly through salivary secretions.

In addition, ixodid ticks possess remarkable blood-feeding features that contribute to successful transmission of pathogens. First, ticks select a suitable attachment site on hosts and cut into the skin with chelicerae. Once the tick inserts its hypostome, it secretes a substance into the

wound during the first 48-72 hours that strengthens the attachment by cementing the hypostome into the host epidermis (Kemp et al. 1982). During the first 48 hours after attachment, little or no blood is taken up by the tick and this time period is termed as the preparatory phase. In addition to the cement-like substance, ticks secrete salivary proteins into the capillary bed that modulate the host's immune response, reduce inflammation, and promote blood flow. Next, the growth phase takes place, during which the attached tick feeds gradually for several days and slowly expands. In all species of hard ticks, adult females are capable of ingesting much larger volumes of blood than adult males and immature stages. Feeding to repletion (i.e., fully engorged) in these ticks is mediated by a rapid phase following the growth phase, during which the tick increases its body weight as much as ten-fold in as little as 12-36 hours. In metastriate species, this rapid phase only occurs in females that mate on the host and is induced by hormonal changes associated with copulation (Sonenshine and Roe 2013b, p. 88).

From a public health perspective, the process of blood feeding is of obvious interest because tick-borne pathogens are transmitted during the process, both to the tick from infected hosts and to the host from infected ticks. However, it is worth noting that for most tick-borne pathogens, transmission from the tick to the host does not occur during the preparatory phase. This is because pathogens tend to stay in the tick midgut following blood feeding and during tick development to the next life stage. Pathogens do not typically begin moving to the salivary glands until after the tick inserts its hypostome into the skin and begins preparing the bite site

(Sonenshine and Roe 2013b, p. 101). Thus, in cases where ticks are removed in the first 48 hours, transmission is unlikely to have occurred.

1.4.2 *Ixodes scapularis and associated pathogens and illnesses*

The blacklegged tick, *Ixodes scapularis*, is the primary vector of agents responsible for Lyme disease, anaplasmosis, babesiosis, and Powassan fever (Table 1.1). Of these, Lyme disease is the most significant in terms of incidence, as Lyme disease is the most commonly reported vector-borne disease in the nation. *Ixodes scapularis* is distributed throughout the eastern U. S., but its medical importance is more pronounced in the Northeast and the Great Lakes region, as this is where the majority of Lyme disease cases occur (Eisen et al. 2016). In these areas, *I. scapularis*

Table 1.1 Major tick vectors in the United States, their pathogens, and associated human diseases.

Vector	Pathogen(s)	Disease(s)
Ixodes scapularis	*Borrelia burgdorferi* *Anaplasma phagocytophilum* *Babesia microti*	Lyme disease Anaplasmosis Babesiosis
Amblyomma americanum	*Ehrlichia chaffeensis* *Ehlichia ewingii* Panola Mountain *Ehrlichia* *Francisella tularensis*[a] Unknown etiology	Ehlirchiosis Tularaemia Southern tick-associated rash illness
Dermacentor variabilis	*Rickettsia rickettsii* *Francisella tularensis*	Rocky Mountain spotted fever Tularaemia
Rhipicephalus sanguineus	*Rickettsia rickettsii*	Spotted fever rickettsiosis
Amblyomma maculatum	*Rickettsia parkeri*	*Rickettsia parkeri* rickettsiosis

[a]*Francisella tularensis* can infect humans through multiple routes of transmission. Tick bites from *D. variabilis* and *A. americanum* have been linked to tularemia but are not the most common source of infection in humans.

usually abundant in wooded forests with well-drained sandy soils, such as oak forests (Guerra et al. 2002, Hamer et al. 2010). Recent studies indicate its expansion and substantial increase in geographic range over the last two decades (Eisen et al. 2016).

Lyme disease is caused by spirochetes belonging to the species *Borrelia burgdorferi*, a type of extracellular bacteria characterized by a corkscrew-shaped morphology (Wormser et al. 2006). Like the majority of tick-borne illnesses, Lyme disease is zoonotic, meaning that the pathogenic agent naturally cycles among ticks and wild animals. The transmission cycle of *B. burgdorferi* is complex and numerous animals have been implicated as important amplifying hosts and natural reservoirs in the eastern U.S., including the white-footed mouse (*Peromyscus leucopus*), the eastern chipmunk (*Tamius striatus*), eastern gray squirrels (*Sciurus carolinensis*), and short-tailed and masked shrews (*Blarina brevicauda* and *Sorex cinereus*) (LoGiudice et al. 2003; Brisson and Dykhuizen 2004, 2006; Brisson et al. 2008). Because ticks hatch infection-free and usually become infected as larvae, the density of questing nymphs is considered a direct measure of Lyme disease risk. Although *I. scapularis* ticks are present in the Southeast, the risk of Lyme disease is much lower due to the relatively low density of *B. burgdorferi*-infected, questing nymphs (Mead 2015, Pepin et al. 2012). Compared with immature ticks in the Northeast, which feed primarily on rodents that are highly reservoir-competent mammals (Giardina et al. 2000), larvae and nymphs in the Southeast frequently feed on lizards of low reservoir-competence for *B. burgdorferi* (Apperson et al. 1993, Clark et al. 2005). Additionally, nymphs from southern populations of *I. scapularis* have been shown experimentally to quest on

average at lower heights compared to northern nymphs and are less prone to biting humans (Arsnoe et al. 2015, Ginsberg et al. 2014). It is also notable that white-tailed deer (*Odocoileus virginianus*), which serve as the primary hosts of the adult black-legged tick, are immune to *B. burgdorferi* but play important roles in tick dispersal and in supporting tick populations (Telford et al. 1988). The latter may explain, at least in part, the increase in tick-borne illnesses in the U. S. over the last few decades since larger deer populations are correlated with larger numbers of *I. scapularis* and other tick species that commonly parasitize deer (Paddock and Yabsley 2007).

Clinically, Lyme disease symptoms can be grouped into early and late phases according to the stage of infection (CDC 2016a). Initially after a tick bite, the spirochetes only spread in the skin but soon enter the general circulation through the cutaneous vasculature (Steere 2001). Early phase symptoms typically occur 3-30 days after exposure (i.e., a tick bite) and most commonly include fever chills, headache, fatigue, swollen lymph nodes, joint and muscle aches, and an erythema migrans rash (Duray 1989, CDC 2016a). Stereotypically, the erythema migrans rash begins at the bite site, expands gradually in size, and often clears as it enlarges, resulting in a "bull's-eye" appearance. The size of the rash varies and may reach up to 0.3 m (12 inches) in diameter. Classically, this has been used in diagnosis, but occurs in 70-80% of infections, so Lyme disease certainly occurs in people who lack this symptom. Lyme disease often responds well to antibiotic treatment in the early phase; but if left untreated, infections spread to other parts of the body, including the joints, heart and nervous system, leading to symptoms of the late phase often months after the initial infection. These symptoms include neck stiffness,

severe headaches, arthritis accompanied by severe joint pain and swelling, irregular heartbeat, episodes of dizziness, and shortness of breath (Paleologo 1991, Wormser et al. 2006).

1.4.3 *Amblyomma americanum and associated pathogens and illnesses*

The lone star tick, *Amblyomma americanum*, is a highly abundant species in the Southeast (Merten and Durden 2000) (Figure 1.1) that has recently expanded into the Northeast and Midwest (Monzón et al. 2016). In the Southeast, it is predominantly found in wooded habitats, particularly young second-growth forest with dense undergrowth (Hair and Howell 1970). The population abundance of the lone star tick is influenced by the availability of habitats and the

Figure 1.1 Geographic distribution of *Amblyomma americanum* in the United States (courtesy of CDC, 2011).

accessibility of hosts as well. This species is a generalist feeder and may be found on birds, rodents, humans, and other mammals (Mullen and Durden 2009), especially in areas with large populations of white-tailed deer, which serve as a major host for adults and nymphs (Paddock and Yabsley 2007). Importantly, in recent decades the lone star tick has become the most common human-biting tick in the Southeast (Stromdahl and Hickling 2012), which until recently was considered a pest of little medical or veterinary importance. Now, however, it is known to be the primary vector of the agents of human and canine Ehrlichiosis (Harris et al. 2016) and an occasional vector of the agents of Rocky Mountain spotted fever (*Rickettsia rickettsii*) and *Rickettsia parkeri* rickettsiosis (Table 1.1) (Berrada et al. 2011, Cohen et al. 2009). In addition, this tick species is commonly associated with "*Candidatus Rickettsia amblyommii*," a bacterial species closely related to *R. rickettsii* and *R. parkeri* but thought not to be pathogenic in humans (Williams-Newkirk et al. 2014).

At least three species of *Ehrlichia* responsible for human ehrlichiosis are vectored by the adult and nymphal stages of *A. americanum*: *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, and Panola Mountain *Ehrlichia* (PME) (McQuiston et al. 1999, Harris et al. 2016). Since becoming a notifiable disease in 1999 (*E. chaffeensis* infections only until 2008 when *E. ewingii* infections were added to the case definition), the average incidence rate of ehrlichiosis has increased from 1.4 cases per million person-years during 2000-2007 to 3.2 cases per million person-years during 2008-2012 (Dahlgren et al. 2011). Moreover, it is worth noting that another ehrlichial species recently emerged as a cause of human illness, *Ehrlichia muris*-like agent, but has only

been detected in Minnesota and Wisconsin since its discovery in 2011 (Pritt et al. 2011). Thus, it will not be discussed further.

Bacteria in the genus *Ehrlichia* are obligately intracellular species and belong to the order Rickettsiales (Paddock and Yabsley 2007). Upon entering the human host through a tick bite, ehrlichiae attack white blood cells, with *E. chaffeensis* and *E. ewingii* infecting monocytes and granulocytes, respectively (Demma et al. 2005a). These pathogens are internalized by a process that involves binding to GPI-anchored proteins on the leukocyte surface, which initiates signaling events that results in internalization (Rikihisa 2010). Once inside, they are maintained inside a membrane-bound vesicle that resists lysosomal degradation (Rikihisa 2010), allowing the bacteria to grow, replicate, and eventually rupture the host cell to infect new cells (McDade 1990). Human ehrlichiosis generally presents as a moderate to severe disease marked by fever, chills, headache, muscle pain and malaise. Leukopenia (low white blood cell count), thrombocytopenia (low platelet count), and elevations in liver enzymes are frequently found in laboratory tests of clinical samples (Dumler et al. 2007). Particularly severe manifestations of *E. chaffeensis* can lead to death as early as the second week of infection (Dawson et al. 2001, Fordham et al. 1998, Martin et al. 1999, Paddock et al. 1997). However, compared to *E. chaffeensis*, infections by *E. ewingii* usually results in milder illness, and no fatal cases have been reported (Goodman and Dennis 2005). As for PME, this recently discovered species (no formal scientific name yet) has been suggested to be an emerging pathogen of human illness since 2008 (Loftis et al. 2006, Reeves et al. 2008). Although few human cases have been

reported, PME has been consistently detected in ticks collected from vertebrate hosts including humans (Sayler et al. 2016, Pompo et al. 2016, Lee et al. 2014, Harmon et al. 2015).

Virtually all cases of ehrlichial transmission to vertebrate hosts are caused by the bites of infected ticks. In the United States, the primary vector of *E. chaffeensis* and *E. ewingii* is *A. americanum*, but the American dog tick, *Dermacentor variabilis*, is also known to be susceptible to infection, although its role in transmission remains unclear (Steiert and Gilfoy 2002, Childs and Paddock 2003). Similar to Lyme disease, ehrlichiosis is zoonotic, but for *E. chaffeensis*, the major host and natural reservoir is the white-tailed deer. Thus, for ehrlichiosis, deer play a central role in both amplifying the number of tick vectors and in maintaining the transmission cycle of the causal agent (Paddock and Yabsley 2007). What's more, both canines and humans are incidental hosts, meaning that although both are susceptible to infection, they are incapable of passing the infection to other ticks (Nair et al. 2014). Compared to *E. chaffeensis*, *E. ewingii* maintains a similar transmission cycle, with both white-tailed deer and possibly wild canines serving as reservoirs (Yabsley et al. 2002, Breitschwerdt et al. 1998). In regards to PME, white-tailed deer and domestic goats are susceptible and suspected to be reservoirs (Loftis et al. 2016).

Another illness associated with lone star ticks in the Southeast is known as southern tick-associated rash illness (STARI), a Lyme disease-like illness that also commonly results in erythema migrans that often has more central clearing (Tibbles and Edlow 2007). Despite years

of research, STARI is still an enigma and causes controversy due to the lack of knowledge about the causative agents and poorly understood diagnostic code (Herman-Giddens 2014). Since 1980s, the erythema migrans lesions suggestive of Lyme disease have been reported from areas with low abundance of *I. scapularis* that are dominated by *A. americanum*. Moreover, the onset of rashes in many cases occurred following the bites of *A. americanum*, which implicated this species as the possible vector of STARI (Barbour 1996, Kirkland et al. 1997, Masters et al. 1998). The expanding distribution of *A. americanum* into northern states that overlaps the area endemic for Lyme disease may be causing overreporting of Lyme disease (Feder et al. 2011). Moreover, the phenomenon exists in the southern states where it is still presumed that little Lyme disease occurs, which may have the opposite effect and cause Lyme disease to be underreported (Herman-Giddens 2014).

The unknown etiology of STARI highlights the limitations of medical diagnosis for this disease. It may be caused by *B. burgdorferi* which has known to be occasionally carried by *A. americanum*. The presence of potent borreliacidal agents in lone star saliva is attributed to the lower prevalence, with less than 13% of saliva-exposed *B. burgdorferi* alive 48h after infection (Ledin et al. 2005, Zeidner et al. 2009). Other studies have also shown the possibility of the pathogenic *Borrelia miyamotoi*, another agent which might cause EM and occasionally be vectored by *A. americanum* (Herman-Giddens 2014, Scoles et al. 2001, Barbour et al. 2009, Scott et al. 2010, Platonov et al. 2011). Widelydistributed microorganisms associated with *A. americanum*, such as *Borrelia lonestari* and *"Candidatus* Rickettsia amblyommii," have also been investigated, but

there is no repeatable evidence connecting these microorganisms to STARI (Stromdahl and Hickling 2012, Nicholson et al. 2009).

The spotted fever group *Rickettsia Candidatus* "Rickettsia amblyommii" (hereafter *Rickettsia amblyommii*) is the last bacterial species of note commonly associated with *A. americanum* (Williams-Newkirk et al. 2014). Species in the genus *Rickettsia* are categorized into two antigenically defined groups: the spotted fever group (SFGR) and the typhus group (Walker 1996). Some members of the SFGR cause severe disease, but to date, little data support the hypothesis that this microorganism is pathogenic to humans. Although *R. amblyommii* has yet to be found in clinical specimens (Nicholson et al. 2009), it has been implicated as a possible pathogen based on serological tests from people recovering from illness after a tick bite (Apperson et al. 2008, Vaughn et al. 2014, Billeter et al. 2007), and on a case where a macular rash developed in a person from which a *R. amblyommii*-infected tick was removed (Billeter et al. 2007).

1.4.4 *Dermacentor variabilis and associated pathogens and illnesses*

The American dog tick, *Dermacentor variabilis*, is the primary vector of *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever in the United States (Table 1.1) and a member of the spotted fever group *Rickettsia*. This tick species is distributed throughout the eastern U.S. and limited areas in the Midwest and California. It is commonly found in meadows and second growth forest, particularly in moist or mesic deciduous forests, old field-forest ecotones and

edges of forests or agricultural areas (Sonenshine 1993, pp. 42-43; Fryxell et al. 2015). In the Southeast, American dog ticks have been found in abundance in clusters in oak-hickory and oak-hickory-pine forests (Sonenshine et al. 1972, Linnemann et al. 1973, Sonenshine and Mather 1994). This significant spatial clustering of ticks may be explained by environmental variables, as well as patterns of host distributions (Fryxell et al. 2015). Immature dog ticks usually quest for small to medium-sized hosts, and adults often feed on similar medium-sized mammals. Common hosts include the raccoon (*Procyon lotor*), white-footed mouse (*Peromyscus leucopus*), striped skunk (*Mephitis mephitis*), prairie vole (*Microtus ochrogaster*), meadow vole (*Microtus pennsylvanicus*), and Virginia opossum (*Didelphis virginiana*), as well as domestic dogs (*Canus lupus familiaris*) in suburban and rural areas where residential areas encroach on tick habitat (Burg 2001, Cohen et al. 2010, Zimmerman et al. 1987, Kollars et al. 2000b). However, only the adults typically bite humans to spread the disease. Larvae and nymphs are rarely collected on drags or flags (Figure 1.2), which are sampling methods that exploit questing behavior (Stromdahl et al. 2011). Rocky Mountain spotted fever (RMSF) is one of the oldest known vector-borne diseases (Parola et al. 2005). It has remained the most severe rickettsial infection in the Western Hemisphere since the first clinical report in 1899 (Maxey 1899, Parola et al. 2005). Nowadays, RMSF is still potentially lethal, but the illness responds well to antibiotic treatment and is easily treated if diagnosed early (Parola et al. 2005). In the United States, most RMSF cases occur in the Southeast and Midwest, particularly in Oklahoma, Missouri, Arkansas, Tennessee, and North Carolina. In a nationwide investigation of RMSF from 2000-2007, approximately two-thirds of reported cases originated in these five states

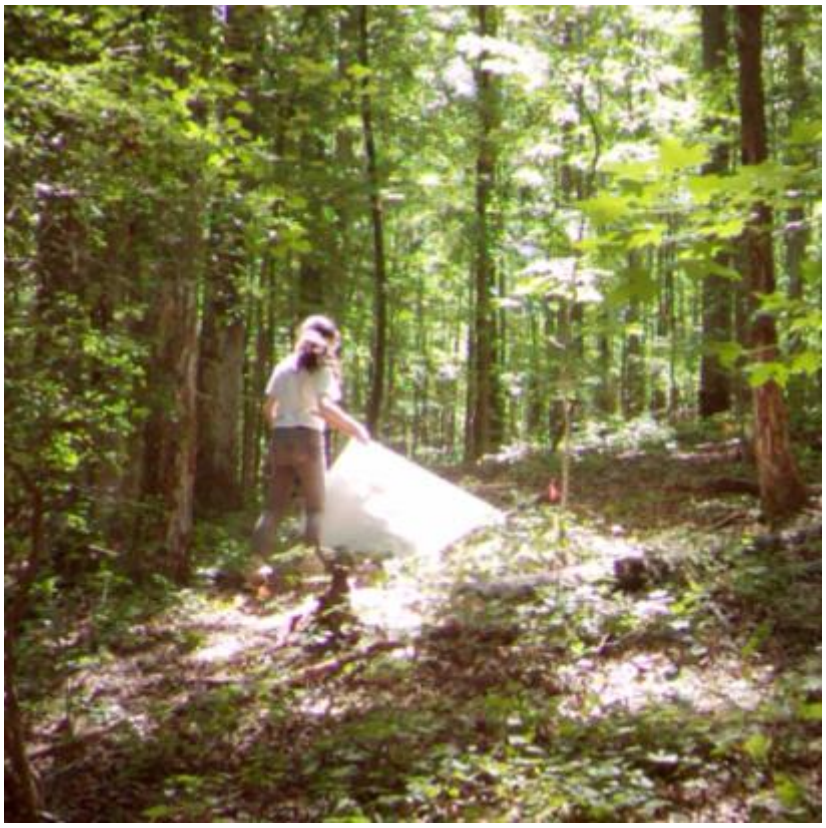

Figure 1.2 A photograph of a tick sampling method called flagging (photo was taken on August 10, 2016).

(Openshaw et al. 2010). In the majority of cases, RMSF involves a sudden onset of high fever accompanied by headache, nausea, vomiting, anorexia, and generalized or focal myalgia (muscle pain), after an incubation period of about seven days (range 2-14 days) (Parola et al. 2005). Unlike many other rickettsial infections, an inoculation eschar is rarely found at the bite site with RMSF (Argueello et al. 2012). Within two to four days after onset of the illness, a characteristic spotted rash develops in 88-90% patients, which appears as small, irregular, pink macules (flat lesions < 1cm) typically on wrists, ankles and forearms (Nawas et al. 2016). These lesions may later evolve to macules, papules (raised lesions), or petechiae (small reddish spots caused by bleeding into the skin) after the fifth day (Sexton 2001). In some cases, RMSF has

severe manifestations including abdominal pain, headaches, pulmonary and renal failure, myocarditis (inflammation of cardiac muscle), neurological manifestations, and focal necrosis or gangrene (Helmick et al. 1984, Buckingham et al. 2007, Minniear and Buckingham 2009). In the present day, the estimated case fatality rate in untreated cases in the United States is 5-10%, with approximately 50% of deaths occur on or before the eighth day of infection (Parola et al. 2005, Paddock et al. 1999).

Regarding the transmission cycle of *R. rickettsii*, several tick species are likely involved in maintaining its transmission and disseminating the pathogen among hosts. Traditionally, *Dermacentor variabilis* is the tick species most often associated with RMSF; however, recent molecular investigations of *D. variabilis* from the geographic range of RMSF showed an extremely low prevalence of *R. rickettsii* (Biggs et al. 2016, Stromdahl et al. 2011). Another tick species, the brown dog tick, *Rhipicephalus sanguineus*, has been shown to have high prevalence for *R. rickettsii* in certain areas, particularly the Southwest, which was not recognized until 2003 (Demma et al. 2005b) (Table 1.1). Additionally, *R. rickettsii* has also been occasionally detected in *Amblyomma americanum*, although a substantial role in transmission is doubtful (Stromdahl et al. 2011).

Inside the tick, rickettsiae ingested with a blood meal escape from the midgut into the hemolymph within approximately five days and disseminate quickly into other tick tissues (Burgdorfer 1988). Unlike other tick-borne bacteria, rickettsiae infect ovaries and oocytes of

female ticks, enabling efficient passage of rickettsiae to their progeny (i.e. transovarial transmission). The occurrence of transovarial transmission results in the tick serving as both the vector and a natural reservoir of *R. rickettsii* (Sonenshine 1993, p. 207; Niebylski et al. 1999). Along with the tick, a number of vertebrate hosts contribute to the maintenance and spread of the pathogen, particularly small rodents such as chipmunks, voles, ground squirrels, and rabbits (Piranda et al. 2011, Demma et al. 2005b, Parola et al. 2005). Humans and dogs, however, are only incidental hosts and do not contribute to the subsequent transmission of *R.* rickettsia despite being susceptible to infection (Socolovschi et al. 2009).

1.4.5 *Amblyomma maculatum and associated pathogens and illnesses*

Amblyomma maculatum is the primary vector of *Rickettsia parkeri,* a member of the spotted fever group *Rickettsia* closely related to *R. rickettsii* and *R. amblyommii*. In terms of human pathogenicity, *R. parkeri* falls in between the other two species, as it causes a relatively mild form of spotted fever (Table 1.1). *Amblyomma maculatum* is commonly called the Gulf Coast tick in accordance with the early description of its distribution along coastal regions of the Southeast (Hooker et al. 1912, Bishopp and Trembley 1945). To date, some established inland populations have also been described, particularly in Arkansas, Oklahoma, and Kansas (Semtner and Hair 1973a, Trout and Steelman 2010, Pagac et al. 2014). These populations have been primarily associated with coastal uplands and tall-grass prairies (Hixson 1940, Semtner and Hair 1973a), which are the preferred habitats of this species and its many hosts. Immature Gulf Coast ticks have been reported to attack birds and rodents, whereas adults have a greater

tendency to feed on large wild and domestic animals (Teel et al. 2010), such as white-tailed deer (Samuel and Trainer 1970), cattle (Barker et al. 2004), horses (Duell et al. 2013) and swine (Greiner et al. 1984). Furthermore, most corresponding human infections of *R. parkeri* rickettsiosis have resulted from bites of adults rather than nymphs (Paddock and Goddard 2015).

Just over a decade ago, *R. parkeri* was recognized to cause disease in humans. At least 40 cases have been identified through 2015 since the first human infection was reported in 2004, and no fatal cases have occurred (Biggs et al. 2016, Paddock et al. 2004). Reported cases of have predominantly occurred in southern states along the Gulf of Mexico and the southern Atlantic coast, roughly approximating the geographic range of *A. maculatum*. Reported prevalences for *R. parkeri* in this species ranges from 5% to 56% (Sumner et al. 2007, Paddock et al. 2010, Trout et al. 2010, Wright et al. 2011, Fornadel et al. 2011, Varela-Stokes et al. 2011, Jiang et al. 2012, Ferrari et al. 2012), while it has only been detected rarely in *A. americanum* (Cohen et al. 2009) and *D. variabilis* (Fritzen et al. 2011, Henning et al. 2014). Similar to *R. rickettsii*, *R. parkeri* is zoonotic and is maintained in transmission cycles involving small rodents and likely transovarial transmission from female ticks to their offspring (Wright et al. 2011).

In cases of human illness, distinguishing symptoms of *R. parkeri* rickettsiosis include the presence of a necrotic inoculation eschar (often more than one) and a low to moderate fever (< 40°C). Indeed, *R. parkeri* rickettsiosis has many clinical features in common with RMSF;

however, although it is milder, diagnosed rickettsial illnesses rarely run their course in the U.S. because treatment immediately follows diagnosis. Therefore, it is possible that *R. parkeri* infections are misdiagnosed as cases of RMSF (Paddock et al. 2008). Moreover, it has been shown that antibodies to SFGR species often cross-react, which in the past contributed to misdiagnosis (Parola et al. 2005) and led to a change in the case definition in 2010 (CDC 2013). All infections *with R. parkeri*, *R. rickettsia*, and potentially *R. amblyommii* are now reported as spotted fever rickettsiosis (CDC 2013).

Chapter 2

Surveillance of Ticks and Tick-borne Pathogens in East Central Alabama

2.1 Introduction

2.1.1 *Ticks and tick-borne diseases endemic to Alabama*

In terms of medical importance, ticks (class Arachnida) rank second to mosquitoes among hematophagous arthropods (de la Fuente et al. 2008). In Alabama, the majority of native tick-borne pathogens are bacterial and are carried by ixodid ticks (family Ixodidae, i.e., hard ticks), including the agents of anaplasmosis, ehrlichiosis, Lyme disease, and Rocky Mountain spotted fever (RMSF) (ADPH 2016). Although all three life stages of the tick can bite people and spread disease, nymphs tend to be the most important for pathogen transmission as a result of usually higher infection rates than larvae and their greater abundance and lower likelihood of detection than adults.

Data from the Alabama Department of Public Health (ADPH) indicates that in each of the last few years approximately 300 cases of tick-borne diseases (TBDs) were reported annually by physicians across the state (ADPH personal communication, Figure 2.1). Despite this number of cases, few formal studies of ticks or TBDs have been undertaken in Alabama since the early 1990s. Therefore, relatively little is known about tick distributions, the prevalence of pathogens associated with each species, or the distributions of tick hosts and competent pathogen

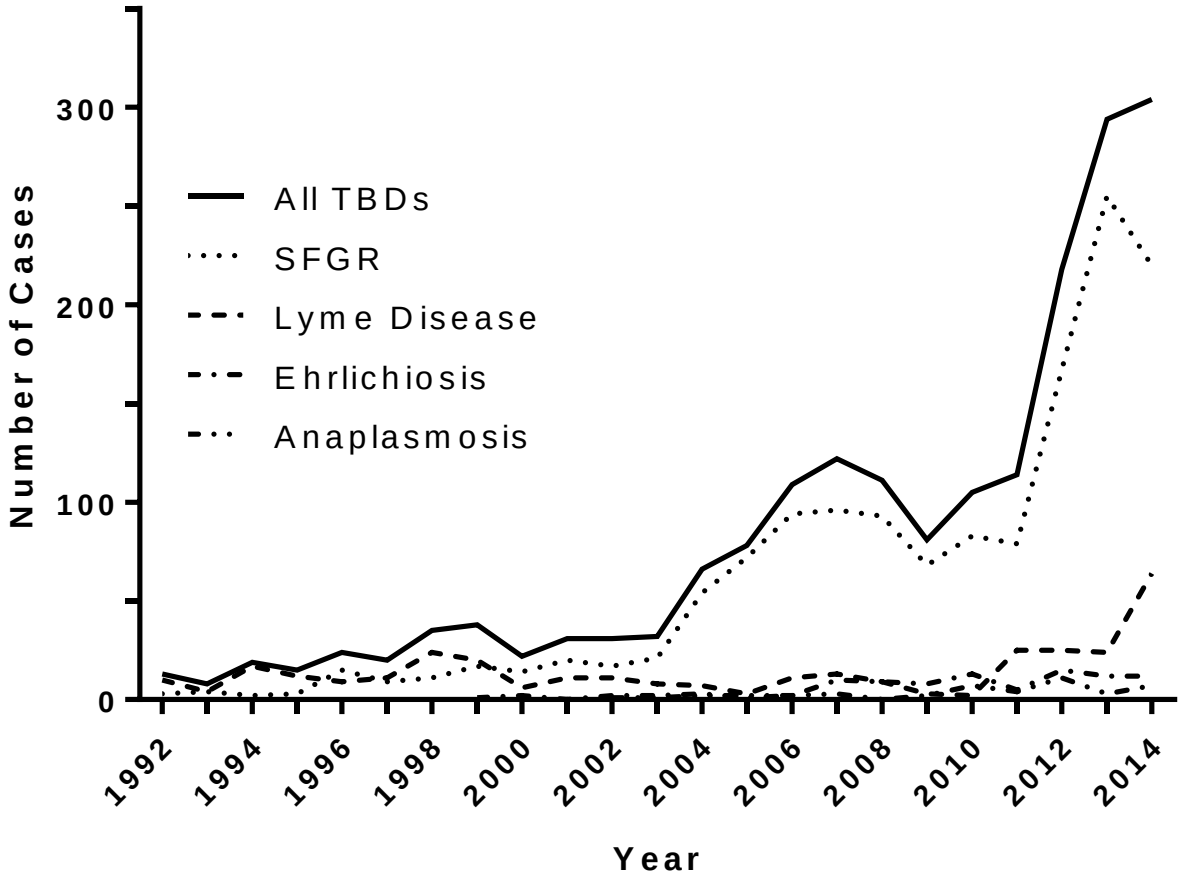

Figure 2.1. Number of cases of tick-borne diseases (TBDs) from 1992-2014. The cumulative number of reported cases for all TBDs during this time frame was 1,892 with more than half (1,035) occurring since 2010.

reservoirs. What we do know, as figure 2.1 demonstrates, is that multiple tick-borne diseases occur each year, particularly spotted fever group *Rickettsia* (SFGR, including Rocky Mountain spotted fever) and Lyme disease.

Although the distribution of ticks and tick-borne pathogens across Alabama is not well defined, data from the Alabama Department of Public Health (ADPH) illustrates the most common TBDs and the counties where they most often occur (Table 2.1). Data reported since 1992 suggest

that rates of human infection with certain pathogens are on the rise (Figure 2.1) and that reports of TBDs vary by county with a greater number of cases from central and northern Alabama (Table 2.1). Despite the limitations of such data (e.g., underreporting), the case reports are valuable indicators of the tick species and pathogens present in the state and highlight potential hotspots of disease activity. Furthermore, the data suggest sharp increases in the incidence of TBDs in Alabama from 2000-2007 and from 2009 to the present (Figure 2.1). The trends are caused primarily by SFGR, although a recent spike in the number of Lyme disease and ehrlichiosis cases contribute to the most recent time frame. The factors underlying the increase in TBDs are unclear, and it is unknown whether the data reflect a true increase in incidence or if cases were underdiagnosed in previous years. Examining the data by county shows that some counties have consistent trends in disease reporting, while others show substantial changes over time. Tuscaloosa County, for example, had three reports of SFGR from 1992-2003. Since 2004, it has had 134 reports of new SFGR cases, a greater than 40-fold increase over a similar period. Another factor that must be considered when interpreting these data is the emergence of SFGR species other than *R. rickettsii*, such as *R. parkeri* and potentially *R. amblyommii*. Reports from other southern states suggest that these less severe *Rickettsia* species, which are impossible to distinguish by serological tests, may be reseanoble for the increased reports of SFGR (Apperson et al. 2008, Moncayo et al. 2010, Stromdahl et al. 2011). From an epidemiological perspective, people who spend time outdoors have the greatest risk of encountering ticks and contracting pathogens that cause disease. Because ticks need vertebrate hosts for blood feeding and relatively humid conditions, woodland areas are potentially risky, since shade, understory, and fallen leaves/limbs provide shelter and appropriate conditions for

Table 2.1. TBDs known to occur in Alabama from 1992-2015. Data are unpublished and courtesy of Kelly Stevens, Alabama Department of Public Health.

Disease (total cases 1992 -2015)[a]	Pathogen(s)	Primary Vector(s)	Alabama Counties w/ Reported Cases (1992 – 2015)
Rickettsiosis/ Rocky Mountain Spotted Fever (1432)[b]	*Rickettsia rickettsii*, *R. parkeri*	*Dermacentor variabilis, Amblyomma maculatum*	**65 counties (97%) with at least 1 case**; counties w/ ≥ 40 cases: Calhoun (42), Jefferson (99), Lamar (48), Lawrence (40), Madison (67), Marion (40), Marshall (50), Mobile (52), Morgan (46), Shelby (53), Tuscaloosa (137), Walker (51)
Lyme Disease (337)	*Borrelia burgdorferi* sensu lato[c]	*Ixodes scapularis*	**53 counties (79%) with at least 1 case**; counties w/ ≥ 10 cases: Baldwin (18), Calhoun (13), Jefferson (34), Madison (44), Mobile (54), Montgomery (12), Shelby (20), Tallapoosa (10), Tuscaloosa (13)
Ehrlichiosis (99)	*Ehrlichia chaffeensis*, *Ehrlichia ewingii*, Panola Mountain *Ehrlichia*	*Amblyomma americanum*	**34 counties (51%) with at least 1 case**; counties w/ ≥ 3 cases: Calhoun (4), Cullman (3), Escambia (3), Franklin (3), Jackson (3), Jefferson (7), Limestone (9), Madison (24), Morgan (6), Walker (4), Winston (4)
Anaplasmosis (45)	*Anaplasma phagocytophilum*	*I. scapularis*	**17 counties (25%) with at least 1 case**; counties w/ ≥ 2 cases: Cullman (2), Jackson (3), Jefferson (5), Limestone (5), Madison (14), Marshall (3), Mobile (2), Tuscaloosa (2)
Babesiosis (3)	*Babesia microti*	*I. scapularis*	**2 counties (3%) with at least 1 case**; Madison (1), Montgomery (2)
Southern Tick-Associated Rash Illness (STARI)[d]	Agent unknown	*A. americanum*	Not a notifiable disease; county distribution unknown

[a]With the exception of STARI, the total number of cases reported in Alabama from 1992-2015 (through March 31, 2015) is given in parentheses. Only Rocky Mountain spotted fever and Lyme disease were notifiable diseases (i.e. part of the National Notifiable Diseases Surveillance System) for the entire time frame.

[b]In 2010, case definitions of Rocky Mountain Spotted Fever (RMSF) and illnesses caused by closely related rickettsial species were combined into a single case definition, spotted fever group rickettsiosis (SFGR). Although RMSF is more severe than other forms of SFGR in Alabama, acute symptoms of rickettsioses are difficult to distinguish and the etiologic agents often cross-react in lab tests, two major reasons for the change in case definition.

[c]*B. burgdorferi* s.l. is a complex of at least 20 closely related genospecies worldwide with 7 known to occur in the U.S. Of these, three have been linked to Lyme disease or Lyme-like borreliosis in the Southeast: *B. burgdorferi* sensu stricto (most common), *B. americana*, and *B. andersonii*.

[d]Acute symptoms of STARI are Lyme-like but no *Borrelia* spp. have been causally implicated. Note that a cluster of cases from Choctaw County in 1999 was reported by Burkot et al. (2001).

both ticks and their hosts. However, despite these generalities, tick distributions tend to vary spatially and seasonally by species (Stromdahl and Hickling 2012, Pfaeffle et al. 2013). As highlighted in Table 1.1 in the previous chapter, the ticks of most concern for public health in Alabama include the American dog tick (*Dermacentor variabillis*), the black-legged tick or deer tick (*Ixodes scapularis*), the lone star tick (*Amblyomma americanum*), and the Gulf Coast tick (*A. maculatum*). Although there is little data on tick distributions across Alabama, three studies from the early 1990s provide the most information. Two of these investigated ticks collected from white-tailed deer (WTD) at hunter check stations in 16 counties (Durden et al. 1991) and 18 counties (Luckhart et al. 1992) from 1988-1990. The former reported collecting 3,633 ticks from 537 deer. Only four species of ticks were represented in the collection: *I. scapularis* (n = 2,060), *D. albipictus* (n = 1,253), *A. americanum* (n = 315), and *A. maculatum* (n = 5) (note that the second most abundant species was a one-host tick that completes its life cycle on a single host and is unimportant for public health). Although *I. scapularis* was the most abundant tick overall (~57%), the number of infested deer, the average number of ticks per deer, and the proportions of tick species in each area varied widely among sites and between years. The second study, performed by Luckhart et al. (1992), focused on determining the prevalence of the agent of Lyme disease, *Borrelia burgdorferi*, in ticks. Although infection prevalence among ticks was low, ranging from 0%-3.8%, the paper reported the presence of *B. burgdorferi* in six counties across central Alabama isolated from the vector *I. scapularis*. The third study from this series of papers focused solely on Lee County in eastern central Alabama to investigate transmission of *B. burgdorferi* in an area where Lyme disease had been reported (Luckhart et al. 1991). In this study ticks were collected by a variety of means, such as sampling for questing

ticks in nature by the drag-cloth method, collecting ticks from hunter-killed WTD, and collecting off of trapped animals, mostly small mammals and lizards. The composition of tick species sampled differed from the studies that only collected from WTD. From non-deer hosts, 222 ticks were recovered and four species were represented: *A. americanum* (48.2%), *Rhipicephalus sanguineus* (26.1%), *D. variabilis* (23.9%), and *I. scapularis* (1.8%). From deer, 390 ticks were collected: *D. albipictus* (70.5%), *I. scapularis* (17.4%), and *A. americanum* (12.1%). This study also screened ticks for *Borrelia* using an antibody test and found spirochetes in *A. americanum* (6 of 144) and *I. scapularis* (5 of 165). In the former, the spirochetes were likely *Borrelia lonestari* and in the latter *B. burgdorferi*, but distinguishing between the two at the time of the study was not possible.

Since these publications few other noteworthy studies of ticks or TBDs in Alabama have been published. However, two have focused on the lone star tick, *A. americanum*. In the first, a cluster of STARI cases in Choctaw County in 1999 prompted an investigation where ticks were sampled near sites of likely exposure (Burkot et al. 2001). STARI patients often present with an erythema migrans rash similar to that of Lyme disease (i.e., a “bulls eye” rash with central clearing that gradually expands from the bite site), but the illness is associated with bites from the lone star tick rather than the black-legged tick, *I. scapularis*. The authors of the study collected 233 ticks total, 204 lone star ticks (21 adults, 183 nymphs) and 29 Gulf Coast ticks (*A. maculatum*). None of the ticks were positive for *B. burgdorferi*, but two of the adult lone star ticks tested positive for *B. lonestari*, the suspected cause of STARI at the time. However, since 2001 investigation of other STARI cases have failed to find evidence of *B. lonestari* in either ticks

or patients (Masters et al. 2008). Nevertheless, the association of STARI with lone star ticks remains strong; and given this tick's status as the primary vector of *Ehrlichia* spp. and its tendency to bite humans, the lone star tick is a medically important species in Alabama. The second and more recent study (Willis et al. 2012) focused on lone star tick ecology rather than its role in pathogen transmission and will be discussed in Chapter 3.

2.1.2 *Research objectives*

As described above, although tick-borne pathogens account for serious illness in Alabama, few studies on tick distributions or pathogen prevalence among ticks have been carried out in the state. Our study was conducted in Lee and Macon Counties in Alabama where our goal was to investigate tick diversity, tick density and pathogen prevalence in recreational areas where people may be at risk of acquiring a TBD. To this end, we collected ticks from forested parks in and around the city of Auburn, from Chewacla State Park, and from Tuskegee National Forest. We then identified them to species, and screened their DNA for pathogens using real-time PCR. Our objective was to understand how the risk of acquiring tick-borne diseases varies among recreational sites across the region.

2.2 Methods

2.2.1 *Tick sampling*

Ticks were collected from eight sites commonly used for recreational purposes in or near Auburn, AL, between May 4 and June 19, 2015 (Table 2.2). Selected sampling locations

Table 2.2. Tick sampling locations and sampling effort at each site.

Location		GPS Coordinates	Total Sampling Effort (hrs)
Chewacla State Park (CH)		32.553°N, 85.468°W	7.0
Hickory Dickory Park (HD)		32.635°N, 85.490°W	3.5
Lake Wilmore (LW)		32.565°N, 85.462°W	5.0
Louise Kreher Forest Ecology Preserve (LK)		32.665°N, 85.486°W	7.0
Tuskegee National Forest (TK)	TK.5	32.443°N, 85.636°W	6.0
	TK.15	32.492°N, 85.604°W	6.0
	TK.20	32.504°N, 85.567°W	7.0
Westview Park (WV)		32.619°N, 85.497°W	7.0

[σ]Tuskegee National Forest is divided into twenty compartments by the U.S. Forest Service. Ticks were sampled from three compartments, which are abbreviated as TK.5, TK.15, TK.20.

consisted of deciduous woodlands with leaf litter and shrubs, including Chewacla State Park, Hickory Dickory Park, Lake Wilmore Park, Louise Kreher Forest Ecology Preserve, Tuskegee National Forest (3 sites) and Westview Park. Ticks were collected by flagging using a 1 m^2 piece of white flannel cloth attached to a wooden dowel. The cloth was pulled through vegetation across the forest floor and checked approximately every three min to remove any attached ticks (Strickland et al. 1976). Ticks were sampled at weekly intervals for 60 min at each site except for Hickory Dickory Park, which was only sampled for 30 min due to its small size. Subsequently, collected ticks were temporarily stored on ice packs, transported to the lab, and then frozen at - 20 ˚C until needed for further study.

2.2.2 *Tick identifications*

Ticks were identified morphologically using both a compound microscope and a dissecting microscope. They were categorized by life stage (adult or nymph) and sex and then identified to

species using dichotomous keys for adult hard ticks (Keirans and Litwak 1989) and for nymphs in the genera *Amblyomma* (Keirans and Durden 1998) and *Ixodes* (Durden and Keirans 1996).

2.2.3 *DNA extraction*

Genomic DNA was extracted using the E.Z.N.A.® Tissue DNA Kit (OMEGA Biotech, Norcross, GA), following the manufacturer's instructions with one modification: DNA was eluted with ultra-pure water instead of elution buffer for the second elution to allow for concentration of the samples if necessary. Prior to extraction adult ticks were cut into four equal parts with a razor blade, while nymphs were bisected longitudinally due to their smaller size. Between samples, the razor blade and cutting surface were cleaned with 10% bleach and 70% ethanol. Ticks were then transferred to 1.5 ml microcentrifuge tubes and homogenized with a pestle in 200 µl of lysis buffer provided with the kit. Twenty-five µl of protease solution was then added to each tube and incubated at 55 ˚C for 2.5 h. RNase A (100 µg/ml) was then added to adult (4 µl) and nymph (2 µl) samples and incubated at room temperature for five min. Tubes were centrifuged at 17,000 x *g* and the supernatant transferred to new 1.5 ml tubes. Next, 220 µl binding buffer was added to each sample, which was then vortexed and incubated at 70 ˚C for 10 min. Following incubation, 220 µl of 95% ethanol were mixed with the contents of each tube and the entire solution was transferred to a HiBind® Mini spin column. Samples were centrifuged at 17,000 x *g* and filtrates were discarded. Spin columns were washed with HBC buffer and then twice with DNA wash buffer. Subsequently, DNA was eluted with 50 µl of pre-warmed (70 ˚C) elution buffer, followed by a second elution with 50 µl of ultra-pure water, and then stored at

- 20 ˚C until used for PCR.

2.2.4 *Pathogen screening*

DNA extracted from samples of *A. americanum* were tested for three ehrlichial and two rickettsial species by a multiplex real-time PCR assay which was modified from that of Gaines et al. (2014) using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The genes targeted in the assay include 16S ribosomal RNA (*Ehrlichia chaffeensis*, *Ehrlichia ewingii*), (Panola Mountain *Ehrlichia*), and outer membrane protein B (*Rickettsia amblyommii*, *Rickettsia parkeri*). Reactions were performed with 4 µl of 5x PerfeCTa® MultiPlex qPCR ToughMix® (Quanta Biosciences, Gaithersburg, MD) in a total volume of 20 µl with 1.0 µl DNA template. Primers and probes (Sigma-Aldrich, St. Louise, MO) were used at final concentrations of 0.3 µM and 0.1 µM per well, respectively (Table 2.3). Reaction conditions involved a 3 min denaturation at 95 ˚C, followed by 40 cycles each of a 15 s denaturation at 95 ˚C and a 1 min annealing/extension at 61 ˚C. Each set included two "no-template controls", with nuclease-free water instead of DNA template, and DNA from three ticks as positive controls of bacterial targets for *E. chaffeensis*, *E. ewingii*/*R. parkeri* (a doubly infected tick), and *R. amblyommii* (kind gifts from Dr. D. E. Norris, Johns Hopkins School of Public Health). Post-reaction thresholds were adjusted manually based on reaction curves for no-template controls. To minimize false positives, one fixed threshold of 2×10^3 relative fluorescence units (RFU) was set for all reactions of *R. amblyommii* based on relatively high RFUs in both positive controls and DNA samples from this study.

Table 2.3 Primers and probes used in the multiplex real-time PCR assay.

Bacterial Target	Gene Target	Type	Oligo Name	Sequence (5'→3')	Amplicon Size
E. chaffeensis/ E. ewingii	16S rRNA	Fwd primer	Ech_16S_17f	GCGGCAAGCCTAACACATG	81bp
E. chaffeensis	16S rRNA	Rev primer	Ech_16S_97r	CCCGTCTGCCACTAACAATTATT	
		Probe	Ech_16S_38bp	[Cy5]AGTCGAACGGACAATTGCTTATAACCTTTTGGT[BHQ3]	
E. ewingii	16S rRNA	Rev primer	Eew_16S_97r	CCCGTCTGCCACTAACAACTATC	
		Probe	Eew_16S_38bp	[6FAM]AGTCGAACGAACAATCCTAATAGTCTCTGAC[BHQ1]	
Panola Mountain *Ehrlichia*	*gltA*	Fwd primer	PMEhr_gltA_214f	TGTCATTTCCACAGCATTCTCATC	121bp
		Rev primer	PMEhr_gltA_334r	ATTAGCGCAATCATACTTGCAA	
		Probe	PMEhr_gltA_266pb	[HEX]TGCCTTAGCTGCACATTATTGTGAT[BHQ1]	
R. amblyommii	*OmpB*	Fwd primer	Ra_OmpB_477f	GGTGCTGCGGCTTCTACATTAG	142bp
		Rev primer	Ra_OmpB_618r	CTGAACTTGAATAAATCCATTAGTAACAT	
		Probe	Ra_OmpB_532pb	[TxRd]TCCTCTTACACTTGGACAGAATGCT[BHQ2]	
R. parkeri	*OmpB*	Fwd primer	Rp_OmpB_127f	CAAATGTTGCAGTTCCTCTAAA	98bp
		Rev primer	Rp_OmpB_224r	AAAACAAACCGTTAAAACTACCG	
		Probe	Rp_OmpB_162bp	[Cy5.5]AATTAATACCCTTATGARCASCAGCAG[BHQ3]	

2.2.5 *Statistical analyses*

For comparisons of tick density between locations, an index of density was calculated as the average number of ticks sampled in each sampling hour. Density was analyzed by one-way

Analysis of Variance (ANOVA), followed by Tukey's multiple pairwise comparisons test using GraphPad Prism® 7 (GraphPad Software Inc., La Jolla, CA). For comparisons of pathogen prevalence between tick life stages, a *z*-test compared two proportions of pathogen prevalence for nymphs and adult ticks. The *z* statistic was calculated as $z = (p1 - p2)/\sqrt{pt(1-pt)(\frac{1}{n1}+\frac{1}{n2})}$, where $p1$ was pathogen prevalence in adult ticks, $p2$ was the prevalence in nymphs, pt was the total prevalence, *n1* was the adult sample size, and *n*2 was the nymph sample size. The significance of *z* scores was determined using an online *z*-test calculator (http://www.socscistatistics.com/tests/ztest/Default2.aspx). To compare prevalence of *R. amblyommii* among sampling locations, logistic regression was used to model prevalence with *R. amblyommii* as the dependent variable and location as the independent variable. Logistic regression was performed using the statistical program Stata® v. 11.0 (StatCorp LP, College Station, TX).

2.3. Results

2.3.1 *Tick density and diversity*

Between May 5 and June 19 of 2015, a total of 1310 ticks were collected from eight field sites (Table 2.4). Ticks were most abundant at LW, yielding 461 ticks, followed by WV, CH, and LK, each of which resulted in 220-284 ticks. The remaining four sites produced fewer than 40 ticks each. When normalized by sampling effort, the number of ticks collected per hour varied dramatically among sites ($F_{7,42}$ = 15.03, P < 0.0001), ranging from 2.6 at TK.15 to 115.3 at LW (Figure 2.2). Multiple comparisons of mean densities (ticks sampled per hour) between

locations revealed that density at LW was significantly greater than all other sites (Tukey's multiple pairwise comparisons q statistic ranged from 8.097 to 11.99, $P < 0.0001$). The only other significant difference between sites was found between WV and HD ($q = 4.559$, $P < 0.05$), with WV significantly higher.

A total of seven species were identified from the collections. By location, HD and WV had the highest tick diversity, as four species were found at each location. Sites TK.15 and LK each had three species, while sites TK.5 and TK.20 each had two. At CH and LW only the lone star tick was found, which also dominated the samples from all locations (Table 2.4). Of 1310 ticks identified to species, 1280 (97.71%) were *A. americanum*, while the other six species combined were represented by 30 (2.29%) specimens. *I. scapularis* was present at six sites, and although second in abundance, only 17 specimens were collected. The only other medically important species identified was *D. variabilis*, which was collected at a single site in Tuskegee National Forest (TK.15). Most (1213 or 92.6 %) ticks collected were nymphs with the remaining (97 or 7.4 %) being adults.

2.3.2 *Pathogen Infection prevalences in A. americanum*

Because *A. americanum* was so abundant, we chose to focus pathogen-screening efforts solely on this species. Of 1119 samples tested, the overall infection prevalences for *E. chaffeensis*, *E. ewingii*, Panola Mountain *Ehrlichia, R. amblyommii*, and *R. parkeri* were 0.27%, 0.45%, 0%, 54.51%, and 0% respectively. When comparing by life stage, 1.12% of adult lone star ticks

Table 2.4 Tick samples by geographic location and species.

Location[a]	Total Samples	*Am.*[b] *amer.*	*Dm. vari.*	*Hm. lepo.*	*Ix. brun.*	*Ix. cook.*	*Ix. dent.*	*Ix. scap.*
CH	234 (17, 217)[c]	234 (17, 217)	0	0	0	0	0	0
HD	33 (5, 28)	23 (5, 18)	0	2 (0, 2)	6 (0, 6)	0	0	2 (0, 2)
LW	461 (9, 452)	461 (9, 452)	0	0	0	0	0	0
LK	220 (20, 200)	212 (17, 195)	0	0	0	0	1 (0, 1)	7 (3, 4)
TK.5	25 (0, 25)	23 (0, 23)	0	0	0	0	0	2 (0, 2)
TK.15	13 (2, 11)	11 (0, 11)	1 (1, 0)	0	0	0	0	1 (1, 0)
TK.20	40 (3, 37)	39 (2, 37)	0	0	0	0	0	1 (1, 0)
WV	284 (41, 243)	277 (41, 236)	0	0	0	1 (0, 1)	2 (0, 2)	4 (0, 4)

[a]Geographic locations are abbreviated as specified in Table 2.2.
[b]Abbreviations for tick species are as follows*: Am. amer*. = *Amblyomma americanum*, *Dm. vari*. = *Dermacentor variabilis*, *Hm. lepo*. = *Haemaphysalis leporispalustris*, *Ix. brun*. = *Ixodes brunneus*, *Ix. cook*. = *Ixodes cookei*, *Ix. dent* = *Ixodes dentatus*, *Ix. scap*. = *Ixodes scapularis.*
[c]Numbers in parentheses denote the number of adult samples followed by the number of nymphs.

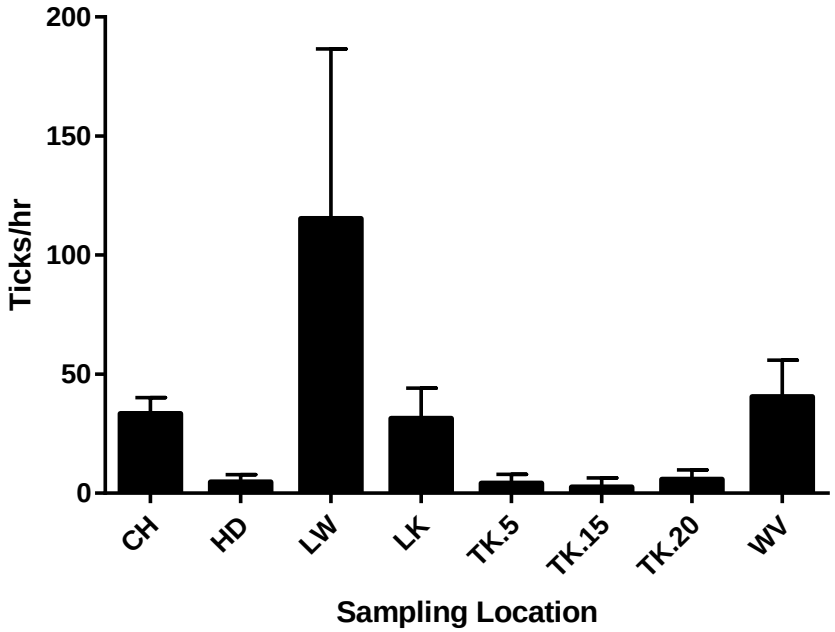

Figure 2.2 Variation in average ticks collected per hour among sampling locations. Abbreviations are in accordance with those given in Table 2.2.

tested positive for both *E. chaffeensis* and *E. ewingii*, whereas infection prevalences among nymphs were 0.19% and 0.39%, respectively. Neither of these differences between adults and nymphs were statistically significant (z = 1.627, P = 0.1038 and z = 0.998, P = 0.3184, respectively). For *R. amblyommii*, adults and nymphs showed much higher infection rates than for either of the two ehrlichial species. Infection prevalences were similar between life stages, as 49.44% and 54.95% of adults and nymphs tested positive, respectively. These differences were not statistically significant (z = - 0.9843, P = 0.3251).

When examining the prevalence data by location, *E. chaffeensis* was only found at two sites, with detection rates for nymphs < 1% (Table 2.5). Moreover, at LK adults had a significantly higher infection rate for adults compared to nymphs (z = 2.196, P = 0.0281). Infection prevalences for *E. ewingii* < 1% among positive sites, and infected at three locations. Adult ticks infected with *E. ewingii* were only found in CH. As for *R. amblyommii*, infection prevalence for lone star ticks irrespective of life stage or sampling location was 54.51%, but prevalence varied significantly from 9.09% in TK.15 to 69.68% in WV (likelihood ratio χ^2 = 95.0, df = 7, P < 0.0001) (Table 2.5). For each location, the 95% confidence interval of the logistic regression coefficient was used to determine which locations differed from one another as reported in Table 2.5. The influence of life stage on infection prevalence was also investigated by logistic regression but showed no effect (likelihood ratio χ^2 = 0.98, df = 1, P = 0.3220).

Lastly, we compared infection status of each positive tick for all three pathogens to investigate

Table 2.5 Pathogen infection prevalence in tick samples by geographic location.

Location[a]	*Ehrlichia chaffeensis* Infection Prevalence[b] (%)			*Ehrlichia ewingii* Infection Prevalence (%)			*Rickettsia amblyommii* Infection Prevalence (%)		
	Total	**Adult**	**Nymph**	**Total**	**Adult**	**Nymph**	**Total[c]**	**Adult**	**Nymph**
CH	0 (234)[d]	0 (17)	0 (217)	0.43 (234)	5.88 (17)	0 (217)	54.27 (234)	41.18 (17)	55.30 (217)
HD	0 (23)	0 (5)	0 (18)	0 (23)	0 (5)	0 (18)	65.22 (23)	20.00 (5)	77.78 (18)
LW	0.33 (300)	0 (7)	0.34 (293)	1.00 (300)	0 (7)	1.02 (293)	35.67 (300)	28.57 (7)	35.84 (293)
LK	0.94 (212)	5.88 (17)	0.51 (195)	0 (212)	0 (17)	0 (195)	64.15 (212)	52.94 (17)	65.13 (195)
TK.5	0 (23)	NT[e]	0 (23)	0 (23)	NT	0 (23)	30.43 (23)	NT	30.43 (23)
TK.15	0 (11)	NT	0 (11)	0 (11)	NT	0 (11)	9.09 (11)	NT	9.09 (11)
TK.20	0 (39)	0 (2)	0 (37)	0 (39)	0 (2)	0 (37)	57.50 (39)	100.00 (2)	56.76 (37)
WV	0 (277)	0 (41)	0 (236)	0.36 (277)	0 (41)	0.42 (236)	69.68 (277)	56.10 (41)	72.03 (236)

[a]Geographic locations are abbreviated as specified in Table 2.2.
[b]Prevalence for each pathogen was calculated as (number of positive ticks)/(number of ticks tested)*100.
[c]*Rickettsia amblyommii* infection prevalence varied significantly between locations by logistic regression (LR $\chi^2 = 95.00$, $df = 7$, $p < 0.0001$). To assess significance between sampling locations, location was modeled as an indicator variable and the 95% confidence interval of the regression coefficient for each site was compared. Sites with non-overlapping confidence intervals are considered different and are denoted by a different capital letter. Sites sharing a letter indicates overlapping confidence intervals, and hence, no difference in *R. amblyommii* infection prevalence.
[d]Numbers in parentheses specify the sample size tested by qPCR.
[e]NT = none tested.

rates of coinfection. Although no ticks were infected with all three, two ticks from LK were coinfected with *R. amblyommii* and *E. chaffeensis* and the two from LW were infected with *R. amblyommii* in combination with *E. ewingii*. This indicated that even though *Ehrlichia* was not frequently found in ticks across sampling areas, coinfection rates for *Ehrlichia*-positive ticks with *R. amblyommii* was relatively high, ranging from 40% for *E. ewingii* to 66.7% for *E. chaffeensis*.

2.4 Discussion

2.4.1 *Tick density, diversity, and disease risk*

Questing tick density varied among sampling locations. LW accounted for approximately one third of all specimens, despite a lower sampling effort that was 82.5% of all sites averaged. WV, LK, and CH also had high densities compared to HD and all three TK sites. A key difference between TK sites and the rest is a history of prescribed burning. The U.S. Forest Service conducts prescribed burns to manage the forest every few years, which is a possible explanation for low tick densities since it is not performed at the other sampling sites. Prescribed burning significantly changes physical conditions of the habitat and tends to decrease tick populations (Gleim et al. 2014). Another potential reason for low tick densities at TK is excessive soil moisture from flooding. Typically, high temperatures during summer months increase the rate of water loss in questing ticks (Estrada-Pena et al. 2012), which means that sufficient soil moisture improves survival. However, periods of standing water can cause tick mortality, and in fact, TK.15 is a flat area adjacent to a steam that flooded areas of the forest in proximity to the sampling site. Therefore, it is possible that TK.15 flooded in early spring or during the seven-week sampling period. Since we only visited the site once per week and there were days with heavy rainfall during this period, we cannot rule out brief flooding events at TK.15.

Among the seven species, *A. americanum* dominated all of the recreational locations, which indicated that lone star ticks are the most likely species encountered in deciduous forests during late spring and early summer. *Ixodes scapularis* was also present in 6 out of 8 sites, but

only comprised 1.3% of the samples. Although this species is the primary vector of Lyme disease, it was a low priority for pathogen screening because it was rarely collected. A third species of medical importance, *D. variabilis*, was also found but at even lower frequency, as only a single specimen was collected during the sampling period (at TK.15). Of the remaining four tick species collected, no studies have shown associations with human disease. Therefore, due to the frequency of *A. americanum* and its tendency to bite humans, ehrlichiosis is the tick-borne disease of most concern for recreational users of hardwood forests in east-central Alabama. Despite low infection rates with *Ehrlichia* spp. in these ticks, high encounter rates between lone star ticks and people suggest a low to moderate risk of exposure in Lee County.

2.4.2 *Potential significance of Rickettsia amblyommii*

In the lone star ticks screened in this study, *R. amblyommii* was found in more than half. Other studies have shown that *R. amblyommii* can be maintained by transovarial (vertical) transmission (Azad and Beard 1998), which likely accounts for the high infection rates often seen in the field. Although its pathogenesis in humans remains questionable (Hermance et al. 2014), some studies have shown that it may cause mild illness (Dasch et al. 1993, 2001; Sanchez et al. 1992). Researchers have also observed that *R. amblyommii* may cross react in serological tests for pathogenic *Rickettsia* spp., such as *R. rickettsii* and *R. parkeri* (Apperson et al. 2008), therefore causing confusion for diagnostic tests. Moreover, with the exception of the maculopapular rash often (but not always) seen with rickettsial infections, ehrlichial and rickettsial illnesses share symptoms and can be easily confused (Carpenter et al. 1999). Thus,

since lone star ticks transmit *Ehrlichia* spp. and are often infected with *R. amblyommii*, bites from co-infected ticks may lead to the misdiagnosis of ehrlichiosis as Rocky Mountain spotted fever or *R. parkeri* rickettsiosis. Additional studies in the eastern U.S. have shown comparable infection rates of *R. amblyommii* in *A. amblyomma* from Virginia (Gaines et al. 2014) and North Carolina (Apperson et al. 2008), and the authors of these reports raised similar concerns about the role of *R. amblyommii* in the potential misdiagnosis of human ehrlichial infections.

In addition to the above, *R. amblyommii* may have public-health consequences due to effects on other pathogens when co-infecting ticks. Multiple studies suggest that endosymbiotic *Rickettsia* spp. can reduce or inhibit *R. rickettsii* infections (Stromdahl et al. 2008). It has been hypothesized that this phenomenon may also apply to *R. parkeri* and explain why infections with this species are rare in lone star ticks. None of the *A. americanum* samples in our study, which had high rates of *R. amblyommii* infections, tested positive for *R. parkeri*. In this study, co-infections with *R. amblyommii* and either *E. chaffeensis* or *E. ewingii* occurred in four ticks suggesting that *R. amblyommii* has little effect on these species of bacteria. Due to the low number of ehrlichial infections, this question cannot be addressed from our data but is worth investigating in the future.

Chapter 3

Influence of Ecological Factors on Questing Behavior of Lone Star Ticks

3.1 Introduction

3.1.1 *Medical importance of lone star ticks in the Southeast*

The lone star tick, *Amblyomma americanum* (Acari: Ixodidae), has been recognized as a vector for multiple pathogens affecting humans and mammals, and is commonly found in the southeastern and eastern United States (Monzón et al. 2016). Within the Southeast, it is by far the most frequently encountered tick species (Merten and Durden 2000) that bites humans at each stage (larva, nymph, adult) (Childs and Paddock 2003). *Amblyomma americanum* ticks undergo a three-host life cycle like other medically important species in family Ixodidae, in that each ectoparasitic stage must find a new host and take a single blood meal. The process of transmitting tick-borne pathogens to and from hosts is tied to this behavior (Randolph 2004). In addition, the risk of acquiring tick-borne diseases is related to tick abundance within a specific geographic area and tick activity within a particular time period (Randolph 2000). Over the last few decades, the number of reported human infections associated with bites from lone star ticks has increased, including ehrlichiosis, tularemia (rare in Alabama) and southern tick-associated rash illness (Stromdahl and Hickling 2012, CDC 2016b). Evidence suggests that the prevalences of disease agents among lone star tick populations may be increasing (Randolph 2000, Stromdahl and Hickling 2012, Savage et al. 2013, Springer et al. 2014, Monzón et al.

2016).

3.1.2 *Factors influencing lone star tick density*

Both environmental and host-related factors determine densities and distributions of lone star ticks, yet these factors vary substantially over space and time. Arguably, tick ecology is more complex than that of other arthropod vectors, and it is a challenge to model the dynamics of the factors that predict tick abundance. However, because the density of host-seeking ticks is correlated with risk of human encounters with infected ticks, understanding tick ecology is key to reducing or preventing the transmission of tick-borne pathogens. Thus, Randolph (2004) proposed a simplified population model for the abundance of hard ticks focusing on four essential processes. First, unfed ticks of each stage must enter the population by successfully developing from the previous stage or by immigration through host movement. Second, under specific microclimatic scenarios, a certain percentage of ticks will quest actively for a host. However, when a tick quests, its probability of attachment to a host (the third process) varies and is affected by host-related factors, such as host diversity and density. Last, each life stage and state (questing, feeding and engorging) have habitat-dependent rates of mortality and emigration (also through host movement) that remove ticks from the population (Randolph 2004).

3.1.3 *Reproduction, survival and immigration*

The contribution of each ixodid female to population growth varies among species. Generally, engorged females take enormous blood meals of long duration and lay thousands of eggs (Oliver 1989). For *A. americanum*, an average of 6,000 eggs are usually produced through 16-31 days until the female senesces and dies (Sacktor et al. 1948, Lancaster and McMillan 1955, Sonenshine and Tigner 1969, Drummond et al. 1971, Koch 1983). This reproductive performance is influenced by the physical environment. Although egg production within the female tick is little affected by relative humidity (RH), both RH and temperature significantly influence periods of pre-oviposition (Lancaster and McMillan 1955, Sonenshine and Tigner 1969, Patrick and Hair 1979). Similar effects were also found in egg hatching. Within a habitat, the incubation period is longer in the cooler spring than in the warmer summer (Patrick and Hair 1979). Additionally, as many insects rely on water as a hatch stimulus (Hinton 1981), the yield of tick larvae is greatly improved when the eggs are placed at RH close to saturation (Lancaster and McMillan 1955, Sonenshine and Tigner 1969, Yoder et al. 2004, 2012).

After hatching from the eggs, both the larval and nymphal stages of *A. americanum* must blood feed on a host for successful development and molting to the next stage. Adult male and female lone star ticks also must feed and usually mate on the host so that fully engorged females drop off and lay eggs to maintain the population. Because they spend only a small portion of their life attached to the host, lone star tick survival strongly depends on the environment of their non-feeding periods and especially their ability to maintain water balance.

Lone star ticks are prone to desiccation and lose water through integumentary loss, the respiratory system, oral or anal excretion, and other related physiological processes (Knülle et al. 1982). However, because exhaustion of water reserves means death, hard ticks have adapted to live against the drying power of the atmosphere through physiological mechanisms, such as a thickened waxy coating on their cuticles (e.g., *A. americanum*) and absorbing water vapor from saturated or mildly sub-saturated atmospheres (e.g., *Ixodes scapularis*) (Sonenshine 2005). Hard ticks also use behavioral mechanisms to preserve water, including movement to microhabitats where rates of water loss fall below critical levels or where uptake of water vapor can occur (Knülle and Rudolph 1982, Needham and Teel 1991).

Because lone star ticks have such high fecundity, their reproductive output would lead to exponential population growth if mortality were not similarly high. Most hard tick species have remarkably constant population sizes with characteristic stage-specific mortality rates (Randolph 1994, 1997). Although it is not realistic to estimate the absolute mortality rates for natural tick populations, in theory, the combined mortality rates of all stages should exceed 99.9% of eggs laid if the population is at a state of equilibrium, i.e., only one egg-laying female tick per egg clutch survives to lay eggs in the next generation (Sonenshine and Mather 1994, p. 33). In fact, Randolph (1997) investigated one subtropical species, *Rhipicephalus appendiculatus*, and found results supporting this hypothesis. Randolph (1998) also reported the observation that mortality rates of interstadial phases (period between blood feeding and molting) increases with increasing tick density of the previous stage. In addition, Randolph

(1994) compared potential ecological factors relevant to mortality rates among different life stages and suggested that the stage from engorged females to emergence of larvae is most sensitive to unfavorable climatic conditions, whereas mortality for the other stages are more strongly influenced by density-dependent factors although climate also played a role (Randolph 2004).

In addition to successful egg hatching and development between stages, tick population density can be influenced by tick immigration. Because they are constrained by small body size and are unable to fly, ticks have very limited capacity for dispersal on their own. Movement over short distances (e.g., questing, seeking favorable microclimates) generally occurs through crawling, while long-distance movement most commonly occurs through passive transport on hosts. Thus, the density of populations in the area where feeding ticks detach from hosts may increase (Sonenshine and Mather 1994, p. 34). Given the high fecundity of lone star ticks, it is easy to see how immigration of blood fed adult females, for example, could bolster a tick population.

Although ticks usually have high mortality rates, unfed ticks of most species may survive a remarkably long period of time through behavioral and morphogenetic diapause, which are effective strategies to overcome adverse environmental conditions (Belozerov 1982). Typically, the life cycle of most three-host ixodid ticks takes one to four years. The duration of the life cycle varies among species and is influenced by a range of factors, including the length of the growing season and availability of hosts (Service 2012, p. 240). Interestingly, limited host

abundance can lead to longer lifespans because of delays in feeding and maturation (Sonenshine and Mather 1994, p. 26).

3.1.4 *Temporal and spatial variation in tick abundance*

Seasonally, life stages of lone star ticks show characteristic peaks in abundance, although there is overlap between the three ectoparasitic stages. In general, peaks of larval activity occur in late summer, while nymphal and adult activities peak in early summer and spring, respectively. Seasonal activity patterns, either within or outside the peak periods, vary geographically in relation to climatic conditions. In warmer southern states, adults become active earlier than in northern states, typically in March, and peak from April to May. Nymphs have two primary activity periods, (i) May to July for overwintering nymphs and (ii) August to September for current year progeny (i.e., larvae that successfully fed in July or August). In contrast, larval peaks occur in July to August (Semtner and Hair 1973b, Ludwig et al. 2016). Tick densities also change spatially at small scales. For example, some studies of *A. americanum* have shown that individuals tend to cluster within apparently uniform habitats (Patrick and Hair 1978, Jackson et al. 1996, Goddard 1997, Schulze et al. 2002). These spatial differences in tick density might reflect tick natality and mortality rates, movement by themselves orienting to favorable microhabitats, and by host activity as a result of detaching from and dropping off of hosts (Ostfeld et al. 1996, Schulze et al. 2002).

3.1.5 *Importance of environmental factors on tick biology*

Lone star ticks are vulnerable to environmental conditions, whether they are host-seeking or in the process of developing after blood feeding. Although these ticks largely depend on hosts for dispersal, they are free-living, non-nidicolous, and spend most of their lives off the host. Therefore, density-independent factors, such as abiotic characteristics of the physical environment greatly influence lone star tick activity, development, reproduction, and survival, and hence, its demography and geographic distribution (Sonenshine and Mather 1994, p. 91). Indeed, it has been proposed that tick spatial patterns are more limited by climatologic variables than by host-related factors (Klompen et al. 1996, Cumming 2002, Léger et al. 2012). Differences in temperature, humidity, and photoperiod play important roles in distributions of different ixodid species with varying degrees of influence (Daniel and Dusbábek 1994, Schulze and Jordan 2005). In one study, for example, the desiccation-tolerant species *Amblyomma maculatum* was more abundant in grasslands, while in contrast *I. scapularis* and *A. americanum* were most frequently encountered in brushy wooded areas (Sonenshine 1993, pp. 47-55). However, the actual space where a tick lives is recognized as its microenvironment. It is a small component of the larger habitat that usually consists of the lower part of vegetation, the leaf litter, and the upper layers of the soil (wood humus) (Sonenshine 1993, p. 28). The corresponding microscale abiotic factors are characterized as microclimate, which strongly influence short-term tick activities. In contrast, the climates of whole regions, which roughly delineate habitat types, is expected to affect tick populations in the medium- or long-term. Herein, it is important to distinguish the climatic conditions among different scales. The most common measurements of atmospheric conditions, those below the forest canopy but above

the microhabitat (i.e. mesoclimate), can only be used to describe variation in abiotic conditions between habitat types (Daniel and Dusbábek 1994). The major reason is that different compositions of forest overstories affect the character of the shrub layer vegetation and the leaf-litter layer, and concomitantly alter microclimatic conditions (Havens 1979). Therefore, measurements in the microsites where ticks host seek and undergo development more accurately characterize conditions that influence population performance (Schulze and Jordan 2005). Even in apparently uniform forest stands, subtle differences in vegetative cover that mostly result from patchy structure of shrubs may still have a moderating effect on the environmental conditions below the forest canopy (Havens 1979, Daniel and Dusbábek 1994). Denser shrub cover helps to maintain lower temperatures as well as reduce moisture and evaporation within the shrub understory. Thus, it can be inferred that microclimatic conditions at leaf litter and soil layers are also affected by the shrub layer vegetation and further by the groundcover (Schulze et al. 2002). Consequently, the distribution of certain tick species is often closely associated with specific types of vegetation (Service 2012, p. 241).

Collectively, most ticks have adapted to a specific favorable habitat (i.e. the optimum habitat) so that they tend to distribute non-randomly or non-uniformly (Sonenshine and Mather 1994, p. 26). Previous studies have suggested several potential indicators of tick distributions. For example, Semtner and Hair (1973b) investigated the influence of tree diversity on the population of *A. americanum* in Oklahoma and found that more ticks were found in sassafras, persimmon and winged elm habitats, while low numbers occurred in hay meadow, native

prairie, maple, pine and white oak habitats (Semtner and Hair 1973b). Another study conducted in southwestern Tennessee revealed that the abundance of lone star ticks was positively associated with increasing ground cover and basal area (Hendricks 2013). In addition, shrub cover and leaf litter depth were, respectively, negatively and positively associated with the number of adult and nymphal *I. scapularis* (Jordan and Schulze 2005, Schulze and Jordan 2005). It has also been reported that larval burdens of *Ixodes dammini* (now recognized as a geographic variant of *I. scapularis*) on white-tailed deer were positively related to the density of woody vegetation and negatively related to herbaceous vegetation (Adler et al. 1992).

Abiotic factors also constrain where, how and when ticks seek hosts (i.e., quest). Most inactive ticks live in sheltered places in the lowest layers of vegetation or in the leaf litter before they begin to seek hosts. There are two strategies for ticks to find a host, which is likely regulated by photoperiod (Belozerov 1982). The first, called questing, is an ambush and wait strategy in which ticks climb up vegetation and wait for direct contact with hosts as they brush by. While questing, they may lose water because humidity in the microenvironment is often well below saturation, particularly at warmer temperatures. Therefore, even though ticks may remain in an active state for several days or even weeks (Lees and Milne 1951, Loye and Lane 1988; Sonenshine and Mather 1994, p. 6), they retreat from the vegetation to moist surroundings frequently to regain water vapor (Rudolph and Knülle 1974, Kahl and Alidousti 1997). Once hydrated, they will climb the vegetation again. On the contrary, for species that have adapted to xeric habitats, they emerge from shelters to hunt hosts actively when animals are nearby

rather than sitting in the open and passively waiting (Randolph 2004, Estrada-Pena et al. 2012).

In addition, diurnal variation in host-seeking, which may be photoperiod-dependent and associated with temperature or humidity, influence tick temporal and spatial distributions as well. In experimental settings, both *I. scapularis* and *Ixodes ricinus* were primarily active during darkness. Such behavior might be a means to diminish water loss while selecting suitable questing locations through crawling (Carroll et al. 1998, Perret et al. 2003, Herrmann and Gern 2015). A field study conducted in New Jersey reported that *I. scapularis* adults tended to quest earlier and later in the day during periods of lower temperatures and higher humidity, whereas *A. americanum* were more frequently collected in late morning and early afternoon when temperatures were higher and humidity was lower (Schulze et al. 2001). Furthermore, it has been shown that different populations of the same species in different geographic areas or physiological states may behave differently. For example, *I. scapularis* from southern populations were less likely to emerge from the leaf litter when questing compared with northern populations (Arsnoe et al. 2015). Interestingly, Alekseev and Dubinina (2000) reported that infection status influenced questing, as different environmental conditions led to questing behavior in *Borrelia*-infected *Ixodes persulcatus* compared to uninfected ticks.

3.1.6 *Tick-host interactions*

One of the most important determinants of tick abundance is the accessibility of suitable hosts,

as blood meals are required for successful development and reproduction. Ticks do not feed equally well on all vertebrates; therefore, it is not surprising that some hard ticks are very specialized in their choice of hosts. It is noteworthy that the host immune system reacts to tick saliva during the period of feeding; and consequently, tick-host specializations are often the long-term outcome of an evolutionary "arms race" between the organisms (Sonenshine 1993). In addition, at the local scale, host availability and utilization are tightly related to the ecological conditions in which the ticks and hosts operate. For example, the microhabitats and times of day in which ticks quest must overlap in space and time with host activity. Lone star ticks are known to quest throughout the day (Schulze et al. 2001), and although they tend to feed on a variety of hosts, they are often found more frequently on a select few. Kollars and colleagues (2000a), for instance, investigated host associations of *A. americanum* in Missouri. They found that adult lone star ticks were most frequently collected from white-tailed deer. Nymphs were also commonly found on white-tailed deer, as well as on wild turkeys and raccoons. For larval ticks, eastern cottontail rabbits, white-tailed deer, raccoons, and squirrels were the most frequent hosts.

Animal hosts play a large role in the establishment and maintenance of tick populations. For example, white-tailed deer, the major host of *A. americanum* throughout its range, serve as a preferred food source which can support enormous numbers of ticks in favorable environments (Paddock and Yabsley 2007). In Arkansas, as many as 2,550 ticks per ear have been recorded on white-tailed deer (Goddard and Mchugh 1990). Therefore, the increase in white-tailed deer

abundance over the last few decades has likely caused an increase in tick abundance, particularly for lone star and black-legged ticks (Paddock and Yabsley 2007). In support of this idea, an experimental reduction in the abundance of white-tailed deer led to a substantial decrease in the number of adult lone star ticks (Adler et al. 1992). Furthermore, as mentioned above, hosts are important for tick dispersal. Ticks attached to hosts can be transported out of local populations through host movement and then re-localized to a new habitat (Paddock and Yabsley 2007). Semtner and Hair (1973) reported that the locations where engorged lone star ticks dropped off from deer in the spring and early summer predicted the distribution of hatched larvae later that summer (Semtner and Hair 1973b). In contrast, it has been shown that the higher the host density during a questing period, the greater the probability of ticks attaching (Randolph 2004). Therefore, host activity can have different effects in tick abundance depending on the season, as they can remove ticks during questing periods (emigration) but also act as a source of new ticks (immigration) when they drop from the host.

Given the above, it follows that host specificity and the corresponding activities of preferred hosts influence patterns of tick spatial and temporal distribution. The center of an area where the host undertakes most of its activities is called the home range. Hence, it is where engorged ticks are most likely to drop off. Additionally, the host home range is generally not fixed but varies with climatic conditions, seasons, and resources (Sonenshine and Mather 1994, p. 158). Hosts with small home ranges and habitat specificity likely have the greatest impact on tick distributions. For those which have a generalized affinity for habitats, detached ticks will likely

be found in mixed environments (Fryxell et al. 2015).

3.1.7 *Research objectives*

Ticks that live within a given habitat type possess physiological and behavioral characteristics that have been influenced by the climatic conditions and the types of vegetation and hosts typical of those habitats (Randolph 2004). Regardless, distributions of questing ticks tend to be unequally distributed within a habitat, which begs the question of which factors best predict questing behavior. Our study focused on a single forested habitat to investigate factors that influence spatial variation of the density of questing lone star ticks. The hypothesis was that tick questing behavior within a hardwood forest is driven by a combination of factors associated with microclimate, vegetation and animal hosts. A statistical modeling approach was used to examine relationships among questing nymph density and a variety of biotic and abiotic factors.

3.2 Methods

3.2.1 *Site selection and tick collection*

The study was conducted in a deciduous forest (33.088602° N, - 86.114859° W) north of Auburn, AL, managed by the School of Forestry and Wildlife Sciences of Auburn University. Based on a pilot study, *Amblyomma americanum* was known to occur at this location, which also provides suitable habitat for a variety of animals that may serve as hosts for ticks. Sixteen plots were established in the mid-slope elevation arranged in two arrays of eight plots, with a

minimum distance of 5 m between plot edges (Figure 3.1). Each plot was circular with a diameter of 15 m and was divided into four equal quadrants along cardinal directions.

Tick sampling was conducted consecutively for three days every other week from May 31 to September 12, 2016, encompassing 24 sampling sessions per plot. Collections were not performed when the vegetation was wet from rain or heavy dew. A conventional flagging method was used to sample questing ticks from vegetation and the forest floor using flags constructed from a 1 m^2 piece of white flannel cloth fastened to a wooden dowel (Strickland et al. 1976). In each plot, flags were checked for ticks after flagging each quadrant. Ticks were removed with forceps and preserved in coded vials of 99% ethanol. Vials were placed on ice packs and transported to the laboratory, where they were stored at 4 °C until identified to species, life stage, and sex by microscopy using taxonomic keys (Keirans and Litwak 1989, Durden and Keirans 1996, Keirans and Durden 1998, Coley 2015).

3.2.2 *Abiotic variable measurement*

Climatologic variables including temperature (T) (°C) and relative humidity (RH) (%) were recorded hourly throughout the sampling season on each plot using a HOBO® Pro v2 Logger (Onset Computer Corporation, Bourne, MA). The data logger was placed horizontally at the center of each plot approximately two inches above the forest floor. Saturation deficit (SD) (kPa), which is a measure of the drying power of the atmosphere, was calculated from the temperature and relative humidity data using the following formula: SD = (1–RH/100) x

Figure 3.1 The arrangement of 16 sampling plots at the North Auburn site, AL. The inset map shows the location of the North Aubrn site relative to the city of Auburn, AL.

$4.9463e^{0.0621T}$ (Schulze et al. 2001).

Abiotic variables including forest gravimetric moisture (GM) (%) and forest-floor depth (mm) were measured at the last sampling session of each month. Forest gravimetric moisture provides a measure of water content in the forest floor layer. A total of three samples of the forest floor were collected per plot from 0.1 m^2 areas chosen immediately adjacent to the western, southern and eastern edges of each plot (cardinal directions selected at random *a*

priori). Each sample was gathered by removing the forest floor debris (e.g. leaves, twigs, duff, loose soil) by hand and placing the materials in a paper bag. Care was taken to avoid areas that had been excessively compacted by foot traffic. Subsequently, forest-floor samples were weighed, placed in a drying oven at 68°C for 48 h, and then weighed again. The gravimetric moisture was determined from the sample wet (m_w) and dry (m_d) masses using the following formula: GM = $(m_w – m_d)/m_d$ x 100. The depth of forest floor was recorded using the probe of a vernier caliper by pushing it into the leaf litter until encountering the surface of the mineral soil. Measurements were made at 20 locations in each plot (five per quadrant), including three points at 2.5 m intervals along a transect from plot center to the midpoint of the quadrant arc along the outer edge and two points to the right and left of the transect at the 5 m point.

3.2.3 *Vegetation characterization*

Vegetation-related factors were classified as discrete or continuous variables. Within each transect, vegetation characteristics were characterized by quadrant including the number and species of mature trees (greater than 1.37 m in height), saplings, seedlings, and canopy characteristics. Vegetation data were recorded for every plot during July 2016, when canopy cover was at or near annual peak. For trees taller than 1.37 m, data were recorded for crown class (e.g., dominant, codominant, intermediate, suppressed) and diameter at “breast height” (DBH; cm) using a DBH tape measure (Forest Suppliers, Jackson, MS). The height of each tree was estimated using a clinometer (Forest Suppliers, Jackson, MS). To determine the vertical structure, basal area (m^2) was estimated by using a 10x factor wedge prism (Forestry Suppliers,

Jackson, MS). In addition, the openness of the forest canopy (%) was determined with a densiometer (Forestry Suppliers, Jackson, MS). For the latter, measurements were taken from four cardinal directions at the center of each plot. Woody stems between 0.3 and 1.37 m in height were considered saplings and seedlings, each of which throughout the plot was identified to species and measured for height. Within each quadrant, ground cover was characterized in a 0.1 m^2 quadrat. For each sampling event, the order, direction, and distance from the center by number of paces were selected at random using a deck of playing cards. The composition and structure of the plant community, as well as the percent cover of leaf litter, rocks, downed wood (i.e., logs, sticks), and bare mineral soil, were recorded simultaneously.

3.2.4 *Vertebrate data collection*

During the sampling season (May 31 to September 12 2016), 16 game cameras (Moultrie® A-5 Gen2, EBSCO Industry Inc., Calera, AL) were installed on trees along the northern (downslope) boundary at every plot to investigate activities of large- or medium-sized hosts. The cameras were specified to cover a sampling radius of 50 m, and each was placed at an appropriate height focused on plot center. Cameras were set to operate 24 h a day upon motion detection and were set to Multi-Shot mode with a 10 s delay, so that a three-photo burst was captured per event with a 10 s period elapsing before the next event could be detected.

In addition, small mammals were trapped three nights per week every other week from May 31 to September 7, 2016, to match tick-sampling effort. Four 35.6 cm Sherman live traps (H.B.

Sherman Traps Inc., Tallahassee, FL) were placed in the quadrant of each plot approximately 3.75 m from the center in cardinal directions. Bait for these traps was peanut butter mixed with rolled oats and suet. All animals collected were identified and then released.

3.2.5 *Statistical analyses*

Climate variables (T, RH, SD) for each plot were averaged to yield weekly mean values, and observed weekly maximum and minimum values were also used in the analyses. The average depth of forest floor layer was estimated by averaging all 20 measurements among four quadrants to yield a value for each plot for each of the three sampling events. As for vegetation variables, data from overstory inventories yielded tree diversity (number of tree species), tree density (tree number per plot), dominant tree species (ranked by the sum of DBH of conspecific trees) for each plot. Similarly, the composition of saplings,seedlings, and ground cover in quadrants were averaged to yield the percent value for each plot or summed to yield the total number. For host abundance, the number of large- and medium-sized animals captured by photo was calculated by the total count of each event, where the repeated presence of the same species within one hour was considered the same event. The number of small animals captured by Sherman trap was added to the photo captures to approximate the total number of available hosts.

To examine relationships between questing nymph density of lone star ticks and our selected explanatory variables, a stepwise Poisson regression model was applied using Stata® v. 14.2

(StatCorp LP, College Station, TX). Modeling began with simple models using two to three variables which were hypothesized *a priori* to influence questing behavior (e.g., mean RH, mean T). For each model, a likelihood ratio test was performed to test the hypothesis that at least one regression coefficient in the model was significantly different from zero (α = 0.05 applied as the level of significance). In addition, for models with significant P-values, *Z*-tests were used to assess whether each regression coefficient significantly differed from zero (α = 0.05 applied as the level of significance). Following the stepwise addition procedure, variables were added singly to significant models and the likelihood ratio test was repeated. Models were compared for relative goodness of fit to one another using the Akaike information criterion (AIC) and the Bayes information criterion (BIC). These model selection criteria estimate how well each model maximizes the likelihood functions while preventing overfitting; and therefore, the smallest AIC or BIC values are used to select the best model. Furthermore, variables were examined for possible collinearity by determining the correlation coefficient (*r*) for each pairwise combination of variables, using a threshold of $|r| > 0.5$. Thus, any variables with $|r| > 0.5$ were not allowed to be included in the same model.

3.3 Results

3.3.1 *Density and seasonal activity of nymphs*

A total of 1348 lone star ticks were captured from May 31 to September 12, 2016, which included 18 (1.34%) adults, 480 (35.60%) nymphs and 849 (62.98%) larvae (Figure 3.2). For the adults, both males and females had 9 specimens collected. Two peaks of the number of nymphs

were observed over the sampling season, with the first peak occurring in early June (weeks 1-3) and the second relatively smaller peak in August (weeks 5-8) (Figure 3.3). Nymphs encountered in week 4 were not included in either peak due to the likely temporal overlap of the two cohorts. During the first peak, a total of 285 nymphs were collected with values ranging from a low of 1 (plot 2) and a high of 45 (plot 13). Similarly, a total of 269 nymphs were collected during the second peak, with the greatest number of nymphs collected in plot 8 (n = 36) and the lowest number collected in plots 6 and 7 (n = 3). When comparing the difference between the two peaks, the largest increase and decrease of sampled ticks occurred in plot 8 ($|n_d| = 35$) and plot 5 ($|n_d| = 7$), respectively. In addition, larvae only emerged from July to September and the majority were collected in a single week in July (n = 485). Large numbers of ticks in this stage were collected in plot 3 (n = 257) and plot 4 (n = 321). Because only a small number of adults were encountered and because of the higher medical importance of nymphs among all three life stages, only the density of questing nymphs was considered in the following analyses.

3.3.2 *Summary of data collected for selected explanatory variables*

Selected environmental variables measured between May and July are shown in Table 3.1. For abiotic factors, forest-floor gravimetric moisture ranged from the lowest of 8.1% in plot 6 to 19.8% in plot 9, and mean forest-floor depth varied from a minimum of 33.5 mm in plot 7 to a maximum of 52.0 mm in plot 12. For biotic factors, a total of 23 tree species were present Also, since two peaks of nymphs represent different cohorts, independent analyses were conducted for each. Only the analysis for the first cohort is presented here.

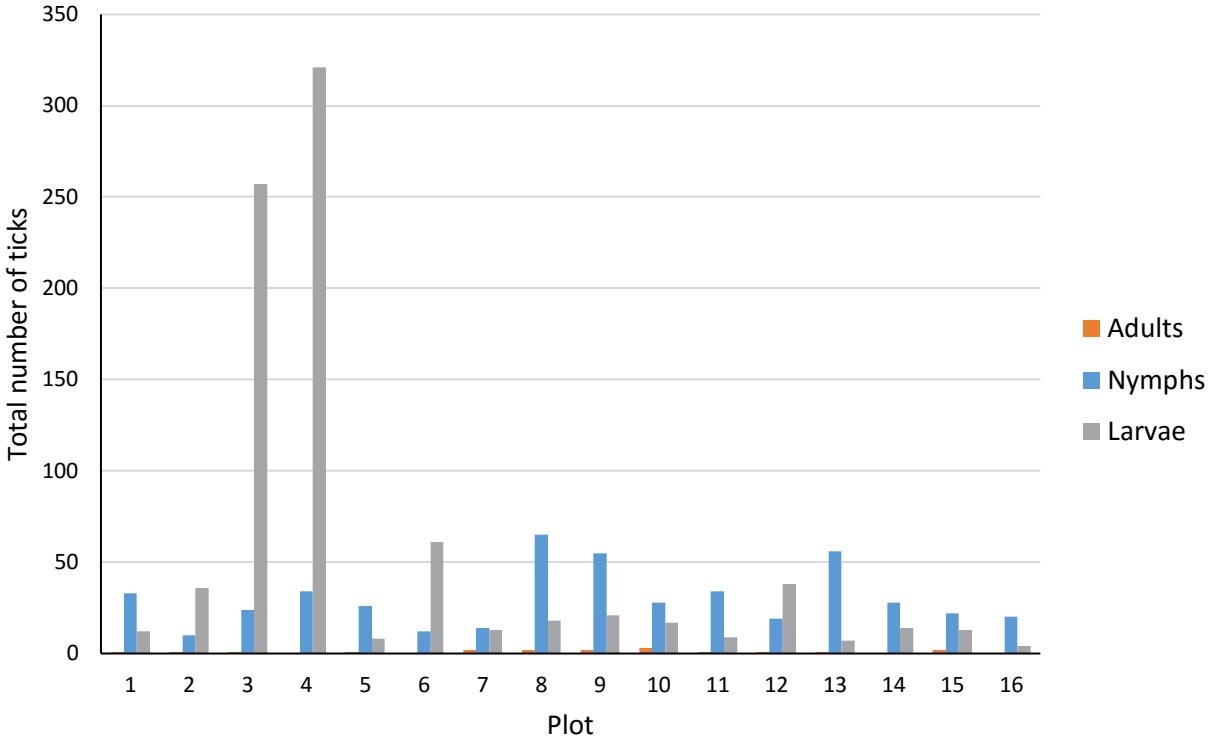

Figure 3.2 Total number of questing *Amblyomma americanum* by plot and stage from May 31 to September 21, 2016 in North Auburn site, AL**.**

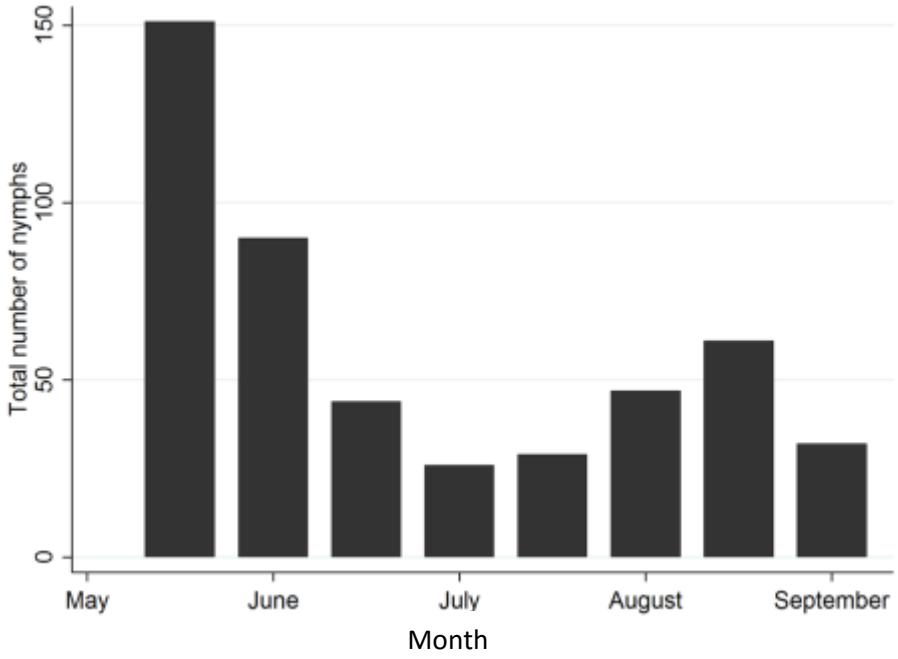

Figure 3.3 Total number of questing *Amblyomma americanum* nymphs collected by week from all 16 Plots at the North Auburn site, AL, from May 31 to September 12, 2016.

throughout all plots, with 14 species as the maximum in plot 10 and 8 species as the lowest in plot 15 (Table 3.2). Specifically, the top five species, which accounted for 70% of all trees present, included white oak, sweet gum, mockernut hickory, red oak and American beech. Canopy cover ranged from 91.7% in plot 4 to 77.9% in plot 5. As for animal activity, total trapping effort (including Sherman traps and game cameras) yielded at least four mammalian species (48 total captures) (Table 3.3). The species with the highest frequency was the raccoon (*Procyon lotor*) with 21 captures, followed by the white-footed mouse (*Peromyscus leucopus*) with 12 captures, eastern gray squirrels (*Sciurus carolinensis*) with 4 captures, and white-tailed deer (*Odocoileus virginianus*) with 4 captures. Of the remaining photo capture, animals from 8 occurrences were unable to be identified. No animals were observed in plots 5, 6, 9 and 11, while plot 2 had the highest occurrence of potential hosts with 15 total captures, 9 of which were raccoons. Moreover, results of three climatic variables were also listed. Plot 8 had the highest weekly mean relative humidity (80.12%), lowest weekly maximum temperature (33.26°C) and lowest weekly mean saturation deficit (4.55 kPa) together among all of the plots. Other than that, weekly maximum temperature (37.70°C) was highest in plot 14, whereas both minimum weekly mean relative humidity (77.7%) and maximum weekly mean saturation deficit (5.22 kPa) were observed in plot 1.

3.3.3 *Optimal regression model*

To better understand the relationship between the number of questing nymphs and environmental factors, the top three Poisson regression models are presented according to AIC

Table 3.1 Total number of nymphs and values of selected *a priori* environmental variables by plot from May 31 to July 2, 2016.

Plot	Total Number of Nymphs	Forest-Floor Gravimetric Moisture (%)	Mean Forest-Floor Depth (mm)	Tree Diversity	Canopy Cover (%)	Total Number of Hosts	Weekly Mean RH (%)	Weekly Maximum T (°C)	Weekly Mean SD (kPa)
1	24	17.2	40.7	9	84.1	4	77.70	34.94	5.22
2	1	14.1	38.9	10	87.5	15	79.17	36.42	4.82
3	7	17.1	35.9	10	88.3	2	NA[a]	NA	NA
4	21	16.3	48.7	7	91.7	1	79.38	33.73	4.72
5	7	16.0	39.0	11	77.9	0	79.43	34.45	4.79
6	7	8.1	34.6	6	90.1	0	79.03	33.54	4.81
7	11	18.8	33.5	10	80.0	1	78.73	33.80	4.93
8	26	17.2	40.8	10	89.3	2	80.12	33.26	4.55
9	29	19.8	50.9	10	81.5	0	78.03	34.42	5.07
10	20	16.1	44.5	14	78.9	4	79.14	33.77	4.81
11	20	14.0	49.1	8	85.2	0	77.89	34.77	5.15
12	13	11.9	52.0	11	82.8	8	78.56	33.95	5.00
13	45	14.5	51.6	13	90.9	3	77.80	33.86	5.15
14	22	17.5	50.2	9	85.7	2	78.17	37.70	5.12
15	16	18.2	41.9	8	85.2	1	77.94	35.14	5.13
16	16	13.6	47.0	11	86.7	6	77.86	35.62	5.21

[a]NA=not available due to data logger malfunction during May and June 2016.

Table 3.2 Total number and ranking of each tree species by plot at the North Auburn field site, AL.

Tree Species	Total Number of Each Tree Species (Rank[a])															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
American beech	1(6)	5(4)	0	8(1)	0	8(1)	2(5)	2(6)	2(6)	4(7)	0	3(7)	1(10)	5(4)	1(7)	2(6)
Ash	0	0	0	0	0	0	0	0	0	1(5)	0	0	0	0	0	0
Basswood	0	0	0	0	0	0	0	0	0	0	0	3(6)	1(9)	2(6)	0	0
Buckeye	0	0	0	0	0	0	1(9)	0	0	0	0	0	0	0	0	1(11)
Cherry	1(7)	0	0	0	0	0	0	0	2(5)	2(9)	0	0	0	0	0	0
Chinese privet	3(9)	1(10)	2(9)	0	3(9)	1(6)	0	1(10)	3(8)	0	0	1(9)	1(12)	2(8)	0	2(10)
Dogwood	0	0	1(7)	0	1(11)	0	1(7)	6(3)	0	1(13)	0	0	0	0	0	0
Elm	3(4)	6(7)	1(10)	1(7)	2(8)	0	1(4)	1(9)	2(7)	2(10)	1(8)	1(10)	2(8)	0	1(8)	4(7)
Hawthorn	0	0	0	0	0	0	0	0	0	0	0	0	1(13)	0	0	0
Hornbeam	0	1(9)	1(8)	0	0	0	0	0	0	0	1(7)	0	0	0	0	0
Mockernut hickory	0	0	0	0	0	0	3(10)	3(2)	3(3)	7(1)	4(1)	4(2)	7(2)	2(1)	0	1(4)
Pignut hickory	2(8)	1(8)	2(5)	0	1(6)	2(4)	2(3)	2(4)	1(10)	1(11)	3(4)	1(11)	3(4)	3(2)	1(6)	3(3)
Pine	0	1(5)	3(2)	0	0	0	0	0	0	0	0	0	0	0	0	0
Red bud	0	0	0	0	0	0	0	0	1(9)	1(14)	0	0	3(1)	0	0	1(9)
Red cedar	0	0	0	0	0	0	0	0	0	0	0	0	0	1(9)	0	0
Red maple	0	0	0	1(6)	0	0	0	0	0	0	0	0	0	0	0	0
Red oak	0	1(3)	0	1(3)	2(2)	0	0	0	3(1)	1(6)	0	2(3)	0	0	1(3)	0
Sweet gum	9(1)	15(1)	8(1)	1(5)	4(3)	1(3)	3(6)	4(7)	10(2)	3(4)	8(2)	3(5)	10(3)	2(7)	7(2)	3(2)
Tupelo	0	0	0	0	2(5)	0	1(8)	6(5)	0	2(12)	2(6)	2(8)	7(7)	0	3(5)	3(5)
Water oak	2(3)	3(2)	1(4)	0	1(4)	0	0	0	0	0	0	0	1(11)	0	0	0
White oak	2(2)	0	2(3)	3(2)	5(1)	1(5)	2(2)	3(1)	3(4)	3(3)	3(3)	3(1)	0	1(5)	4(1)	4(1)
Yellow poplar	0	0	0	0	1(7)	0	1(1)	0	0	1(2)	0	0	2(5)	0	0	0
Unknown	2(5)	3(6)	1(6)	2(4)	1(10)	3(2)	0	1(8)	0	2(8)	4(5)	4(4)	3(6)	5(3)	2(4)	1(8)

[a]Each tree species was ranked by the sum of DBH of all conspecifc trees in the plot.

Table 3.3 The occurrence of different sized mammalian hosts by week and plot from May 31 to September 12, 2016 in North Auburn site, AL**.**

Plot	Occurrence of Hosts																							
	Week 1			Week 2			Week 3			Week 4			Week 5			Week 6			Week 7			Week 8		
	L[a]	M	S	L	M	S	L	M	S	L	M	S	L	M	S	L	M	S	L	M	S	L	M	S
1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	2	0	0	7	0	1	1	0	0
2	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	6	0	2	3	0	0
3	1	2	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	NA	NA	NA	NA	NA	NA
4	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
5	0	3	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	3	0	0
6	0	2	0	0	0	0	0	0	0	0	0	0	3	0	0	3	0	0	3	0	0	2	0	0
7	0	0	0	0	NA[b]	NA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	3	0	0	NA	NA	0	0	0	0	0	0	2	0	0	0	0	0	3	0	0	0	0	1
9	0	2	2	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	2	0	0	1	1	0
10	0	4	2	1	0	0	0	0	0	0	0	1	1	0	0	0	1	0	2	0	1	2	3	0
11	0	3	0	0	0	0	0	0	0	0	0	0	1	0	0	0	2	1	0	0	1	0	1	0
12	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	2	0	2	0	0	1	1	0
13	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	1	1
14	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0
15	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	3	0	0	3	1	0
16	0	2	1	1	0	0	0	0	0	2	0	1	0	0	0	0	0	0	1	0	0	1	3	0

[a]Hosts were grouped by size as follows: Large-sized (L) animals included white-tailed deer; medium-sized (M) animals included coyotes and raccoons; small-sized (S) animals included white-footed mice and squirrels.

[b]NA indicated the loss of animal data.

and BIC values (Table 3.4). In comparing the models, five predictor variables were consistently represented: forest-floor gravimetric moisture (GM), forest-floor depth (FFD), tree diversity (TrDiv), canopy cover (CanCo) and the number of available hosts (TotHst). Additional variables in at least one of the top three models included weekly mean RH, weekly mean SD, and the combination of weekly mean RH (MeanRH) and weekly maximum T. Although the regression coefficient of MeanRH is non-significant, it appears to show a weak negative relationship with number of nymphs (Figure 3.4a). Moreover, its inclusion in the model decreased AIC and BIC values, which, in turn, indicated that the model with MeanRH is a better fit than those without it. In conclusion, based on selection criteria (AIC and BIC), the best-fit model from our data can be written as:

$$\text{Log}(\#\text{Nymphs}) = b_0 + b_1(\text{GM}) + b_2(\text{FFD}) + b_3(\text{TrDiv}) + b_4(\text{CanCo}) + b_5(\text{TotHst}) + b_6(\text{MeanRH})$$

A summary of this regression model is shown in Table 3.5. The regression coefficient for each variable can be interpreted as the difference in the log expected number of nymphs for a one unit change in the predictor variable given that the other predictors in the model are held constant. The analysis implies a positive relationship between forest-floor gravimetric moisture and the number of nymphs sampled (Figure 3.4b), suggesting that more ticks quest when their shelters had higher water content. However, in contrast, as alluded to above, MeanRH had a negative relationship. Admittedly, the coefficient is non-significant, but it does suggest that perhaps there is an optimal RH, above which questing is reduced. Similar to GM, a positive relationship was found between tick numbers and forest-floor depth (Figure 3.4c). In other words, more nymphs quested in the plots in which more leaf litter had accumulated. Questing

nymph density was also significantly influenced by tree diversity (Figure 3.4d), with more ticks sampled on average in habitats with a greater number of tree species. In the model, canopy cover also showed a positive relationship with number of questing lone star ticks (Figure 3.4e), which indicated that more nymphs tend to be found in shaded areas. The last variable in the model, TotHst, showed a negative relationship with the total number of nymphs sampled (Figure 3.4f). For this variable, fewer nymphs were found on plots with greater host activity. Finally, to test whether any predictor variables were collinear (i.e., non-independent), correlation coefficients were calculated for every pair of six predictor variables. None had $|r| > 0.5$ suggesting no strong relationships among any of the predictor variables in the model (Table 3.6).

3.4 Discussion

3.4.1 *Abiotic variables*

To date, few studies have investigated the combination of host-related factors, climate, and vegetation on questing tick densities. The top regression model indicates that both biotic and abiotic variables play roles in influencing questing behavior of *A. americanum* nymphs. Of the variables tested, traditional climatic variables surprisingly had the weakest effects on questing nymph densities while forest-floor gravimetric moisture had a significant effect and may be a better indicator of humidity in the leaf litter. However, since including either of weekly mean relative humidity, weekly maximum temperature, or weekly mean saturation deficit did improve performance of the regression model when added to the other five variables, it

Table 3.4 Top three Poisson regression models selected by AIC and BIC for the relationship between total number of questing *Amblyomma americanum* nymphs and eight explanatory variables, consisting of forest-floor gravimetric moisture (GM), forest-floor depth (FFD), tree diversity (TrDiv), canopy cover (CanCo), the number of available hosts (TotHst), weekly mean relative humidity (MeanRH), weekly maximum temperature (MaxT) and weekly mean saturation deficit (MeanSD) from May 31 to July 2, 2016.

Poisson Regression	P > |z|									df	AIC	BIC
	GM	FFD	TrDiv	CanCo	TotHst	MeanRH	MaxT	MeanSD	Cons[a]			
Model 1	0.010	0.042	0.000	0.000	0.001	0.133	--[b]	--	0.561	7	98.75803	103.7144
Model 2	0.010	0.037	0.000	0.000	0.001	--	--	0.140	0.000	7	98.84415	103.8005
Model 3	0.005	0.022	0.003	0.000	0.023	0.056	0.200	--	0.205	8	99.03517	104.6996

[a]Cons represents the model constant. This is the Poisson regression estimate when all variables in the model are evaluated at zero.
[b]The dash indicates that this variable is excluded in the model.

Table 3.5 Summary of Poisson regression model for the relationship between total number of questing *Amblyomma americanum* nymphs and eight explanatory variables, consisting of forest-floor gravimetric moisture (GM), forest-floor depth (FFD), tree diversity (TrDiv), canopy cover (CanCo) and the number of available host (TotHst) and weekly mean relative humidity (MeanRH), from May 31 to July 2, 2016.

Total Nymphs	Coef.[a]	$e^{coef.}$ [b]	P > |z|
GM	0.075	1.078	0.010
FFD	0.028	1.029	0.042
TrDiv	0.139	1.149	0.000
CanCo	0.069	1.072	0.000
TotHst	- 0.086	0.917	0.001
MeaRH	- 0.140	0.870	0.133
Constant	4.371	79.120	0.561

[a]Coef is the estimated Poisson regression coefficient for the model, which can be interpreted as the difference in the logs of expected number of nymphs for a one unit change in the predictor variable given that the other predictors in the model are held constant.
[b]e^{coef} represents log transformed regression coefficient as expected tick counts.

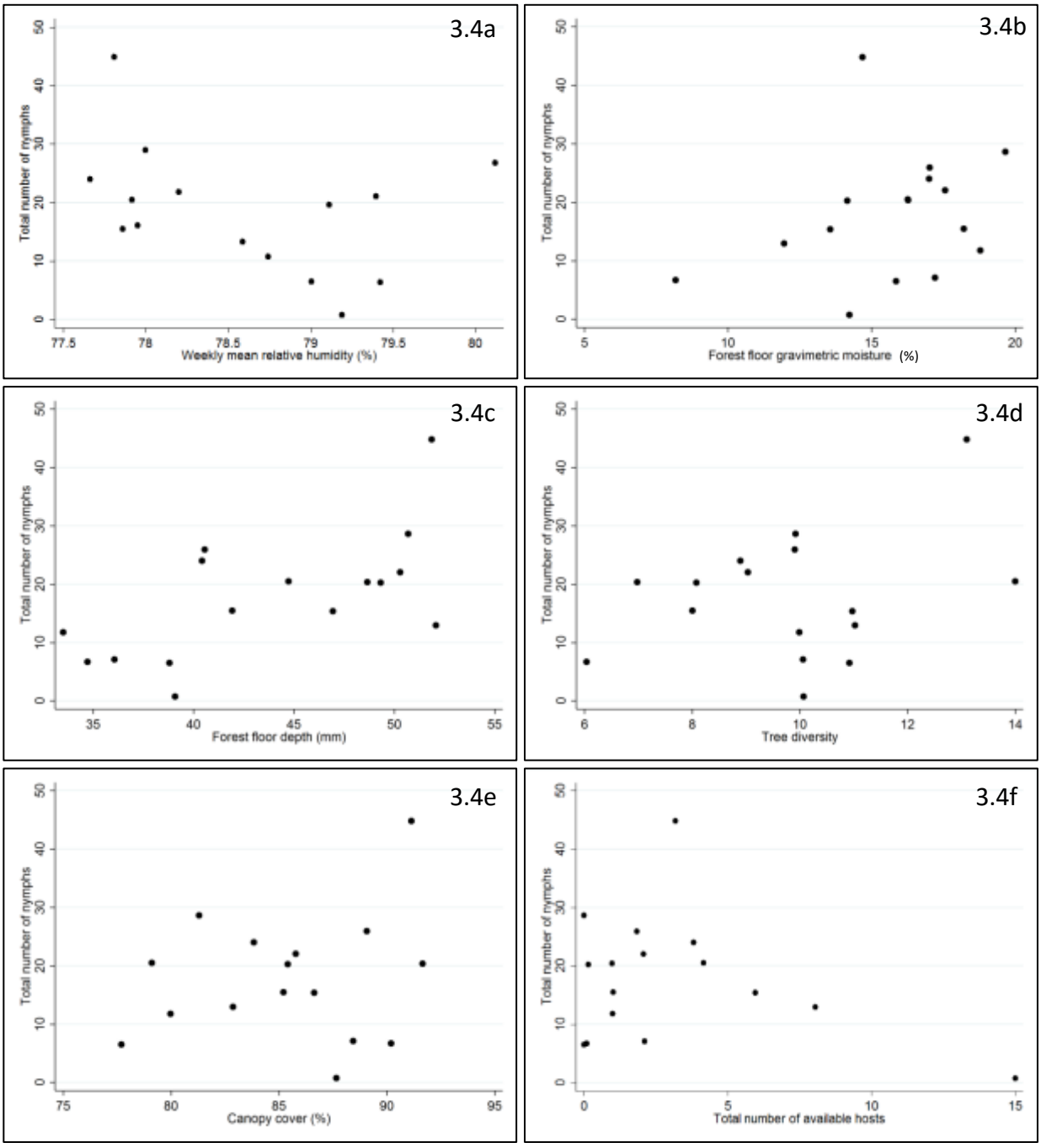

Figure 3.4 Scatterplots showing relationships between the number of questing *Amblyomma americanum* nymphs and a) weekly mean relative humidity, b) forest-floor gravimetric moisture, c) forest-floor depth, d) tree diversity, e) canopy cover, f) the number of available hosts, from May 31 to July 2, 2016.

suggests that microclimate just above the leaf litter influences questing activity to a certain extent. *A priori*, we suspected that more ticks would be found in more humid environments; and although a non-significant, negative relationship was found between weekly mean relative humidity and the number of nymphs, an optimal range of relative humidity that is favorable for questing might exist. Moreover, an interaction may occur between temperature and relative humidity so that their independent effects are difficult to tease apart. This has been consistently found in previous studies (e.g., Schulze et al. 2001), so dismissing this result may be premature. For example, it was reported that the expansion of lone star ticks was directly affected by climate, especially when considering the covariance of temperature (minimum temperature and maximum temperature) and rainfall together (Cumming 2002). The other significant abiotic variable, forest-floor depth, had a positive effect on nymph numbers and likely influences the developmental success and activity of nymphs, as leaf litter provides important shelter for ticks to buffer against extreme microclimatic conditions. It also provides a better environment for engorged larvae, which are more prone to water loss, to molt into nymphs. In addition, a study conducted in east-central Alabama near Jacksonville has also

Table 3.6 Pearson correlation coefficient for each pairwise combination of six predictor variables in the best-fit model. Variables include forest-floor gravimetric moisture (GM), forest-floor depth (FFD), tree diversity (TrDiv), canopy cover (CanCo), the number of available hosts (TotHst), and weekly mean relative humidity (MeanRH).

	MeanRH	**GM**	**FFD**	**TrDiv**	**CanCo**	**TotHst**
MeanRH	1.0000					
GM	-0.0898	1.0000				
FFD	-0.4275	0.0735	1.0000			
TrDiv	-0.0086	0.1779	0.2511	1.0000		
CanCo	0.0415	-0.3786	0.1524	-0.4277	1.0000	
TotHst	0.0596	-0.2564	0.0129	0.3124	0.0969	1.0000

reported a positive relationship between the mass of leaf litter and questing tick density (Wills et al. 2012). However, there may exist a range of the forest-floor depth that benefits their survival. When Schulze and colleagues investigated the distribution of both *I. scapularis* and *A. americanum*, they reported that the *A. americanum* were more frequently collected in sites with significantly less litter-layer depth (the average of leaf-litter depth was 69.1 mm) compared with *I. scapularis* (the average of leaf-litter depth was 86.0 mm) (Schulze et al. 2001).

3.4.2 *Biotic variables*

3.4.2.1 *Host-related variables*

The Poisson regression model also provides insight into the potential effects of the density of available hosts. According to our data, a higher number of hosts visiting an area in June causes the number of *A. americanum* nymphs to decrease. This negative relationship is best represented by data from plot 2, which had the highest number of host captures (n = 15) as well as the lowest number of nymphs sampled (n = 1). This suggests that host activity during the peak period of tick questing (and period of sampling in this case) is more likely to remove *A. americanum* nymphs from plots than to bring new ticks in from other locations. It has been reported that different hosts vary in their ability to harbor lone star ticks (Kollars et al. 2000a). Generally, medium-sized mammals are large enough to sustain high tick burdens, whereas relatively smaller hosts, such as rodents, tend to carry a smaller fraction of the lone star tick population (Talleklint and Jaenson 1994, Craine et al. 1995, Randolph 2004). However, the number of animals in each size class may vary dramatically, so it's difficult to estimate the total

tick load based on averages from individual hosts. In addition to host size, other host characteristics likely account for variation in tick infestation levels. Some such factors investigated for mice and *I. scapularis* included gender, body mass, and home range sizes (Devevey and Brisson 2012).

Another factor to consider is that the cohort of lone star tick nymphs we sampled likely quested near where they dropped from their hosts as larvae in the previous active season. Therefore, investigating the activity of animals with high larval burdens could improve our understanding of the associations between questing nymphs and host activity, particularly for tick dispersal. Although our vertebrate data captured only part of the annual activities of the potential hosts, it still gives us insight into the animal communities at our field site. In general, ticks that employ mesomammals for dispersal, such as raccoons, will occupy a more limited distribution compared to ones feeding on large-sized animals due to variation in home range sizes. Other studies show that animals with larger home range sizes, such as white-tailed deer and coyote, tend to disperse lone star ticks widely (Lockhart et al. 1995, Keirans and Lacombe 1998, Yabsley et al. 2003). The role of these mammals for tick movement also has implications for disease risk, as they can introduce infected ticks into areas where infections had not previously occurred (Kocan et al. 2000, Paddock and Yabsley 2007).

3.4.2.2 *Vegetation variables*

Other biotic variables in the model that influenced the number of questing *A. americanum*

nymphs was related to vegetation, namely tree diversity and canopy cover. These variables may impact the presence of questing nymphs in a number of ways. For example, they could influence host activity as vegetation type is known to affect the ability of certain animals to establish and maintain populations (e.g., food, shelters) in different environments (Ostfeld and Keesing 2000, Despommier et al. 2006, Halos et al. 2010, Rynkiewicz and Clay 2014). In the model, the variable of canopy cover was positively associated with the number of questing nymphs. This factor may influence microclimate, as a denser canopy layer likely provides protection from direct sunlight and creates more stable conditions in the microhabitat for ticks. For example, the temperature gradient of a meadow measured at the soil surface and 15.2 cm above the ground had an inverse value compared with an oak-hickory forest (Robertson et al. 1975). Moreover, the number of ticks were predicted to be higher in the plots with higher tree diversity. One explanation of the potential effect is that different tree communities influence microclimatic conditions experienced by questing ticks as mentioned in the introduction (Havens 1979). In support of this hypothesis, Semtner and Hair (1973b) showed that tree diversity altered the population abundance of ticks by life stage. Nevertheless, a handful of studies on the lone star tick suggest only weak relationships between vegetation characteristics and tick density when only considered one variable at a time (Fryxell et al. 2015, Wills et al. 2012).

Chapter 4

Conclusions

The work completed through this thesis has investigated tick distributions, densities and pathogen prevalences among forested sites in east-central Alabama. Our results are supportive of the fact that the lone star tick is widely distributed in the Southeast, which is also the species of most concern across our sampling sites. It is also the first study that specifically focused on the risk of tick-borne diseases for recreational users in Alabama and resulted in an estimate of the encountered rate of *Ehlirhica*-infected lone star ticks in parts of Lee and Macon Counties. Above all, people should take precaution against the bite of the lone star ticks in forested sites throughout the late spring and summer, but particularly from May through June. Further studies conducted throughout the year to more definitively delineate the duration of seasonal peaks in tick abundance in accordance with human activity patterns would be worthwhile to estimate the risk of disease more comprehensively.

In regards to the third chapter of this thesis, which focused on the influence of environmental and host-related factors on questing tick density, our Poisson regression model provided support for at least five important variables, including forest-floor gravimetric moisture, forest-floor depth, tree diversity, canopy cover, and the number of available hosts. Weekly mean relative humidity, the remaining variable of the best-fit model, may have influenced tick

numbers in our study although the regression coefficient was non-significant. In addition, our modeling approach provides insight into the influence of spatial heterogeneity on questing behavior in *A. americanum* nymphs. A habitat that appeared homogeneous to the naked eye, turned out to be quite heterogeneous on the scale important for ticks. For future studies, looking beyond questing and determining the most important climatic, vegetation- and host-related factors for the establishment and maintenance of tick populations is important for our understanding of tick-population dynamics, as well as the risk of tick-borne diseases. Moreover, a more continuous characterization of microenvironmental conditions and host activities across seasons and an increase in the number of sampling plots, may better characterize the relationships between tick density and the variables investigated.

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