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Off-Host Biology and Ecology of Immature Gulf Coast Ticks (Amblyomma Maculatum Koch) in Mississippi

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Off-host biology and ecology of immature Gulf Coast ticks (*Amblyomma maculatum* Koch) in Mississippi

By

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Little is understood about off-host behavior and ecology of immature *Amblyomma maculatum* Koch (Gulf Coast tick). A more complete understanding of this tick is essential to protect human and animal health. My research focused on seasonality and distribution of immatures in Mississippi, potential suitability of some insect and human hosts to larvae, and aspects of nymphal questing behavior.

A single larva was collected (third off-host collection reported) when sampling *A. maculatum* habitat using a novel device. Collection of this larva in November expands the stage's known seasonality and confirmed a prediction concerning seasonality of larval *A. maculatum*. Low frequency of immatures (8.3%) confirmed that they're incredibly difficult to collect off-host. Nymphal collections peaked in March, and known seasonality was extended for both nymphs and adults.

I examined known records, elucidating seasonality and distribution of *A. maculatum* in Mississippi. Either multiple generations per year or diapause are responsible for observed bi-modal distribution of immature collections. Additionally, I

compiled the most extensive host record of immature *A. maculatum* in Mississippi and investigated seasonality patterns using USDA plant hardiness zones.

I compiled the most complete record of ticks found on arthropods. *Amblyomma americanum* and *A. maculatum* were both confirmed to crawl onto arthropods, giving support to occasional, unintentional dispersal by phoresy. There was no conclusive evidence that larval *A. maculatum* feed on arthropods, however data supported feeding by larval *A. americanum*. These results have interesting implications regarding evolution of pathogens/endosymbionts.

I provided the first evidence that larval *A. maculatum* can attach to humans. *Rickettsia parkeri*, a human pathogen transmitted by this species has recently been shown to be capable of transovarial transmission. Therefore, larval *A. maculatum* may provide another avenue of transmission.

I have demonstrated that *A. maculatum* are difficult to collect off-host in part because they prefer to quest low to the ground. In choice studies, 5-cm-tall stems were most likely to be occupied by nymphs released into an array of stems. Low vapor pressure deficit encouraged questing, while higher *VPD* and warmer temperature increased questing height. These results may have implications in understanding host-seeking behavior in other tick species as well.

DEDICATION

This dissertation is dedicated to my family, as they have been my support and motivation throughout my academic journey. My mother LuAnn Portugal and my father José Santos Portugal Aguilar II worked hard to instill in me a strong work ethic, a desire to learn, and a love of reading. My little brother and sister Jon-Mikel and Stephanie Renée Portugal inspired me during my youth, and I am in awe of the incredible adults they have grown to become. My intelligent, beautiful wife Nicole Marie Portugal was the driving force behind me completing my undergraduate degree and throughout graduate school; I cannot imagine my life without her. Finally, my amazing son Cameron Santos Portugal. You inspire me to be better, and I hope that the work I put into this dissertation in some way serves as an inspiration to you as well. *Deo Gratias!*

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As an undergraduate student at Texas A&M University, I was fortunate to have worked with Dr. Pete Teel. Introducing me to the *Rhipicephalus* (*Boophilus*) ticks and bovine babesiosis started me on a course that has led me to this point. Sir, you were spot on when you suggested I reach out to Jerome for graduate school, and I thank you for that advice. Your kind words and mentorship allowed me to see something in myself that I'd never seen before.

My minor advisor and the remainder of my committee have helped guide me though this journey. Your suggestions, revisions, comments, and scrutiny contributed to this work and my success as a graduate student. I would also like to thank Dr. Scott Willard, the faculty staff and students of the Mississippi State University Department of Biochemistry, Molecular Biology, Entomology, & Plant Pathology, Dr. Jeffrey L. Ullman, and the teachers/mentors throughout every level of my education.

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CHAPTER I

A REVIEW OF LITERATURE AND BACKGROUND INFORMATION ON THE GULF COAST TICK, *AMBLYOMMA MACULATUM* KOCH (ACARI: IXODIDAE), WITH EMPHASIS ON THE IMMATURE STAGES

Background and history

There are approximately 900 species of ticks (subclass Acari) worldwide, consisting of three families (Ixodidae, Argasidae, and Nuttalliellidae) (Guglielmone et al. 2010). "Hard ticks" (possessing a scutum) in the family Ixodidae, have been well-documented as competent vectors of numerous human and animal pathogens. These ixodid ticks utilize one of two strategies to obtain a host, either the ambush strategy or the hunter strategy (Sonenshine 2005). Actively hunting for a host requires energy, the ability to move quickly, and eyes in position and of the acuity to observe and obtain the host (Kaltenrieder et al. 1989). Typically, ticks that utilize this strategy are of the genus *Hyalomma* and are found in arid regions of northeast Africa, Asia, and Europe (Kaltenrieder et al. 1989) with a lower vegetative density to obscure the sightline of potential hosts (Uspensky 2002). Most ticks however, including many species that do not possess eyes, utilize the ambush strategy to obtain a host. This method involves vertical positioning of a tick on a surface (such as vegetation) in anticipation of a preferred host coming into proximity of that surface. Forelegs of ticks contain a sensory organ (Haller 1881) known as "Haller's organ" allowing them to detect various stimuli exuded by

potential hosts (Foelix and Axtell 1972, Waladde 1982, Steullet and Guerin 1992). Their ability to quest or hunt for hosts, as well as their exceptional fecundity, has almost certainly contributed to the family's success over evolutionary time.

The tick genus *Amblyomma* has been demonstrated to utilize both the ambush strategy of host procurement, as well as to a lesser extent, the hunter strategy. This genus contains numerous species associated with zoonotic pathogens which they propagate and transmit to other hosts in both the Old and New World. *Amblyoma* spp. are known for their long mouthparts, ornate scutum, eyes, festoons, and are commonly encountered in tropical and sub-tropical regions. With the exception of *Amblyomma rotundatum* which uses parthenogenesis (Rhor 1909), members of this genus reproduce sexually. *Amblyomma* spp. ticks are common in the southeastern United States, and are not only the most common genus of tick collected from animals in Mississippi (White 1955), they are also the most common genus of tick removed from humans in Mississippi (Goddard 2002).

Amblyomma maculatum Koch, 1844 (the Gulf Coast Tick), (Figure 1.1), is a New World hard tick species that utilizes three hosts in its life cycle, and has been observed feeding on humans and animals alike. This tick is an emerging vector threat (Teel et al. 2010, Paddock and Goddard 2015), which has been relatively unstudied, especially in the immature stages. Diseases associated with this tick, as well as the various medical conditions it can induce, are all of moderate to severe medical and veterinary concern. In addition, the Gulf Coast tick has an expanding geographic range allowing this species to come into contact with an increasing number of people and animals (Goddard and Norment 1983, Teel et al. 2010).

Figure 1.1 Comparison of *Amblyomma maculatum* life stages.

Amblyomma maculatum larva (left), nymph (center), and adult female (right) in relation to size of a penny (USA).

Carl Ludwig Koch, a German entomologist, first officially described *Amblyomma maculatum* in 1844 from a specimen collected in "Carolina" in the United States (Koch 1844, Kohls 1956). Koch described four additional species of *Amblyomma* (*A. rubripes* Koch, 1844, *A. ovatum* Koch, 1844, *A. tigrinum* Koch, 1844, and *A. triste* Koch, 1844) from South America later that year (Koch 1844). These specimens were later believed by various authors to all be synonymous with *Amblyomma maculatum* (Neumann 1899, Kohls 1956). Kohls (1956) was able to visually examine all of Koch's 1844 type specimens with the exception of *A. rupribes*, a species that has been rarely mentioned in literature. Kohls states that he was informed by a "Prof. Dr. A. Kaestner" that type specimens of *A. rupribes* could not be found in their collection at the Zoologisches Museum in Berlin (Kohls 1956). Further work on the *A. maculatum* group, performed in

the mid-twentieth century, determined that only three species existed. This was done by combining the now defunct *A. ovatum* with *A. tigrinum* (Kohls 1956). Camicas et al. (1998) redefined the *Amblyomma maculatum* group as consisting of: *Amblyomma maculatum*, *Amblyomma triste*, *Amblyomma tigrinum*, *Amblyomma parvitarsum* Neumann, 1901, and *Amblyomma neumanni* Ribaga, 1902. Advances in molecular phylogenetics combined with traditional morphological methods, have further clarified the taxonomic status of *A. maculatum*. Currently per Estrada-Pena et al. (2005), the *Amblyomma maculatum* group consists of *A. maculatum*, *A. triste*, and *A. tigrinum*. Not only have both *A. parvitarsum* and *A. neumanni* been excluded from the group, but have also been removed from the subgenus *Anastosiella* Santos Dias, 1963 (Estrada-Pena et al. 2005). Estrada-Pena et al. (2005) further proposed removing members of the *A. ovale* group from this subgenus, thereby suggesting that *Anastosiella* is comprised solely of the *A. maculatum* group. A recent study has noted that *A. triste* collected in Arizona are genetically indistinguishable from *A. maculatum* in the Southeastern U.S. (L. Beati, USNTC, personal communication), which may suggest synonymy.

During the early portion of the 20th century, *A. maculatum* was already known to be a major pest of animals. Bishopp and Hixson (1936) described mortality from exsanguination in small birds and mammals in the laboratory, however ground-dwelling birds in a natural environment are not known to suffer any significant health issues from infestation by immature Gulf Coast ticks (Hixson 1940, Williams and Hair 1976, Teel et al. 1988, Teel et al. 2010). In addition, *A. maculatum* was designated as the second most economically significant pest of cattle after *Rhipicephalus* (formerly *Boophilus) annulatus* (Bishopp and Hixson 1936).

Amblyomma maculatum is a large, ornate, three-host tick, that is known to be an aggressive feeder. Female Gulf Coast ticks mate while on the host, and have been shown to be attracted to hosts containing successfully feeding males via an aggregation pheromone (Gladney et al. 1974, Sleeba et al. 2010). To acquire a host, Gulf Coast ticks will quest vertically on foliage and grasses, extending their front legs in anticipation of a host; or in some cases may actively move towards (hunt) a host. In one study performed in Mississippi, adults quested from 20cm-75cm above the ground with an average of 36cm (Goddard et al. 2011). When a suitable host approaches, CO_2 expelled during respiration alerts the tick, which will then attempt to attach and feed, typically around the head and neck area (Felz and Durden 1999). Larval and nymphal Gulf Coast ticks will also react to stimuli from an approaching host and may move toward it (Hixson 1940), however they do not utilize a pure "hunter" strategy as they will quest as well. Since *A. maculatum* is a three-host tick, it will perform this action three different times, detaching each time to molt after taking a blood meal. After the engorged female drops off of the host, she seeks a suitable location on the ground or in debris to oviposit. The act of oviposition generally occurs within 3-9 days of blood feeding (Wright 1971), however some studies with photoperiod have shown variation in this time range (Wright 1971, Lohmeyer et al. 2009). Observations have further demonstrated that laden females will oviposit directly on to the soil (Bishopp and Hixson 1936), and in some instances may create a small invagination in the soil to shelter the eggs (Hixson 1940). Gravid females can oviposit tremendous number of eggs in a short period of time. For example, one laboratory experiment demonstrated 9473.1 eggs/female in a 25 day period with a single female producing a one day maximum of 1810 eggs (Drummond and Whetstone 1970).

In an observation conducted in the field, *A. maculatum* oviposition ranged from 4,560 to 11,265 eggs/female with an average of 8,282 eggs/female deposited (Hooker et al. 1912). Current understanding of how newly emerged larvae react to their environment is unclear, however they have been observed gathered en masse at the base of various plants (Hixson 1940). At this life stage, larval ticks are at a high risk of desiccation, and surrounding foliage plays a major role in determining the microclimate (Fleetwood 1985). Early work on another hard tick, *Ixodes ricinus*, suggested that some ticks will flee to a biomass layer of soil, containing leaves and decaying vegetation in order to preserve moisture (Milne 1950a, b). In addition, adult *Amblyomma tuberculatum*, a close relative of *A. maculatum*, often bury themselves in soil to protect against desiccation (Hooker et al. 1912), thus displaying intentional humidity regulation by digging underground.

Distribution of the Gulf Coast tick

The Gulf Coast tick has historically ranged from countries in South and Central America, various Caribbean islands, into portions of the southeastern United States (Goddard and Paddock 2005). *Amblyomma maculatum* typically occurs in regions directly bordering the Atlantic Ocean and Gulf of Mexico (Figure 1.2) (Estrada-Pena et al. 2005). Although occasionally described as being "essentially Nearctic" or being "originally a Nearctic species," lack of extensive collection records as well as morphological similarities within the *A. maculatum* group do not allow for this designation (Evans et al. 2000, Voltzit 2007, de Azevedo Gomes et al. 2016). Based on records available, (Hooker et al. 1912, Vogelsang and Cordero 1940, Bishopp and Trembley 1945) it is appropriate to describe both the historical and current distribution as

Neotropical-Nearctic (Estrada-Pena et al. 2005, Nava et al. 2007). In the United States, this species has traditionally been encountered in areas near the Gulf of Mexico, hence the common name "Gulf Coast tick." Within the last century however, the range of *A. maculatum* in the United States has expanded, with specimens being collected beyond the traditional range of ~160km inland (Snoddy and Cooney 1984, Teel et al. 2010). *Amblyomma maculatum* in the United States are routinely collected in Texas, Louisiana, Mississippi, Alabama, Georgia, South Carolina, North Carolina, Florida and Eastern Oklahoma (Figure 1.3) (Bishopp and Hixson 1936, Goddard and Norment 1983, Sumner et al. 2007, Teel et al. 2010).

Figure 1.2 Reported distribution of *Amblyomma maculatum* group in the Western Hemisphere

Reported distribution of *Amblyomma maculatum* group (*A. maculatum, A. tigrinum, and A. triste*) in the Western Hemisphere. *Amblyomma maculatum* in this figure are displayed as white circles. From Estrada-Pena et al. (2005)

States not bordering the Gulf of Mexico, but directly bordering coastal southern states (Tennessee and Oklahoma) have the earliest records of increased range with specimens identified from 1942 in Oklahoma and 1945 in Tennessee (Cooley and Kohls 1944, Bishopp and Trembley 1945). *Amblyomma maculatum* specimens in other states

are first identified chronologically in a pattern that generally radiates outwards from the original, natural range. Gulf Coast ticks were identified in 1965 from Virginia (Sonenshine et al. 1965), in 1972 from Kansas (Bell Jr 1972, Gates and Brooks 1972), in 1973 from Arkansas (Lancaster 1973), and in 1984 from Kentucky (Snoddy and Cooney 1984).

Figure 1.3 Classic and hypothetical range of *Amblyomma maculatum* in the United States

Classic range (dark blue) and hypothetical range (pale blue), based on published information and personal communications. Dots represent locations of confirmed (shaded circles) and probable (unshaded circles) cases of American boutonneuse fever (*R. parkeri* rickettsiosis) (See next section). From Paddock et al. (2008).

States not following this distribution pattern were Arizona in 1944 (Cooley and Kohls 1944), Illinois in 1945 (Bishopp and Trembley 1945) and New York in 1981 (Wiedl 1981). A single male *Amblyomma maculatum* was collected by Marx in

California (Lancaster 1973), however it is probable that this specimen was transported by a bovine shipped to California from an endemic region (Hooker et al. 1912). In a recent survey for *Ixodes scapularis* by the State Entomologist of West Virginia, a single *A. maculatum* specimen was collected and determined to be the first Gulf Coast tick identified from the state (Dotseth 2012). Researchers have proposed that many of these outlying specimens collected in northern states could be the result of migratory birds and not from a breeding population (Smith Jr et al. 1996, Teel et al. 2010). In addition, the cattle industry may be involved in dispersal of the tick, with high numbers of *A. maculatum* being reported in herds and at cattle sale barns (Bishopp and Trembley 1945, Semtner and Hair 1973, Barker et al. 2004, Edwards 2010, Edwards et al. 2011b) as well as other potential anthropogenic factors. Currently, *A. maculatum* is considered "established" as far north along the Atlantic coast as Delaware, and possibly Maryland (Florin et al. 2014). Although the specific causes of the Gulf Coast tick's range expansion are not fully understood at this time, it is likely that the range is steadily moving inland towards the north (Goddard and Norment 1983, Teel et al. 2010).

Pathogens and diseases associated with the Gulf Coast tick

Amblyomma maculatum has been identified in the literature as a natural vector for American canine hepatozoonosis (*Hepatozoon americanum)* (Vincent-Johnson et al. 1997), and American boutonneuse fever (*Rickettsia parkeri*) (Parker et al. 1939, Parker 1940, Goddard 2004) in animals and humans respectively, as well as an experimental vector for heartwater disease (*Ehrlichia ruminantium*) (previously *Cowdria ruminantium*) (Uilenberg 1982). Heartwater disease presents a major threat to the American cattle industry. In 1982, Uilenberg first determined that *A. maculatum* was a competent vector

of the heartwater bacterium (Uilenberg 1982). This rickettsial pathogen is known for producing an accumulation of pericardial and pleural fluids in the thoracic cavity of cattle and causes much morbidity and mortality in cattle herds. The disease has been transferred from Africa to the Caribbean and is suspected to be island hopping via cattle egrets (*Bubulcus ibis*) with infected ticks (*Amblyomma variegatum*) attached to them (Barré et al. 1987, Barré et al. 1995).

American canine hepatozoonosis is an emerging disease most commonly associated with domestic dogs. The causative agent, a protozoan, is transmitted by *Amblyomma maculatum*, but is contracted by accidental ingestion of infected ticks by canines during grooming (Ewing and Panciera 2003) rather than via tick feeding. Canine hepatozoonosis is a wasting disease of dogs and is seen typically in the American Southeast (Vincent-Johnson et al. 1997). Domestic dogs are suspected of being a dead-end host for the protozoan, which is believed to naturally occur in a cycle among immature Gulf Coast ticks and their bird or small mammal hosts (Ewing et al. 2002).

The only known human disease agent transmitted by the Gulf Coast tick is *Rickettsia parkeri*, also referred to in literature as "Maculatum agent" or erroneously as "Maculatum virus," which is the causative agent of "American boutonneuse fever," "*Rickettsia parkeri* rickettsiosis," "Tidewater spotted fever," or "Maculatum disease" (Parker et al. 1939, Parker 1940, Philip and White 1955, Goddard 2004, Wright et al. 2011, Paddock and Goddard 2015). *Rickettsia parkeri* was originally isolated from *A. maculatum* (Parker et al. 1939) and was later detected in *Amblyomma americanum* specimens from Mississippi and Kentucky (Goddard and Norment 1986). Although it is possible in the laboratory to infect the more common *A. americanum* with *R. parkeri*,

high mortality rates in the tick suggest that *A. americanum* cannot survive the infection over an extended period of time (Goddard 2003). A unique strain of *R. parkeri* was recently identified in *Dermacentor parumapertus* Neumann from Texas; however, it is not known whether this tick plays a role in human transmission, as its preferred host is the black-tailed jackrabbit (*lepus californicus*) (Paddock et al. 2017). Currently, the only known competent North American vector of *R. parkeri* is the Gulf Coast tick, however *A. triste* may occasionally pose a threat (Mertins et al. 2010, Herrick et al. 2016). *Rickettsia parkeri* is a member of the spotted fever group (SFG) of *Rickettsiae* of which only three others are known to be pathogenic in the United States (*R. rickettsii*, *R. akari* and *R. felis*). Ralph R. Parker was the first scientist to suggest the possibility of a new pathogen after isolating the agent from Gulf Coast ticks collected in Liberty County, TX in 1937 (Parker et al. 1939). At the time, Parker noticed cross-reactivity with the agent of Rocky Mountain spotted fever (*R. rickettsii)*, however he reported *R. parkeri* as being pathogenic only in guinea pigs. Parker was also limited in his research due to the technology available to late 1930's scientists, referring to Rocky Mountain spotted fever as a "virus," yet he was the first to compare this agent to boutonneuese fever (*Rickettsia conorii)*. *Rickettsia parkeri* was first documented to be pathogenic to humans in 2004 from a patient in Virginia (Paddock et al. 2004). In humans, the disease is characterized by eschars (spots of necrosis) at the site of the infection, as well as a rash, headache malaise and fever (Paddock et al. 2008, Goddard and Varela-Stokes 2009). In the state of Mississippi, *R. parkeri* was first isolated in 1986 from *A. americanum* ticks (Goddard and Norment 1986) and was first identified from a human patient in 2006 (Finley et al. 2006).

Very little is known about what stages of *A. maculatum* transmit *R. parkeri* to humans. In a 10-year study of human tick bites in Mississippi, despite the fact that Gulf Coast ticks were the second most commonly encountered tick species attached to humans (11.8% of total), only adult and nymphal *A. maculatum* were removed from patients (Goddard 2002). These data support the belief that larval *A. maculatum* do not feed on humans, and thus are not a significant contributor to American boutonneuse fever cases. However, it is also possible that due to the small size of larval Gulf Coast ticks, as well as their suspected overlapping seasonality and distribution with larval *Amblyomma americanum*, they may be less likely to be seen attached, correctly identified, and reported. This is of importance, as *R. parkeri* has recently been demonstrated to be capable of transovarial transmission in *A. maculatum* under laboratory conditions (Wright et al. 2015)

In addition to these pathogenic agents, *A. maculatum* is infamous in the sheep, goat, and cattle industries for its tendency to attach to ears often resulting in a condition known as "gotch ear" (Drummond and Whetstone 1970, Edwards 2011, Edwards et al. 2011a). Gotch describes the severe structural damage to the cartilage of an animal's ear resulting from an infestation of ticks such as *A. maculatum*, causing the ear to flop over (Figure 1.4) swell and excrete a yellowish exudate which later crusts over (Drummond and Whetstone 1970). Another result of damage that has been attributed to *A. maculatum* on cattle is myiasis from the "primary screwworm" *Cochliomyia homnivorax* (previously *Cochliomyia americana)* (Spicer and Dove 1938). The bite damage caused by Gulf Coast ticks on livestock may attract the female primary screwworm to oviposit in these spots (Keirans and Durden 1998). Although the primary screwworm has mostly been

eradicated from the United States, it is still a threat to livestock and people in Central and South America (often in countries that have *A. maculatum* populations).

Figure 1.4 Gotch ear in a goat caused by *Amblyomma maculatum*

Gotch ear in a Saanen doe goat (ear facing downwards), with concurrent infestation of four *Amblyomma maculatum* attached to the inner pinna. Note characteristic drooping and swelling of ear that are among the diagnostic signs for this condition. From Edwards (2011).

Amblyomma maculatum has also been reported to cause "tick paralysis" (Paffenbarger Jr 1951, Espinoza-Gomez et al. 2011). Tick paralysis occurs when an attached tick is associated with an ascending paralysis in its host (Rose 1954). Although it is suspected that tick paralysis is dependent on attachment location of *A. maculatum* (usually on the head, neck, back and groin) (Paffenbarger Jr 1951), studies with other ticks have shown this not to be the case (Rose 1954, Dworkin et al. 1999). In fact, the

site selection associated with *A. maculatum* and tick paralysis may simply be a factor of their ability to be undetected in hairy parts of the body, as seen in other ticks (Schmitt et al. 1969). The paralysis is caused by a neurotoxin released into the host by the tick as it feeds, however the exact mechanisms are not fully understood (Grattan-Smith et al. 1997). Tick paralysis has been observed in livestock, canines, and humans, and may be lethal if the tick is not promptly removed (Felz et al. 2000, Dryden and Payne 2004, Van Gerpen and Caruso 2005, Remondegui 2012).

Natural history of immature *Amblyomma maculatum*

As mentioned, the Gulf Coast tick is a three-host tick, requiring a suitable host for each life stage. Often both immature forms of *A. maculatum* can be found on the same animals; however, it is rare to see all three life stages on the same host. With exception of coyotes and domestic sheep, it is not known if any hosts are fed upon by all three life stages (Teel et al. 2010). The Gulf Coast tick has been recovered from at least 71 species of warm-blooded hosts consisting of both birds and mammals (Teel et al. 2010). Adult *A. maculatum* ticks are often observed feeding on cattle (Bishopp and Hixson 1936), domestic dogs (Hooker et al. 1912), white-tailed deer (Travis 1941) and various other large mammals including man. In contrast, immature *A. maculatum* (nymphs and larvae) have been reported feeding on small mammals and ground dwelling birds such as meadowlarks (Hooker et al. 1912, Bishopp and Hixson 1936, Teel et al. 1988), Northern bobwhite quail (Bishopp and Hixson 1936, Semtner and Hair 1973) the Lincoln's Sparrow (Rainwater et al. 2007), cotton rats (Barker et al. 2004, Moraru et al. 2013) and various other similarly-sized animals. In addition, Gulf Coast tick nymphs occasionally have been reported to feed on dorsal body areas of cattle (as compared to adults which

typically feed around the head and ears) (Ketchum et al. 2005). Experimental studies support natural observations that immature *A. maculatum* do not utilize reptiles as hosts at either immature stage (Moraru et al. 2012). Although immature forms of this tick have been reported from various hosts across its range, there are no thorough studies of the natural history of *A. maculatum* immatures.

Purpose of this study

Currently there is a dearth of ecological and behavioral information pertaining to immature stages of the Gulf Coast tick. As a competent vector of medical concern, as well as its potential as a vector of pathogens produce high mortality in animals, further elucidation of the off-host biology and ecology of this tick is warranted. Immature stages of this tick are especially important to study not only because so little is known about them, but also because *R. parkeri* has recently been demonstrated to be transmitted transovarially in *A. maculatum.* This dissertation will fill in gaps of knowledge concerning immature stages of this species. Results concluded from this dissertation, combined with what is currently in literature as well as with future research, will be used to mitigate nuisance and disease threats posed by this species in Mississippi, the United States, and anywhere else in the world where this tick is established.

Dissertation objectives

1. Determine the seasonality of larval and nymphal Gulf Coast ticks in Mississippi by systematically sampling from two sites (northern and southern MS) by multiple methods over two years during periods of suspected peaks of immature activity.

2. Conduct an exhaustive review of literature and records of immature *Amblyomma maculatum* in Mississippi to determine historical seasonality. Combined with results from Objective 1, this will be the most complete assessment and analysis of larval and nymphal activity in Mississippi.
3. Under laboratory conditions, evaluate the capacity of larval *Amblyomma maculatum* ticks to respond to and imbibe hemolymph from three species of insects under laboratory conditions.
4. Under laboratory conditions, investigate whether larval *Amblyomma maculatum* ticks can successfully attach to humans.
5. Evaluate how varying temperature, relative humidity, vapor pressure deficit, and wind movement affect the host-seeking behavior of nymphal *Amblyomma maculatum* ticks under laboratory conditions.

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CHAPTER II

COLLECTIONS OF IMMATURE *AMBLYOMMA MACULATUM* KOCH (ACARI: IXODIDAE) FROM MISISSIPPI, U.S.A.

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Abstract

Immatures of *Amblyomma maculatum* have historically been difficult to collect when not on an animal host, thus a definite seasonality of the immatures has never been determined. In this study, I made tick collections at two sites during 2012 – 2014. Site A (Oktibbeha County) in northern Mississippi, and Site B (Jackson County) in southern Mississippi, were sampled from September-May and October-May, respectively. Sampling was made from vegetation with a 1 m^2 weighted white corduroy drag cloth, dry ice traps, and also a novel method known as "swabbing." A total of 157 ticks was collected, with 42% (n=65) being *A. maculatum*. Of these 65, only 20% (n=13) were immatures, with 18.5% (n=12) being nymphs, and one larva (1.5%) (collected by swabbing). To our knowledge, this is only the third time that larvae of this species have ever been collected from vegetation in the United States. The larva is the first collected from Mississippi in November, and expands the known seasonality of this stage. Of the

twelve nymphs collected, one was found during May, becoming the first collected south of U.S. Route 84 in Mississippi during this month.

Introduction

Amblyomma maculatum Koch, known as the "Gulf Coast tick", is a commonly encountered tick in Mississippi, as well as throughout the southern United States, extending southward into Central and South America and some Caribbean islands (Goddard and Paddock 2005). In the United States, this tick was historically only reported within ~160 km from the coast (Bishopp and Hixson 1936). However in Mississippi, whether due to the movement of livestock, increase of white-tailed deer, or changes in climatic conditions, *A. maculatum* are now increasingly found far north of that historical boundary (Goddard and Norment 1983, Paddock and Goddard 2015).

Compiling behavioral and seasonality data for this species in Mississippi is essential to future medical and veterinary disease control and prevention programs. This tick is a vector of American boutonneuse fever (*Rickettsia parkeri*), a bacterial disease that results in fever, rash, and escars at the bite site in humans, and which has been identified in Mississippi (Parker et al. 1939, Paddock et al. 2004, Goddard and Varela-Stokes 2009, Ekenna et al. 2014). An additional bacterial agent transmitted by *A. maculatum* is *Candidatus* Rickettsia andeanae (Ferrari et al. 2012, Ferrari et al. 2013), however pathogenicity of this agent is unknown at this time. This tick is a known vector of *Hepatozoon americanum* (American canine hepatozoonosis), a wasting disease in domestic dogs and other canines (Vincent-Johnson et al. 1997), and potentially a vector of *Ehrlichia ruminantium* (Heartwater disease), a high-mortality disease affecting cattle and other ruminants (Uilenberg 1982).

Adult Gulf Coast ticks are often found on White-tailed deer, cattle and other livestock (Bishopp and Hixson 1936, Bishopp and Trembley 1945, Teel et al. 2010). All three feeding stages are usually not found on the same host, with exception of domestic sheep and coyotes (Teel et al. 2010). Immature *A. maculatum* have been reported from small mammals as well as ground-dwelling birds (Hooker et al. 1912, Cooley and Kohls 1944, Teel et al. 2010); attempts to feed immatures of this species on reptiles have not been successful (Moraru et al. 2012).

To date, the most productive method of collecting immature Gulf Coast ticks has been by direct examination of small animal hosts. Although adult *A. maculatum* can be collected quite easily from vegetation using a drag cloth, researchers have had to employ mist nets, animal traps, and even firearms, to collect bird and small mammal hosts in order to obtain immatures (Teel et al. 1998). Immature *A. maculatum* are very difficult to collect when not on a host, and a systematic collection to determine seasonality has never been conducted in Mississippi (Goddard 2007). This current project was an intensive effort to collect larvae and nymphs of *A. maculatum* from two sites in Mississippi known to have large populations of adults.

Materials and Methods

Two locations were selected for this study, one representing northern Mississippi (Site A), and the other representing southern Mississippi (Site B). Collections were made twice a month from September through May at Site A, and once a month from October through May at Site B during the years 2012-2014. All collections were made between 1100-1500, based upon literature suggesting that larval activity of newly emerged *A. maculatum* peaks early-to mid-afternoon (Fleetwood and Teel 1983).

Site A (Northern Mississippi)

Site A (Figure 2.1, Figure A.1, Figure A.2) was located in Oktibbeha County, MS (33.496 N, -88.959 W) at ~90 m elevation (Google Earth © 2013 Google Inc.) in the Blackland Prairie ecoregion in the Southeastern Plains of Mississippi (Chapman et al. 2004). The collection area encompassed a mixed pine/hardwood forest, as well as the gradient between a recently clear-cut area (~5 years) with natural regeneration and manicured grass.

Figure 2.1 Collection Site A, rural Oktibbeha County, MS

Flagging vegetation gradient at Collection Site A. Photo credit: Jerome Goddard, Ph.D.

Site B (Southern Mississippi)

Site B (Figure 2.2) was located in Jackson County near Moss Point, MS at the Grand Bay National Wildlife Refuge (30.432 N, -88.426 W) at ~2.3 m elevation (Google Earth © 2013 Google Inc.). This site is located in the Gulf Coast Flatwoods ecoregion in the Southern Coastal Plain of Mississippi (Chapman et al. 2004) and the collection area is considered a Pine Savannah (Longleaf and Slash) which is sporadically subjected to controlled burns. This area runs adjacent to an electric line transmission right-of-way clearing.

Figure 2.2 Collection Site B, Grand Bay NWR, near Moss Point, MS

Vegetation gradient in utility right-of-way at Collection Site B. Photo credit: Jerome Goddard, Ph.D.

Sites were sampled using a 1 m^2 weighted white corduroy drag cloth for 200 m (total) (Figure A.3), examining the cloth approximately every 3-5 m (Figure A.4). Based on vegetation type, both flagging and dragging methods were used in sampling. Flagging with a cloth targets ticks above ground-level on the stems and leaves of plants, whereas dragging is directed at ticks on shorter vegetation near the ground. A recent study of nymphal *Ixodes scapularis* concluded that both flagging and dragging did not produce significantly different results, thus allowing for their interchangeable use (Rulison et al. 2013).

In addition to flagging and dragging, a novel method known as "swabbing" (Figure 2.3) was performed in underbrush for 50 m, the swab being examined every 2-3 m. Additional, supplementary collections were made on some occasions using dry ice placed on a white sheet (Figure A.5), as such traps have been shown to attract immature *A. maculatum* (Semtner and Hair 1975). All tick specimens collected during this study were placed in vials containing 95% ethanol and identified at Mississippi State University in Starkville, MS using standard keys (Keirans and Litwak 1989, Keirans and Durden 1998). Identification of all immature *A. maculatum* specimens was verified by Dr. Richard G. Robbins (Armed Forces Pest Management Board, Silver Spring, MD).

Novel "Swabbing" Method

This method was developed in 2012 by Dr. Jerome Goddard at Mississippi State University and uses a "swab" consisting of a wooden dowel rod with a swatch of white corduroy attached to one end (similar to a large cotton swab) (Figure 2.3)

Figure 2.3 Novel tick collecting "swab"

Photo credit: Jerome Goddard, Ph.D.

By twisting and turning this swab into underbrush, along rodent trails and inside of small burrows (Figure 2.4), access to immature ticks is theoretically improved. Since immature *A. maculatum* may quest lower on vegetation, use of a drag cloth alone may not be adequate when sampling for them (Goddard 2007).

Figure 2.4 "Swabbing" a rodent runway for ticks.

Photo credit: Jerome Goddard, Ph.D.

Results and Discussion

A total of 157 ticks were collected during this study, 106 from Site A and 51 from Site B. Of these, 42% (n=65) were *A. maculatum* with 67.7% (n=44) being collected at Site A and 32.3% (n=21) at Site B. Although *A. maculatum* was the most common tick collected being 42% of the total (n=65), three other species were also collected: *Ixodes scapularis* (Figure A.6) 28.7% (n=45), *Amblyomma americanum* (Figure A.7) 22.9% (n=36), and *Dermacentor variabilis* (Figure A.8) 6.4% (n=10). No immature *I. scapularis* or *D. variabilis* were collected, while 32 of the *A. americanum* were immatures. The fact that species other than *A. maculatum* were also collected (and

immatures of *A. americanum*) during this survey demonstrates efficacy of our tick collecting methods.

Only 13 (20%) of the *A. maculatum* collected were immatures: being 12 nymphs (18.5%) and one larvae (1.5%), all collected from Site B. No *A. maculatum* immatures were collected from Site A despite the fact that a larger number of adults were collected from Site A. The forested area of Site A did not yield any Gulf Coast ticks. All three methods (dragging/flagging, swabbing, and dry ice) were successful in collecting adult *A. maculatum*, although dragging/flagging were the most productive (Table 2.2). Only two *A. maculatum* (adult male and female) were collected (Site B) using dry ice in this study, suggesting that this method is not particularly effective in collecting Gulf Coast ticks.

Table 2.2 Relative efficacy of collection methods for *Amblyomma maculatum* used in this study

	Flag/Drag	Swab	Dry Ice
Larvae	0	1	0
Nymphs	12	0	0
Adults	49	1	2

Note that larval *A. maculatum* was collected using swab apparatus, as well as relative ineffectiveness of dry ice traps for collecting this species.

A single larval *A. maculatum* was collected with the swab method from Site B (Figure 2.5). To the best knowledge of the authors, this is only the third time that a larva of this species has been collected from vegetation in the United States, with the first being a larval mass collected from a dry ice trap in Oklahoma (Semtner and Hair 1975) and the second being 25 collected by drag cloth from Mississippi on 05 June 2003 (Goddard unpublished data). Due to the large numbers of larvae collected in a

concentrated area, both of these previous instances were probably encounters with the larval mass soon after eclosion. The single specimen in the present study was collected in November, which is the first record for Mississippi during this month, and appears to lengthen the known seasonality of this life stage in the state (Goddard and Paddock 2005). Additionally, November correlates with predicted larval seasonality range in Mississippi based on known biology and the seasonality of adults in the state (Goddard 2007). *Aristida beyrichiana* (Wiregrass) was dominant vegetation type in the site, and this single larva was collected from what appeared to be a rodent runway in the grass.

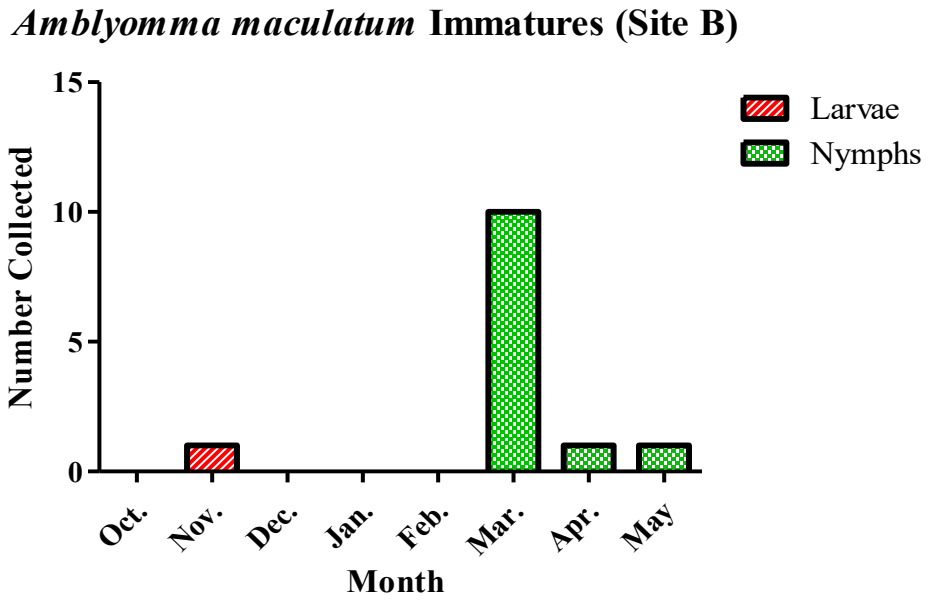

Figure 2.5 Immature *Amblyomma maculatum* collected from Site B

Larval (red) and nymphal (green) *A. maculatum* collected in this study by month. Note: all immatures were collected from Site B.

Twelve Gulf Coast tick nymphs were collected during March (10), April (1), and May (1) (Figure 2.5). March and April correlate with the known seasonality of this life stage, while this is believed to be the first time a nymphal *A. maculatum* has been collected in Mississippi in May south of U.S. Route 84 (Goddard and Paddock 2005). No nymphs were collected during February despite being reported in literature from a Swamp sparrow during that month (*Melospiza georgiana*) (Goddard and Paddock 2005). *Aristida beyrichiana* (Wiregrass) was the dominant vegetation type from which these nymphal specimens were collected.

Although adult Gulf Coast ticks are not central to this study, reporting these data helps construct a more complete understanding of this vector's seasonality. Adults were collected at both sites with Site A collections in September and May and Site B collections October and April (Figure 2.6). Though counter-intuitive, adults typically appear earlier in the central (cooler) portion of the state compared to the southern area (Goddard and Paddock 2005); however, environmental conditions are not substantially different, and factors such as vegetation type and available hosts could also have an impact. In this current study, not only were adult Gulf Coast ticks collected earlier at Site B than Site A, this was the earliest (April) that adults have been reported south of Hattiesburg, Mississippi (Goddard and Paddock 2005, Goddard 2007). It should be noted that of the 44 adult *A. maculatum* collected at Site A, 31 were from a single May collection.

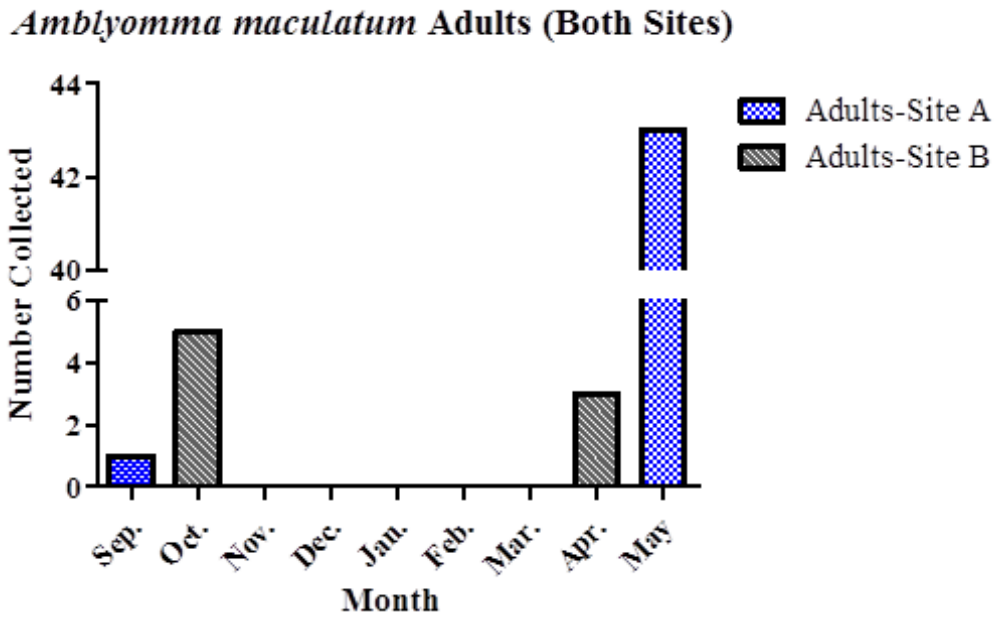

Figure 2.6 Adult *Amblyomma maculatum* collected from both sites

Adult *A. maculatum* collected from Site A (blue), and Site B (gray) by month during this study. Note: no collection attempts were made in September at Site B dues to logistics.

Results of this study reinforce the fact that collection of immature *A. maculatum* from the field when not on a host is extremely difficult. This limited seasonality study helps fill in some gaps of known *A. maculatum* activity in Mississippi. Additionally, a novel method is described for attempting collection of immature ticks, and which obtained a single specimen of the elusive larval stage of *A. maculatum*. The threat of both human and animal pathogens, coupled with incomplete seasonality data for this tick in Mississippi, shows the need for further investigation.

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CHAPTER III

SEASONALITY AND DISTRIBUTION OF IMMATURE *AMBLYOMMA MACULATUM* KOCH (ACARI: IXODIDAE) IN MISSISSIPPI, USA: A REVIEW AND NEW RECORDS

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Abstract

The Gulf Coast tick (*Amblyomma maculatum* Koch), a known vector of medical and veterinary concern, is well established in Mississippi. Although seasonality and distribution patterns of adult *A. maculatum* are well documented, those of immatures (larvae and nymphs) have not. In this study, a review of literature was combined with new and unpublished data to identify trends in immature *A. maculatum* activity. Compiled data from dates ranging from 1920-2014 consisted of 2,368 total specimens of *A. maculatum* collected in Mississippi, some published and some not. Of those, 2,295 (96.92%) were adults while only 27 (1.14%) were nymphs and 46 (1.94%) were larvae. Only 4 separate larval collections have been recorded (one each in June and November and two in October). Seventeen nymphal collections were recorded with peaks in March and August, roughly corresponding to bi-modal distribution observed in larval records. This bi-modal distribution suggests that there may be two batches of *A. maculatum* per

year, or that immatures go through a stage of inactivity during periods of both winter and summer months. As expected, these data show that nymphs were collected in southern portions of Mississippi earliest, but unexpectedly, adults were collected earlier further north. Surprisingly, it was noted that larvae were collected progressively later in the year further south.

Introduction

Numerous species of hard ticks (Acari: Ixodidae) have been documented from Mississippi (White 1955, Goddard 2006, Goddard and Layton 2006). Of these, members of the genus *Amblyomma* are the most common ticks removed from humans, and are encountered most frequently when field-collecting (White 1955, Goddard 2002). *Amblyomma* ticks threaten health of both animals and humans in Mississippi by transmitting various pathogens, and can economically impact livestock production throughout the state (Bishopp and Hixson 1936, Edwards 2011).

Amblyomma maculatum Koch, known as the "Gulf Coast tick," is an aggressive, three-host tick known to utilize both mammals and birds as hosts (Paddock et al. 2010, Teel et al. 2010). Historically in the United States, the Gulf Coast tick's range reportedly did not extend further inland than ~160 km bordering the Gulf of Mexico and Atlantic Coast (Bishopp and Hixson 1936). This range in the United States has slowly expanded north of its historically understood extent (Goddard and Norment 1983, Snoddy and Cooney 1984, Paddock and Goddard 2015). An article published in 1945 alluded to potential range expansion of this tick due to its feeding habits, calling it "surprising" that more ticks had not yet been found "north of the normal range" (Bishopp and Trembley

1945). In Mississippi, this range expansion has been especially conspicuous as the Gulf Coast tick is now frequently encountered in the northern two-thirds of the state.

Gulf Coast ticks are the vector of *Rickettsia parkeri* Lackman (Parker et al. 1939, Parker 1940, Lackman et al. 1965), a bacteria which has been demonstrated to be transmitted transovarially (Wright et al. 2015), that causes a disease of humans (Paddock et al. 2004) sometimes referred to as "American boutonneuse fever" (Goddard 2004). Additionally, *A. maculatum* is known to transmit *Hepatozoon americanum* Vincent-Johnson (Vincent-Johnson et al. 1997). This pathogen is the causative agent of American canine hepatozoonosis, a wasting disease of dogs and other canines. *Amblyomma maculatum* has also been noted to be a competent vector of heartwater disease (*Erlichia ruminantium* (Dumler), a highly virulent disease of cattle and other ruminants, at least in laboratory settings (Uilenberg 1982).

Due to difficulty collecting these ticks off-host, little is known of the behavior, distribution, and seasonality of immature *A. maculatum* (larvae and nymphs) in Mississippi (Portugal III and Goddard 2015). Immatures of this species have been reported to utilize ground-dwelling birds (such as meadowlarks and bobwhite quail) and small mammals (such as the cotton rat) as hosts (Hooker et al. 1912, Bishopp and Hixson 1936, Moraru et al. 2013). Although collection and observation of these animal host species can aid in determining seasonality and distribution of this tick, systematic collection efforts of this tick from vegetation have not been very successful in Mississippi (Goddard 2007, Portugal III and Goddard 2015). Additionally, seasonality records from other states are not useful due to variations in geography, climate, and other factors. For example, a study in Texas identified peak larval (December and January) and nymphal

(February) collections of the Gulf Coast tick (Teel et al. 1998) which did not match results from the neighboring state of Oklahoma for peak larval (July) and nymphal (August) collections (Semtner and Hair 1973). This present study examines all available records of immature *A. maculatum* from Mississippi to help determine its seasonality and geographic distribution.

Materials and Methods

We obtained data for this study from three majors sources: 1) The United States National Tick Collection, 2) literature available from online databases, and 3) unpublished university data. A request was submitted to the U.S. National Tick Collection (USNTC) (formerly the Rocky Mountain Laboratories collection), located in Statesboro, GA, for all historical host and biting data involving *A. maculatum* in Mississippi. Records were provided as both a summarized list of identifying information for each collection submitted, as well as photocopies of the original USNTC identification forms. Photocopies were carefully examined to ensure accuracy of compiled records. Various Internet databases were also searched for publications (journals and books) that contained information on this subject. Combinations of keywords such as '*Amblyomma maculatum*,' 'immatures,' 'seasonality,' 'Mississippi,' 'Gulf Coast tick,' and 'distribution' were used in searches. Databases included Google Scholar, the Armed Forces Pest Management Board (AFPMB), PubMed, and EBSCO Host. Lastly, any available unpublished university or USNTC data were also used. These included one additional collection of 25 larval *A. maculatum* which was made by drag cloth from Oktibbeha Co., MS in 2003 and confirmed by a USNTC employee. This

collection has to-date not received a unique accession number entering it into the USNTC (Goddard and Paddock 2005).

Results and Discussion

Collection data from all sources showed dates ranging from 1920-2014 consisting of 2,368 total specimens of *A. maculatum* collected in Mississippi. Of those, 2,295 (96.92%) were adults while only 27 (1.14%) were nymphs and 46 (1.94%) were larvae (Figure B.1). Larval records consisted of only 4 collections with one in June, two in October, and one in November (Figure B.2). October and November collections correspond with previous estimates of activity in Mississippi from September to November (Goddard 2007). Additionally, the peak in October/November corresponds with data from Georgia, although that study did not report any larval ticks collected in June (Hixson 1940). There was a single USNTC record (A.P. 25130) from July that noted 400± larvae from a sheep, however closer inspection of the original USNTC form revealed that the larvae resulted from several engorged females (A.P. 25108) in small jars containing moss (Figure B.3). Because these specimens were not actually collected from the field, these data were excluded. Nymphal records consisted of 17 collections with two discernible peak periods (Goddard and Paddock 2005, Moraru et al. 2013, Portugal III and Goddard 2015). The first peak period extended from February through May (22 nymphs collected), and a second smaller peak during August and September (5 nymphs collected) (Fig. 1). March had the most specimens collected (16 nymphs), with August second (4 nymphs). This primary nymphal peak from February to May corresponds with the peaks reported from Georgia (March-May) (Hixson 1940) and Texas (February) (Teel

et al. 1998). Only a single nymph was collected (May) in the northern two-thirds of Mississippi, not allowing any conclusions to be made about seasonality in that region.

Due to variation in number of specimens collected in each life stage (Figure B.1), combined data were plotted logarithmically ($\log_{10}(x)$) (Figure 3.1). Although fewer immatures were collected than adults, collection dates can be used to determine seasonality compared to adults. Adult records demonstrate a large peak of activity in August (26 collections) with two smaller peaks in May (11 collections) and November (3 collections). Combined records suggest that immature *A. maculatum* populations are less active during summer and winter and are most active during spring and fall months. Activity of *A. maculatum* immatures suggests a bi-modal distribution, mirroring behavior of the closely-related *A. americanum* (L.) nymphs in Mississippi, which also display bi-modal peaks in spring and again in late summer/early fall (Goddard 2007). Bi-modal activity patterns of the immatures (Fig. 1), suggest either a period of inactivity, or multiple batches of ticks per year. Activity of larval *A. maculatum* in Mississippi begins in June, seems to cease from July-September (hot summer months), and begins again in fall (October-November). Gulf Coast ticks have been shown to be the most susceptible to desiccation while in the larval stage (Yoder et al. 2008), which may account for these activity patterns. Larvae which become active early and feed before the summer period of inactivity probably emerge as nymphs in August and September, while those larvae that are active after the summer period of inactivity probably emerge as nymphs the following February to May. These data suggest that *A. maculatum* likely overwinters as both larvae and nymphs in Mississippi.

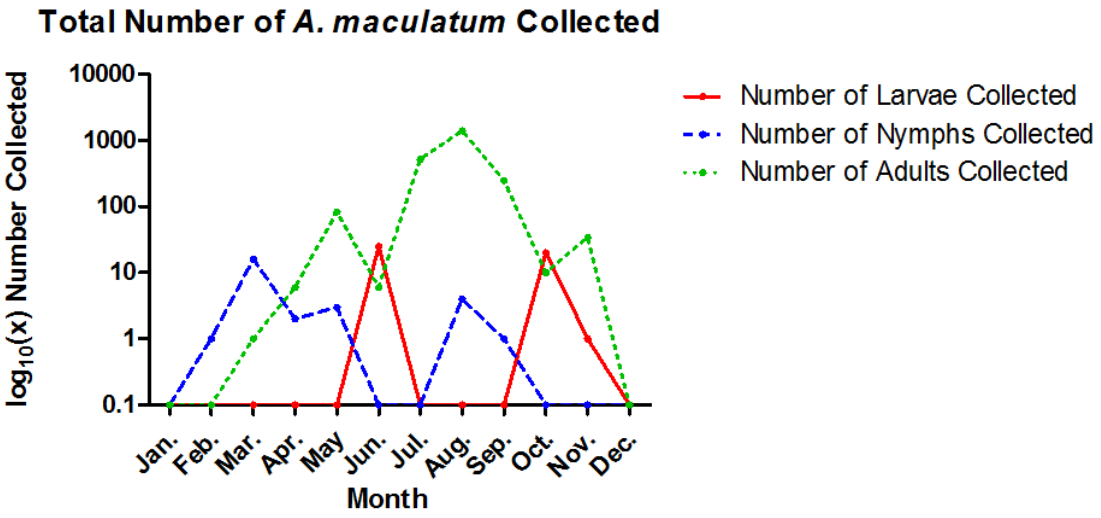

Figure 3.1 Seasonality of each life stage of *Amblyomma maculatum* in Mississippi

Note: plotted $\log_{10}(x)$, months with zero collections of a particular life stages were plotted as 0.1

Combined Gulf Coast tick activity data for Mississippi does not take into account variations in environmental conditions throughout the state. The northernmost third of the state experiences lower temperatures and less humidity on average than the southernmost third. The fact that this particular tick originated from warm and humid climates suggests that it might be more adapted to conditions in the southern third of the state as opposed to the northern third. This is supported by the traditional distribution of *A. maculatum* in the U.S., which is within coastal regions of the warm and humid North American Coastal Plain. It is important to note that the gradient in environmental conditions within Mississippi is not particularly extreme, and *A. maculatum* have been known to successfully establish populations in areas that are much cooler and dryer (Oklahoma and Kansas) (Cooley and Kohls 1944, Bell Jr 1972, Gates and Brooks 1972).

Amblyomma maculatum collections reported herein were from 17 of 82 counties in Mississippi- two counties from the northern third of the state, 6 from the middle third, and 9 from the southern third. These divisions correspond roughly to the USDA's Plant hardiness map, which characterizes zones based on average minimum temperatures. An additional zone is included in this map comprising most of the small strip of land between Interstate 10 and the coast, which represents extreme southern Mississippi. Zones correlate as: northern third (7b), middle third (8a), southern third (8b) and I-10 to the coast (9a) (Figure 3.2).

Using the combined data, nymphs were recorded earliest in zone 9a (February). Adult activity was not observed after July in zone 7b, and August in zone 8a, while more southern zones 8b and 9a continued to see adult activity until November. Overall, more collections of *A. maculatum* were made in zones 9a and 8b. Collections from zone 7b were very few compared to the southern zones, which is expected, as average minimum temperature is higher further south. However, when analyzing these data by zone, despite the fact that nymphs were identified earliest in the most southern zone (9a), adults appeared earliest in a zone further north (8a). Additionally, collections of larvae were made earlier in 8a (June), than 8b and 9a (October and November respectively). I have no explanation for these patterns.

Hosts of larvae and nymphs described in the literature and USNTC records included Swamp sparrows (*Melospiza Georgiana* (Latham)), Hispid cotton rats (*Sigmodon hispidus* Say and Ord), Eastern meadowlarks (*Sturnella magna* (L.)), Eastern woodrats (*Neotoma floridana* (Ord)), Emus (*Dromaius novaehollandiae* (Latham)) and humans (*Homo sapiens* L.) (Table 3.1). With exception of emus, all their hosts correlate

well with previously reported larval and nymphal hosts being either small mammals (Cooley and Kohls 1944), ground-dwelling birds (Hooker et al. 1912, Teel et al. 2010), or humans (nymphs) (Goddard 2002).

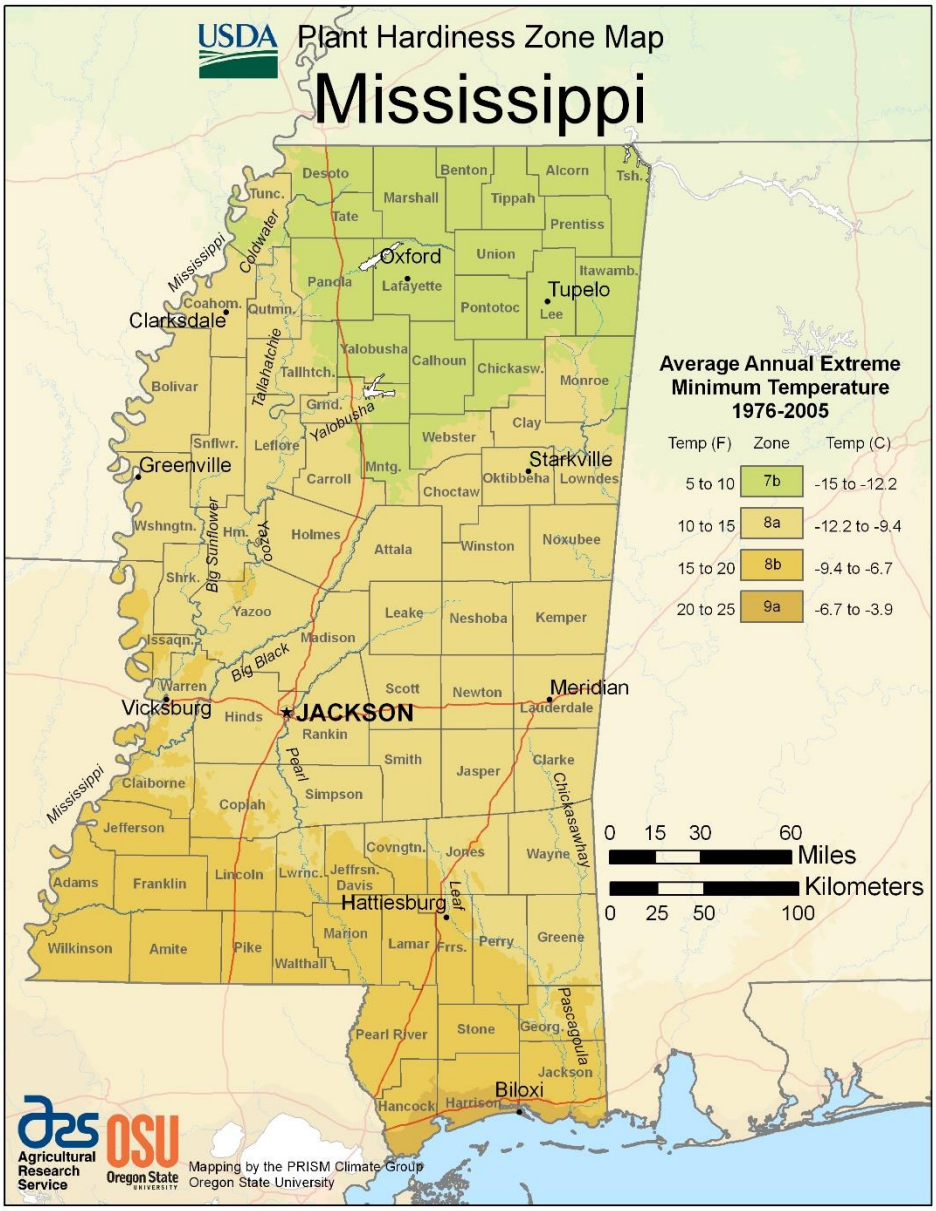

Figure 3.2 USDA Plant Hardiness Zone map of Mississippi

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Table 3.1 Combined records of immature *Amblyomma maculatum* collected in Mississippi

Life Stage	Date	Record Source	Number	Collected From	County
Larvae	05 Jun 2003	Goddard and Paddock 2005	25	Vegetation	Oktibbeha
Larvae	?? Oct 1998	USNTC 123084	19	*Neotoma floridana*	Holmes
Larvae	23 Oct 1994	USNTC 121763	1	*Dromaius novaehollandiae*	Covington
Larvae	21 Nov 2013	Portugal and Goddard 2015	1	Vegetation	Jackson
Nymph	17 Feb 1940	USNTC 57682	1	*Melospiza georgiana*	Harrison
Nymph	14 Mar 2014	Portugal and Goddard 2015	3	Vegetation	Jackson
Nymph	15 Mar 2010	Moraru et al. 2013	2	*Sigmodon hispidus*	Jackson
Nymph	20 Mar 2000	USNTC 123013	1	*Homo sapiens*	Rankin
Nymph	20 Mar 2009	Goddard Unpublished	2	Vegetation	Jackson
Nymph	21 Mar 2014	Portugal and Goddard 2015	6	Vegetation	Jackson
Nymph	24 Mar 1990	USNTC 120743	1	*Sigmodon hispidus*	Jackson
Nymph	26 Mar 2013	Portugal and Goddard 2015	1	Vegetation	Jackson
Nymph	06 Apr 2013	Portugal and Goddard 2015	1	Vegetation	Jackson
Nymph	18 Apr 2003	USNTC 123521	1	Vegetation	Copiah
Nymph	05 Apr 1993	USNTC 46130	1	Unknown	Noxubee
Nymph	11 May 1989	USNTC 119665	1	Vegetation	Copiah
Nymph	16 May 2014	Portugal and Goddard 2015	1	Vegetation	Jackson
Nymph	02 Aug 1948	USNTC 25693	2	*Sturnella magna (2)*	Jackson
Nymph	03 Aug 1991	USNTC 120400	1	*Homo sapiens*	Harrison
Nymph	04 Aug 1991	USNTC 120402	1	*Homo sapiens*	Pike
Nymph	07 Sep 1989	USNTC 119725	1	*Homo sapiens*	Harrison

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There are limitations when analyzing historical collection records from Mississippi to determine seasonality and distribution of immature *A. maculatum*. The most glaring is sample size. Due to the fact that immatures of this species are difficult to collect in nature, there are only a small number of specimens recorded in the USNTC and

in published literature. Additionally, only specimens that were actually collected are recorded, and not failed collection attempts, which adds bias to the analysis. Seasonality and distribution data may also be affected by sampling bias, such as giving preferential treatment to specific times of year or places for collection. Further systematic research is necessary to mitigate these limitations as well as to update the USNTC records with new information concerning immature *A. maculatum* activity and their distribution in Mississippi.

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CHAPTER IV

ATTEMPTS TO FEED LARVAL *AMBLYOMMA MACULATUM* KOCH AND *AMBLYOMMA AMERICANUM* (L.) (ACARI: IXODIDAE) ON THREE ARTHROPOD HOSTS

Results from this chapter have been previously published in Journal of Vector Ecology (2015) 40(1): 202-204. The publication is available at http://dx.doi.org/10.1111/jvec.12154

Abstract

Ticks occasionally have been found attached to arthropods such as beetles, flies, and bees; however, the frequency and success of such feedings have never been investigated. This study evaluated whether the larvae of two tick species: *Amblyomma maculatum* Koch and *Amblyomma americanum* (L.), can feed on three commonly encountered insects: the European honey bee (*Apis mellifera*), the house cricket (*Acheta domesticus*) and larvae of the Fall armyworm (*Spodoptera frugiperda*), whose distributions overlap that of these ticks. Larval ticks were placed inside clear plastic tubes containing the test insects and then observations were made every 24 hours until 72 hours, when the tube was frozen and the insects examined microscopically for attached ticks. This procedure was repeated three times with all three insect species. In the cricket and fall armyworm experiments, red food coloring was injected into the insects to help determine if ticks fed (or not). Both species of ticks initially moved towards and

crawled upon all three insects used in this experiment. Although one tick (*A. maculatum*) appeared to be attached to a cricket, attachment could not be confirmed. Sixteen *A. americanum* ticks exposed to crickets appeared slightly larger than other (unexposed) ticks from the colony. These were washed three times with a PBS solution and compared to normal appearing ticks from the same tubes. Six ticks had a red hypostome and gut, indicating they had fed on the crickets. Further investigation is needed to confirm laboratory feeding of ticks on arthropod hosts.

Introduction

Ticks (subclass Acari) are closely related to mites, many of which utilize invertebrates such as arthropods for feeding as well as for phoresy (transport). Therefore, it is reasonable to assume that ticks may occasionally infest invertebrates. Indeed, they are recorded from arthropods in literature (Table 3.1). In 1900 an *Amblyomma fimbriatum* was noted "to occur" on a beetle (*Aulacocyclus kaupi*) (Keirans 1985). Although the authors did not provide further details, this report presented an interesting observation. Additionally, a United States Department of Agriculture manual on ticks reported a beetle as a host for an *Amblyomma maculatum* tick (Hooker et al. 1912), again, no further details were given. More recently, an adult male *Rhipicephalus annulatus* was found on a pinned horsefly specimen (Leprince et al. 1988), and two attached larval *Ixodes persulcatus* (Taiga tick) were discovered on two piophillid flies from Siberia (Petrova and Basikhin 1993). Additionally, in 2010 seven *Dermacentor variabilis* (American dog tick) larvae were collected from a carpenter bee (*Xylocopa virginica*) in Louisiana (Goddard and Bircham 2010). Of the seven ticks found on the

carpenter bee, one appeared partially engorged although none were actually seen attached.

Table 4.1 Combined records of ticks found on or attached to arthropods

Tick Species[a]	Number	Arthropod Host	Stage	Location
Amblyomma spp.				
A. fimbriatum[1,b]	1	*Aulacocyclus kaupi*	Adult	New South Wales, Australia
A. gervaisi[2,c]	5	*Calliphora erythrocephala*	Larva	Delhi, India
A. maculatum[3]	1	Coleopteran	Unknown	Unknown
Dermacentor spp.				
D. nitens[4,d]	5	*Lucio castelnaui*	3 Larvae 2 Nymphs	Mato Grosso do Sul, Brazil
D. variabilis[5]	7	*Xylocopa virginica*	Larva	Louisiana, USA
Ixodes spp.				
I. persulcatus[6]	2	Piophilidae	Larva	Yamal, Siberia, Russia
I. ricinus[7,8]	5	*Trypocopris pyrenaeus*	2 Nymphs	Errenteria, Guipúzcoa, Spain
		Nicrophorus vespilloides	1 Nymph	Errenteria, Guipúzcoa, Spain
		Anoplotrupes stercorosus	1 Larva	Berkshire, United Kingdom
		Hydrotaea dentipes	1 Nymph	Bratislava-Kopáč, Slovakia
Rhipicephalus spp.				
R.(B.) annulatus[9]	1	*Tabanus americanus*	Adult	Louisiana, USA
R. turanicus[10]	2	*Tabanus leleani*	Adult	Ashkhabad, Turkmenistan

All tick species names are current per Guglielmone et al. 2010

[a,1] Keirans 1985; [2]Nagar and Raizada 1977; [3]Hooker et al. 1912; [4]Flechtmann and Baggio 1993; [5]Goddard and Bircham 2010; [6]Petrova and Basikhin 1993; [7]Saloña-Bordas et al. 2015; [8]Mašán and Krištofík 1992; [9]Leprince et al. 1988; [10]Boshko and Skliar 1981.

[b]Originally reported in 1901 as *Aponomma ecinctum* (Neumann 1901)

[c]Originally reported as *Aponomma ophiophilum* (synonym) (Santos-Dias 1958)

[d]Originally reported as *Anocentor nitens*

Amblyomma maculatum (Gulf Coast tick) and *Amblyomma americanum* (Lone Star tick) are common ticks in the southeastern United States. Both are aggressive feeders (Goddard and Varela-Stokes 2009, Paddock et al. 2010) whose larvae will respond to movement as well as CO_2 from respiration (Ginsberg and Ewing 1989, Teel et

al. 2010). Accordingly, these species are good candidates for potential tick-arthropod infestations. There are no documented instances of *A. americanum* ticks attaching or feeding from invertebrates. This current study was performed to evaluate whether these two tick species will feed on three commonly encountered arthropods: European honey bees (*Apis mellifera*), house crickets (*Acheta domesticus*), and larval fall armyworms (*Spodoptera frugiperda*), all of whose distributions overlap that of the ticks. Crickets and larval fall armyworms could encounter larval ticks while feeding and moving through vegetation, honeybees may encounter them while foraging at flowers such as clover.

Materials and Methods

Crickets

Prior to testing, crickets purchased from a local sporting goods store (sold as bait for fishing) were housed in a wire screen container and provided a supply of potato and tropical fish food to fulfill nutritional requirements (Figure 4.1. A 1:20 dilution of dye (McCormick® red food color) in phosphate buffer solution (Fisher BioReagents®) was injected between the abdominal segments (1 cc) which tinted the hemolymph bright red without killing the cricket.

Figure 4.1 Crickets (*Acheta domesticus*) feeding on potato in wire enclosure.

Crickets were provided potato and tropical fish flakes to feed on. A small cardboard structure was provided as a shelter to reduce stress to crickets. Photo credit: J. Santos Portugal III

Honey Bees

Honey bees were taken from a hive located on the campus of Mississippi State University and bees chosen were considered newly emerged (approximately 0-5 hours old) which had not yet been exposed to any foraging stress. The hive consisted of Italian strain bees of the Cordovan recessive color variation (C.F. Koehnen & Sons, Inc. stock) which were selected for this study due to their mild temperament. To provide sustenance

for the bees, “queen candy” was pressed into the lids of the tubes (Figure 4.2. Queen candy is a combination of corn syrup and powdered sugar kneaded to the point of being firm and can be molded as necessary to feed bees when in captivity. Experiments began the day bees were removed from hives, and no red dye was injected due to difficulties related to handling the bees.

Figure 4.2 Bee (*Apis mellifera*) feeding on “queen candy” pressed into lid.

Photo credit: J. Santos Portugal III

Fall Armyworms

Larval fall armyworms were obtained from a colony at Mississippi State University. Red dye (McCormick® red food color) was given to the larvae via their diet (Ward's Science® Stonefly Heliothis diet) (Figure 4.3, Figure C.2). Two hundred µl dye/gram of diet was sufficient to tint hemolymph without killing the larvae. Newly-eclosed larvae were placed on this diet and reared for 12 days (4th instar). Rearing was conducted at the Insect Rearing Center (Mississippi State University), with environmental conditions of 80°F (26.7° C) and 60% relative humidity.

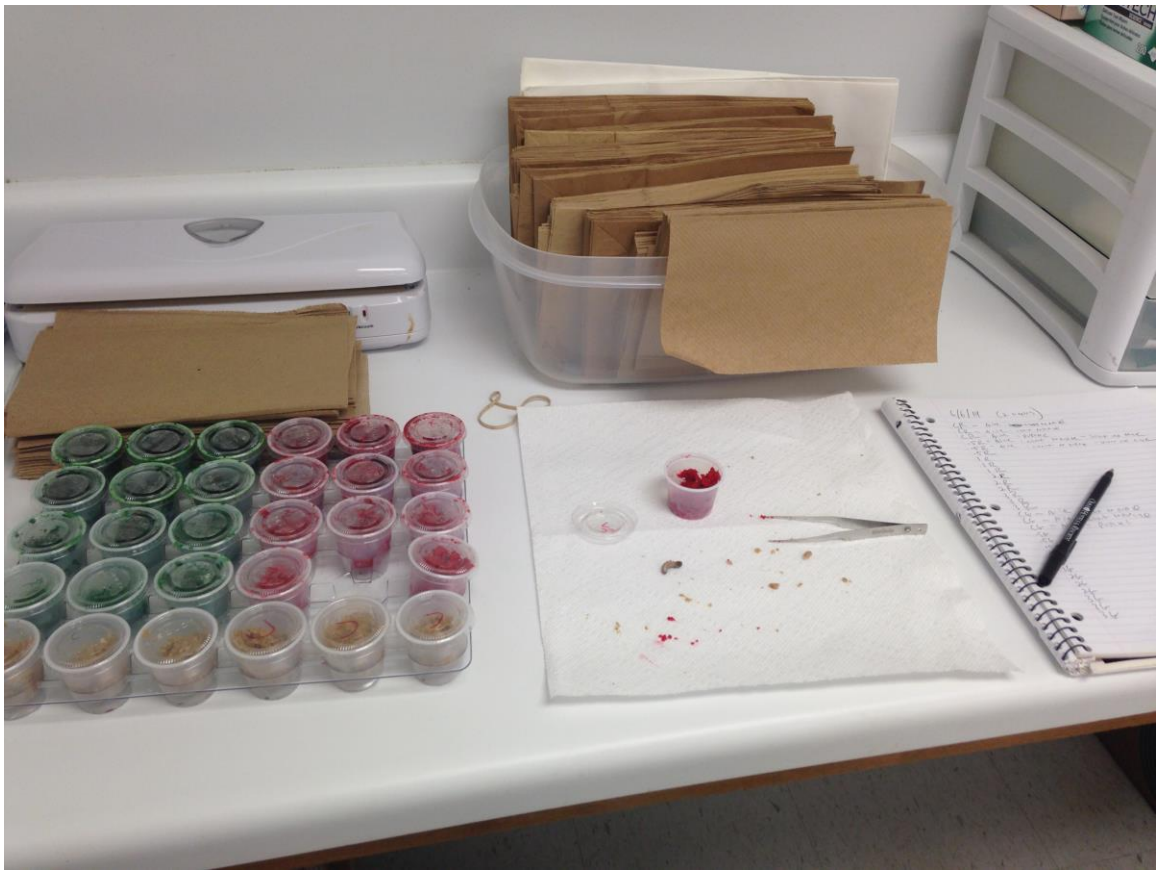

Figure 4.3 Larval fall armyworms (*Spodoptera frugiperda)* reared on diet containing various concentrations and colors of dye.

Photo credit: J. Santos Portugal III.

Arthropod hosts were placed one per tube, in clear plastic vacuum aspirator tubes measuring 8cm in length and 2.5cm in diameter (Figure 4.2). The tubes had a removable screw-cap metal lid on one end and a screen barrier on the other. During each replication, approximately 25 larval ticks (obtained from a colony at Oklahoma State University) were placed inside the tubes with the arthropod host. Tubes were then placed in resealable plastic bags with a moistened paper towel to prevent desiccation (Figure C.3). Observations were made every 24 hours and at 72 hours, each arthropod host was frozen (-20°C) and subsequently examined microscopically for tick attachment. I looked for any evidence of engorgement such as swelling or red color contained in the gut. This procedure was repeated three separate times with larval *Amblyomma maculatum* and then three times with larval *Amblyomma americanum*.

Results and Discussion

No ticks definitively attached to the crickets, honeybees, or fall armyworms at any of the observational periods. One *Amblyomma maculatum* appeared to be attached near the base of the forewing of a cricket, but it could not be confirmed that its hypostome was embedded in the integument (Figure 4.4). Many of the ticks did seem to react to movements of all three arthropods, and were often observed crawling on, or sequestered in, crevices and areas under appendages of the arthropod hosts (both *A. americanum* and *A. maculatum*). Movement by the arthropod hosts may have triggered this response, but CO_2 released during respiration as well as other volatiles and compounds produced by the hosts may have also played a role. Both tick species were observed to quest occasionally on the crickets and fall armyworms but not the bees. Sixteen ticks (*A. americanum*) held in tubes with crickets appeared to be slightly larger than other ticks from the colony and

were washed thoroughly three times with phosphate buffer solution. These ticks, along with normal-sized specimens from the same tubes, were then examined for red color. Six of these ticks had a red hypostome and gut (Figure 4.5, Figure C.4). This is significant in that if the dye had only topically stained the integument, palps, scutum, and legs would also be red. Although metabolites of hemoglobin include reddish pigments that have been demonstrated to tint hemolymph in some tick species (Wigglesworth 1943, Hamdy et al. 1974, Hamdy and Sidrak 1982), I saw no color difference between ticks prior to the experiments.

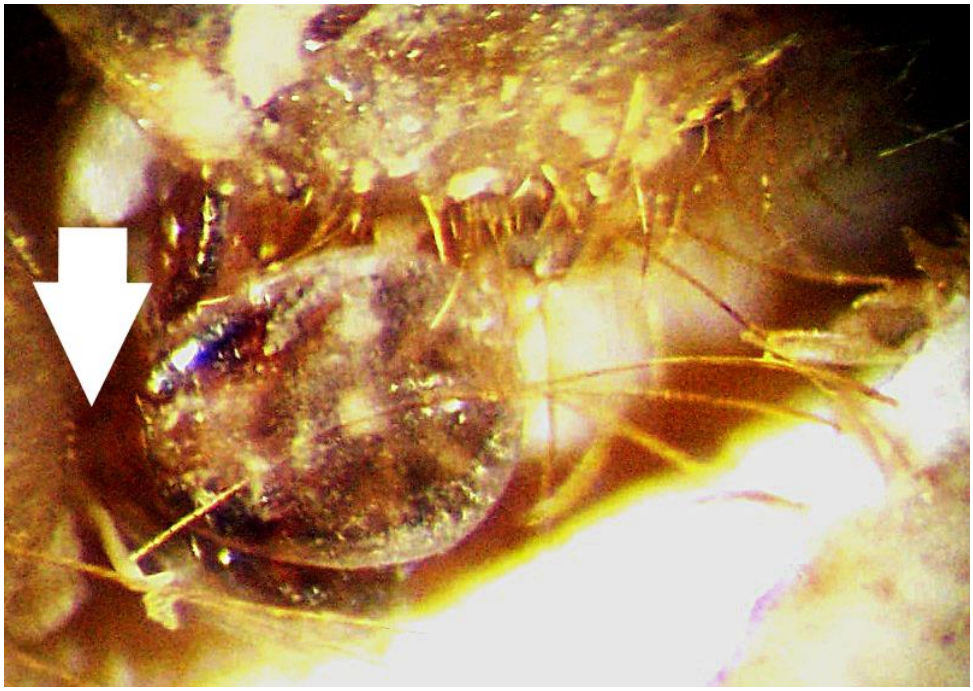

Figure 4.4 Larval *Amblyomma maculatum* tick appearing to be attached to cricket

Tick located under forewing near point of wing attachment, arrow pointing to mouthparts. Photo credit: Jerome Goddard, Ph.D.

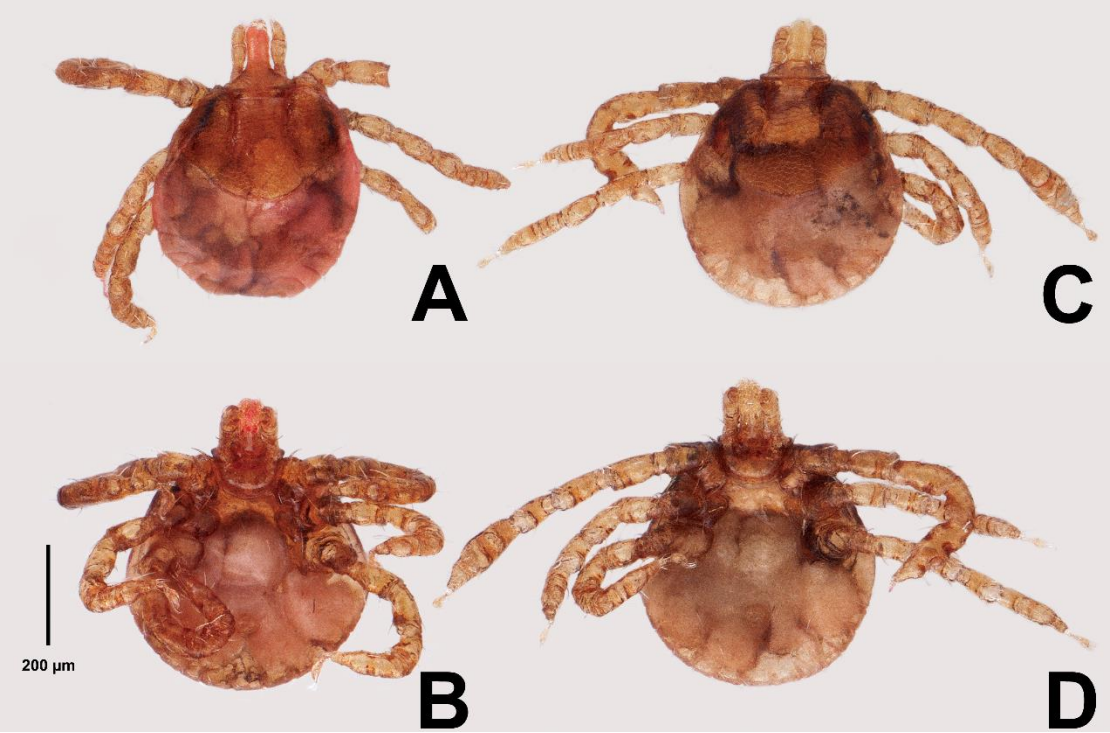

Figure 4.5 Red gut and hypostome of ticks that imbibed hemolymph from cricket (left) compared to ticks that did not (right)

Dorsal (A) and ventral (B) view of larval *Amblyomma* americanum ticks suspected of feeding from crickets, versus ticks that did not (C, D). Photo credit: Joe MacGown.

These data show that under experimentally controlled conditions, *A. americanum* larvae may attach to crickets and imbibe some volume of hemolymph. Although ticks have been previously identified with their mouthparts embedded in arthropods (Boshko and Skliar 1981, Leprince et al. 1988), actual feeding by ticks upon arthropods has not been demonstrated. Earlier work (Milne 1949) operated under the assumption that invertebrates are not a suitable host for ticks, as ticks had not been found previously attached to invertebrates in natural conditions. However, it has been suggested that ticks will occasionally attempt to feed from insects to complete a blood meal (Hoogstraal

and Aeschlimann 1982). Indeed, if immature ticks readily fed upon invertebrates, researchers across multiple disciplines of entomology and biology would have come across this phenomenon more frequently than it has been documented. However, it is possible that larval ticks may in some instances be confused with mites if not closely examined. Another possibility may be that taking a hemolymph meal reduces the threat of desiccation facing unfed immature ticks under adverse conditions.

Reaction to movement displayed by ticks in this experiment, may allow for the possibility that ticks could unintentionally utilize arthropods as a method of dispersal. Although this behavior may not be considered "true" phoresy, as it has not been demonstrated that ticks intentionally use arthropods to relocate, behavior demonstrated in this experiment may be advantageous as an unintentional, passive dispersal method. This method of dispersal is exemplified in an instance where 3 larval and 2 nymphal *Dermacentor nitens* (Neumann, 1897) were collected from the setae of a *Lucio castelnaui* (Lampyridae) captured in midflight (Flechtmann and Baggio 1993).

There are currently approximately 900 species of ticks known to date, and an ever-increasing number of invertebrate species described; therefore, interaction of these two groups is inevitable. Our work may have interesting implications concerning the ecology and evolution of invertebrate endosymbionts and their link to vertebrate pathogens (Dale and Moran 2006). Additionally, vertebrate pathogens have been found in non-hematophagous insects (Thepparit et al. 2011), which could possibly be transferred to ticks. Further investigation should be undertaken to explore the possibility of ticks utilizing arthropods as hosts, for dispersal, and their interaction with various endosymbionts and other microbial organisms.

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CHAPTER V

EVALUATION OF HUMAN ATTACHMENT BY LARVAL *AMBLYOMMA MACULATUM* KOCH (ACARI: IXODIDAE)

Results from this chapter have been previously published in Journal of Medical Entomology (2016) 53(2): 451-45, and Journal of the American Medical Association (JAMA) Dermatology (2015) 151(12): 1373-1375. The publications are available at http://dx.doi.org/10.1093/jme/tjv185 *and* http://dx.doi.org/10.1001/jamadermatol.2015.2388

Abstract

The tick, *Amblyomma maculatum* Koch (Gulf Coast tick), has recently been shown to be an important disease vector of both medical and veterinary concern. Although much is known about the behavior and ecology of adults, little is known of the immatures. Larval feeding on humans has never been demonstrated (and thus, there are no collection records from humans). In this experiment, approximately 10 larval *A. maculatum*, *Amblyomma americanum* (L.) (a positive control), and *Dermacentor variabilis* (Say) (a negative control), were applied to both forearms of 10 human volunteers (five male, five female). Ticks were placed in plastic caps and secured to skin with medical-grade adhesive tape, and volunteers remained sedentary during the experiment. After 15 min, caps were removed, and attachment was determined using fine-tipped forceps. Any *Amblyomma maculatum* that were attached were then removed

and subsequently examined microscopically to verify identification. A total of 34 ticks attached to the subjects, including 11 *A. maculatum* (5.5%), 23 *A. americanum* (11.5%), and no *D. variabilis*. *Amblyomma maculatum* attached to six volunteers, and no apparent association between gender and attachment rate was noted. No skin lesions developed in any of the human volunteers bit by *A. maculatum*. This is the first report of larval *A. maculatum* attaching to humans, and is significant in that *Rickettsia parkeri*, a human pathogen transmitted by this species, has recently been reported to be transmitted transovarially. If *A. maculatum* are infected as larvae, they could potentially transmit *R. parkeri* to people.

Introduction

Amblyomma maculatum Koch, also known as the Gulf Coast tick, is an ornate, large tick commonly encountered in the United States along the Gulf and Atlantic Coasts. Adult *A. maculatum* typically utilize large mammals such as cattle as hosts, while immatures (larvae and nymphs) usually feed from small mammals and ground dwelling birds (cotton rats, meadowlarks, and Bobwhite quail) (Hooker et al. 1912, Bishopp and Hixson 1936, Teel et al. 2010, Moraru et al. 2012). Both adults and nymphs will readily feed on humans, but it has not been determined whether larval *A. maculatum* will bite people (Goddard 2002, Teel et al. 2010). This species has been described in literature as a competent vector of the human pathogen *Rickettsia parkeri*, a spotted fever group rickettsia (Paddock et al. 2004). Originally isolated from *A. maculatum* ticks in 1939, *R. parkeri* or the "Maculatum agent" caused mild fever and scrotal swelling in guinea pigs (Parker et al. 1939). *Rickettsia parkeri* was not known to be a human pathogen for approximately 70 years, when cases of a "new" rickettsiosis were identified by

researchers in the U.S. (Paddock et al. 2004). This new disease is variously known as American Boutonneuse fever (ABF), "maculatum disease," or Tidewater fever (Lackman et al. 1949, Goddard 2004, Wright et al. 2011) and is characterized by eschars at the bite site, fever, rash, and headache (Paddock and Goddard 2015). Thus far, adult and nymphal *A. maculatum* have been implicated in cases of ABF, but not larvae (Paddock and Goddard 2015). Larvae of many other hard tick species such as *A. americanum* are known to feed on humans (Cooley and Kohls 1944), whereas others such as *Dermacentor variabilis* do not (Bishopp and Trembley 1945). There are no records of *A. maculatum* larvae feeding from humans (Goddard 2002, Teel et al. 2010). The objective of this study was to determine if and at what frequency larval *A. maculatum* attach to humans compared with a species known to bite people and one which does not.

Materials and Methods

Three species of larval ticks were used in this experiment: *Amblyomma americanum* (L.) (a positive control), *Dermacentor variabilis* (Say) (a negative control), and *A. maculatum* (the species in question). Ticks were obtained from colonies maintained by the Centers for Disease Control and Prevention, and were certified "free of known rickettsial pathogens" and "specific pathogen free" (Figure D.1). Ticks were less than a month old when received, placed in a desiccator jar with saturated salt solution to maintain high humidity (avg. 97%), and kept under laboratory conditions (22°C and 8:16 L:D photoperiod). All testing was performed within 10 days after ticks were received. Ten human volunteers, five of both sexes, were recruited for this experiment, and all participants were briefed on the procedure and associated risks (Figure D.2).

Plastic caps from 20mL glass scintillation vials (Model 74516-20, Kimble®, Vineland, NJ) were used to contain ticks during testing. Ten larval ticks were placed into each of three caps (one species per cap). Ticks were applied to caps using a fine-tipped paintbrush, and *A. maculatum* were applied first to avoid contamination with our positive control. These caps were then secured to the palmar (ventral) surface of the forearm with cloth first aid tape (CVS Pharmacy®, Woonsocket, RI) (Figure 5.1, Figure D.3). Arms were chosen as the site of exposure simply due to accessibility and replication. This procedure was then duplicated on the other arm for a total of twenty of each tick species. Caps containing *A. americanum* were placed close to the cubital fossa, *D. variabilis* were placed close to the wrist, and *A. maculatum* were placed in the middle. Ticks were left in place 15 min, allowing for ample time to attach, but not sufficient time for transmission of most tick-borne pathogens (Moore 1911, Piesman et al. 1987, Katavolos et al. 1998). Alcohol swabs and adhesive bandages were provided to volunteers after testing when requested.

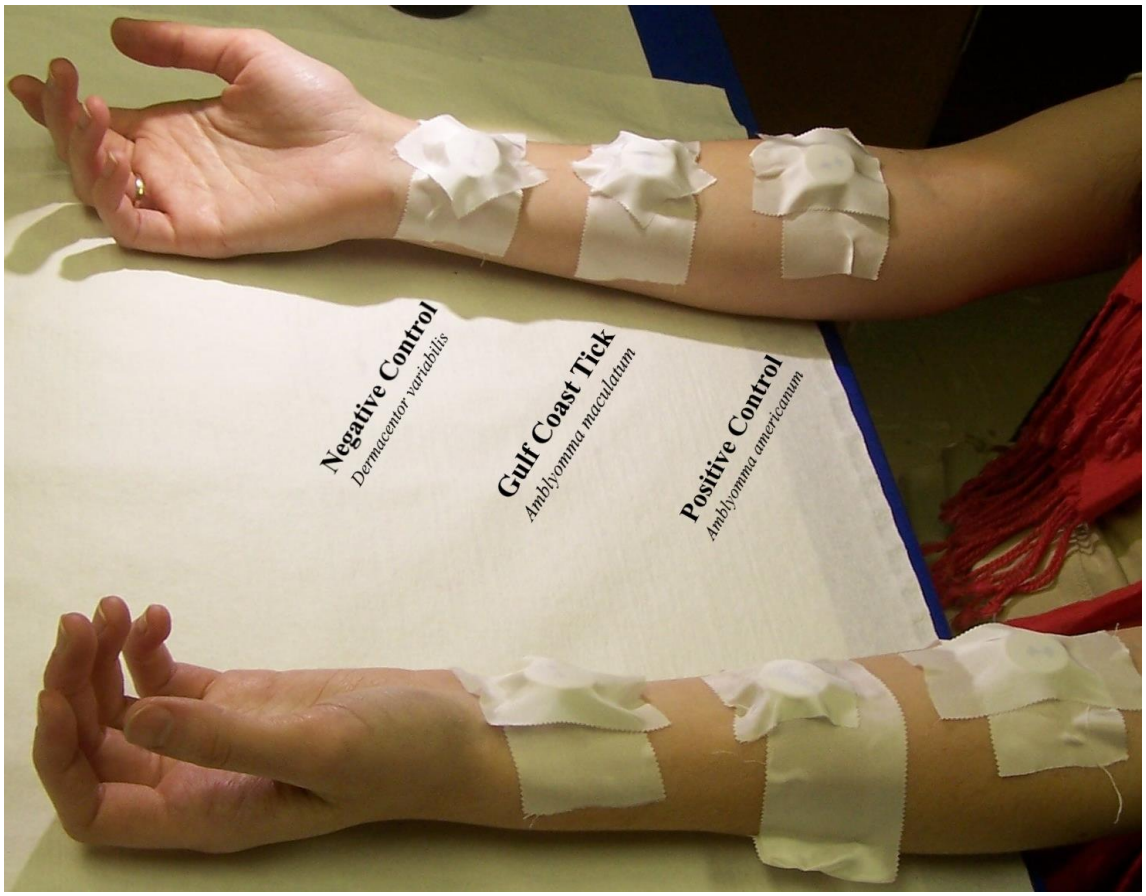

Figure 5.1 Positioning of caps containing ticks of all three species on forearms

Amblyomma maculatum were in the center cap, while *Amblyomma americanum* were in the cap proximal on arm, and *Dermacentor variabilis* were in cap distal on arm. Note: ticks were tested on both left and right arm. Photo credit: Jerome Goddard, Ph.D.

When caps were removed from volunteers, unattached (crawling) ticks were collected with masking tape. Ticks that appeared to be attached or had not moved from the application site were challenged with fine-tipped forceps and photographed. Slight pressure was applied to the posterior end of these ticks, moving them if not attached. Attached ticks were noted and removed, then reexamined microscopically to verify

identification. Data were square root transformed, and analyzed using PROC GLIMMIX in SAS 9.3 (SAS Institute, Cary, NC). P-values <0.05 were considered significant.

Results and Discussion

Researchers have occasionally used human volunteers in tick-biting experiments without any major medical or health issues (Sheele et al. 2013, Marques et al. 2014, Sheele et al. 2014), as was the case in this study. A total of 34 larval *A. americanum* (Figure D.4) and *A. maculatum* (Figure 5.2) clearly attached to humans during the 15 min. testing period**.** No *D. variabilis* (negative control) attached, which was expected (Figure D.5). I observed that larval *A. americanum* ticks frequently selected feeding sites near where the cap contacted skin (Figure D.6). Twenty-three *A. americanum* (11.5%) attached to 8 of 10 volunteers, and 11 *A. maculatum* (5.5%) were confirmed attached to 6 of 10 volunteers (Figure 5.2, Table 5.1). Six volunteers reported papular lesions and mild itching at *A. americanum* bite sites (Goddard and Portugal III 2015), which resolved within 14 days, but none developed lesions or itching at any of the *A. maculatum* bite sites. Although approximately twice as many *A. americanum* attached as did *A. maculatum*, the difference was not significant ($t = 1.89$; df = 1, 27; $P = 0.699$).

Figure 5.2 Larval *Amblyomma maculatum* attached to forearm of human volunteer Next to penny (USA) for size comparison. Photo credit: Jerome Goddard, Ph.D.

Table 5.1 Attachment rates for all three species of larval ticks by individual volunteer

Volunteer	*Amblyomma americanum* (Positive control)	*Dermacentor variabilis* (Negative control)	*Amblyomma maculatum*
1	0 (0%)	0 (0%)	1 (5%)
2	1 (5%)	0 (0%)	0 (0%)
3	2 (10%)	0 (0%)	1 (5%)
4	3 (15%)	0 (0%)	5 (25%)
5	3 (15%)	0 (0%)	0 (0%)
6	1 (5%)	0 (0%)	2 (10%)
7	6 (30%)	0 (0%)	1 (5%)
8	3 (15%)	0 (0%)	0 (0%)
9	0 (0%)	0 (0%)	1 (5%)
10	4 (20%)	0 (0%)	0 (0%)
Total	23/200 (11.5%)	0/200 (0%)	11/200 (5.5%)

This is the first report of larval *A. maculatum* ticks attaching to a human subject, and is significant in that *R. parkeri* has been recently reported to be transmitted transovarially (Wright et al. 2015). Since the agent sometimes may be found in larval *A. maculatum*, this experiment suggests they potentially could transmit *R. parkeri* to people. In contrast, although transovarial transmission of *R. rickettsii* may sometimes occur in *D. variabilis,* larvae of this species do not bite people and thus are not a threat (Goodman et al. 2005). As for *A. americanum*, the larvae readily bite humans, but *Ehrlichia chaffeensis* is not transovarially transmitted (Goodman et al. 2005). Although larval *A.*

maculatum are rarely encountered by humans in the field and do not readily attach, they may be misidentified as *A. americanum* due to overlapping seasonality and similar appearance (Goddard and Paddock 2005, Goddard 2007, Portugal III and Goddard 2015, 2016). As a newly recognized potential threat to human health, the extent to which larvae of this species will attach and feed from humans and potentially transmit *R. parkeri* should be further investigated.

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CHAPTER VI

LABORATORY STUDIES OF HOST-SEEKING BEHAVIOR IN COLONIZED NYMPHAL *AMBLYOMMA MACULATUM* KOCH TICKS (ACARI: IXODIDAE)

Abstract

Many environmental factors can affect host-seeking behavior in ticks. Eighty nymphal *Amblyomma maculatum* Koch, a vector of animal and human pathogens, were released in a glass aquarium observation arena containing 4 different heights of broomsedge stems (*Andropogon virginicus* L.) anchored in a sand substrate. Observations were made over a three-day period on proportion questing, distribution by stem height, and distribution by questing height. This process was performed three times with different sets of ticks for each of three combinations of temperature and humidity: High Temperature/High Humidity (*HTHH*), High Temperature/Low Humidity (*HTLH*), and Low Temperature/High Humidity (*LTHH*). A fourth set of environmental conditions included periods of Wind (*HTHHW+/-*) by alternating days of wind and no-wind conditions over four days. Mean questing height for *HTHH*, *HTLH*, and *LTHH* ranged from 4.45-6.03 cm with ticks questing significantly higher in *HTHH*. A significantly lower proportion ticks quested in *HTLH* (8.64%) than *HTHH* (14.06%) and *LTHH* (15.33%). In *HTHH* and *LTHH*, a significantly higher proportion of ticks were observed questing on 5-cm stems. Wind significantly reduced average tick questing height. When

wind was absent, ticks on 20 and 30 cm stems quested significantly higher. I was not able to determine if orientation (head up or down) was biologically significant in this study. Additionally, these data suggest that *A. macuatum* nymphs may randomly select stems on which to quest and that they climb upward until adverse conditions are prohibitive or until an "ideal" height is reached. Conditions with reduced vapor pressure deficit encouraged higher questing frequency, but higher temperature with Vapor Pressure Deficit (*VPD*) nearly constant saw increased mean questing height.

Introduction

Ticks include ca. 900 species and are found on every continent (Guglielmone et al. 2010). They are the primary vector of animal pathogens worldwide and, as a medical threat to humans, are surpassed only by mosquitoes. For this reason, it is important to elucidate tick behavior and ecology. Hard ticks (Acari: Ixodidae), are of particular concern as vectors of pathogens. They are typically non-nidiculous and utilize either ambush or hunter strategies to obtain their hosts (Sonenshine 2005). *Amblyomma* are frequently encountered in tropical and subtropical regions of both hemispheres, including the United States (Cooley and Kohls 1944). In the southeastern United States, *Amblyomma* is one of the most common genera of ticks encountered while field-collecting, as well as removed from humans (White 1955, Goddard 2002). Many *Amblyomma* spp. are capable of transmitting both human (Paddock et al. 2004, Goddard and Varela-Stokes 2009, Breitschwerdt et al. 2011), and animal pathogens (Walker and Olwage 1987, Vincent-Johnson et al. 1997).

Amblyomma maculatum, the Gulf Coast tick, is a 3-host tick typically found in the North-Central Atlantic coast; which encompasses coastal portions of the mid-Atlantic and

southeastern United States, Central America, and northern South America (southern Nearctic, northern Neotropical realms). Known for its ornate scutum and large size, *A. maculatum* is a vector of both medical and veterinary concern (Teel et al. 2010, Paddock and Goddard 2015) This tick predominantly feeds on medium to large mammals as an adult and small mammals and ground-dwelling birds as an immature, i. e. larva and nymph (Hooker et al. 1912, Bishopp and Hixson 1936, Teel et al. 2010). Nymphs in particular have been shown in the laboratory to have very high engorgement and molting success on bobwhite quail (*Colinus virginianus*) and even higher success on both cotton rats (*Sigmodon hispidus*) and wood rats (*Neotoma floridana*) compared to larvae (Koch and Hair 1975). All mobile stages of this tick will attach to humans, while only nymphs and adults have been documented feeding from humans outside the laboratory (Teel et al. 2010, Portugal III and Goddard 2016a). A competent vector of the human pathogen *Rickettsia parkeri* (causative agent of American boutonneuse fever) (Parker et al. 1939, Goddard 2004, Paddock et al. 2004), transovarial transmission has been described in this species (Wright et al. 2015); and an infection rate of ca. 17 % of questing nymphs in the field was observed in one study (Goddard et al. 2016).

Although known to move aggressively towards a potential host, this tick will also quest on vegetation for long periods of time. Questing by hard ticks is an ambush strategy that involves moving vertically on vegetation or other surfaces to a height that is ideal for obtaining its preferred host(s) (Camin and Drenner 1978, Goddard 1992, Mejlon and Jaenson 1997, Oorebeek et al. 2009). Conditions such as temperature, relative humidity (*RH*), wind, and photoperiod have been demonstrated to have some influence on questing behavior (Loye and Lane 1988, Perret et al. 2003, Schulze and Jordan 2003).

As hard ticks can spend over 90% of their time off-host, environmental conditions and their complementary responses can significantly affect survivability (Norval 1977, Needham and Teel 1991). In particular, immature hard ticks are susceptible to desiccation in unfavorable conditions, especially when questing (Needham and Teel 1991). Due to increased risk of desiccation, immatures typically do not quest as high as adults, perhaps in order to be able to retreat from adverse environmental conditions (Mejlon and Jaenson 1997). This tendency to quest lower than adults may restrict the size of host utilized by immatures to smaller species. Vapor pressure deficit (*VPD*) (sometimes known as "saturation deficit") (Yoder et al. 2017), is the difference between the water vapor pressure (kPa) at saturation and the water vapor pressure actually in the air. *VPD* is another environmental factor that can have an effect on questing behavior. A high *VPD* is typically observed in conditions where *RH* is low and temperature is high, although this is not always the case. Also referred to as the "drying power of air", high *VPD* can interfere with tick questing behavior by causing retreat to lower litter layers and topsoil where humidity levels are higher (Milne 1950, Perret et al. 2004, Tomkins et al. 2014). Higher humidity at the soil level may be in part why gravid *A. maculatum* females have been observed ovipositing directly on to and in some instances into the soil (Bishopp and Hixson 1936, Hixson 1940), as eggs and larvae of this species lose water at a rate ca. nine times as fast as adults (Yoder et al. 2008).

Once humidity drops below an organism's critical equilibrium humidity/activity (*CEH*/*CEA*) it is unable to actively uptake water vapor and must respond behaviorally or face desiccation (Fleetwood and Teel 1983, Needham and Teel 1986, Perret et al. 2004, Yoder et al. 2008). Moving downward in the face of desiccating conditions should

reduce the threat of desiccation. Immature *A. maculatum* are rarely collected in the field, therefore very little is known about them in regards to off-host behavior (Goddard 1992, Goddard and Paddock 2005, Portugal III and Goddard 2015, 2016b). This current experiment was performed to better understand how varying *RH*, temperature (*VPD*), and air movement, affect host-seeking behavior in nymphal *A. maculatum*.

Materials and Methods

The *Amblyomma maculatum* nymphs used in this study were obtained from a colony maintained at the Oklahoma State University Tick Rearing Laboratory, Stillwater, Oklahoma. Upon receipt the nymphs were immediately placed in a desiccator jar with a saturated solution of KNO_3 to maintain 97 % relative humidity, and kept at 22°C and 9L:15D until needed. All ticks were used within three weeks of receipt, six weeks of molting to the nymphal stage, and were unfed. Experiments were performed in an insect rearing chamber at the Mississippi State University Insect Rearing Center (Figure E.1). These walk-in chambers allow for maintaining a constant temperature, relative humidity, and photoperiod. Tick behavior was observed in a 75 gallon (283.9 L) Top Fin® glass aquarium arena containing purified pool sand (HTH® #20 grade silica sand) to a depth of 10 cm (Figure E.2, Figure E.3). Anchored in this substrate was a series of stems, whose length above the sand was 5, 10, 20, and 30 cm (Figure 6.1). These stems were field-collected broomsedge (*Andropogon virginicus*) from which the leaves had been removed and had an average base diameter of 1.73 ± 0.42 mm (SD). Broomsedge was selected because the authors have previously observed *A. maculatum* questing on this plant species in the field. A total of 496 stems, 124 of each length, were arranged in 4 squares, beginning immediately adjacent to a central square area of grid paper 8 cm on a side

(Figure 6.1). Each square had different length stems on its four sides, was 2.5 cm away from the central area or next smaller square, and had no sides with stems of the same length as the adjacent sides of neighboring squares. Individual stems were positioned 0.5 cm from each other

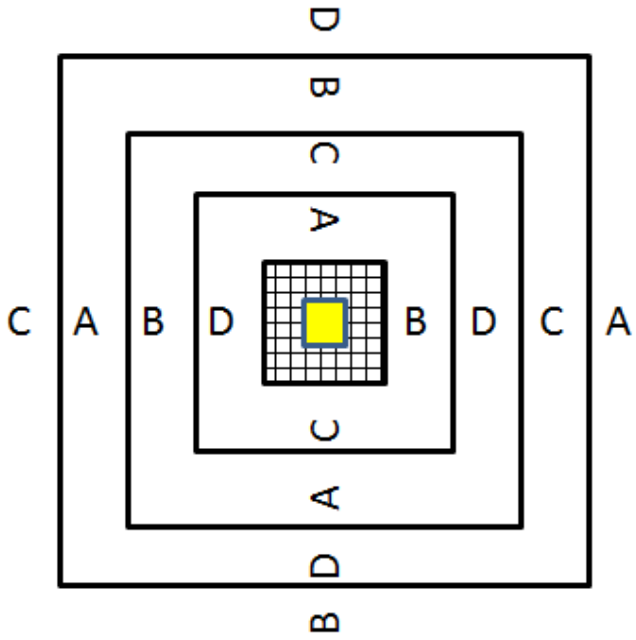

Figure 6.1 Arrangement of alternating stem heights in the observation arena.

A= 30-cm stems, B= 20-cm stems, C= 10-cm stems, and D= 5-cm stems. The central square (yellow) was release point for ticks in this experiment.

Four sets of environmental conditions were used: High Temperature/High Humidity (*HTHH*), High Temperature/Low Humidity (*HTLH)*, *LTHH*, and *HTHH* plus alternating periods of Wind (*HTHHW+/–)* (Table 6.1). Relative humidity was limited by the capacity range of our environmental control system. To help maintain higher relative

humidity during the *HTHH*, *LTHH*, and *HTHH* +/- treatments, a humidifier (Kaz, Inc., Model V3600, Hudson, NY) was placed inside the rearing chamber.

Table 6.1 Environmental conditions associated with each of four sets of treatments.

Treatment[1]	Temperature ± SE (°C)	Relative Humidity ± SE (%)	Vapor Pressure Deficit (kPa)[2]	Wind Velocity ± SE (km/h)
HTHH	26.67 ± 0.002	65.01 ± 0.176	1.22	0
HTLH	26.66°C ± 0.004	29.08 ± 0.045	2.48	0
LTHH	18.22°C ± 0.016	53.96 ± 0.091	0.96	0
HTHHW+/-	27.62°C ± 0.006	67.43 ± 0.148	1.20	5.37 ± 0.231

Treatments HTTH, HTLH, and LTHH were analyzed separately from *HTHHW+/-* due to differing number of observation days.
[1]See text for abbreviations.
[2]Standard errors of calculated estimates for *VPD* could not be calculated by the method of combining variances (Barford 1967). The assumption of independence could not be met, as *RH* was calculated using the temperature recorded in the rearing chamber.

For *HTHH*, *HTLH*, and *LTHH* conditions, testing took place over 5 days (108 hours) (Figure 6.2), while for *HTHHW+/–* testing was over 6 days (132 hours) (Figure 6.3). The photoperiod was maintained at 12L:12D consistent with the photoperiod during peak nymphal activity in Mississippi (March) (Portugal III and Goddard 2016b). Each day, overhead lights came on at 0800, and remained on until 2000. Temperature and *RH* were constant, as the objective was to observe behavioral responses for specific environmental conditions. Conditions for the *HTHH* and *HTHHW+/–* treatments depended on limitations of the environmental control system, but the conditions achieved were within the range experienced by nymphs on the U.S, Gulf Coast in the spring. Both temperature and *RH* were monitored by a recorder (HOBO® Pro v2 temp/RH onset, Part no. U23-001). Vapor pressure deficit was calculated using the formula for saturation water vapor pressure (e_s) provided by Tetens (1930) (Equation 6.1), ambient water vapor

pressure (e_a) calculated from e_s and *RH* s (Equation 6.2), and *VPD* calculated as the difference between e_s and e_a (Equation 6.3) (Snyder 2005).

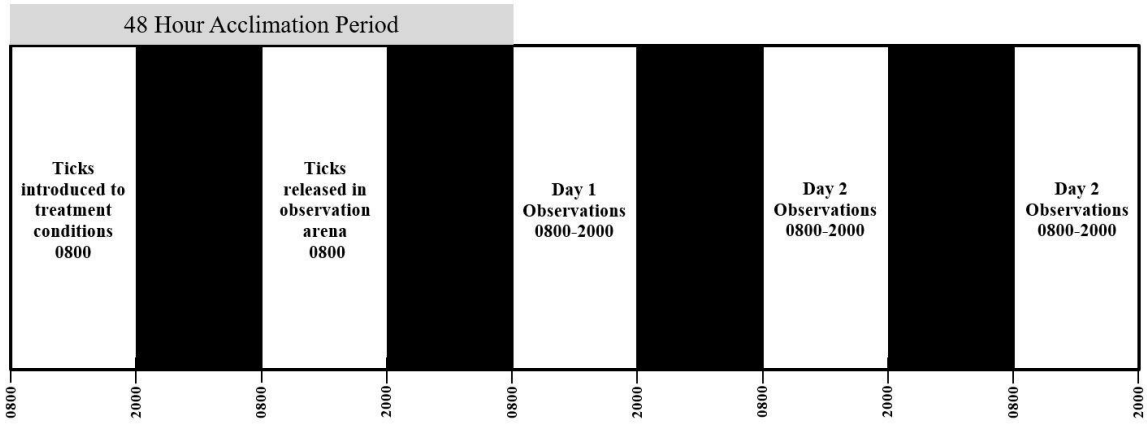

Figure 6.2 Piano chart depicting the sequence of events within each replication of the main treatments *HTHH*, *HTLH*, and *LTHH* (see text).

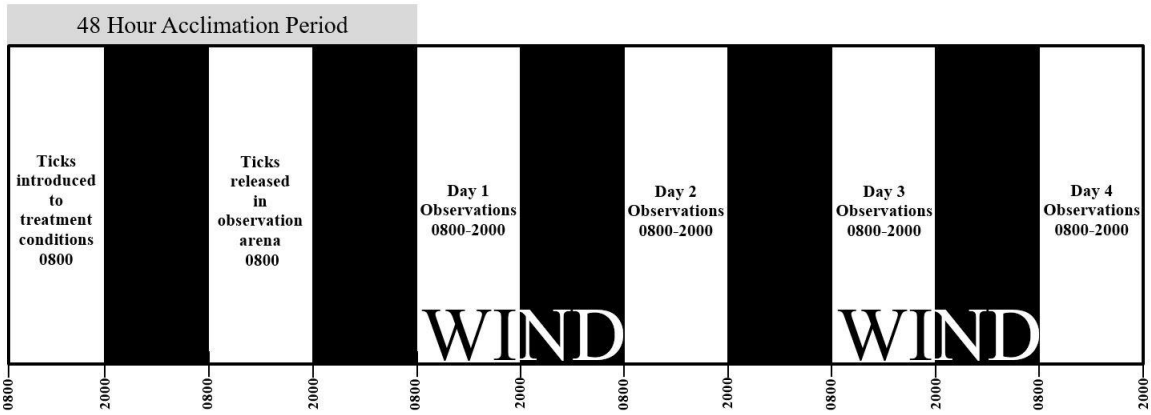

Figure 6.3 Piano chart depicting the sequence of events within each replication of the treatment *HTHHW+/–* (see text).

Note that this treatment has one extra day to allow for an even number of alternating days of wind and no wind.

$$e_s(T) = 0.6108 \text{ kPa} * \exp^{(17.27 * T/(T+273.2)} \quad (6.1)$$

$T \equiv$ ambient temperature (°C)

$$e_a = e_s(T) * RH/100\ \% \quad (6.2)$$

$RH \equiv$ relative humidity (%)

$$VPD \equiv e_s(T) - e_a \quad (6.3)$$

Each of the three replications for each set of environmental conditions began with a 48-hour acclimation period. For the first 24 hours, 80 nymphal *Amblyomma maculatum* were enclosed in a small plastic tube in the rearing chamber to acclimate to ambient environmental conditions of the treatment. At 0800 of the second 24-hour period, the nymphs were dislodged from the vial onto a square area 2-cm on a side in the center of the grid paper in the observation arena (Figure 6.1). Beginning 24 hours after release, observations were made at 0800, 0900, 1100, 1400, and 2000 during the three or four subsequent photophase periods. Observations at 2000 were made just before light off. Data recorded during every observation included: number of ticks seen questing, orientation of those ticks (upward or downward), height (cm) above sand substrate, and stem height selected. Observations were made from the side of the tank to allow for more accurate height observation as well as to avoid expelling CO_2 and other compounds from observer respiration into the tank. Larvae of this species have been reported to strongly respond to stimuli under increased *VPD* conditions (Fleetwood and Teel 1983), and I presume this may also be true of nymphs. Time spent for each observation was limited to 3 minutes or less to reduce elicitation of a response by the ticks to movement and casting of shadows. Questing in this experiment was defined as a nymphal tick

positioning itself above the sand substrate on a stem. Ticks that did not quest could not be observed due to the coloration and consistency of the sand substrate.

For the *HTHHW*+/– treatment, a small fan (Lasko® Type 6-1, style MLCS, West Chester, PA) was placed 60 cm away from the nearer edge of the stem array to simulate wind across the testing area (Figure 6.4). The middle of the fan was centered 15 cm above the substrate and turned on 24 hours at a time on days 1 and 3 after initial release (Figure 6.3). Air velocity was 13.8 km/h at the fan and, and slowed to 5.4 km/h at the stems. A Pocket Hygro-Thermo-Anemometer-Light Meter (Model 45170, EXTECH Instruments, Nashua, NH) was used to measure air velocity with an accuracy of ±3% (manufacturer's specification).

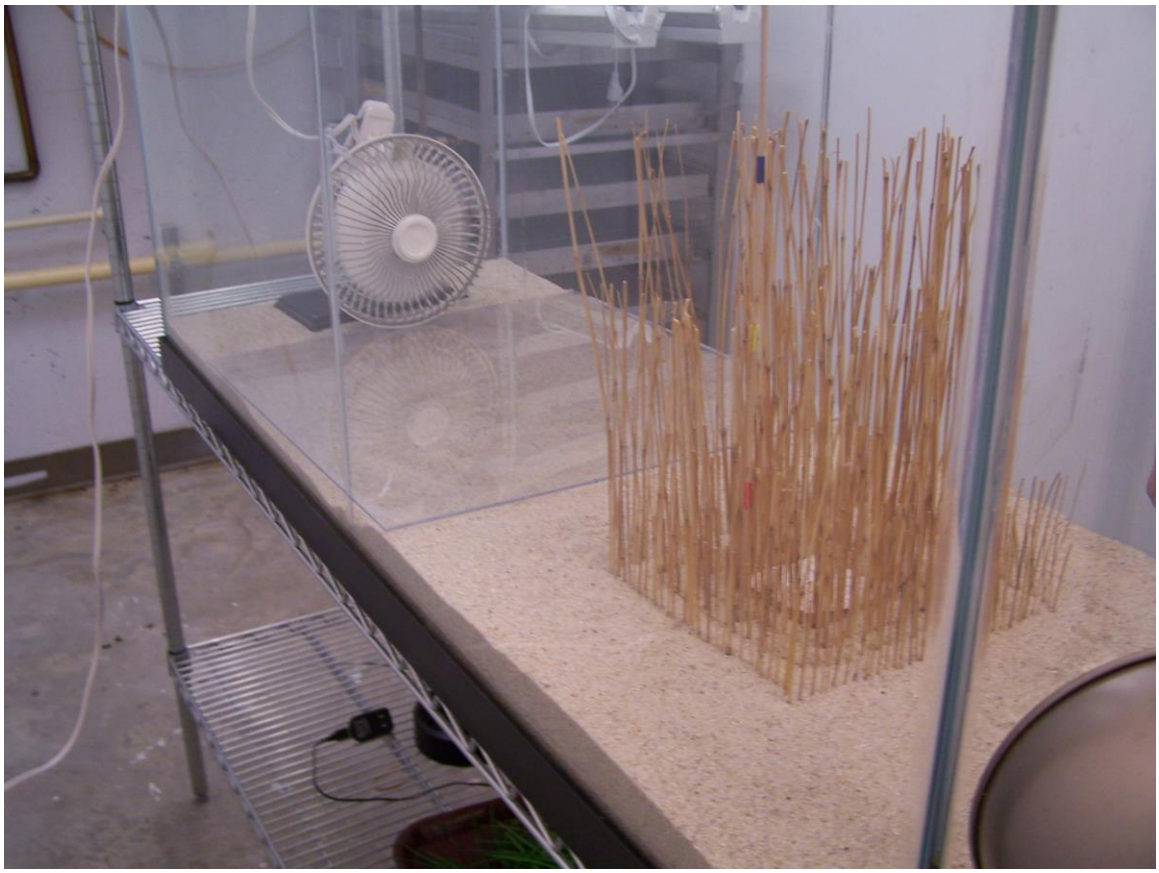

Figure 6.4 The observation arena with the fan in place for the *HTHHW+/-* treatment (see text).

Stem arrangement is identical for all treatments. Note Plexiglas on sand substrate between fan and testing arena was present in *HTHHW+/-* treatment to prevent sand from blowing across the stems. Photo credit: Jerome Goddard, Ph.D.

Mixed model analyses were conducted using PROC MIXED in SAS for Windows v9.4 (SAS Institute, Inc., Cary, NC, USA) for observations of numbers of ticks questing and tick questing height. The fixed effects initially included in the model were Treatment, Day, Time of Day, Stem Height and all two-way interactions. Treatment within replication was included as a random effect with a variance components covariance structure. To develop more parsimonious models, interaction terms and main effects not included in an interaction, with the largest *P*-value were sequentially removed

until only significant [$P < 0.05$ (here and for all other analyses] interaction terms and main effects whether significant or not remained. An LSMESTIMATE statement with the SIMULATE option was used to adjust p-values for multiple comparisons.

Mixed model logistic regression using PROC GLIMMIX was used for the tick orientation observations. The initial model and random effect structure were the same as used for the tick height and tick count data. A similar variable selection process was used to achieve a more parsimonious model. Although all observation times were included initially, the final model included only the 800 and 1400 time points because of model convergence problems with inclusion of the other time points.

For the studies investigating the effect of wind on tick height, tick count, and tick orientation, similar methods as described above were used. Two separate analyses were performed for each dependent variable: one with Time of Day limited to 0800 and one with time point 0800 excluded. This was done because observations taken at 0800, i.e. when the fan went on or turned off, might not be representative of the conditions. Initial models excluding observations at 0800, included Wind, Trial, Time of Day, Stem Height, and the Wind*Trial, Wind*Time, Wind*Stem Height, and Time*Stem Height interactions as fixed effects. The initial models limited to time point 0800 included Wind, Trial, Stem Height, and the Wind*Trial, and Wind*Stem Height interactions as fixed effects. Trial within replication was included as a random effect with a variance components covariance structure.

Results

A total of 1,369 observations of nymphal *A. mac*ulatum were made across the treatments *HTHH*, *LTHH*, and *HTLH* (Table 6.2). For questing height, Day and Time of

Day were not significant ($P > 0.05$), but there was a significant interaction between Treatment and Stem Height ($F = 27.30$; df = 1345; $P < 0.0001$) (Table 6.3). For the number of nymphs observed questing, there was again a significant interaction between Treatment and Stem Height ($F = 29.12$; df = 464; $P < 0.0001$) while Day and Time of Day were not significant ($P > 0.05$). Orientation varied significantly by Stem Height ($F = 7.01$; df = 535; $P = 0.0001$) as well as by the interaction of Treatment and Day ($F = 3.80$; df = 535; $P = 0.0047$).

Data for *HTHHW+/–* excluding observation time 08:00 were analyzed separately. For questing height, there was a significant interaction between Wind and Stem Height ($F = 22.19$; df = 477; $P < 0.0001$). For the number of nymphs observed questing, only Stem Height was significant ($F = 20.82$; df = 163; $P < 0.0001$). For orientation, there was a significant Wind*Stem Height interaction ($F = 5.85$; df = 477; $P = 0.0006$). Data for *HTHHW+/–* from the 0800 observation time only were also analyzed separately. For questing height, there was a significant Wind*Stem Height interaction ($F = 3.00$; df = 112; $P = 0.0337$) as was the case for observation times other than 08:00. For number of nymphs observed questing and for orientation, no significant effects were found, which is dissimilar to the results for observation times other than 08:00.

Table 6.2 Questing behaviors of nymphal *Amblyoma maculatum* under various environmental conditions.

Treatment[1]	n=	% Observed	Questing Height AVE ± SE (cm)	% Head Upwards
HTHH	506	14.06	6.03 ± 0.119	89.13
HTLH	311	8.64	4.45 ± 0.145	88.75
LTHH	552	15.33	4.76 ± 0.116	82.97
HTHHW+/-	490	12.76	5.98 ± 0.200	76.53
HTHHW-	259	13.49	7.22 ± 0.328	77.61
HTHHW+	231	12.03	4.58 ± 0.171	75.32

[1]See text.

Table 6.3 Questing height and (number of nymphs questing per observation) (AVE ± SE) for each Treatment by Stem Height combination.

	Treatment				
Stem Height (cm)	***HTHH***	***HTLH***	***LTHH***	***HTHHW–***	***HTHHW+***
5	4.54 ± 0.047BC (5.80)a	4.01 ± 0.084BC (2.16)cd	4.05 ± 0.069BC* (4.36)ab	4.58 ± 0.069CD (4.04)	3.75 ± 0.149D (3.17)
10	7.27 ± 0.369A (1.53)d	7.85 ± 0.343A (1.57)d	6.82 ± 0.202A (2.53)cd**	7.22 ± 0.221B (4.04)	5.88 ± 0.259BC (3.08)
20	7.95 ± 0.363A (2.21)cd	3.83 ± 0.309BC (2.53)cd	5.09 ± 0.521B* (1.68)d	10.42 ± 1.150A (1.29)	4.04 ± 0.264CD (1.13)
30	7.50 ± 0.272A (2.40)cd	3.24 ± 0.175C (2.05)d	4.08 ± 0.221BC (3.84)bc**	11.85 ± 1.865A (1.42)	4.24 ± 0.542CD (2.25)

*Denotes that values are significantly different from each other despite same letter.
**Denotes that values are significantly different from each other despite same letter.

Questing Height (*HTHH*, *HTLH*, *LTHH*)

Mean questing height of ticks in treatment *HTHH* was 6.03 ± 0.119 cm, in *HTLH* was 4.45 ± 0.145 cm, and in *LTHH* was 4.76 ± 0.116 cm (Table 6.2). Questing height of ticks in *HTHH* was significantly higher ($F = 15.74$; df = 6; $P = 0.0041$) than ticks in both the *HTLH* and *LTHH* treatments, while there was no significant difference in mean questing height between *HTLH* and *LTHH*. Ticks on 5 cm stems quested significantly lower than those on 10 cm stems across all treatments, and quested significantly lower

than 20 and 30 cm stems (t = 12.93; df = 1345; P < 0.0001 and t = 11.02; df = 1345; P < 0.0001 respectively) in *HTHH* and 20 cm stems (t = 3.41; df = 1345; P = 0.0175) in *LTHH*. Ticks on 20 and 30 cm stems in *HTHH* quested significantly higher than in the other two treatments (P < 0.0001). In treatment *HTLH*, ticks on 10-cm stems quested significantly higher than those on 20 and 30-cm stems (t = 4.95; df = 1345; P < 0.0001 and t = 10.04; df = 1345; P < 0.0001 respectively), while ticks on 20-cm quested significantly higher than those on 30-cm stems (t = 3.17; df = 1345; P =0.0380).

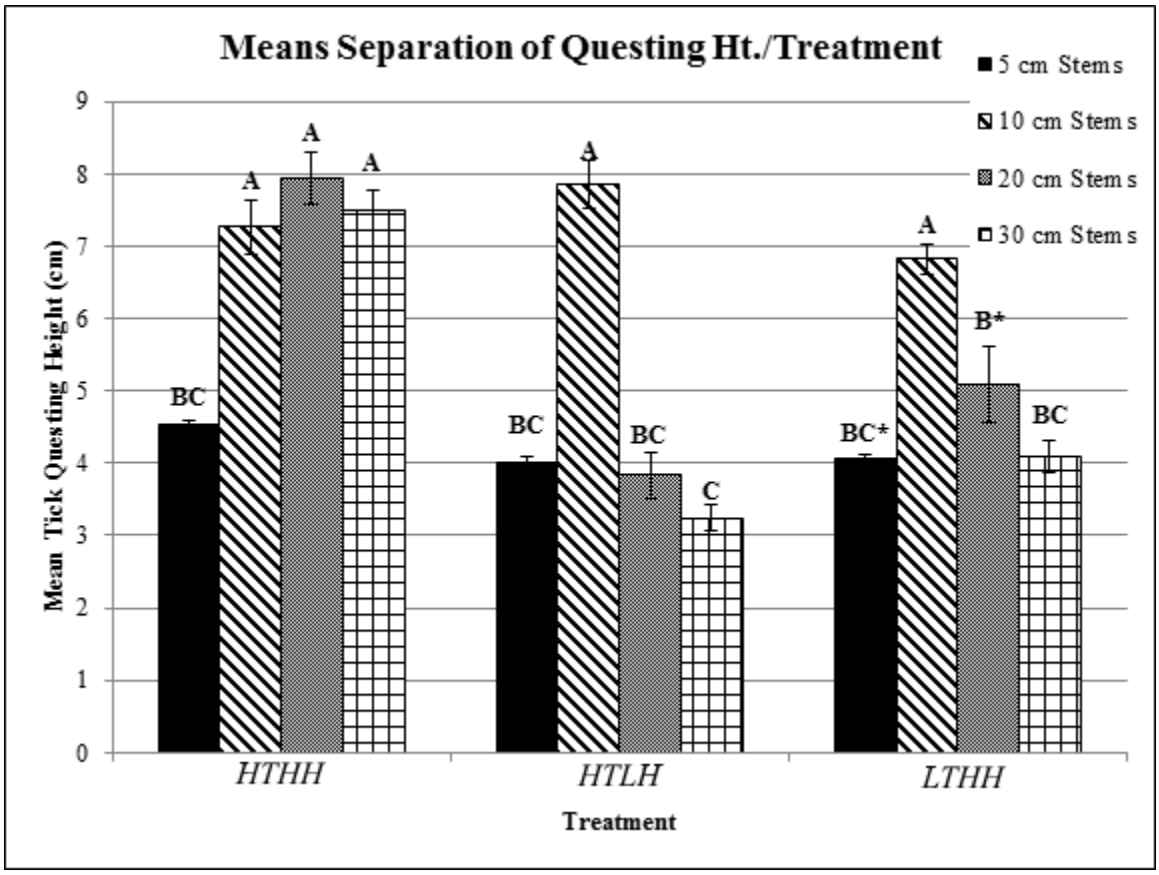

Figure 6.5 Means separation graph of tick questing height by treatment for three main treatments (*HTHH*, *HTLH*, *LTHH*).

LSMESTIMATE, SIMULATE option in SAS 9.4. Note: Asterisk (*) denotes that values are significantly different despite same letter.

Frequency of Ticks Questing (*HTHH*, *HTLH*, *LTHH*)

There were 3,600 opportunities to observe a questing tick for each of the treatments *HTHH*, *HTLH*, and *LTHH* (80 ticks/obs. x 5 obs. /day x3 days x 3 reps.), and in 14.06 % of these opportunities a tick was observed for *HTHH*, 8.64% for *HTLH*, and 15.33% for *LTHH*. Except for the 5-cm stems (4.356) in *LTHH*, significantly more ticks quested on the 5-cm stems in (5.80) *HTHH* than any other stem/treatment; while there was no significant difference between 5 and 30-cm stems (3.844) in *LTLH* (Table 6.3). There was no significant difference Significantly more ticks quested on 5-cm stems for both the *HTHH* (3.64) (t = 6.74; df = 464; $P < 0.0001$) and *LTHH* (2.20) (t = 4.07; df = 464; $P = 0.0012$) treatments than in the *HTLH* treatment. There was no significant difference between average numbers of ticks questing on other stem heights in all treatments.

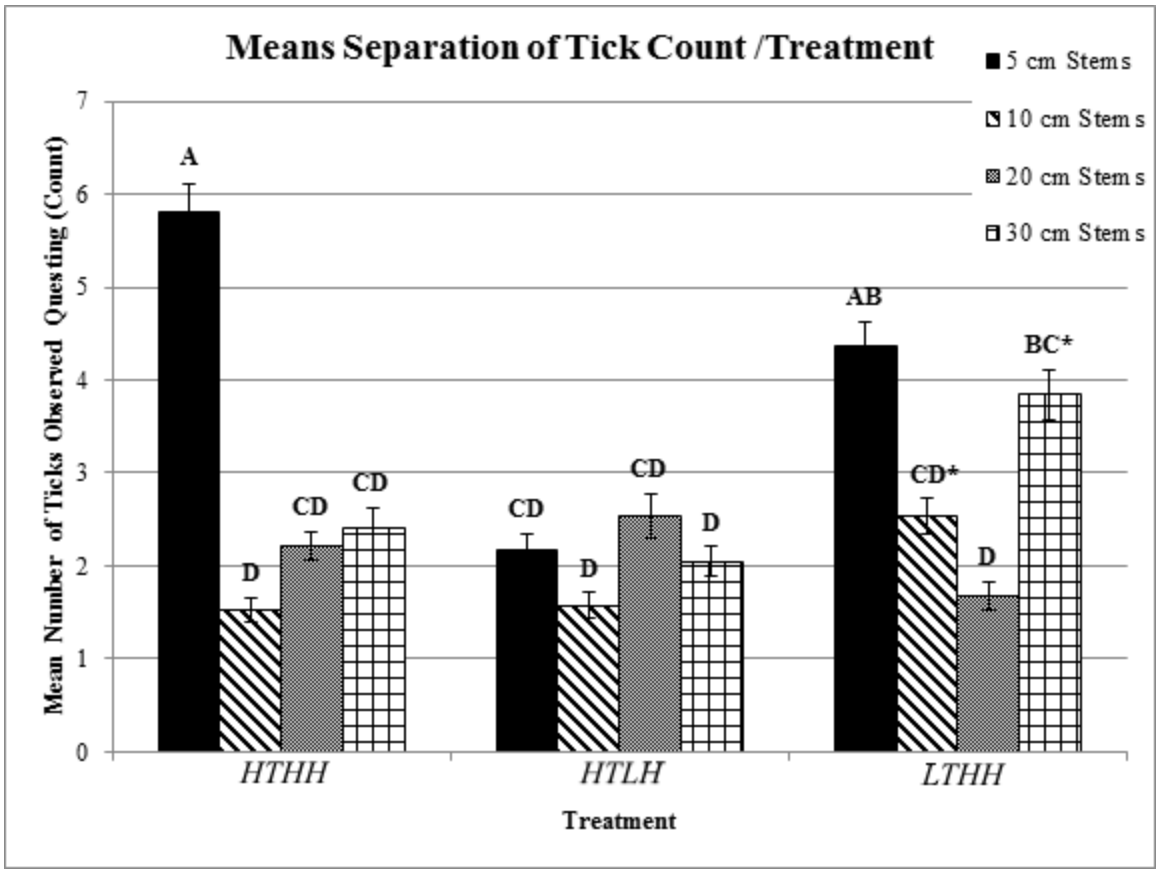

Figure 6.6 Means separation graph of tick count by treatment for three main treatments (*HTHH*, *HTLH*, *LTHH*).

LSMESTIMATE, SIMULATE option in SAS 9.4. Note: Asterisk (*) denotes that values are significantly different despite same letter

Orientation of Ticks (*HTHH*, *HTLH*, *LTHH*)

For tick orientation, Stem Height ($P = 0.0001$) as well as Treatment*Day interaction were significant ($P = 0.0047$), while Time of Day was not. The frequency of observation of ticks head upward on 5-cm stems was 3.47 times greater than on 10-cm stems ($t = 3.51$; df = 535; $P = 0.005$). For Treatment*Day, the frequency of observation of ticks head upward in *HTHH* on Day 1 was 5.50 times greater than that in *HTLH* ($t = 2.01$; df = 535; $P = 0.045$) and 7.02 times greater than that in *LTHH* ($t = 2.42$; df = 535; $P = 0.016$). On Day 3, the frequency of observation of ticks head upward in *LTHH* was

4.19 times greater than in *HTHH* (t = 2.56; df = 535; P = 0.011) and 3.67 times greater than in *HTLH* (t = 2.00; df = 535; P = 0.046). Ticks in treatment *HTHH* were 8.82 times more likely to face upwards on Day 1 vs. Day 3 (t = 2.75; df = 535; P = 0.006), and 3.08 times more likely on Day 2 vs. Day 3 (t = 2.09; df = 535; P = 0.037). Interestingly, for ticks in *LTHH*, the frequency of observation of ticks head upward was 3.3 times greater Day 3 vs. Day 1 (t = 2.20; df = 535; P = 0.028).

High Temperature/High Humidity/Wind (*HTHHW+/-*)

A total of 490 individual tick observations were made in *HTHHW+/–*; 259 without wind (*HTHHW–*), and 231 with wind (*HTHHW+*) (Table 6.2, Figure 6.7). There were 1,920 opportunities to observe a questing tick per condition (80 ticks/obs. x 4 obs. /day x 2 days x 3 reps.); 13.49% of ticks quested in *HTHHW–*, and 12.03% in *HTHHW+*. Although Stem Height significantly affected number of ticks questing in this treatment, Wind did not. Significantly more ticks quested on both 5 and 10-cm stems than on 20 and 30-cm stems ($P < 0.0001$), while there was no significant difference between ticks on 5 vs 10-cm stems, as well as 20 vs 30-cm stems.

Overall mean questing height by stem height for *HTHHW+/-* ranged from 3.80-11.79 cm (Figure 6.7). Ticks on 20 and 30-cm stems in *HTHHW-* quested significantly higher than all other stems in *HTHHW+/-* ($P < 0.0001$); additionally, these stem heights were the only instances where there was a significant difference between the *HTHHW-* and *HTHHW+* conditions. In *HTHHW+* mean tick questing height by stem height ranged from 3.80 (5-cm stem) to 6.13 cm (10-cm stem); with ticks on 10-cm stems questing significantly higher than on 5-cm stems (t = 3.83; df = 477; P = 0.0022). During *HTHHW-*, ticks on 10-cm stems quested significantly lower than on the 20 and

30-cm stems ($t = 3.54$; df = 477; $P = 0.0065$ and $t = 5.80$; df = 477; $P < 0.0001$ respectively), and ticks on 5-cm stems quested significantly lowest of the condition ($P < 0.0001$).

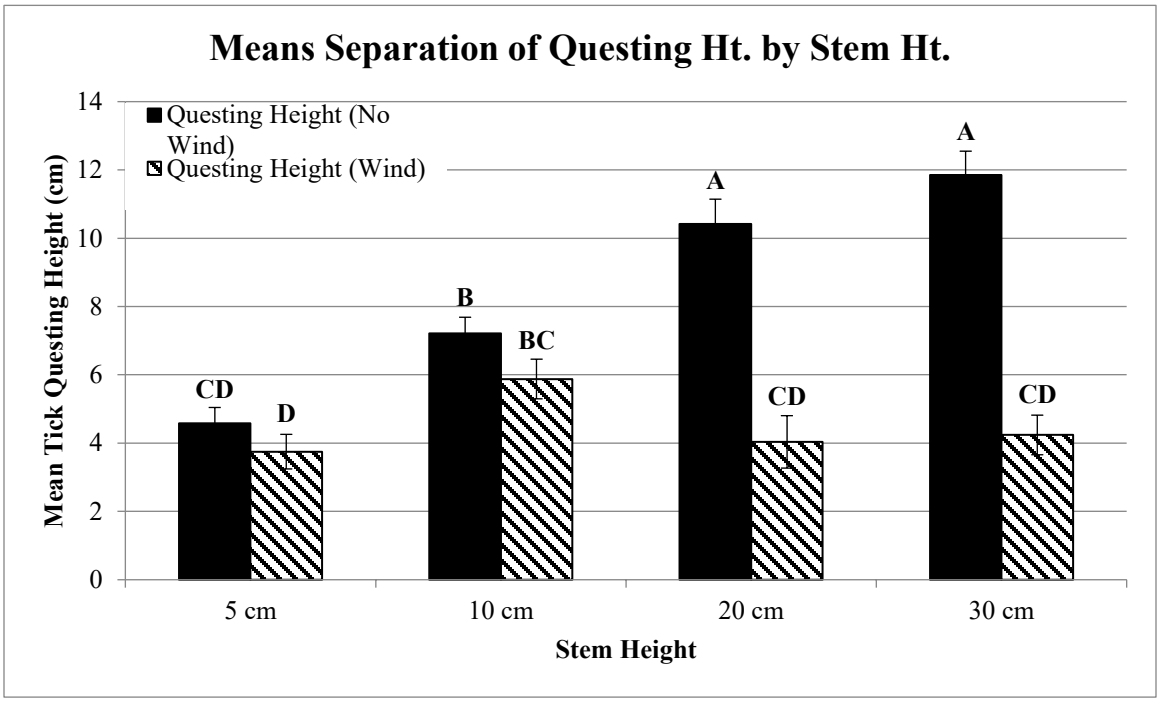

Figure 6.7 Means separation graph of questing height by stem height for wind treatment (*HTHHW+/-*)

LSMESTIMATE, SIMULATE option in SAS 9.4.

Although the Wind*Stem Height interaction was significant ($P = 0.0006$) in regards to orientation as was seen in the three main treatments (*HTHH*, *HTLH*, and *LTHH*), Day was not despite being significant in the previous treatments. The frequency of observation of ticks head upward on 10-cm stems was 2.52 times higher for *HTHHW+* versus *HTHHW–* ($t = 2.60$; df = 477; $P = 0.0096$), while those on 30 cm stems were actually 3.95 times more likely to face upwards when wind was present than when it

wasn't (t = 2.73; df = 477; P = 0.0065). On 5-cm stems in *HTHHW–*, the frequency of observation of ticks head upward was 2.60 times greater than on 20-cm stems (t = 2.01; df = 477; P = 0.0450) and 4.32 times greater than on 30-cm stems (t = 3.28; df = 477; P = 0.0011). The frequency of observation of ticks head upward on 10-cm stems was 3.47 times higher than on 30-cm stems in *HTHHW–* (t = 2.87; df = 477; P = 0.0043). In *HTHHW+*, the frequency of observation of ticks head upward was 3.03 times greater on 20-cm stems than 10-cm stems (t = 2.01; df = 477; P = 0.0445), and 2.87 times greater on 30-cm stems than 10-cm stems (t = 2.41; df = 477; P = 0.0163).

Discussion

In this study, temperature, relative humidity, and *VPD* all significantly affected tick questing height. As suspected, *A. maculatum* nymphs exposed to higher temperature and relative humidity conditions (*HTHH*) quested significantly higher overall than when exposed to either a lower relative humidity or temperature. Mean questing heights observed in this laboratory study were similar to the ~3-5 cm seen with *A. maculatum* nymphs in the field (Portugal III and Goddard 2016c). High temperature/low humidity conditions during the *HTLH* treatment resulted in a substantially lower number of ticks observed questing overall as compared to the other treatments. However, due to the significant Treatment*Stem Height interaction in this analysis, one cannot interpret variation due to Stem Height alone. In contrast to questing height results, questing frequency in this study was 6-10-fold higher than the 1.5% frequency observed in the field (Portugal III and Goddard 2016c). This difference can presumably be attributed to uncontrolled factors in the field (e.g. precipitation, fluctuating temperatures and relative humidity, host acquisition, predation, pathogens, etc.) and differences in experimental

design (e.g. number of observations, time between observations, etc.). As Day was not significant in this experiment, and both mean questing height and questing frequency of ticks at 0800 (lights on) and 2000 (lights off) did not significantly differ, I speculate that darkness does not induce or restrict movement or host-seeking behavior in nymphs of this species, in contrast to what has been observed in *Ixodes ricinus* and *I. scapularis* ticks (Carroll et al. 1998, Perret et al. 2003). After analyzing orientation results, I could not determine any biological significance in our study.

In *HTHH*, ticks on 10, 20, and 30-cm stems quested significantly highest (Table 6.3, Figure 6.5), which substantially contributed to the higher overall mean questing height in the treatment. During periods of lower relative humidity or temperature (*HTLH*, *LTHH*), the 10-cm stem is where ticks quested significantly highest. Ticks on 5-cm-tall stems quested significantly lower or at least not significantly higher than those on taller stems for all treatments, suggesting that shortness of the 5-cm stems may limit height quested. Questing around 4.45-4.76 cm above the surface may represent a level at which conditions are less than "ideal" but are not so deleterious as to warrant further energy utilization in pursuit of more humid conditions at the expense of not being in position to acquire a host. A similar range of questing heights was observed during periods of wind application in *HTHHW+/–*, with an overall mean (4.58 cm) within the range described (Table 6.2, Table 6.3).

Under warm, humid conditions (*HTHH*), *A. maculatum* nymphs quested significantly more frequently on 5-cm stems than on the other heights. It is not definitively known if selection of this stem height was made visually by the ticks or if ticks quested upon multiple stems but tended to "decide" to stay on 5-cm stems and leave

taller stems. A greater frequency of observation of ticks on 5-cm stems was also shown in the *LTHH* treatment, however, this frequency was not significantly different from that of ticks on 30-cm stems. As stem height preference was similar for both *LTHH* and *HTHH* conditions, which had comparably low *VPD*s, *VPD* may play a role in stem height selection. Even though ticks were limited in questing height by the 5-cm stems during *HTHH* (and to a lesser extent *LTHH*), they still seemed to prefer 5-cm stems over the three other available heights. The reason for this is unknown, but may be biologically significant. Mean questing height of ticks on 5-cm stems ranged from 4.01 (*HTLH*) to 4.54 cm (*HTHH*), which is close to the stem tips. This tendency to quest near the tip of vegetation might increase their chances of successful interaction with a potential host, as they may face less obstruction than experienced lower on vegetation. Stems may taper as a tick approaches the tip, increasing the ratio of tick size to stem diameter. Preference for questing toward or at the tip of a particular height stem/blade of grass is a behavior that has been observed in other tick species (Lees and Milne 1951, Loye and Lane 1988).

Frequency of ticks questing on 5-cm stems in the *HTLH* treatment was significantly lower than in the other two treatments, suggesting that *RH* and/or *VPD* may influence the number of these ticks that will quest. A study with nymphal *I. ricinus* demonstrated that high *VPD* limits duration of questing (Perret et al. 2003), therefore it is possible that ticks in the *HTLH* treatment initially quested at a higher rate prior to observation. Conditions under this treatment could potentially have also increased mortality during observations, contributing to the significantly lower observed questing frequency. Mortality would be increased if *VPD* in the *HTLH* treatment was above that at the CEA/CEH for these ticks (Fleetwood and Teel 1983).

These results confirm that more desiccating conditions limit questing height, as has been documented previously with this and other species (Robertson et al. 1975, Mejlon and Jaenson 1997, Tomkins et al. 2014). Additionally, I believe that with nymphal *A. maculatum*, it is *VPD* that plays a more important role in questing activity versus temperature or RH alone. In *A. americanum*, Robertson et al. (1975) concluded that temperature was the key driver of activity, however it is noted that in his study *VPD*s increased at the higher temperatures tested, but this effect was not discussed. As immatures of this species lose water 3-9 x faster and are more susceptible to desiccation than adults (Yoder et al. 2008), a positive hygrotactic response when exposed to drier conditions is imperative for survival. This involves retreating to lower layers of vegetation closer to the soil, where moisture level is higher. However, in our study it must be noted that ticks overall quested significantly higher vertically in *HTHH* (*VPD*=1.22 kPa) than *LTHH* (*VPD*=0.96 kPa) with only a 0.22 kPa difference in *VPD*. Perhaps, low temperature suppresses questing height independently of its effect on *VPD*

Although the *HTHHW+/–* experiment was somewhat artificial, it did provide insight into the behavioral responses of nymphal *A. maculatum* to moving air. Air moving over a tick increases the moisture gradient immediately surrounding it. This increased gradient draws water from the tick, thus increasing the rate of transpiration. The air that moved through the tick arena in *HTHHW+* was of the same temperature and relative humidity as the still air in *HTHHW–*, but moving air appeared to suppress questing. Ticks quested progressively higher (statistically higher between all but 20 and 30-cm stems) as stem height increased during *HTHHW–* (Figure 6.7). This suggests that as soon as wind was removed, ticks climbed upward well beyond what was seen during

HTHH for both 20 and 30-cm stems. Little substantial difference between questing height (~4-6 cm) during *HTHHW+* among all 4 stem heights suggests that either: (1) wind combined with ambient temperature and relative humidity of the treatment was not so detrimental to water balance as to force further retreat down the stems or (2) that air movement at this height was not as forceful than higher on the stems. The latter effect is possible, as the base of all four stem heights probably provided shelter from wind within this range; however, 4-6 cm was within the range of mean questing heights observed during the *HTHH* and *LTHH* treatments. Although not included in this analysis, observations from 0800 time point data of questing height by stem height were almost identical to what was observed in *HTHHW+/-*.

Retreating from desiccating conditions (increased *VPD*, increased temperature, air movement, etc.) to conditions potentially more humid and favorable to maintaining adequate body water level/water balance, is not represented by any current taxic term. Ticks in this experiment did not move in response to any directional stimulus, they simply appeared to obey the rule that they move downward when exposed to desiccating conditions. As sand has no adsorption properties and does not act as a desiccant, the sand substrate did not provide a moisture gradient that ticks would experience in the field. Thus, I believe that the simple response to desiccating conditions observed in this experiment is most likely a Fixed Action Pattern/ Fixed Behavior (Tinbergen 1951). Simply put, ticks that retreated downward when confronted with desiccating conditions were selected for over evolutionary time, and this behavioral response has perpetuated.

Due to the nature of this experiment, as well as facilities available, conditions for all treatments were below the *CEA* for nymphal *A. maculatum* of a_v = 0.85-0.93 at 25 ºC

(*VPD* = 0.48-0.22 kPa) (Yoder et al. 2008). Additionally, these treatments were above the critical saturation deficit (*CSD*) of this species (4 mm Hg or 0.53 kPa); beyond which, ticks and other arthropods lose water to the atmosphere (Fleetwood 1985). One may speculate that equilibrium with ambient water vapor pressure in nymphs of this species may be experienced somewhere between 0.22 and 0.53 kPa. However, it is important to note that this does not take into account how varying RH, temperature, and other factors affect cuticle permeability, water balance, and other physiological processes by tick stage (Lees 1946, Mead-Briggs 1956). It is not known at what point active water uptake based on cuticle permeability begins (pump threshold) for nymphs of this species, and temperature may play some role in this (Mead-Briggs 1956, Teel et al. 1982, Needham and Teel 1991, Gibbs 1998). Broadly speaking, increased *VPD* (be it due to low *RH* or increased temperature), is correlated in arthropods with increased transpiration (Mead-Briggs 1956, Teel et al. 1982, Needham and Teel 1991), however this may not the case if *RH* is not below *CEA*/*CEH* (Lees 1946).

Questing behavior observed during this experiment may only be representative of colony or Oklahoma *A. maculatum* nymphs, and not necessarily the species overall. Additionally, variations in microbiome or other uncontrolled factors might possibly influence questing behavior. It is important to note that *A. maculatum* from the Oklahoma State University colony have previously tested positive for the bacterial agent, *Candidatus* Rickettsia andeanae, at a high frequency (Moraru et al. 2013). I do not know if this was true of ticks in this experiment, but it has been demonstrated previously that bacterial infections can substantially affect questing behavior in ticks (Lefcort and Durden 1996). Due to the weight of the tick arena (> 100 kg), I were not able to rotate it

between replications; however, I believe conditions within the rearing chamber were spatially uniform. Yoder et al. (2017) concluded that water loss rates in immature *A. maculatum* are affected by photoperiod, with short-day conditions (10L:14D) decreasing the rate of water loss. Photoperiod was constant in the present study and thus not a source of variation.

A single cohort (shipment) of ticks was used for each treatment due to logistical reasons. I believe that this issue is mitigated by the fact that these ticks are from a colony, and thus variation between ticks should be minimal. It has been demonstrated that critical equilibrium humidity (*CEH*) of a tick increases with tick age (Knulle and Rudolph 1982), and water-loss rate in the closely related *A. americanum* increases at a given *VPD* with age (Needham and Teel 1991, Sigal et al. 1991). However, the impact on the current study should be minimal as each treatment was conducted within three weeks of ticks being received, and replication was not significant in any treatment.

It was not possible to determine whether ticks not questing on stems were in fact alive. Some level of mortality should be expected, while adverse conditions could have increased mortality in treatments detrimental to maintaining water balance. However it is also possible that ticks not questing were alive and had crawled into crevices below the substrate, as seen previously in laboratory with adult *Ixodes brunneus* (Goddard 2013). It was assumed measurements of tick behavior were independent events; although, it is recognized that there is likely some degree of autocorrelation due to repeated measures of the same ticks. I included a random statement to account for potential autocorrelation, however it was impossible to include tick identity as a random effect in this experiment.

As seen in other tick species (Camin and Drenner 1978, McPherson et al. 2000), *A. maculatum* nymphs tended to quest in a height range putting them into position to obtain their "preferred" hosts, which are small mammals and ground-dwelling birds (Teel et al. 2010). However, is it preference for particular hosts that dictates questing height/stem height preference and selects for various physical and behavioral adaptations; or do abiotic factors that increase risk of desiccation such as wind, lower *RH*, and higher *VPD* dictate questing height and thus determine host usage? Ginsberg et al. (2017). speculate that the latter scenario is more likely in the case of larval *Ixodes scapularis*; however, they call for further study. With so much still unknown about the off-host behavior and ecology of immature Gulf Coast ticks, it is imperative that future research be conducted to mitigate medical and veterinary threats posed by this vector species.

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CHAPTER VII

OVERALL DISCUSSION AND CONCLUSIONS

Results from this dissertation contribute new information to what is known about the ecology and behavior of immature *A. maculatum* ticks. The objectives for all the individual experiments were met, and all but one have already been published in scientific journals. These studies ranged from seasonality and distribution within Mississippi, to behavioral and ecological studies that are applicable to this species throughout its range.

After field collections, as well as an in-depth review of records and literature, we now better understand the activity of immature *A. maculatum* in Mississippi. March is confirmed as the peak of questing activity for nymphs, and there appears to be a bi-model seasonality for immatures in the state. A novel method of collecting immature ticks known as swabbing was first implemented, and it was determined that dry ice trapping was not a productive method for collecting any stage of Gulf Coast tick. A single larval *A. maculatum* was collected with the swab method; only the third successful collection attempt for this stage in literature, and the only collection of an individual larva as opposed to a larval mass.

Experiments with larval *A. maculatum* and arthropod hosts did not definitively prove that they will imbibe hemolymph; however, it was discovered that they will move towards and crawl upon other arthropods. This fact suggests that unintentional phoresy

by utilizing arthropods may occasionally occur in the field. Concurrent testing with larval *A. americanum* did demonstrate consumption of arthropod hemolymph; a fact that may have interesting implications regarding the evolution of endosymbionts and pathogens.

It is now confirmed that larval Gulf Coast ticks will attach to humans under laboratory conditions. Potential attachment of larval *A. maculatum* to humans in the field is further supported due to failure of the negative control to attach in this experiment. As the bacterium *R. parkeri* has been shown to be transmitted transovarially, these results suggest the possibility of a new route of transmission to humans.

Laboratory experiments investigating nymphal *A. maculatum* host-seeking behavior resulted in an overall mean questing height ranging from 4.45 to 6.03 cm, and a questing frequency ranging from 8.64-15.33 %. For the two treatments with higher *RH* and decreased *VPD*, 5-cm stem heights were significantly "preferred" over other stem heights. As expected, wind significantly reduced mean questing height. Results from this experiment failed to demonstrate biological significance of tick orientation on stems. Conditions with reduced vapor pressure deficit encouraged higher questing frequency, but higher temperature with Vapor Pressure Deficit (*VPD*) nearly constant yielded increased mean questing height. These results answer the question of why collection of immature *A. maculatum* from vegetation in the field is difficult. Traditional tick collection utilizes a drag cloth, which does not get low enough to sample for immature *A. maculatum*. Improved methods such as swabbing, as well as further ecological and behavioral studies are needed to better ascertain and mitigate the potential threat posed by immature *A. maculatum*.

APPENDIX A

SUPPLEMENTAL MATERIAL FOR CHAPTER II

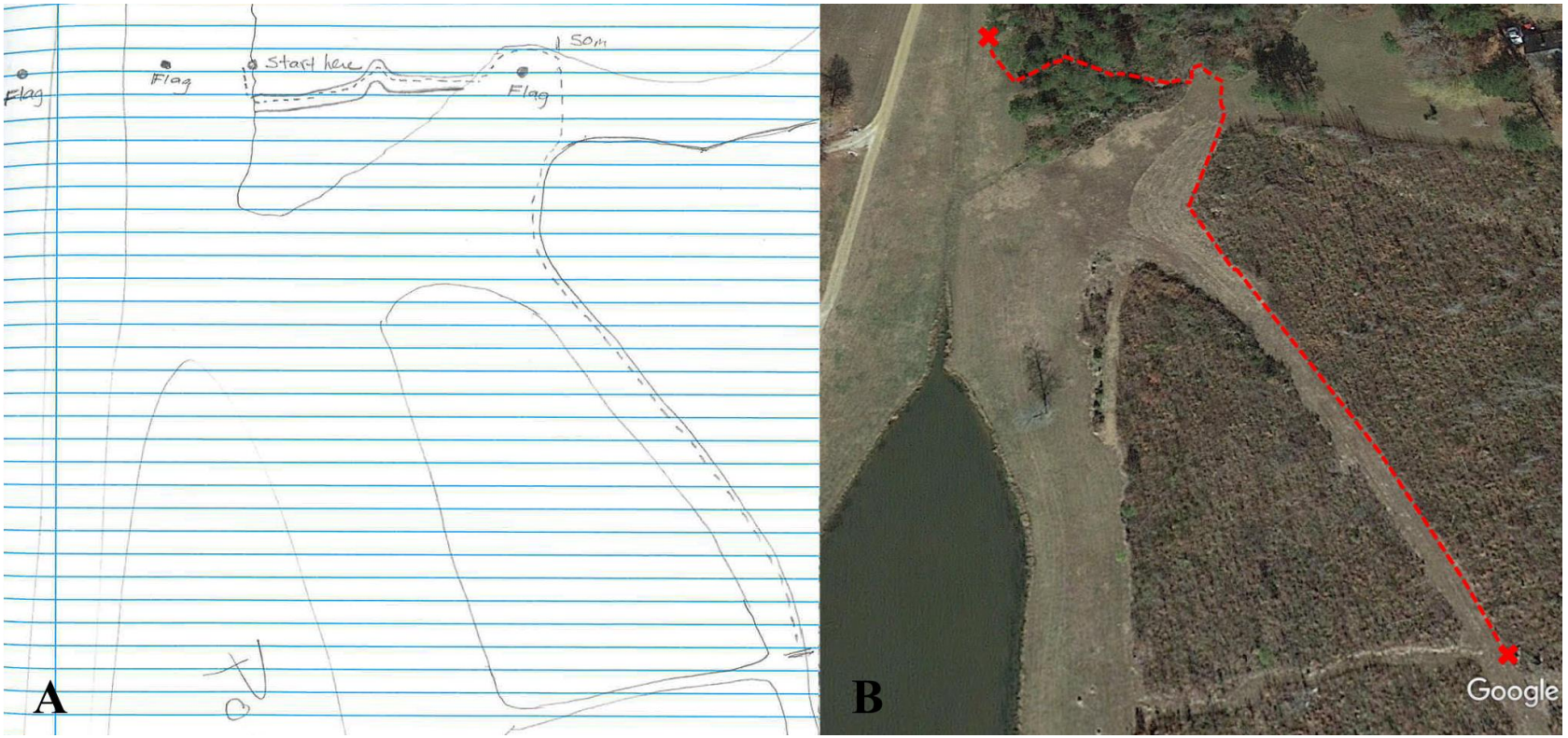


Figure A.1 Sampling transect at Collection Site A.

Hand drawn (A) and satellite imagery (B) of collecting area for Site A. Dotted black line (A) and red line (B) denotes actual path used for tick collection. Drawing credit: Jerome Goddard, Ph.D., satellite image taken from Google Earth 7.1.7.2606 and modified with paint.net 4.0.12.

Figure A.2 Trail cut specifically for sampling ticks at Collection Site A.

Portion of collection path at Site A running through mixed pine/deciduous woods (Figure A.1). No *Amblyomma maculatum* ticks were collected from this portion of the transect in this experiment as expected. Photo credit: Jerome Goddard, Ph.D.

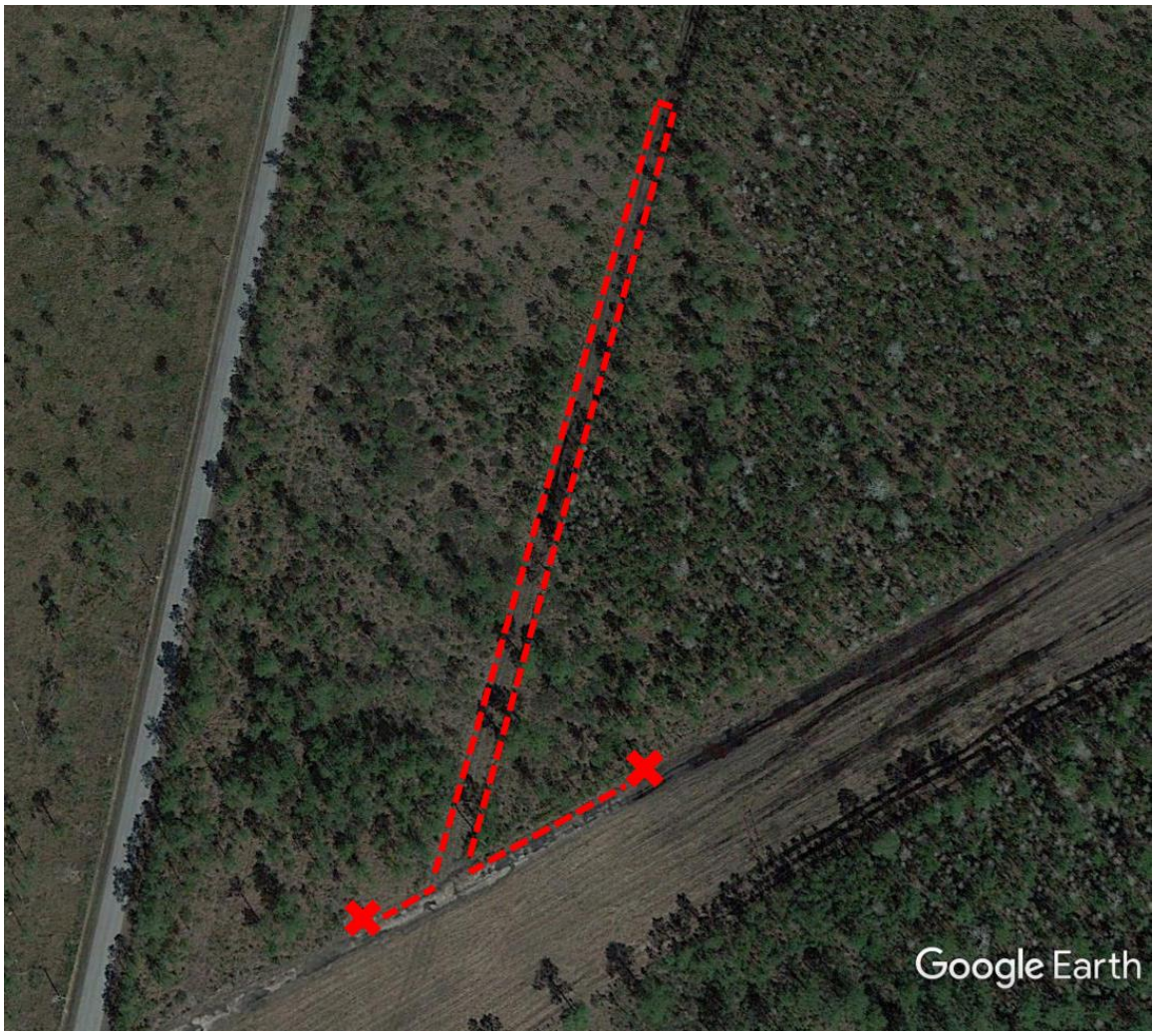


Figure A.3 Sampling transect at Collection Site B.

Satellite imagery of collecting area for Site B. Dotted red line denotes actual path used for tick collection. Satellite image taken from Google Earth 7.1.7.2606 and modified with paint.net 4.0.12.

Figure A.4 Examining dragcloth at Collection Site B.

Drag cloths were visually examined ever ~3-5 meters, and ticks present were removed with forceps and placed in vials containing 70-90% ETOH solution. Photo credit: Jerome Goddard, Ph.D.

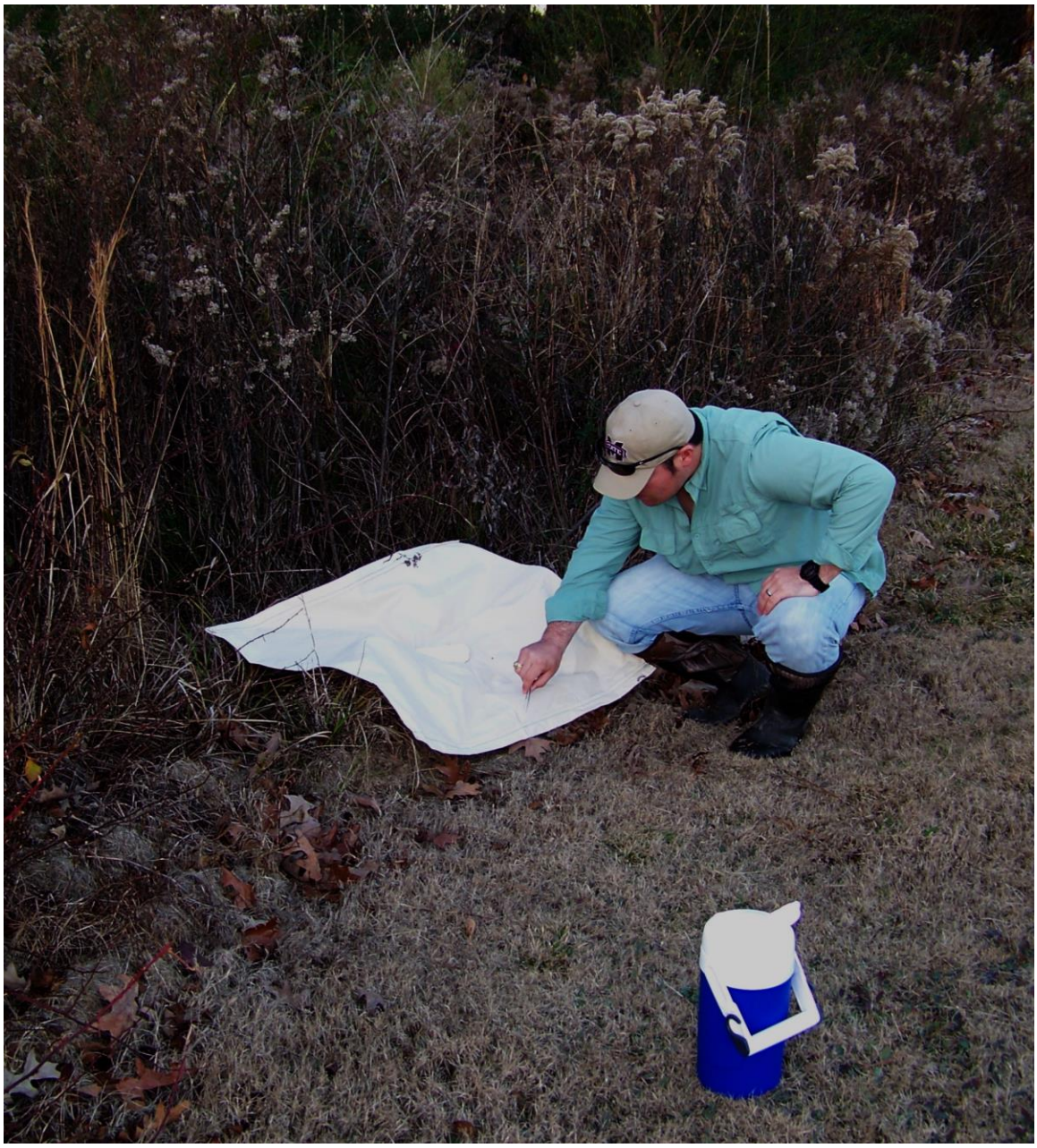


Figure A.5 CO_2 tick trap at Collection Site A.

Dry ice was placed in the center of a white canvas sheet, and gaseous CO_2 sublimed, eliciting a response ticks within the immediate vicinity. Ticks climbed onto white sheet, were immediately identified, and were placed into a vial containing a 70-90% ETOH solution. Photo credit: Jerome Goddard, Ph.D.

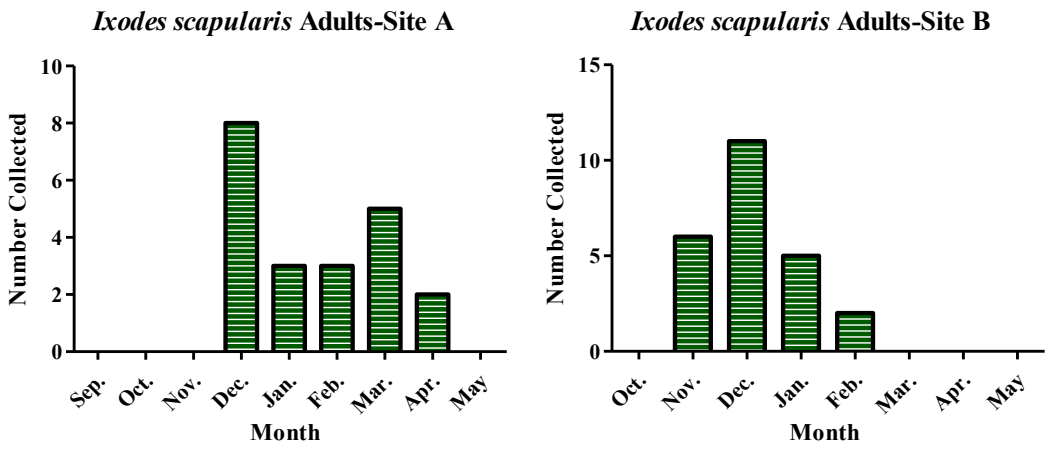


Figure A.6 *Ixodes scapularis* collections from both sites in experiment.

Ixodes scapularis ticks collected from Site A (left) and Site B (right) in this study. Note that only adults were collected.

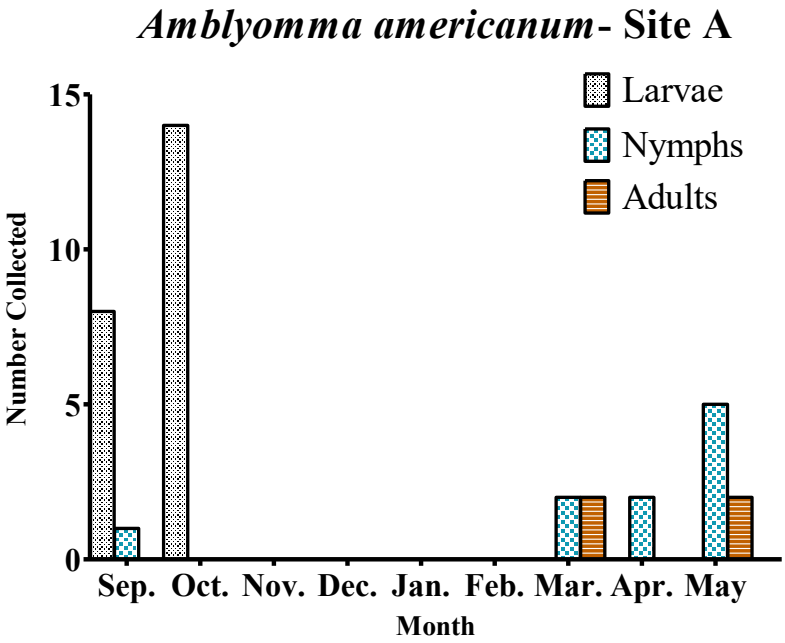


Figure A.7 *Amblyomma americanum* collected in this experiment.

Note that all were collected from Site A- presumably due to dense canopy cover.

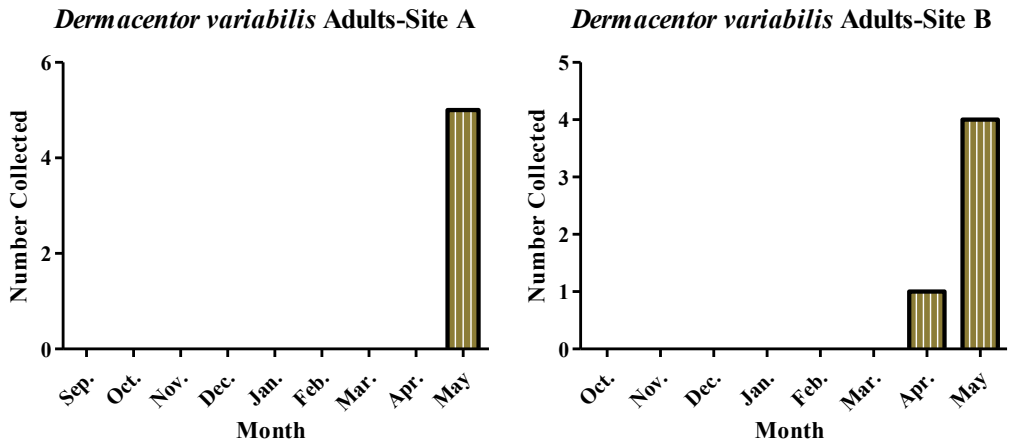


Figure A.8 *Dermacentor variabilis* collected during this experiment.

Note that only adults were collected.

APPENDIX B

SUPPLEMENTAL MATERIAL FOR CHAPTER III

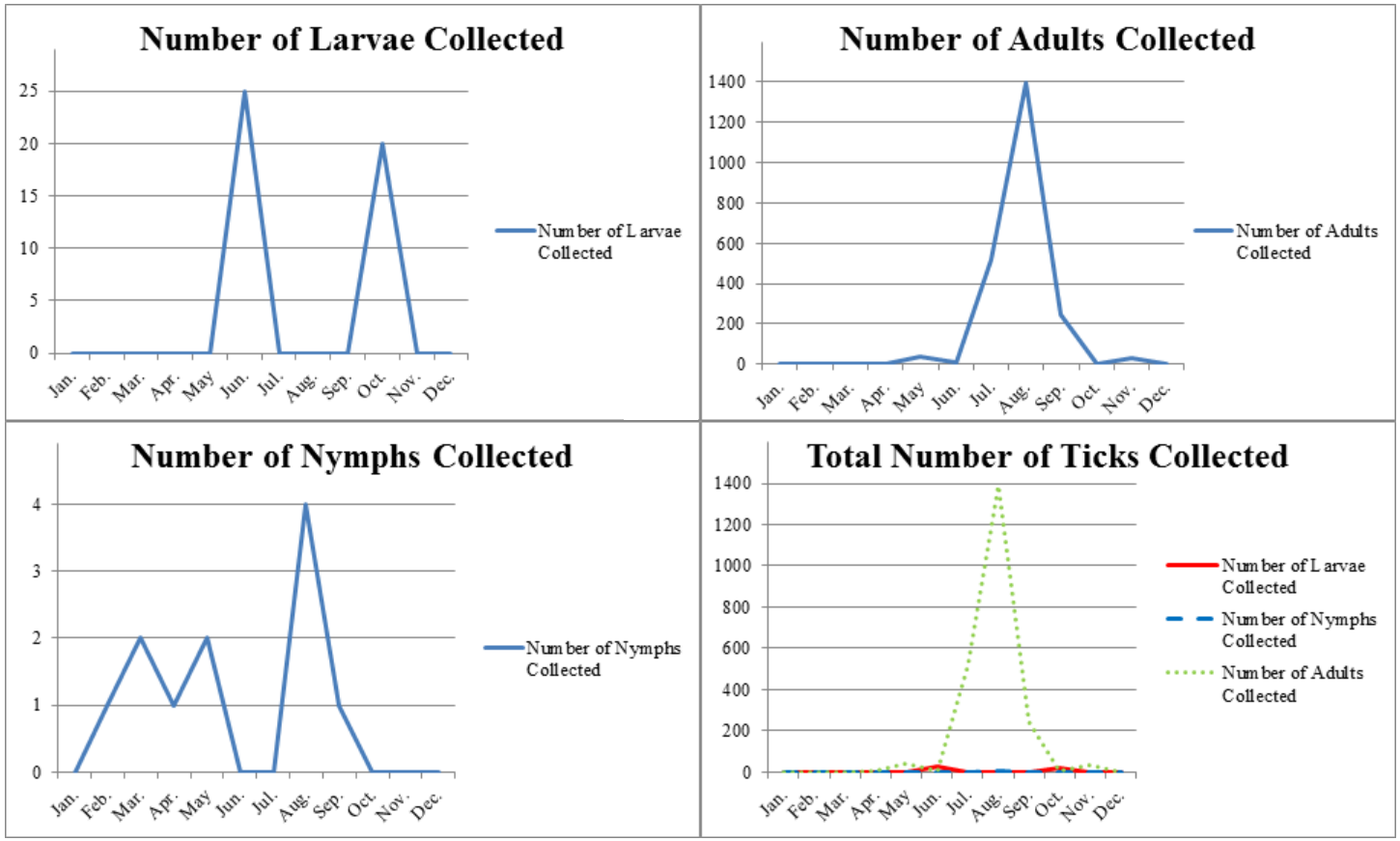


Figure B.1 Number of *Amblyomma maculatum* ticks collected by life stage.

Number of larvae (A), nymphs (B), adults, (C), and combined (D) from this study. Note that exceptionally high adult count obscures larval and nymphal counts in D.

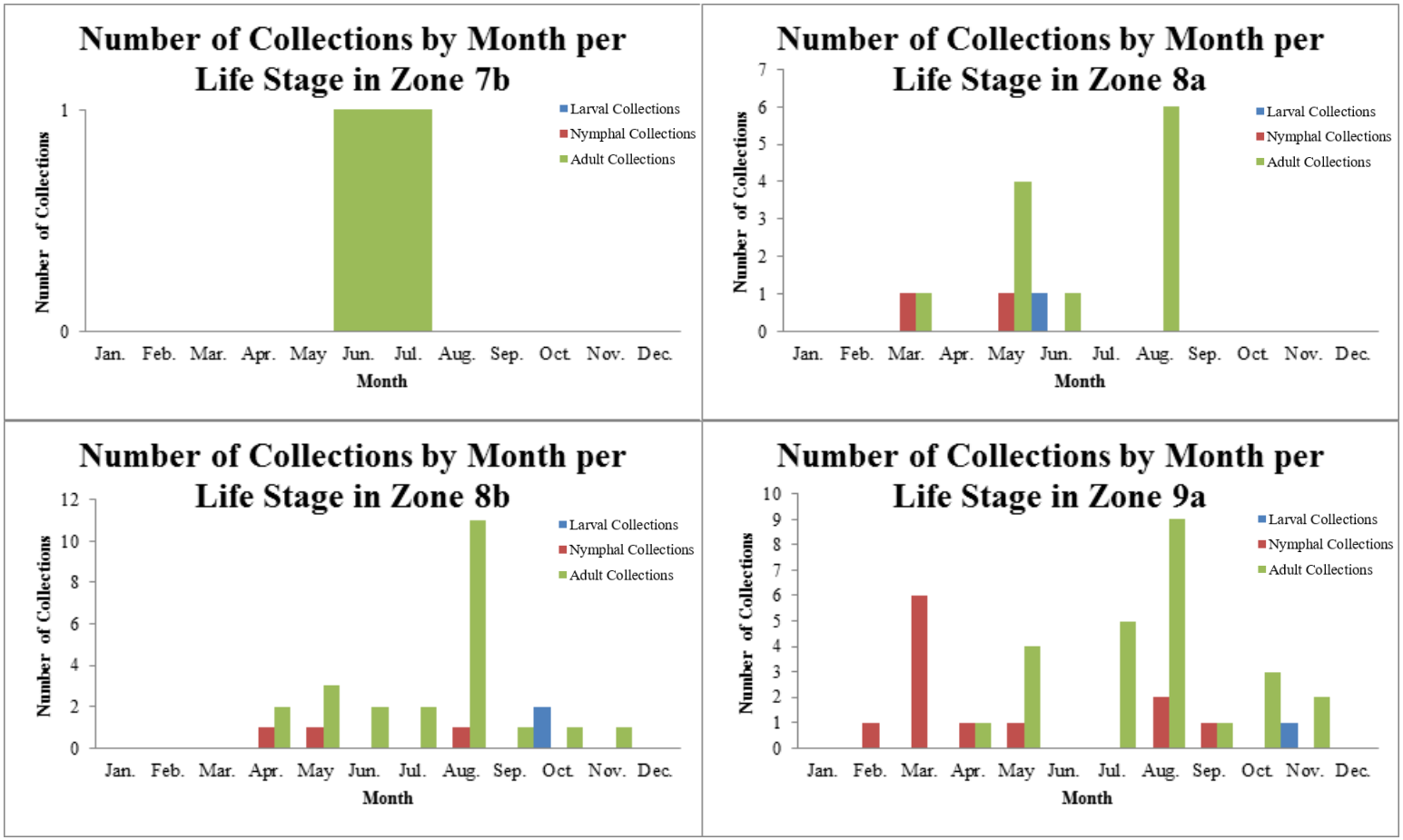
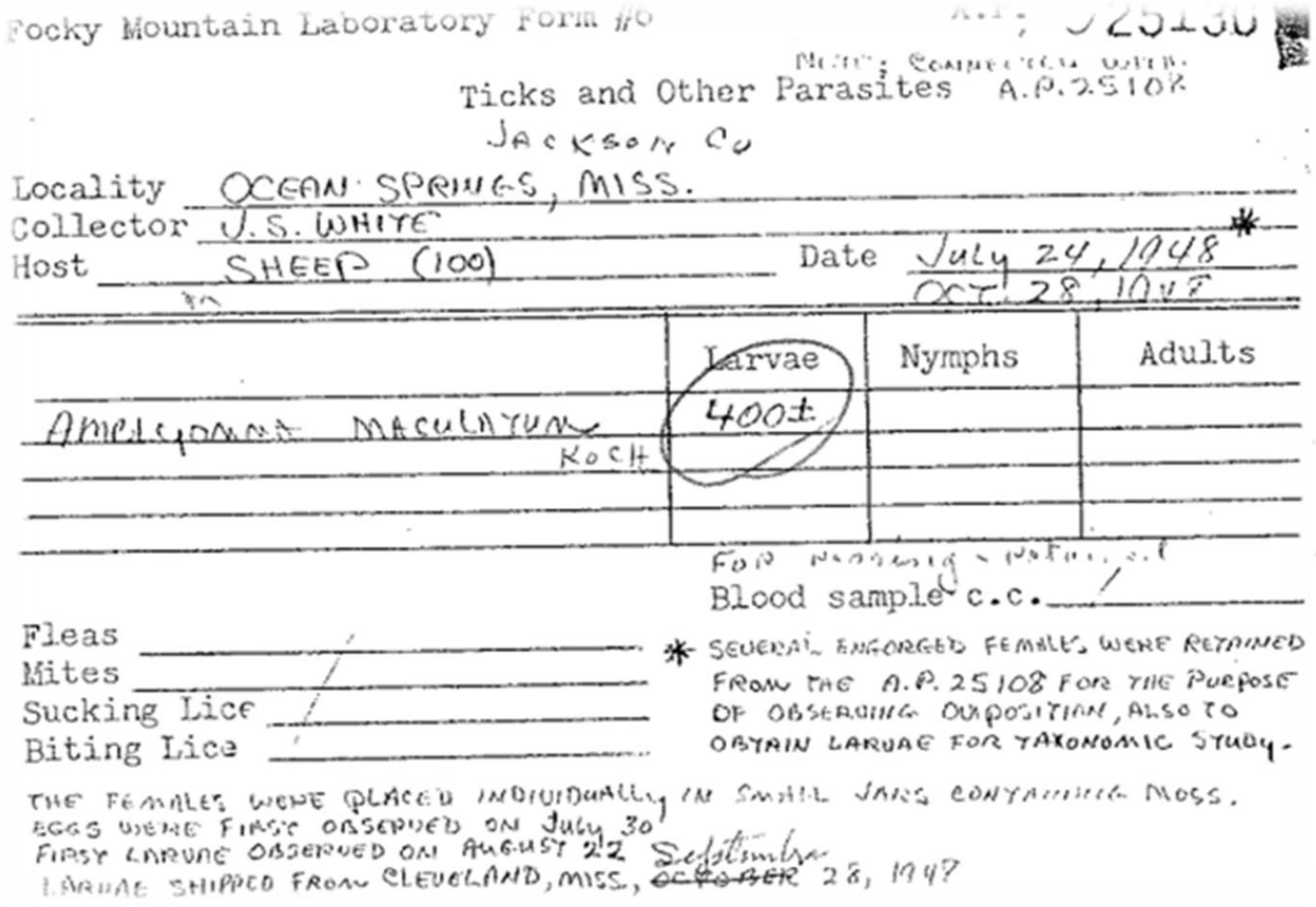


Figure B.2 Number of collections by month of each *Amblyomma maculatum* life stage.

Number of collections for all stages from north to south,7b (A), 8a (B), 8b (C), 9a (D), in Mississippi by plant hardiness zone (Figure 3.2). Note that no immatures were collected in the 7b, the most northerly zone (A).



Rocky Mountain Laboratory Form #6

Ticks and Other Parasites A.P. 25108

JACKSON Co

Locality OCEAN SPRINGS, MISS.

Collector J.S. WHITE

Host SHEEP (100) Date July 24, 1948 * OCT. 28, 1947

	Larvae	Nymphs	Adults
AMBLYOMMA MACULATUM KOCH	400±		

FOR [illegible]

Blood sample c.c.

Fleas

Mites

Sucking Lice

Biting Lice

* SEVERAL ENGORGED FEMALES WERE RETAINED FROM THE A.P. 25108 FOR THE PURPOSE OF OBSERVING OVIPOSITION, ALSO TO OBTAIN LARVAE FOR TAXONOMIC STUDY.

THE FEMALES WERE PLACED INDIVIDUALLY IN SMALL JARS CONTAINING MOSS.
EGGS WERE FIRST OBSERVED ON July 30
FIRST LARVAE OBSERVED ON AUGUST 22 September
LARVAE SHIPPED FROM CLEVELAND, MISS., ~~OCTOBER~~ 28, 1947

Figure B.3 Photocopied United States National Tick Collection records from 1948.

Note that 400 larvae previously reported were actually reared in laboratory from gravid field-collected females.

APPENDIX C

SUPPLEMENTAL MATERIAL FOR CHAPTER IV

Figure C.1 Removing young worker bees directly from beehives with assistance from Dr. Jeff Harris

Young bees were removed directly from frames, and had not been exposed to foraging stresses. Interestingly, a substantial number of ticks were encountered in the grass during this process, but these were excluded from this dissertation.

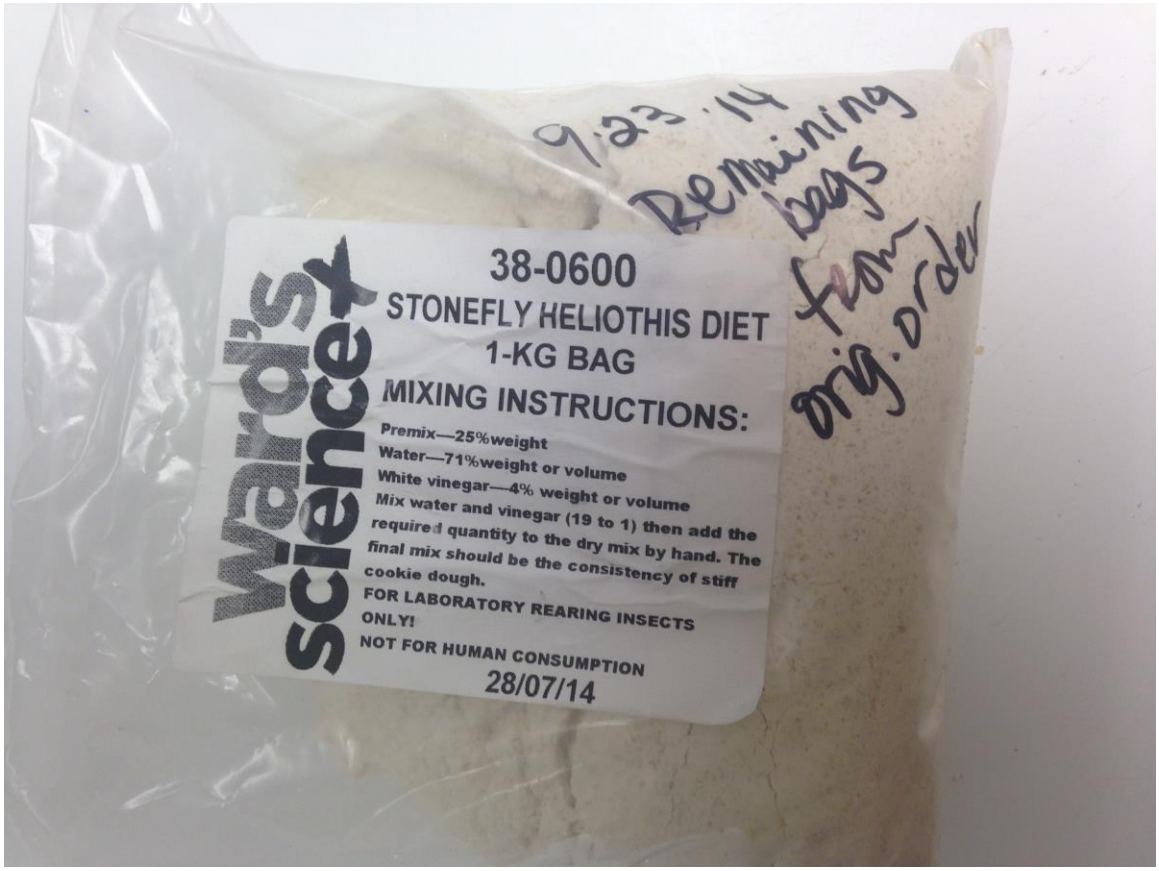


Figure C.2 Diet mix used for rearing fall armyworms (*Spodoptera frugiperda)* in laboratory.

Mixing instructions on bag were followed as posted, and diet was prepared within 24 hours of use.

Figure C.3 Cricket and ticks secured in tube, within resalable plastic bag containing moist paper towel.

Note that bag was briefly opened to allow exchange of gasses daily.

Figure C.4 Magnified view of nymphal *Amblyomma americium* ticks suspected of feeding (left) and control (right). Red on hypostome and gut as seen with tick on left confirms ingestion of dyed hemolymph.

APPENDIX D

SUPPLEMENTAL MATERIAL FOR CHAPTER V

Centers for Disease Control and Prevention (CDC)
National Center for Emerging and Zoonotic Infectious Diseases (NCEZID)
Division of Vector-Borne Diseases (DVBD)
Rickettsial Zoonoses Branch (RZB)
Medical Entomology Laboratory

VALIDATION OF THE PATHOGEN-FREE STATUS OF TICK COLONIES

Live ticks included in this shipment are free of known rickettsial pathogens and considered Specific Pathogen Free.

Laboratory colonies of ixodid ticks of the following species are currently being maintained in the Medical Entomology Laboratory: *Amblyomma americanum, Amblyomma maculatum, Amblyomma mixtum, Dermacentor variabilis, Haemaphysalis leporispalustris, Ixodes ricinus, Ixodes scapularis, Ixodes persulcatus, Ixodes pacificus, and Rhipicephalus sanguineus.*

Uninfected colonies are maintained in the laboratory by feeding of all life stages upon tick- and pathogen-naive New Zealand white rabbits according to previously published methods (Troughton & Levin 2007) and the IACUC-approved protocols. Uninfected ticks are fed only on uninfected animals in a dedicated room.

The pathogen-free status of every individual colony is routinely monitored and confirmed by appropriate PCR assays in representative samples of ticks and serological assays in serum samples from every animal used for colony maintenance.

All individual adult ticks (post-reproduction) of every generation and representative samples of progeny of each individual female tick (100-150 eggs or larvae) are tested by PCR for the presence/absence of the following tick-borne agents: pan-*Rickettsia, Rickettsia rickettsii, R. parkeri, R. amblyommii, Borrelia lonestari, Ehrlichia chaffeensis, E. ewingii, Borrelia burgdorferi, Anaplasma phagocytophilum*. Sera of all rabbits used in tick maintenance are tested by IFA for antibodies against the same pathogens. Only cohorts of ticks that produce unequivocal negative results both in PCR and serology are used for maintenance and proliferation of uninfected colonies.

Certification of the SPF status must not be interpreted as a release from standard safety requirements applicable to work with live ticks.

Michael L. Levin, Ph.D.
Medical Entomology Laboratory Director
Rickettsial Zoonoses Branch, DVBD
Centers for Disease Control and Prevention
1600 Clifton Road, MS G-13, Atlanta, GA 30333
Phone: (404) 639-3639
Fax: (404) 639-4436
E-mail: MLevin@cdc.gov

Figure D.1 Validation of pathogen-free status of tick colonies from Dr. Levin at CDC

All volunteers were provided a copy of this validation.

Mississippi State University
Informed Consent Form for Participation in Research

Title of Research Study: Attempted Feeding of Larval *Amblyomma maculatum* (Koch) Ticks on Humans

Study Site: Mississippi State University, Clay Lyle Entomology Building, Room 155

Researchers: **Dr. Jerome Goddard**, Extension Professor of Medical and Veterinary Entomology, Department of Biochemistry, Molecular Biology, Entomology, and Plant Pathology, Mississippi State University, and **Mr. José Santos Portugal III**, Graduate Research Assistant and Ph.D. Student, Department of Biochemistry, Molecular Biology, Entomology, and Plant Pathology, Mississippi State University.

Purpose

The purpose of this research is to determine if, and to what extent larval (immature) *Amblyomma maculatum* ticks attempt to attach and feed on humans.

Procedures

If you participate in this study, you will have six small plastic caps, each containing ~10 larval ticks (about the size of poppy seeds) taped to your arm. This is only a one-time event. The ticks will be removed after being exposed to your skin for 15 minutes. All ticks used will be certified by the Centers for Disease Control and Prevention (CDC) as "pathogen free" for your safety.

Risks or Discomforts

There is some risk of localized mild itching and reddness. You could also possibly develop a secondary infection at the tick feeding site, especially if you scratch it. You should seek medical care if you develop any unusual or long-lasting skin reaction or infection at the site.

Benefits

Recently, a human pathogen (*Rickettsia parkeri*) resulting in a disease known as American Boutonneuse fever, has been demonstrated to be transmitted by this species of tick in Mississippi and other parts of the United States. It is not known if the larval (immature) stage of this tick species will attempt to attach and feed on humans. This experiment will determine if they will, and therefore, if the larval stage of this tick species could possibly transmit this new human disease.

Incentive to participate

There are no incentives for participating in this study.

Figure D.2 Mississippi State University Informed Consent Form for Participation in Research.

Confidentiality

No identifiers will be collected or allowed in the processing or reporting of these data, nor will there be any DNA analysis or disease analysis of the ticks.

Please note that these records will be held by a state entity and therefore are subject to disclosure if required by law. Research information may be shared with the MSU Institutional Review Board (IRB) and the Office for Human Research Protections (OHRP).

Questions

If you have any questions about this research project, please feel free to contact Jerome Goddard at 662-325-2085.

For questions regarding your rights as a research participant, or to express concerns or complaints, please feel free to contact the MSU Regulatory Compliance Office by phone at 662-325-3994, by e-mail at irb@research.msstate.edu, or on the web at http://orc.msstate.edu/participant/.

Research-related injuries

MSU will not provide any payment to you or for your treatment if you are harmed as a result of taking part in this study.

In addition to reporting an injury to Jerome Goddard at 662-325-2085 and to the Regulatory Compliance Office at 662-325-3994, you may be able to obtain limited compensation from the State of Mississippi if the injury was caused by the negligent act of a state employee where the damage is a result of an act for which payment may be made under §11-46-1, et seq. Mississippi Code Annotated 1972. To obtain a claim form, contact the University Police Department at *MSU UNIVERSITY POLICE DEPARTMENT, Williams Building, Mississippi State, MS 39762, (662) 325-2121.*

Voluntary Participation

Please understand that your **participation is voluntary.** Your **refusal to participate will involve no penalty or loss** of benefits to which you are otherwise entitled. You **may discontinue your participation** at any time without penalty or loss of benefits.

Figure D.2 (Continued)

Options for Participation
Please initial your choice for the options below:

___The researchers may contact me again to participate in future research activities.

___The researchers may NOT contact me again regarding future research.

Please take all the time you need to read through this document and decide whether you would like to participate in this research study.

If you agree to participate in this research study, please sign below. You will be given a copy of this form for your records.

_____ _____
Participant Signature Date

_____ _____
Investigator Signature Date

Figure D.2 (Continued)

We have continued to retain signed consent forms, and no substantial negative health issues were reported to us by participants.

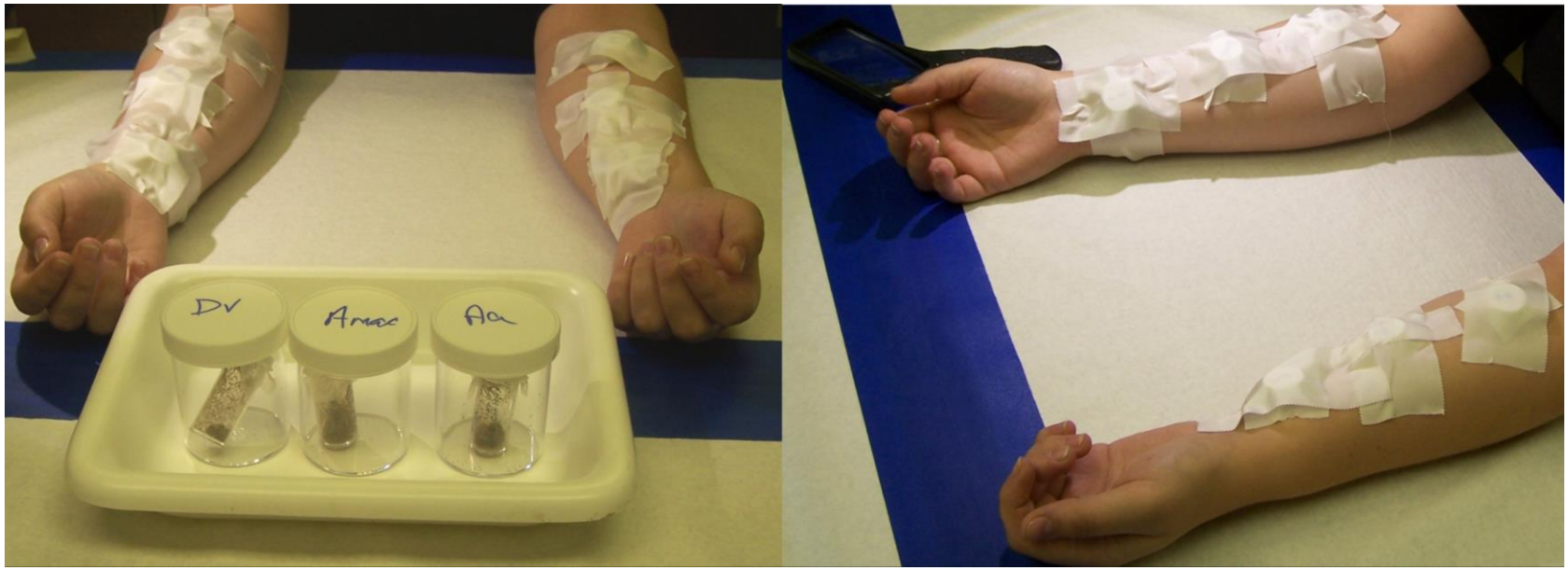


Figure D.3 Front (left) and side (right) view of caps containing ticks secured to palmar forearm of volunteer.

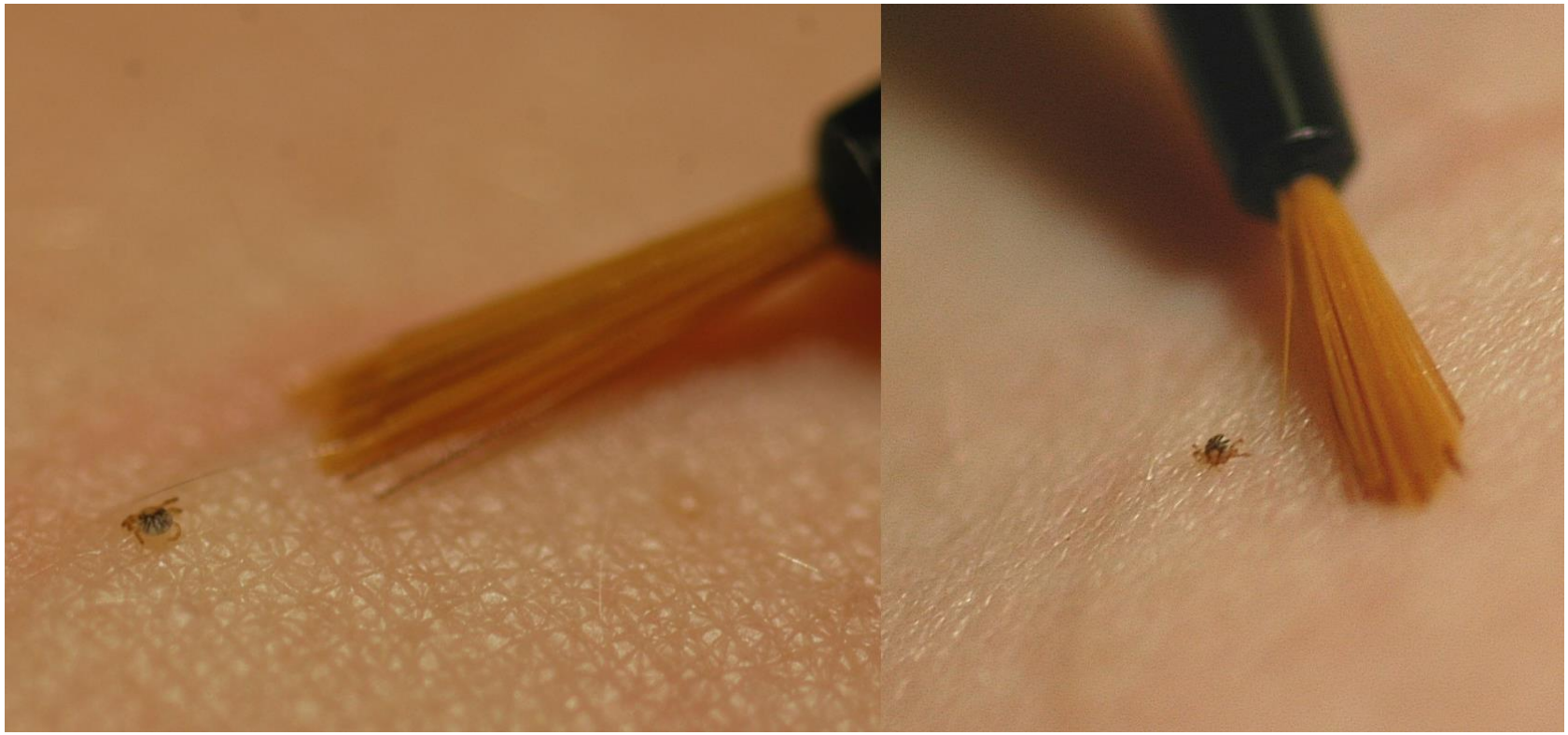


Figure D.4 Magnified view of larval *Amblyomma americanum* feeding from volunteer in experiment.

Ticks pictured were challenged with fine-tipped forceps to confirm attachment.

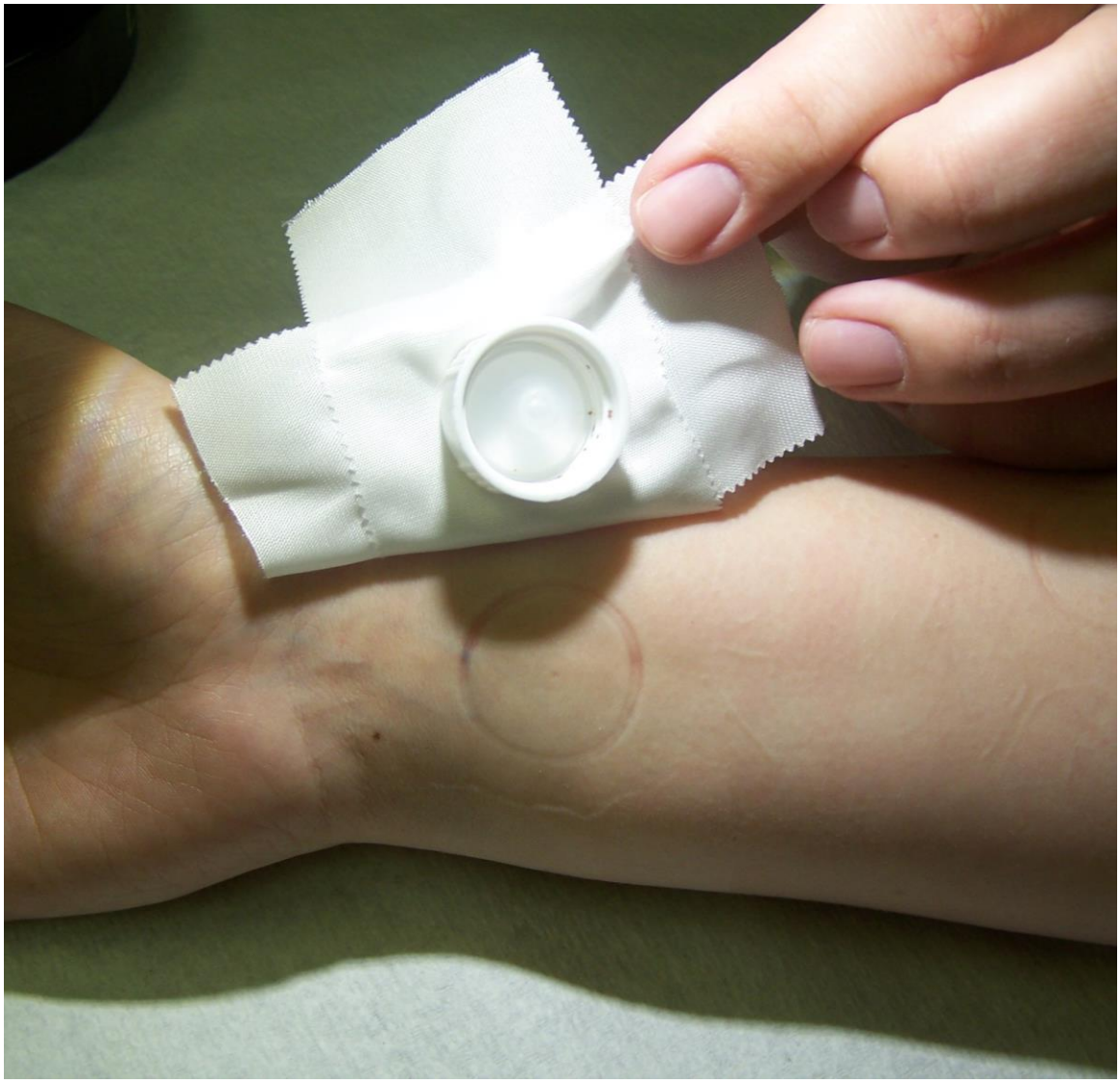


Figure D.5 No observed attachment by negative control (larval *Dermacentor variabilis*) ticks as expected.

Larval *Dermacentor variabilis* did not attach at any point during testing.

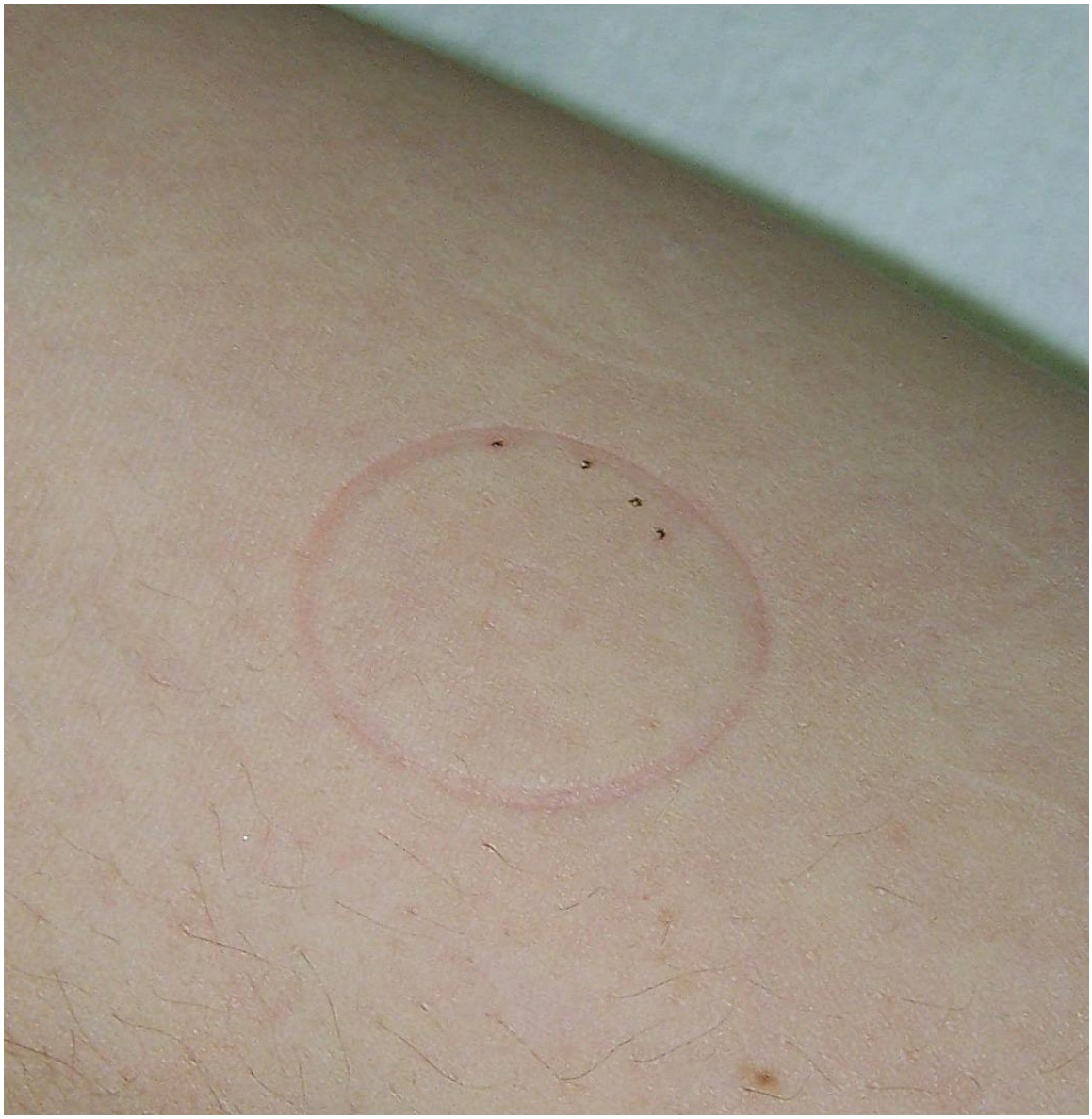


Figure D.6 Feeding larval *Amblyomma americanum* ticks congregating where cap met skin.

Ticks pictured were challenged with fine-tipped forceps to confirm attachment.

APPENDIX E

SUPPLEMENTAL MATERIAL FOR CHAPTER VI

Figure E.1 Insect rearing chamber used in this study.

Note that light source is directly overhead testing arena.

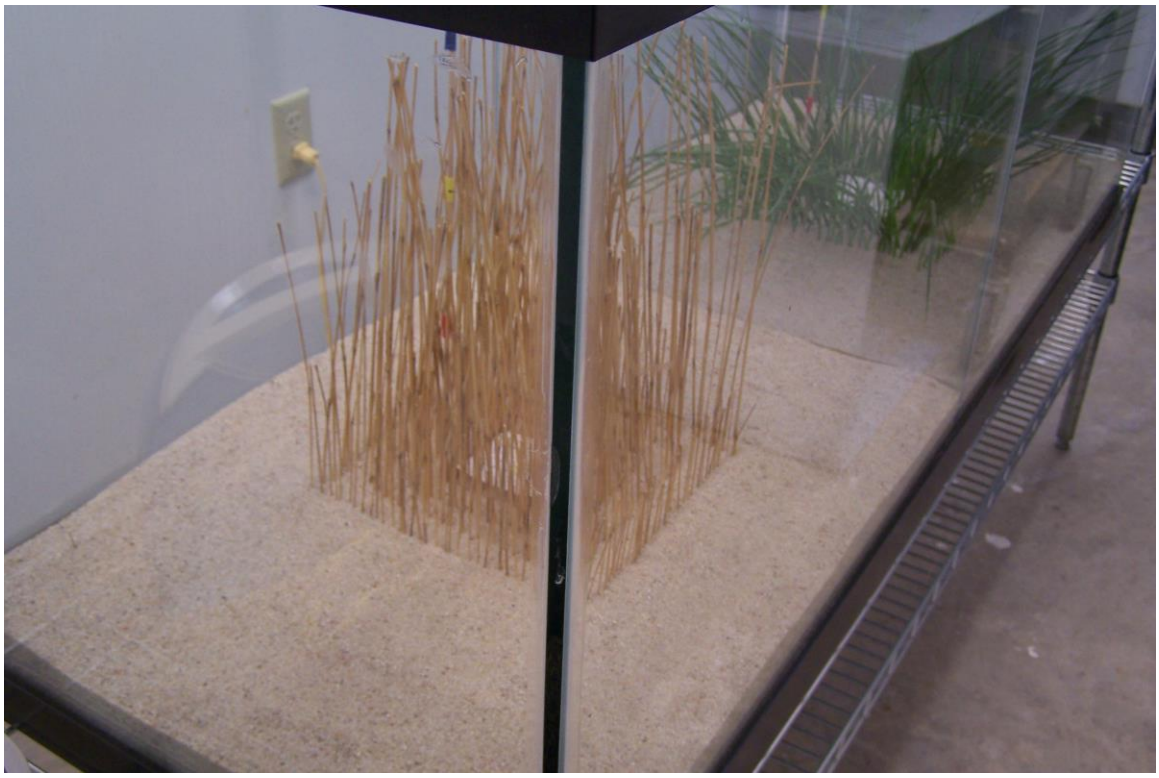


Figure E.2 Side view of observation arena inside insect rearing chamber.

On metal rack, inside of insect rearing chamber (Figure E.1). Note sand substrate anchoring stems.

Figure E.3 Closer view of observation arena used in this study.

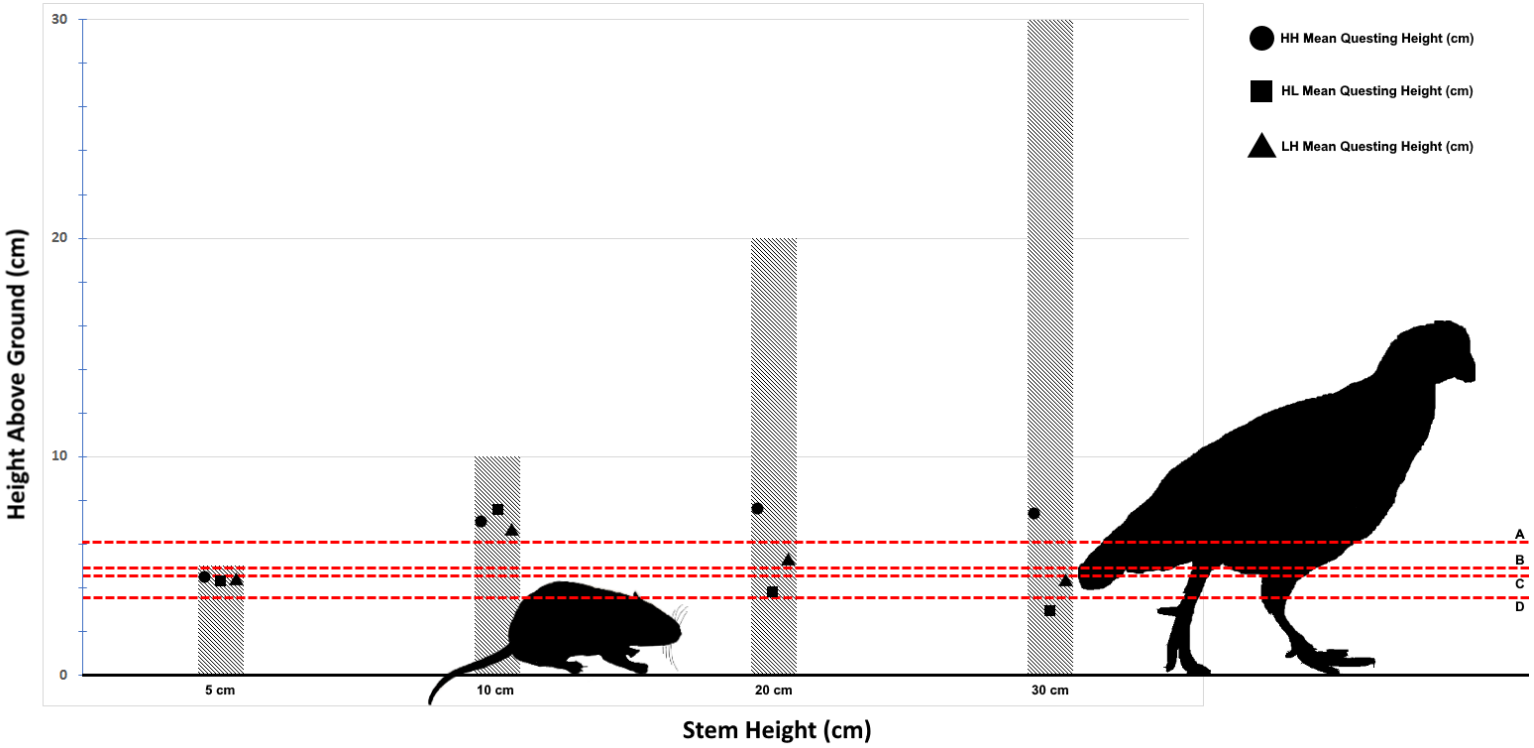


Figure E.4 Mean questing heights by treatment and stem in relation to preferred hosts.

Mean overall questing height for nymphal *A. maculatum* in HTHH (line A), LTHH (B), HTLH (C), and from Portugal and Goddard 2016b (D). Image of rodent on left is hispid cotton rat (*Sigmodon hispidus*), and image on right is bobwhite quail (*Colinus virginianus*). Both images are to scale