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Trapping Methods for Ixodid Ticks and Pathogen Associations of *Amblyomma maculatum* (Gulf Coast Tick) in Western Tennessee

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To the Graduate Council:

I am submitting herewith a thesis written by Sarah Elizabeth Mays entitled "Trapping Methods for Ixodid Ticks and Pathogen Associations of *Amblyomma maculatum* (Gulf Coast Tick) in Western Tennessee." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

Rebecca T. Trout Fryxell, Major Professor

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Abstract

Ticks are vectors of disease agents and pests of humans and animals. Various methods are used for tick monitoring and pathogen surveillance to assess tick distributions, pathogen prevalence and control measures, such as monitoring the changing geographic distribution of the Gulf Coast tick, *Amblyomma maculatum*. This project (1) compared the effectiveness of six trapping methods for the collection of hard (Ixodid) ticks in a typical grassland-forest habitat in southwestern Tennessee, and (2) examined pathogen associations of *A. maculatum* collected in western Tennessee.

To compare trapping methods across time and habitat types, a temporal study was conducted in 2013 and a habitat study was conducted in 2014. Conventional tick collection methods (dragging, flagging, dry ice trapping, and sweep-netting) and novel methods (carbon dioxide (CO_2)-reinforced dragging and flagging) were compared across five monthly sampling periods. Dragging, CO_2 dragging, CO_2 flagging and dry ice trapping were then compared across four habitat types (grassland, upland deciduous, bottomland deciduous, and coniferous). Significant interactions between trapping method and sampling period (2013) and between trapping method and habitat (2014) were identified. In both studies, the novel methods were comparable to their conventional counterparts; the addition of CO_2 did not significantly increase the number of ticks collected. Dry ice trapping and dragging were effective methods of tick collection across time and habitat types, and were among the most effective methods for all species collected.

To detect pathogens associated with *A. maculatum* and identify the best surveillance methods for monitoring infected ticks, questing and host-feeding *A. maculatum* (n = 265) collected in the 2013 and 2014 trapping studies and other concurrent studies were PCR-screened

for *Rickettsia*, *Ehrlichia*, and *Borrelia* species. Of the *A. maculatum* screened, none were *Borrelia* positive, 2 were *Ehrlichia* positive, and 60 were infected with *R. parkeri* (a pathogenic *Rickettsia*). No particular surveillance technique (e.g. habitat type or collection source) was significantly more effective for detection of infected *A. maculatum*. The results of this project demonstrate the importance of monitoring and surveillance methods based upon habitat, target species, and research objectives, and the need for continued monitoring and surveillance of ticks, including *A. maculatum*.

Keywords: Ixodidae, field collection, trapping, *Amblyomma maculatum*, *Rickettsia*, *Ehrlichia*

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1. Introduction

Introduction

Ticks: Ticks are the primary vectors of human arthropod-borne diseases in the United States, and are second only to mosquitoes world-wide in the transmission of arthropod-borne diseases to humans (Spach *et al.*, 1993; Parola & Raoult, 2001). Ticks also transmit pathogens affecting livestock, pets, and wildlife. In the southeastern U.S., the status of tick-borne disease is uncertain due to factors such as the introduction and identification of new diseases, the difficulty of tick-borne disease diagnosis, habitat fragmentation, and warming weather trends (Moncayo *et al.*, 2010; Stromdahl & Hickling, 2012; Léger *et al.*, 2013).

Ticks of Tennessee: Several human-biting tick species associated with pathogen transmission are found in Tennessee. *Amblyomma americanum* (Linnaeus), the lone star tick, transmits the agents of ehrlichiosis (Cohen *et al.*, 2010a). *Amblyomma maculatum* Koch, the Gulf Coast tick, transmits an agent of rickettsiosis (Paddock *et al.*, 2004). *Dermacentor variabilis* (Say), the American dog tick, is the primary vector of the agent of Rocky Mountain spotted fever (RMSF) in the eastern U.S. (Burgdorfer, 1975). *Ixodes scapularis* Say, the black-legged tick, transmits the agents of anaplasmosis, babesiosis, and Lyme disease (Adelson *et al.*, 2004).

Tick Monitoring: Tick monitoring allows measures and comparisons of relative abundance of tick species, evaluation of control methods, identification of pathogen vectors, estimation of the risk of encounter with infected ticks, and establishment of spatial distribution of ticks and their associated pathogens (Jameson & Medlock, 2011; Carr *et al.*, 2013). A number of different trapping methods are used for tick collection, including the collection of questing ticks and host-feeding ticks. These may vary in their efficiency and associated biases, including estimates of pathogen prevalence (Ginsberg & Ewing, 1989; Schulze *et al.*, 1997; Petry *et al.*, 2010). One typical method of tick collection is dragging, which collects ticks that are questing on

vegetation or leaf litter. Flagging, another traditional tick trapping method, functions similarly to the drag, but may allow for the sampling of multiple layers of vegetation, while dragging samples primarily the top, or tallest layer of vegetation (Ginsberg & Ewing, 1989; Cohnstaedt *et al.,* 2012). Dry ice trapping is a method for tick collection involving a lure to attract ticks to a specific location. As the dry ice sublimes carbon dioxide (CO_2), a questing stimulant in some tick species, is released as a gas (Schulze *et al.,* 1997). Because of species differences in questing strategies, collection method efficiency may vary in the numbers of ticks or the proportion of individual species that are collected (Ginsberg & Ewing, 1989; Schulze *et al.,* 1997). Sweep-netting is sometimes used for tick collection (Semtner & Hair, 1975), and is a common tool employed by many entomologists for surveillance protocols such as the development or monitoring of economic thresholds in Integrated Pest Management systems.

More recently, researchers have attempted to develop methods with alternative sources of CO_2 (Niebuhr *et al.,* 2013), partly due to the sometimes prohibitive cost of dry ice and the difficulty in obtaining large quantities, or to combine traditional collection methods such as flagging with a source of CO_2 to increase method efficiency by increasing the attractiveness of the sampling material (Gherman *et al.,* 2012). Both the Gherman *et al.,* (2012) and the Niebuhr *et al.,* (2013) studies used a tank of compressed CO_2 as a gas source, and a perforated hose system to release the CO_2 at precise locations (Table 1.1).

Pathogen Surveillance: A number of tick-borne diseases have been diagnosed in Tennessee, including Rocky Mountain spotted fever (RMSF), caused by *Rickettsia rickettsii*; ehrlichiosis, caused by *Ehrlichia chaffeensis* and *E. ewingii*; and Lyme disease, caused by *Borrelia burgdorferi* (Fig. 1.1) (TNDOH, 2015). Although the state of Tennessee accounts for only 2.4% of RMSF cases in the U.S., southwestern Tennessee contributes to 26% of RMSF

fatalities nation-wide (Adjemian *et al.*, 2009). In spite of this high number, the causative agent of RMSF has not been identified in ticks collected in Tennessee (Moncayo *et al.*, 2010; Stromdahl *et al.*, 2011; Hendricks, 2013). Ehrlichiosis is common, yet poorly understood. Multiple *Ehrlichia* spp., including *E. chaffeensis*, *E. ewingii*, and Panola Mountain *Ehrlichia*, have been identified in ticks in Tennessee (Cohen *et al.*, 2010a; Hendricks, 2013; Mays *et al.*, 2014; Harmon *et al.*, 2015). Though Lyme disease has been repeatedly diagnosed in patients in Tennessee (average of 34.6 cases/year over the last 15 years) (TNDOH 2014), the causative agent for Lyme disease has not been identified in ticks from Tennessee (Rosen *et al.*, 2012, Mays *et al.*, 2014). Lyme disease is more prominent in northeastern and midwestern regions of the U.S., but is considered non-endemic in Tennessee and is thought unlikely to pose a high threat in southern and southeastern states (Diuk-Wasser *et al.*, 2012). The absence of the causative agents of these two tick-borne diseases indicates that many patients may be misdiagnosed. The current diagnostic test for RMSF can be cross-reactive, and could indicate infection with *R. rickettsii* when a patient has antibodies to another Spotted Fever Group *Rickettsia* (SFGR), including non-pathogenic species, which do not cause disease (Philip *et al.*, 1976; Hechemy *et al.*, 1989; Raoult 2004). This cross-reactivity can result in the true cause of illness remaining unidentified. There is a critical need for research that identifies tick vectors of concern in Tennessee and identifies pathogens present in tick populations.

Significance of *Amblyomma maculatum*: The Gulf Coast tick is a 3-host tick endemic to the southern U.S. and parts of South America (Teel *et al.*, 2010). The historic distribution of this tick was within 100 miles of the Gulf Coast (Bishopp & Trembly, 1945), with isolated populations established in Kansas and Oklahoma by 1973, likely transported on livestock (Semtner & Hair, 1973). The range of the Gulf Coast tick has since expanded northward and

eastward along the Mississippi river and the Appalachian mountains (Teel *et al.*, 2010). Adults are primarily a pest of cattle, but will also feed on a variety of other medium and large mammals, including a variety of ungulates and canids (Bishopp & Trembley, 1945; Teel *et al.*, 2010). The larval and nymphal stages of this tick feed on a number of small and medium-sized mammal species, as well as birds (Bishopp & Trembley, 1945; Semtner & Hair 1973; Teel *et al.*, 1998; Teel *et al.*, 2010). Heavy infestation of *A. maculatum* in livestock and other mammals has historically been associated with a predisposition to screwworm infection (Bishopp and Hixon, 1936), and has been associated with decreased rate of gain, weight loss, and poor condition (Gladney *et al.*, 1977). Heavy infestations of this tick in young cattle and other livestock can cause a condition called "gotch ear", which results in crumpling of the tips of the ear, and reduced market value (Gladney *et al.*, 1977; Drummond, 1988; Teel *et al.*, 2010; Edwards, 2011). This tick is found primarily in open grassland habitats such as coastal and tall-grass prairie and grasslands with various brush species present, characteristics of early to mid-successional stage habitat. It has also been collected in post oak savannahs (Semtner & Hair 1973; Teel *et al.*, 2010).

The Gulf Coast tick may play a role in the transmission cycles of pathogens such as *R. parkeri*, *Hepatozoon americanum*, and *E. ruminantium* (Mahan *et al.*, 2000; Baneth *et al.*, 2003; Goddard & Varela-Stokes, 2009). A SFGR that causes a spotted fever illness in humans, *R. parkeri* has been associated with *A. maculatum* for some time (Parker *et al.*, 1939) but has been identified only recently as a human pathogen (Paddock *et al.*, 2004). The distribution of *R. parkeri* seems to closely match the distribution of the Gulf Coast tick (Sumner *et al.*, 2007). This pathogen causes an illness in humans similar to RMSF, though typically less severe. Symptoms often include fever, myalgia, malaise, and headache (Paddock *et al.*, 2008; Goddard & Varela-

Stokes, 2009). Patients may present with a rash, which is typically less widespread on the body than the rash associated with RMSF, and usually includes an eschar at the site of tick attachment (Paddock *et al.,* 2008). *Rickettsia parkeri* has been identified in *A. maculatum* in a number of southeastern and eastern states at rates ranging from 11% to 40%, though infection rates of 15-30% are more common (Sumner *et al.*, 2007; Goddard & Varela-Stokes, 2009; Paddock *et al.*, 2010; Varela-Stokes *et al.*, 2011; Wright *et al.,* 2011; Ferrari *et al.,* 2012; and Pagac *et al.*, 2014).

The Gulf Coast tick also transmits *Hepatozoon americanum,* a parasitic apicomplexan, to canids (Baneth *et al.,* 2003; Little *et al.,* 2009). Transmission of the agent occurs when a dog or other canid ingests an infected Gulf Coast tick (Vincent-Johnson *et al.,* 1997). Unlike the hepatozoonosis caused by *H. canis* and transmitted by another tick, *Rhipicephalus sanguineus*, which is common in many regions, *H. americanum* causes a more severe and more often fatal disease in dogs (Baneth *et al.,* 2003; Little *et al.,* 2009). Lab studies have proven *A. maculatum* to be a competent vector of *E. ruminantium,* the causative agent of heartwater (Mahan *et al.,* 2000). This disease of livestock and wild ruminants is endemic to Africa, and has since spread to other areas (Uilenberg *et al.,* 1984; Deem, 1998). Although this pathogen is not currently established in the continental U.S. there is a risk for introduction, which could impact a variety of naive ruminants including livestock (Uilenberg *et al.,* 1984; Deem, 1998; Burridge *et al.,* 2002). Additionally, three species of *Borrelia,* including *B. burgdorferi* (the Lyme disease agent) have been identified in *A. maculatum* (Trout Fryxell *et al.,* 2012; Lee *at al.,* 2014). The ability of *A. maculatum* to transmit these agents remains unknown (Trout Fryxell *et al.,* 2012; Lee *at al.,* 2014).

***Amblyomma maculatum* in Tennessee:** *Amblyomma maculatum* is not a species historically common in Tennessee, though it seems to have become more frequently encountered

in more recent years. Bishopp & Trembley (1945) mention specimens from Tennessee supposedly introduced on livestock shipped from *A. maculatum*-endemic areas. Sampling of foxes and coyotes in Tennessee and Kentucky in 1986 did not identify *A. maculatum* (Bloemer & Zimmerman, 1988). Sampling of medium-sized mammals in southwestern Tennessee in 1990-1991 yielded >2,500 ticks of seven different species, but no *A. maculatum* (Kollars, 1993). Durdan and Kollars (1992) note that *A. maculatum* did not appear to be established in Tennessee, and speculate that the small number collected in the state may have represented immature individuals carried in on birds. During a survey in 2007 and 2008 of medium and large mammal tick hosts in Tennessee, nearly 2,000 ticks were collected from mammals and by dragging. Of this total, two *A. maculatum* were collected from human investigators during sampling, but none were collected by host-sampling or dragging (Cohen *et al.*, 2010b). Sampling of hunter-harvested white-tailed deer, known hosts of *A. maculatum* (Teel et. al. 2010), in 2007 and 2008 also yielded no *A. maculatum* specimens (Rosen *et al.*, 2012). Though collections in the past have consisted of only a small number of specimens, a larger number (n = 20) were collected in southwestern Tennessee in 2012 using dry ice traps and drags (Hendricks, 2013), spurring the investigation into pathogen associations of *A. maculatum* in western Tennessee described in this project.

Current *Amblyomma maculatum* Management Options: A variety of methods have been used for tick control, including the application of acaricides to animals, the application of acaricide to habitat, and various methods of habitat disturbance. Field tests in the mid-1970s indicated that many commercially available products for tick control on cattle, including ear smears, dusts, sprays, and slow-release devices, offered only minimal protection, typically lasting only 1-3 weeks after application to tick-infested cattle, though most demonstrated high

rates of initial post-treatment control (Gladney *et al.*, 1977). A later laboratory study testing the current commercially available products for tick control on livestock using lab-reared *A. maculatum* found that cyfluthrin and permethrin resulted in rapid kill of *A. maculatum* (Burridge *et al.*, 2003).

Acaricides alone are often not useful long-term when applied to existing vegetation because of reinfestation facilitated by animal use of such areas, although the integrated use of vegetation removal along with acaricides can produce habitats that are unsuitable for tick survival and establishment (Hoch *et al.*, 1971). Mechanical clearing of vegetation alone resulted in an immediate reduction of local tick populations, though long-term reduction was not demonstrated (Wilson, 1986).

Various results have been obtained in experiments considering controlled burning as a means of tick control. Hoch *et al*, (1972) indicated that controlled burning of woodlots was not effective for the control of lone star ticks, and other studies have reported similar failure to control tick populations with burning (Padgett *et al.*, 2009); however, some studies indicated a short-term reduction (~1year) in the populations of some species, including *A. maculatum* (Wilson, 1986; Scifres *et al.*, 1988; Cully, 1999; Gleim *et al.*, 2013). Polito *et al.* (2013) found that although rotational burning in pastures had no effect on the numbers of questing ticks collected, tick burdens on cattle were reduced. *Amblyomma maculatum* seems to be more tolerant of periodic burning than lone star ticks, suggesting that this tick may be much less prone to desiccation, and better-suited to an open environment exposed to more direct sunlight than some other species. This may be due to behavioral differences and increased ability of moisture retention due to decreased whole-body permeability in *A. maculatum* (Needham and Teel, 1991;

Gleim *et al.*, 2013). Management of *A. maculatum* will likely not be achieved without a comprehensive, integrated method of control.

Study Site: Ames Plantation Research and Education center (AMES), located in southwestern Tennessee in Hardeman and Fayette counties, is a 7,446 ha University of Tennessee Research and Education Center owned by the Hobart Ames Foundation (Fig. 1.2). AMES is devoted to forestry and ecological research, forage and crop development research, livestock development, and cultural preservation. AMES is home to the fourth oldest Angus cattle herd in the U.S. Additionally, AMES supports hunting clubs for white-tailed deer, turkey, and quail, and hosts the annual National Championship for bird dogs.

Objectives and Hypotheses

The overall goal of this project was to compare the efficiency of four traditional and two novel methods of collection for ixodid ticks, and to investigate the pathogen associations of *A. maculatum* in western Tennessee. The information gained from these studies will help to improve monitoring and surveillance methods for tick vectors by determining which are more effective for tick collection and identifying how they vary across time and habitat, and help to establish what threat *A. maculatum* in western Tennessee may pose to human and animal health.

Trapping Methods: In order to determine what methods are most effective for tick collection, a temporal study (2013) and a habitat comparison study (2014) were carried out to test:

Question 1: What methods are most effective at collecting ticks?

H_O: There is no difference between trapping methods.

H_{A1}: Trapping method efficiency will vary by tick species.

H_{A2}: Trapping method efficiency will vary by sampling month.

H_{A3}: Trapping method efficiency will vary by habitat type.

Pathogen Surveillance: Due to the changing distribution of *A. maculatum* and recent collections in Tennessee, questing and host-collected ticks were screened for tick-borne pathogens diagnosed in Tennessee to test:

Question 2: What pathogens are associated with *A. maculatum* in western Tennessee?

H_O: There are no pathogens associated with *A. maculatum* in western Tennessee.

H_{A1}: *Amblyomma maculatum* in Tennessee are associated with pathogens of humans and animals.

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Appendices

Table 1.1: Advantages and disadvantages associated with conventional and novel methods of tick collection.

Method	Description	Advantages	Disadvantages	Target
Sweep net	Sweeping motion through vegetation	Simple, inexpensive	Can be hindered by vegetation; not target-specific	Passively questing ticks
Drag	Pulling $1m^2$ cloth over vegetation	Representative of human risk, inexpensive	Can be hindered by vegetation; only samples top vegetation layer	Passively questing ticks
Flag	Sweeping motion through vegetation	Representative of human risk; samples multiple vegetation layers, inexpensive	Can be hindered by vegetation	Passively questing ticks
Dry Ice	Dry ice in container, releases CO_2	Not hindered by vegetation	Dry ice access; time (trap removal), expensive	Actively questing ticks
CO_2 Flag	Compressed CO_2 in tank, releases throughout flag	Combines active and passive method	Hindered by vegetation, expensive	Passive and actively questing ticks
CO_2 Drag	Compressed CO_2 in tank, releases along top of drag	Combines active and passive method	Hindered by vegetation, expensive	Passive and actively questing ticks

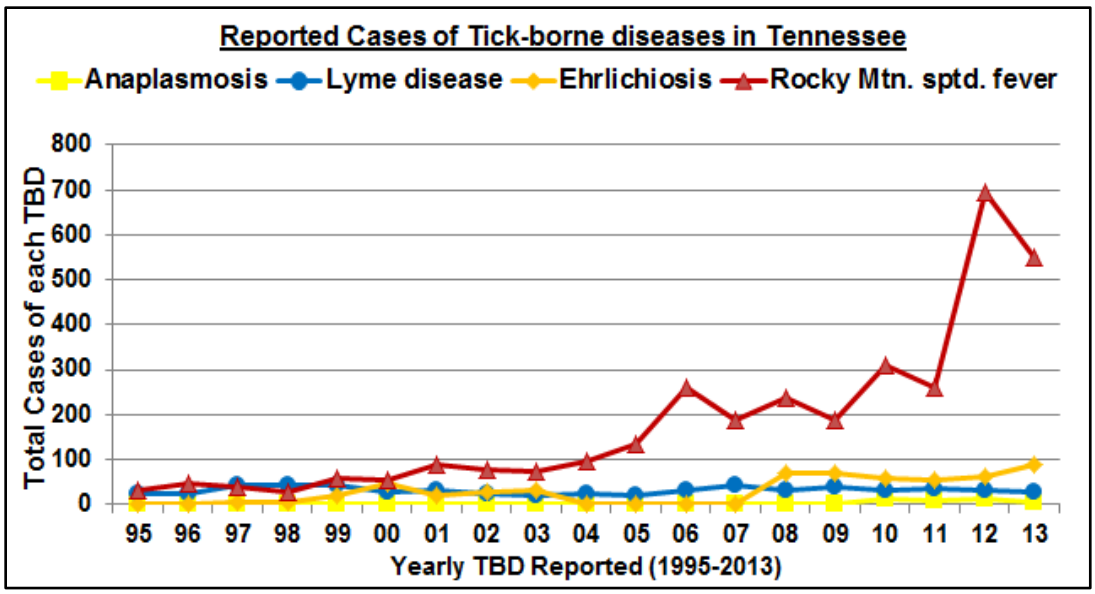

Figure 1.1: Reported cases of 4 common tick-borne diseases in Tennessee. (TNDOH 2015, created by R. Trout Fryxell)

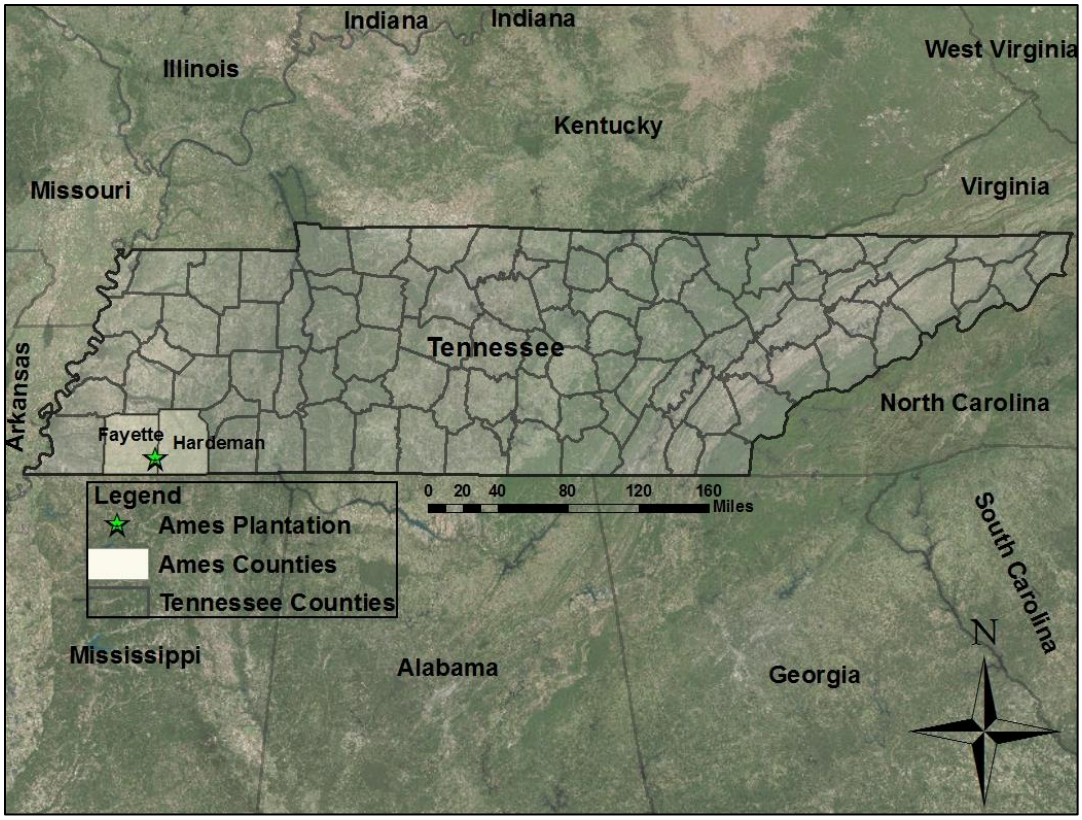

Figure 1.2: Location of Ames Plantation Research and Education Center in relation to Tennessee and neighboring states.

2. Comparison of novel and conventional trapping methods for Ixodid ticks

Abstract

Tick-borne disease surveillance and research rely on cost- and resource-effective methods for tick collection. This study compared the performance of several trapping methods in a mixed grassland-forest habitat in western Tennessee. To test for temporal differences in effectiveness, sites were sampled monthly (April – Aug 2013) with dry ice, dragging, flagging, sweep-netting, carbon dioxide (CO_2) dragging, and CO_2 flagging. To evaluate the effect of habitat on method effectiveness four methods (dragging, CO_2 dragging, CO_2 flagging, and dry ice) were compared in four habitat types (bottomland deciduous, upland deciduous, coniferous, and grassland) in June 2014. In the temporal comparison ticks were most abundant in April and May, and there was a significant sampling period and method interaction, such that method effectiveness varied across sampling period. Sweep-netting was significantly less effective than the other methods. In the habitat comparison, dry ice was the most effective method in upland deciduous and coniferous habitats. CO_2 flagging was significantly less effective than CO_2 dragging and dragging in bottomland deciduous habitats. Collection method success did not differ significantly within grassland habitats. Overall, dry ice trapping and dragging were the most effective methods for tick collection across time and habitat.

Keywords: *Amblyomma americanum*, *Amblyomma maculatum*, *Dermacentor variabilis*, *Ixodes scapularis*, carbon dioxide, dragging, flagging, questing, trapping

Introduction

Ticks are significant pests and pathogen vectors affecting both humans and animals world-wide, and are the primary vectors of arthropod-borne disease in the U.S. (Parola & Raoult, 2001). Emerging pathogens and pathogen interactions complicate the status of tick-borne diseases in the southeast, as do the changing ranges of various tick species (Stromdahl & Hickling, 2012; Léger *et al.,* 2013). Factors contributing to this complication include the difficulty of diagnosing tick-borne disease, the potential for co-infection with multiple pathogens, the cross-reactivity of diagnostic tests, changing weather trends, and increased travel and movement of humans and animals (Mitchell *et al.,* 1996; Adelson *et al.,* 2004; Raoult 2004; Stromdahl & Hickling, 2012; Léger *et al.,* 2013).

Several tick species are commonly encountered in the southeast, and may be contributing to human disease cases. These include species such as the lone star tick, *Amblyomma americanum* (Linnaeus) (Ixodida: Ixodidae); the Gulf Coast tick, *A. maculatum* Koch (Ixodida: Ixodidae), whose range is currently expanding in areas of the southeast; the American dog tick, *Dermacentor variabilis* (Say) (Ixodida: Ixodidae); and the black-legged tick, *Ixodes scapularis* Say (Ixodida: Ixodidae) (Stromdahl & Hickling, 2012). These tick species are associated with various disease agents of concern, including anaplasmosis, borreliosis, ehrlichiosis, and rickettsiosis. The collection of questing ticks is one of the best representations of the risk of human encounter with ticks and tick-borne pathogens (Reye *et al.,* 2012). A number of methods are employed to collect questing ticks, but these methods may vary in the number of ticks collected, in tick species specificity or diversity, and other biases (Ginsberg & Ewing, 1989; Schulze *et al.,* 1997; Petry *et al.,* 2010).

Commonly used methods for collecting tick species include trapping with dry ice, dragging, and flagging. Sweep-netting is a common arthropod collection tool employed by entomologists. Dry ice trapping uses carbon dioxide (given off when the dry ice sublimes) to attract actively host-seeking ticks. Because this method is stationary, it is not as restricted by vegetation type and density when compared to methods such as sweep-netting (Kensinger & Allan, 2011); however, not all species or life stages of ticks are equally attracted (Holscher *et al.*, 1980, Ginsberg & Ewing, 1989, Schulze *et al.*, 1997; Cohnstaedt *et al.*, 2012). Additionally, variables such as wind direction and wind speed make it difficult to determine the actual area being sampled (Adeyeye & Butler, 1991; Cohnstaedt *et al.*, 2012). Dragging involves the movement of a piece of flannel or cotton cloth across vegetation behind an observer, to which ticks will attach as it passes. Dragging can be more easily quantified in terms of area or distance sampled than dry ice trapping, and is more representative of the risk of human encounter with host-seeking ticks (AFPMB, 1998); however, dragging can be more easily inhibited by vegetation than dry ice trapping, and the number of ticks collected with this method may vary by species (Ginsberg & Ewing, 1989). While dragging tends to sample upper vegetation layers, flagging, which involves the use of a smaller cloth than a drag, can be used to sample multiple vegetation levels (Ginsberg & Ewing, 1989; Cohnstaedt *et al.*, 2012). In some instances, sweep-netting can be comparable to other collection methods, such as in grassland habitat types; however, it can be impeded by dense vegetation such as blackberry and greenbrier (Semtner & Hair, 1975).

Tick questing behavior involves responses to multiple stimuli, such as movement, CO_2, light, and temperature (Gherman *et al.*, 2012), but many collection methods function by targeting only one of these responses, such as dragging (movement) or dry ice trapping (CO_2). Gherman *et*

al. (2012) attempted to increase the efficiency of tick collection by combining the stimulus of movement and CO_2 in a traditional flag reinforced with CO_2 dispersed throughout the body of the flag. This combination yielded significantly more *I. ricinus* ticks, but not more *D. marginatus*, than traditional flagging in woody-edge habitat in Romania.

Knowledge about the diversity of species collected with various methods can help improve sampling and surveillance procedures such as estimates of disease exposure risks, evaluation of pathogen prevalence, estimates of relative tick densities, comparison of habitat use, and monitoring of changing populations.

The purpose of this project was to examine the effectiveness of several conventional and novel methods for collection of questing ticks, and whether they vary by tick species, temporally, and by habitat type. This study was conducted in two parts, with a temporal comparison carried out in April-August 2013, and a habitat comparison in June 2014.

Materials and Methods

Study Site: This study was carried out at Ames Plantation Research and Education Center (AMES). Owned by the Hobart Ames Foundation, AMES is located in southwestern Tennessee and operates as a 7,446 hectare University of Tennessee Research and Educational Center devoted to forestry and wildlife ecological research, livestock development, forage and row crop research, and archeological research. Various occurrences of tick-borne disease cases have been reported at AMES, and previous studies have identified human and animal pathogens in *A. americanum*, *A. maculatum*, and *I. scapularis* collected from AMES (Hendricks, 2013; Mays *et al.*, 2014).

Tick Collection Methods: Six methods were selected for comparison: four conventional methods (dry ice, dragging, flagging, and sweep-netting) and two novel methods integrating a conventional method with carbon dioxide (CO_2 dragging and CO_2 flagging) (Gherman *et al.*, 2012; Niebuhr *et al.,* 2013). Dry ice traps consisted of a small cooler filled with dry ice (~3.5lbs) placed on a 1-m^2 white cloth. The dry ice traps operated overnight, and were collected the next morning to maximize the time the trap was active. Ticks on the cloth were removed with forceps, and placed into a vial of 80% ethanol. The tick drag was constructed of 1-m^2 squares of light-colored corduroy sewn onto a dowel rod 30-mm in diameter and 122-cm in length. A rope was attached to either end of the dowel rod, in order to drag it behind the sampler. The flag was a 60 × 80-cm rectangle constructed of the same corduroy material as the drag. The shaft of the flag was a 130-cm-long hollow PVC pipe, 20-mm in diameter. The sweep net was made from canvas, with a 38-cm-diameter net hoop, and a 61-cm-long handle (BioQuip, Rancho Dominguez, CA). The CO_2 drag was constructed as the conventional drag, but with 4.76-mm-diameter vinyl tubing running along the dowel rod, inside the drag. The end of the hose was attached to a 5-lb tank of compressed CO_2 carried in a backpack. The tubing was punctured every 10-cm with a 22-gauge needle to allow for the release of CO_2. The CO_2 flag was constructed based upon a design by Gherman *et al.,* (2012), similarly to the conventional flag. Thin vinyl tubing (1-cm inner diameter) was run throughout the body of the flag in a serpentine pattern and through the PVC shaft, and attached to a tank of CO_2. The tubing was punctured every 10-cm with a 22-gauge needle to allow for the release of CO_2 throughout the body of the flag. One side of the flag was left unsewn and secured with Velcro to allow access to the tubing (Fig. 2.1). All collections were stored in 80% ethanol and ticks were identified to life stage, sex, and species (Cooley & Kohls

1944; Keirans & Litwak 1989; Keirans & Durden 1998) in the Medical and Veterinary Entomology laboratory at the University of Tennessee.

Environmental Data Collection: At each site, temperature (°C) and relative humidity (%) was measured, using a Kestrel 3500 (Nielson Kellerman, Boothwyn, PA) held at knee-height near the dry-ice trap. Temperature and relative humidity were measured again when the dry ice traps were retrieved. Minimum and maximum temperature and precipitation data were collected daily for Ames Plantation by Ames Plantation personnel.

Temporal Study; *Is there variation over time in the effectiveness of collection methods?* Twenty sites were selected and classified as either grassland (n = 14) or woodland (n = 6) habitat types. Sampling was designed to ensure a diverse species collection (*A. americanum, A. maculatum,* and *D. variabilis*). Sites were selected by choosing 10 sites at which a minimum of three species were collected during previous sampling efforts (Hendricks, 2013), and then choosing for each of the 10 an additional site of complementary habitat type at which three species had not been collected. These sites were sampled monthly from April through August in 2013. Each site contained six 20 × 20-m plots, which were sampled in six 20-m segments (Fig. 2.2). All six methods were compared in this study. Ticks were collected from each method at the end of each 20m segment, and stored in 80% ethanol vials. Traps were randomly assigned to a plot at each site upon each sampling trip (Fig. 2.2). For statistical comparisons, total tick counts were analyzed, and each species was analyzed individually in SAS 9.4 (SAS Institute Inc., Cary, NC) using a PROC GLIMMIX procedure with a Poisson distribution. Tukey-Kramer adjustment for multiple comparisons was used for means separation. Because of the high number of *A. americanum* nymphs collected, *A. americanum* adults and nymphs were also analyzed separately. Adult *A. americanum* data, *A. maculatum* data, and *D. variabilis* data were rank

transformed for analyses. Total tick collection data, *A. americanum* nymph and total data were not transformed (raw counts were used).

Habitat study; *Does habitat type affect trapping methods?* For the habitat comparison, 76 sites were selected and classified as grassland (n = 19), coniferous (n = 14), bottomland deciduous (n = 14), or upland deciduous (n = 29) habitat types. Collections were carried out in June of 2014. Each site contained three 100-m parallel transects positioned 10-m apart (Fig. 2.3). For this study, four methods were selected from those used in the 2013 temporal comparison: dragging, dry ice trapping, CO_2 dragging, and CO_2 flagging. One method was randomly assigned to each transect, and the dry ice trap was placed in the center of the middle transect after passive sampling. Traps were checked and ticks collected every 20-m along the 100-m transects. The dry ice trap remained overnight, and ticks were collected from the cloth the following morning. For statistical comparisons, raw total tick collection data, total *A. americanum* data, and *A. americanum* nymph data were used. Adult *A. americanum* data, *A. maculatum* data, *D. variabilis* data, and *I. scapularis* data were rank transformed. Means were compared in SAS 9.4 using a PROC GLIMMIX procedure with a Poisson distribution and the Tukey-Kramer adjustment for multiple comparisons for means separation.

Results

Environmental Data: During the temporal study from April-August 2013, temperatures ranged from 18.3-35.1 ºC (Mean ± SEM 29.34 ± 0.33 ºC) at trap set up, and 18.9-33.8 ºC (26.1 ± 0.93 ºC) at trap collection. The relative humidity ranged from 36.7-97.1% (71.52 ± 1.23%) at trap set up, and 56.3-100% (78.47 ± 0.93%) at trap collection. In April, the mean temperature at trap set was 25.8 ± 0.77 ºC, and the mean relative humidity was 60.61 ± 3.11%. Total rainfall

was 19.41-cm. In May, the mean temperature was 29.97 ± 0.41 ºC, and the mean relative humidity was 69.55 ± 2.82%. Total rainfall was 17.53-cm. In June, the mean temperature was 30.27 ± 0.42 ºC, and the mean relative humidity was 73.67 ± 1.72%. Total rainfall was 5.87-cm. In July, the mean temperature was 31.22 ± 0.57 ºC, and the mean relative humidity was 78.44 ± 1.75%. Total rainfall was 13.18-cm. In August the mean temperature was 29.60 ± 0.74 ºC, and the mean relative humidity was 75.87 ± 2.29%. Total rainfall was 3.56-cm.

During the habitat comparison in June 2014, temperatures ranged from 22.2-36.2 ºC (29.39 ± 0.35 ºC) at trap set up, and from 21.4-32.4 ºC (25.1 ± 0.27 ºC) at trap collection. The relative humidity ranged from 48.6-100% (76.02 ± 1.18%) at trap set up, and from 71-100% (84.91 ± 0.61%) at trap collection. In grassland habitat, the mean temperature at trap set was 31.59 ± 0.73 ºC, and the mean relative humidity was 69.0 ± 2.49%. In upland deciduous habitat, the mean temperature was 28.41 ± 0.51 ºC, and the mean relative humidity was 80.05 ± 1.66%. In bottomland deciduous habitat, the mean temperature was 29.10 ± 0.71 ºC, and the mean relative humidity was 77.54 ± 3.0%. In coniferous habitat, the mean temperature was 28.71 ± 0.62 ºC, and the mean relative humidity was 75.69 ± 1.73%. June 2014 experienced greater precipitation than normal (29.82-cm). The mean rainfall in June at Ames Plantation for 2000-2014 was 11.43 ± 1.83-cm.

Temporal Study; *Is there variation over time in the effectiveness of collection methods?* A total of 2106 ticks were collected, consisting of three species: 1795 *A. americanum* (455 adults and 1340 nymphs), 237 *D. variabilis* (231 adults and 6 nymphs), and 74 *A. maculatum* (adults). Each method collected individuals of all three tick species. For overall tick collection using traditional tick trapping methods, the mean number ± SEM of ticks per site per sampling period collected by dry ice trapping was 4.80 ± 1.08; by dragging 5.47 ± 1.53; by

flagging 2.81 ± 0.62; and by sweep-netting 1.21 ± 0.36. Using the two novel methods, the mean number ± SEM of ticks per site per sampling period by CO_2 dragging was 3.82 ± 0.59, and by CO_2 flagging was 2.95 ± 0.41 (Table 2.1).

For overall tick collection, there was a significant sampling period effect (F = 58.99; df = 4, 462; $P < 0.0001$), such that significantly more ticks were collected in May (likely associated with high numbers of *A. americanum* collections) than any other month except April, and significantly fewer ticks were collected in July and August (likely associated with decreasing *A. americanum* collections) than any other months (Fig. 2.4a). Sweep-netting was significantly less effective than all the other methods (F = 5.75; df = 5, 114; $P < 0.0001$). There were no differences between the other methods (Fig. 2.4b). There was a significant trap by sampling period effect (F = 17.59; df = 20, 462; $P < 0.0001$) for overall tick collection, such that the differences between trapping methods varied across the sampling periods (Fig. 2.4c). In April, there were no significant differences in any of the trapping methods. In May, all methods except dragging were significantly more effective than sweep-netting. In June, only dragging differed significantly from sweep-netting, with dragging being significantly more effective. In July, only CO_2 dragging and CO_2 flagging were significantly more effective than sweep-netting. In August, dragging was significantly more effective than all methods except CO_2 dragging.

For collection of *A. americanum*, there was a significant sampling period effect (F = 54.64; df = 4, 462; $P < 0.0001$), with the number of ticks collected declining across the sampling periods. Significantly more *A. americanum* were collected in April and May, with June collections significantly lower than April, but not May, and July and August collections significantly lower than June. Trapping methods varied significantly (F = 3.64; df = 5, 114; P = 0.0043), with sweep-netting being less effective than all other methods. There was also a

significant trap by sampling period effect (F = 18.14; df = 20, 462; P < 0.0001), such that the difference between the trapping methods varied across the sampling periods (Fig. 2.4d). In April, tick numbers did not differ among trapping methods. In May, all methods except dragging were significantly more effective than sweep-netting. In both June and July, there were no significant differences between trapping methods. In August, dragging was significantly more effective than flagging, CO_2 flagging, and sweep-netting, though not significantly different from dry ice trapping and CO_2 dragging.

Dividing the *A. americanum* collections into adult and nymph life stages for analysis yielded similar results, as there was a significant sampling period effect for the collection of adults (F = 55.87; df = 4, 462; P < 0.0001) and nymphs (F = 31.91; df = 4, 462; P < 0.0001). Adult *A. americanum* collections in April did not differ from collections in May or June, though significantly more adults were collected in May than in June. Adult collections were lowest in July and August. Collections of nymphal *A. americanum* were significantly higher in April, May, and June, than in July and August. There was no significant trap by sampling period effect for adult *A. americanum* (F = 1.39; df = 20, 462; P = 0.1204), though the means (± SEM) are presented in Fig. 2.4e; however, there was a significant trap by sampling period effect (F = 17.97; df = 20, 462; P < 0.0001) for the collection of *A. americanum* nymphs (Fig. 2.4f). In April, there were no significant differences between any trapping methods. In May, all methods except dragging were more efficient than sweep-netting. Methods did not differ in June and July. In August, dragging was significantly more effective than sweep-netting, though not different from CO_2 dragging, dry ice trapping, flagging, or CO_2 flagging.

For collection of *D. variabilis*, there was a significant sampling period effect (F = 146.21; df = 4, 462; P < 0.0001), with significantly more *D. variabilis* collected in April and July,

followed by June. Collections in May and August were significantly lower than all other months. There was a significant trap effect (F = 5.65; df = 5, 114; P < 0.0001), with sweep-netting being less effective than all other methods except flagging. There was also a significant trap by sampling period effect (F = 38.56; df = 20, 462; P < 0.0001), such that the difference between the trapping methods varied across the sampling periods (Fig 2.4g). In April, dragging was significantly more effective than dry ice trapping and sweep-netting, though not different from CO_2 dragging or CO_2 flagging. In May, there were no significant differences between trapping methods. In June, both dragging and CO_2 dragging were significantly more effective than flagging and sweep-netting, though not different from dry ice trapping and CO_2 flagging; no *D. variabilis* were collected with the sweep net. In July, all other trapping methods were significantly more effective than sweep-netting; there were no *D. variabilis* collected with the sweep net. In August, there were no significant differences in trapping method.

There were not sufficient numbers of *A. maculatum* for a sampling period or trapping method comparison but the means (± SEM) are presented in Fig. 2.4h.

Habitat study; *Does habitat type affect trapping methods?* A total of 5040 ticks were collected, consisting of four species: 4893 *A. americanum* (727 adults and 4166 nymphs), 128 *D. variabilis* (adults), 12 *A. maculatum* (adults), and 7 *I. scapularis* (nymphs) (Table 2.2). A total of 271 ticks were collected from the 19 grassland sites (mean ± SEM of 14.26 ± 1.32 ticks per site), 2664 from the 29 upland deciduous sites (91.86 ± 19.9 ticks per site), 411 ticks from the 14 bottomland deciduous sites (29.36 ± 3.48 ticks per site), and 1694 from the 14 coniferous sites (121 ± 17.59 ticks per site). *Amblyomma americanum* and *D. variabilis* were collected with all methods and in all habitats, while *A. maculatum* was collected with all four methods but only in

grassland sites and *I. scapularis* was only collected with CO_2 dragging in deciduous upland and bottomland (Table 2.2).

There was a significant trapping method effect in upland deciduous habitat ($F = 9.65$; df = 3, 100; $P < 0.0001$), bottomland deciduous habitat ($F = 3.56$; df = 3, 40; $P = 0.023$), and coniferous habitat ($F = 11.53$; df = 3, 56; $P < 0.0001$). Trapping methods in grassland habitat did not differ ($F = 1.79$; df = 3, 44; $P = 0.163$). In both upland deciduous and coniferous habitat, dry ice trapping was significantly more effective than dragging, CO_2 dragging, and CO_2 flagging. In bottomland deciduous habitat, the mean number of ticks per site collected by dry ice, dragging, and CO_2 dragging did not differ; however, CO_2 flagging was significantly less effective than dragging and CO_2 dragging (Fig 2.5a).

There was a significant trapping method effect for collection of *A. americanum* in upland deciduous habitat ($F = 8.85$; df = 3, 100; $P < 0.0001$), bottomland deciduous habitat ($F = 3.92$; df = 3, 40; $P = 0.0153$), and coniferous habitat ($F = 10.31$; df = 3, 56; $P < 0.0001$). There was no significant trapping effect for collection of *A. americanum* in grassland habitat ($F = 1.63$; df = 3, 44; $P = 0.1956$). In both upland deciduous and coniferous habitat, dry ice trapping was significantly more effective than all other methods. In bottomland deciduous habitat, CO_2 flagging was significantly less effective than dragging and CO_2 dragging (Fig 2.5b). Dividing and analyzing the *A. americanum* collections by the different life stages yielded similar results. There was a significant trapping method effect for the collection of adults in upland deciduous habitat ($F = 13.55$; df = 3, 100; $P < 0.0001$) and coniferous habitat ($F = 15.33$; df = 3, 56; $P < 0.0001$). There was no significant trapping method effect in bottomland deciduous habitat ($F = 1.65$; df = 3, 56; $P = 0.194$) or in grassland habitat ($F = 0.69$; df = 3, 44; $P = 0.563$). In upland deciduous and coniferous habitat, dry ice trapping was significantly more effective than all other

methods (Fig 2.5c). There was a significant trapping method effect for the collection of nymphs in upland deciduous habitat (F = 5.43; df = 3, 100; P = 0.0017), bottomland deciduous habitat (F = 4.55; df = 3, 40; P = 0.0078), and coniferous habitat (F = 4.10; df = 3, 56; P = 0.0106). There was no significant trapping effect for the collection of nymphs in grassland habitat (F = 2.18; df = 3, 44; P = 0.1036) (Fig. 2.5d).

Most of the tick collection from this trapping period consisted of *A. americanum*, though smaller numbers of three other species were collected (Table 2.2). There was no significant trapping effect in any of the habitats for *D. variabilis*, [(grassland F = 0.32; df = 3, 40; P = 0.811) (upland deciduous F = 1.64; df = 3, 96; P = 0.184), (bottomland deciduous F = 0.15; df = 3, 48; P = 0.929), (coniferous: F = 0.42; df = 3, 56; P = 0.738)] (Fig. 2.5e). *Amblyomma maculatum* was collected with all trapping methods, but only in grassland habitat. Twelve *A. maculatum* were collected; this number was insufficient for a trapping method by habitat comparison (Fig. 2.5f). There was a total of seven *I. scapularis* nymphs collected. Six were collected in upland deciduous habitat, and one was collected in bottomland deciduous habitat; all were collected with the CO_2 drag. The number collected was insufficient for a trapping method by habitat comparison (Fig. 2.5g).

Discussion / Conclusions

These studies compared several trapping methods over time and in four different habitat types to determine if temporal and habitat variations affect trapping efficiency. In the temporal study, there was a significant difference between trapping methods for the collection of ixodid ticks. While all three tick species were collected with all six methods, sweep-netting was the least effective method for collecting ticks. In addition to the lower numbers of ticks collected,

sweep-netting also collected non-target arthropods, which made spotting and collecting ticks from the net more difficult. While sweep-netting is not commonly used in tick collections, it is a tool that many entomologists employ because it is relatively simple, inexpensive, and allows the sampling of a variety of arthropod species (Buffington & Redak, 1998; Yi *et al.*, 2012); however, the other methods used were more effective in targeting ticks while avoiding most other non-target arthropods. The habitat comparison study (2014) identified significant differences in collection method within various habitat types, and demonstrated the importance of selecting a collection method best suited to the habitat type. In upland deciduous and coniferous habitats dry ice trapping was the most effective collection method, while CO_2 flagging was overall less effective than the other methods. When considering only *A. maculatum*, CO_2 flagging seemed to be slightly more effective despite its lower tick numbers overall for both the temporal and the habitat comparison studies; however, insufficient numbers of *A. maculatum* were collected to detect a significant difference in methods.

When the novel methods (CO_2 dragging and CO_2 flagging) were compared with their conventional counterparts in the temporal study, they demonstrated comparable performance. There were no significant differences between each novel method and their conventional counterpart for any species, in any time period, or in any habitat. There was slight monthly variation in method effectiveness, with sweep-netting consistently being one of the least effective. In August, the drag collected significantly more ticks than all other methods, likely a result of encountering a high number of nymphs on one transect, which was not reflective of the decrease in the overall average number of ticks collected per method per site during this sampling period compared to earlier sampling periods. Dry ice was the best method for tick collection in both coniferous and upland deciduous habitats, and was one of the best methods for

collection in bottomland deciduous habitat. As in the 2013 study, the drag and CO_2 drag yielded comparable results. The mean number of ticks per site collected by the CO_2 flag was significantly less than the number of ticks collected by the other methods in bottomland deciduous habitat, and was also less, though not significantly less, than the number collected by both dragging and CO_2 dragging in coniferous and upland deciduous habitats. This suggests that though dry ice trapping is the most efficient method in most instances, in woodland habitat types dragging and CO_2 dragging will perform comparably to each other and both are appropriate methods for tick collection, while CO_2 flagging may be less effective. Any of the four methods appear to be appropriate for tick collection in grassland habitats. Overall, dry ice trapping seems to be the most efficient in overall tick collection across habitat types.

Though there was some slight monthly variation in the most effective collection methods for *A. americanum*, the novel methods were effective for this species as well, and were generally among the most effective methods for each period. Along with the novel methods, conventional dragging, flagging, and dry ice trapping were effective methods for collection. None of these methods differed significantly from each other throughout the sampling periods, with the exception of sweep-netting being significantly less effective than all methods except dragging in the May sampling period, and CO_2 dragging in the August sampling period. The variation in methods across sampling period seemed to be distributed between all methods, with no one method appearing to be more appropriate within each sampling period or across all periods. In both coniferous and upland deciduous habitat, dry ice trapping was significantly more effective than both CO_2 dragging and CO_2 flagging, though not significantly different from dragging. In bottomland deciduous habitat, CO_2 flagging was significantly less effective than all other

methods. Solberg *et al.*, (1992) found dry ice trapping to be more effective than dragging for the collection of *A. americanum* in forested habitats, as did Petry *et al.*, (2010).

There were no significant differences in methods for the collection of *A. maculatum*, though the CO_2 flag collected the highest total number (21), followed by dragging (total of 14), flagging (total of 14), CO_2 dragging (total of 12), dry ice (total of 7), and sweep-netting (total of 6). The inability to detect a difference in sampling method is likely due to the small total number of ticks of this species collected; this tick has only recently been found in Tennessee, and does not occur in such densities as *A. americanum* or *D. variabilis*. There were no detectable differences in trapping methods for the collection of this tick in grassland habitat types, where all of the *A. maculatum* were collected in the 2014 study. The CO_2 flag collected the highest total number (n = 5), followed by dragging and dry ice trapping, each of which collected three, while only one was collected with the CO_2 drag. *Amblyomma maculatum* seems to prefer open grassland habitat (Teel *et al.*, 1998; Goddard & Varela-Stokes 2009; Teel, 2010), which is supported by this data. All *A. maculatum* collected in the temporal and habitat studies were adults. Immature stages are more often collected from host animals rather than questing, although another novel method, involving sampling underbrush and animal burrows with a swab-like device, has been used to collect questing immature *A. maculatum* (Portugal & Goddard 2015).

For collection of *D. variabilis*, there was some slight monthly variation in collection methods. In contrast to *A. americanum* collections, dry ice trapping was typically not as effective as dragging and CO_2 dragging, though only significantly less effective than any other methods in April and June. This slight difference may result because *D. variabilis* does not quest as aggressively as *A. americanum* (Petry *et al.*, 2010). Flagging also seemed less effective than

other methods, though it was only significantly different from dragging and CO_2 dragging in June. Both dragging and CO_2 dragging were consistently effective methods, with no significant differences between the two for any collection period. There was no detectable difference in methods for collection of *D. variabilis* in the habitat comparison study. Petry *et al.*, (2010) found no significant difference in dry ice trapping and dragging for the collection of adult *D. variabilis* in either woodland or grassland habitat types in Missouri. Another study in the Midwestern U.S. collected *D. variabilis* with both dragging and dry ice trapping, with higher numbers of *D. variabilis* collected in woodland habitat than in grassland habitat (Rynkiewicz & Clay, 2014).

Ixodes scapularis was collected in both deciduous habitat types in the 2014 study, but was only collected using the CO_2 drag. Additionally, all seven *I. scapularis* collected were nymphs. While we could not make trap comparisons in our study, another study found no significant difference in traditional flagging and dragging for the collection of *I. scapularis* nymphs (Rulison *et al.*, 2013). Gherman *et al.*, (2012) collected a significantly higher number of *I. ricinus* ticks during spring collections in Romania using a CO_2 flag similar to that used in this study than with a conventional flag. While the addition of CO_2 to the drag method in this study may have contributed to the increased collection of *I. scapularis*, the small collection number prevents an accurate comparison. No *I. scapularis* were collected in the 2013 temporal study. Falco & Fish (1991) indicate that dry ice trapping was less effective for *I. scapularis* than for other species, including *A. americanum*, and attributed this to decreased mobility and less aggressive host-seeking behavior when compared with *A. americanum*. Ginsberg & Ewing (1989) found that dry ice trapping and flagging collected disproportionate numbers of *A. americanum* and *I. scapularis*, and Schulze *et al.*, (1997) also reported that dry ice trapping was more effective for collection of *A. americanum* than for *I. scapularis*. A study comparing dry ice

trapping and dragging however collected greater numbers of *I. scapularis* with the dry ice trap than with dragging in spite of the apparent decreased mobility of *I. scapularis* when compared with other species (Solberg *et al.*, 1992).

Though comparisons among trapping methods for *A. maculatum* and *I. scapularis* could not be made in this study, the unequal efficiency found between sampling methods for various life stages and habitat types for the collection of *A. americanum* and *I. scapularis* (Ginsberg & Ewing, 1989; Schulze *et al.*, 1997), and the apparently decreased responsiveness of *I. scapularis* to dry ice baited traps when compared to both *A. americanum* and *D. andersoni* (Ginsberg & Ewing, 1989; Falco & Fish, 1991) suggests that methods may vary in effectiveness depending upon targeted species. Careful selection of sampling methods with consideration to target species as well as habitat types that will be sampled is necessary when designing experiments. When multiple tick species are targeted, the integration of multiple methods may be necessary to ensure representative samples of all species present are collected (Rynkiewics & Clay, 2014), particularly in carrying out studies that involve measurements of species diversity and relative abundance. Alternatively, if time and resources are limited, the dry ice trap might be the best method for collecting all three species in all habitats. Failure to account for potential differences in species collected with a specific method could result in biased estimations of relative abundance when comparing multiple tick species (Schulze *et al.*, 1997).

Because the dry ice-baited traps were the most consistent across the habitat types sampled, this method may be the most appropriate when sampling areas which may undergo changes in vegetation, such as in areas subjected to periodic prescribed fires, or when comparing habitat types. Kensinger & Allan (2011) found no significant differences in the proportion of ticks recaptured on dry ice traps in a mark-recapture study carried out in grasslands and

deciduous forests, which further suggests that this method may be consistent in the proportions of ticks collected across habitat type. Although species may respond differently to the dry ice trap depending upon questing behavior (Ginsberg & Ewing, 1989; Falco & Fish 1991), it may still be a more efficient method for tick collection than other collection alternatives (Solberg *et al.*, 1992).

The use of effective trapping methods is critical for accurate estimations and comparisons of tick presence and abundance, as well as surveillance for pathogen presence and prevalence. In addition to the effectiveness of each method, practicality must also be considered. Though the novel CO_2-reinforced methods were in most instances comparable to their conventional counterparts, the downfalls of the methods may outweigh any potential benefit. The added weight and bulk of the CO_2-tank carried in a backpack caused increased difficulty when sampling in areas of dense vegetation (e.g., dense woodland undergrowth) or rough terrain (e.g., steep slope). There is also additional maintenance involved with the CO_2-reinforced methods in order to ensure that gas is flowing correctly through the tubing, and consideration must be given to protecting the exposed portion of the hoses which run from the tank to the collection material from getting snagged or punctured by vegetation. The CO_2-reinforced methods are also more expensive than the traditional counterparts.

Dry ice trapping was very effective for collecting ticks; however, the amount of dry ice necessary for large-scale trapping efforts can be difficult and expensive to obtain, and difficult to transport to collection sites. When available, this method is very efficient for tick collection. In most instances, the use of dry ice trapping reduces the amount of time spent at each site, which can reduce the amount of time that collectors are exposed to potentially infected ticks; however, in cases where a large number of ticks are collected on the cloth (some sites had several hundred

ticks collected on the dry ice trap), the time necessary to remove the ticks becomes comparable to the other sampling methods. A slight change in methodology, such as placing the cloth in a sealable bag, storing the bag and contents (cloth and ticks) in a freezer, and removing the ticks at another location may be necessary in situations where high tick densities occur, or in areas with high pathogen prevalence where human exposure is a health concern.

In almost all situations, dragging was among the most effective methods for tick collection. Dragging is simple, comparable with other studies, and less costly than dry ice trapping, and has a lower cost and requires less maintenance than CO_2 dragging. When dry ice is not available, dragging is a suitable replacement.

Knowledge of the most appropriate methods for collection based upon the targeted tick species, time of year, and targeted habitat is important in designing and carrying out protocols for tick and tick-borne pathogen surveillance and monitoring, as well as for estimates of relative tick densities and habitat use when use of the most accurate and representative method is critical. Because of the differences shown, consideration should be given to target tick species, as well as habitat type, when selecting a method for any of these purposes (Table 2.3).

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Appendices

Table 2.1. Temporal study 2013; Total number of ticks collected (mean number ± SEM of ticks per site) collected in each sampling period and each sampling method by species and life stage.

Sampling Difference	*A. americanum* adults	*A. americanum* nymphs	*A. americanum* total	*A. maculatum* adults	*D. variabilis* adults	*D. variabilis* nymphs	Total
Differences by Sampling Period							
April	176 (8.8 ± 0.36)	332 (16.6 ± 1.36)	508 (25.4 ± 1.67)	19 (0.95 ± 0.08)	55 (2.75 ± 0.12)	0	582 (29.1 ± 1.67)
May	164 (8.2 ± 0.26)	416 (20.8 ± 1.74)	580 (29 ± 2.02)	14 (0.7 ± 0.06)	31 (1.55 ± 0.11)	0	625 (31.25 ± 2.05)
June	98 (4.9 ± 0.21)	346 (17.3 ± 2.38)	444 (22.2 ± 2.58)	31 (1.55 ± 0.09)	50 (2.5 ± 0.13)	6 (0.3 ± 0.09)	531 (26.55 ± 2.60)
July	16 (0.8 ± 0.07)	82 (4.1 ± 0.50)	98 (4.9 ± 0.52)	9 (0.45 ± 0.05)	69 (3.45 ± 0.19)	0	176 (8.8 ± 0.65)
August	1 (0.05 ± 0.01)	164 (8.2 ± 2.06)	165 (8.25 ± 2.08)	1 (0.05 ± 0.01)	26 (1.3 ± 0.09)	0	192 (9.6 ± 2.10)
Totals	**455 (22.75 ± 0.23)**	**1340 (67 ± 1.74)**	**1795 (89.75 ± 1.93)**	**74 (3.7 ± 0.06)**	**231 (11.55 ± 0.13)**	**6 (0.3 ± 0.04)**	**2106 (105.3 ± 1.97)**
Differences by Sampling Method							
Drag	82 (0.82 ± 0.10)	389 (3.89 ± 1.46)	471 (4.71 ± 1.52)	14 (0.14 ± 0.03)	62 (0.62 ± 0.07)	0	547 (5.47 ± 1.53)
CO_2 Drag	70 (0.70 ± 0.08)	235 (2.35 ± 0.51)	305 (3.05 ± 0.58)	12 (0.12 ± 0.02)	65 (0.65 ± 0.09)	0	382 (3.82 ± 0.59)
Dry Ice	119 (1.19 ± 0.18)	318 (3.18 ± 0.83)	437 (4.37 ± 1.04)	7 (0.07 ± 0.02)	36 (0.36 ± 0.05)	0	480 (4.80 ± 1.08)
Flag	59 (0.59 ± 0.07)	190 (1.90 ± 0.57)	249 (2.49 ± 0.62)	14 (0.14 ± 0.03)	18 (0.18 ± 0.03)	0	281 (2.81 ± 0.62)
CO_2 Flag	86 (0.86 ± 0.09)	139 (1.39 ± 0.33)	225 (2.25 ± 0.39)	21 (0.21 ± 0.03)	43 (0.43 ± 0.07)	6 (0.06 ± 0.04)	295 (2.95 ± 0.41)
Sweep Net	39 (0.39 ± 0.06)	69 (0.69 ± 0.28)	108 (1.08 ± 0.36)	6 (0.06 ± 0.02)	7 (0.07 ± 0.02)	0	121 (1.21 ± 0.36)
Totals	**455 (4.55 ± 0.10)**	**1340 (13.4 ± 0.78)**	**1795 (17.95 ± 0.86)**	**74 (0.74 ± 0.03)**	**231 (2.31 ± 0.06)**	**6 (0.06 ± 0.02)**	**2106 (21.06 ± 0.88)**

Table 2.2. Habitat study 2014; Total number of ticks collected (mean number ± SEM of ticks per site) collected in each habitat and each sampling method by species and life stage.

Habitat	*A. americanum* adults	*A. americanum* nymphs	*A. americanum* total	*A. maculatum* adults	*D. variabilis* adults	*I. scapularis* nymphs	Total
Differences by Sampling Habitat							
Grassland	54 (2.84 ± 0.18)	190 (10 ± 1.07)	244 (12.84 ± 1.31)	12 (0.63 ± 0.1)	15 (0.79 ± 0.12)	0	271 (14.26 ± 1.32)
Coniferous	248 (17.71 ± 0.79)	1417 (101.21 ± 16.51)	1665 (118.93 ± 17.56)	0	29 (2.07 ± 0.22)	0	1694 (121 ± 17.59)
Upland Deciduous	350 (12.07 ± 0.55)	2248 (77.51 ± 19.35)	2598 (89.59 ± 19.88)	0	60 (2.07 ± 0.20)	6 (0.21 ± 0.07)	2664 (91.86 ± 19.90)
Bottomland Deciduous	75 (5.36 ± 0.31)	311 (22.21 ± 3.23)	386 (27.57 ± 3.47)	0	24 (1.71 ± 0.26)	1 (0.07 ± 0.04)	411 (29.36 ± 3.48)
Totals	**727 (9.57 ± 0.28)**	**4166 (54.82 ± 8.07)**	**4893 (64.38 ± 8.34)**	**12 (0.16 ± 0.03)**	**128 (1.68 ± 0.10)**	**7 (0.09 ± 0.03)**	**5040 (66.32 ± 8.34)**
Differences by Sampling Method							
Drag	102 (1.34 ± 0.16)	656 (8.63 ± 2.24)	758 (9.97 ± 2.46)	3 (0.04 ± 0.03)	26 (0.34 ± 0.08)	0	787 (10.36 ± 2.48)
CO_2 Drag	117 (1.54 ± 0.17)	673 (8.86 ± 2.21)	790 (10.39 ± 2.42)	1 (0.01 ± 0.01)	27 (0.36 ± 0.07)	7 (0.09 ± 0.06)	825 (10.86 ± 2.45)
Dry Ice	423 (5.57 ± 0.44)	2593 (34.12 ± 15.60)	3016 (39.68 ± 16.05)	3 (0.04 ± 0.02)	55 (0.72 ± 0.17)	0	3074 (40.45 ± 16.05)
CO_2 Flag	85 (1.12 ± 0.11)	244 (3.21 ± 0.98)	329 (4.33 ± 1.06)	5 (0.07 ± 0.03)	20 (0.26 ± 0.07)	0	354 (4.66 ± 1.06)
Totals	**727 (9.57 ± 0.28)**	**4166 (54.82 ± 8.07)**	**4893 64.38 ± 8.34)**	**12 (0.16 ± 0.03)**	**128 (1.68 ±0.10)**	**7 (0.09 ± 0.03)**	**5040 (66.32 ± 8.34)**

Table 2.3. Recommended trapping methods for tick collection by species, life stage, month and habitat based upon results of temporal and habitat comparisons.

Tick Species	Life Stage	Month	Habitat	Recommended Method
	Total	April, May	Upland deciduous, coniferous	Dry ice, drag
Amblyomma americanum	Adults	April, May	Upland deciduous, coniferous	Dry ice, drag
	Nymphs	April-June	Upland deciduous, coniferous	Dry ice
Dermacentor variabilis	Adults	April, July	Upland deciduous, coniferous	Dry ice, drag
Amblyomma maculatum	Adults	June	Grassland	*CO_2 flag**
Ixodes scapularis	Nymphs	-	Upland deciduous	*CO_2 drag**
Total ticks		**April - June**	**Upland deciduous, coniferous, grassland**	**Dry ice, drag**

*Trends suggest that these methods may be effective, though there were not enough individuals of these species collected to detect a significant difference in trapping methods.

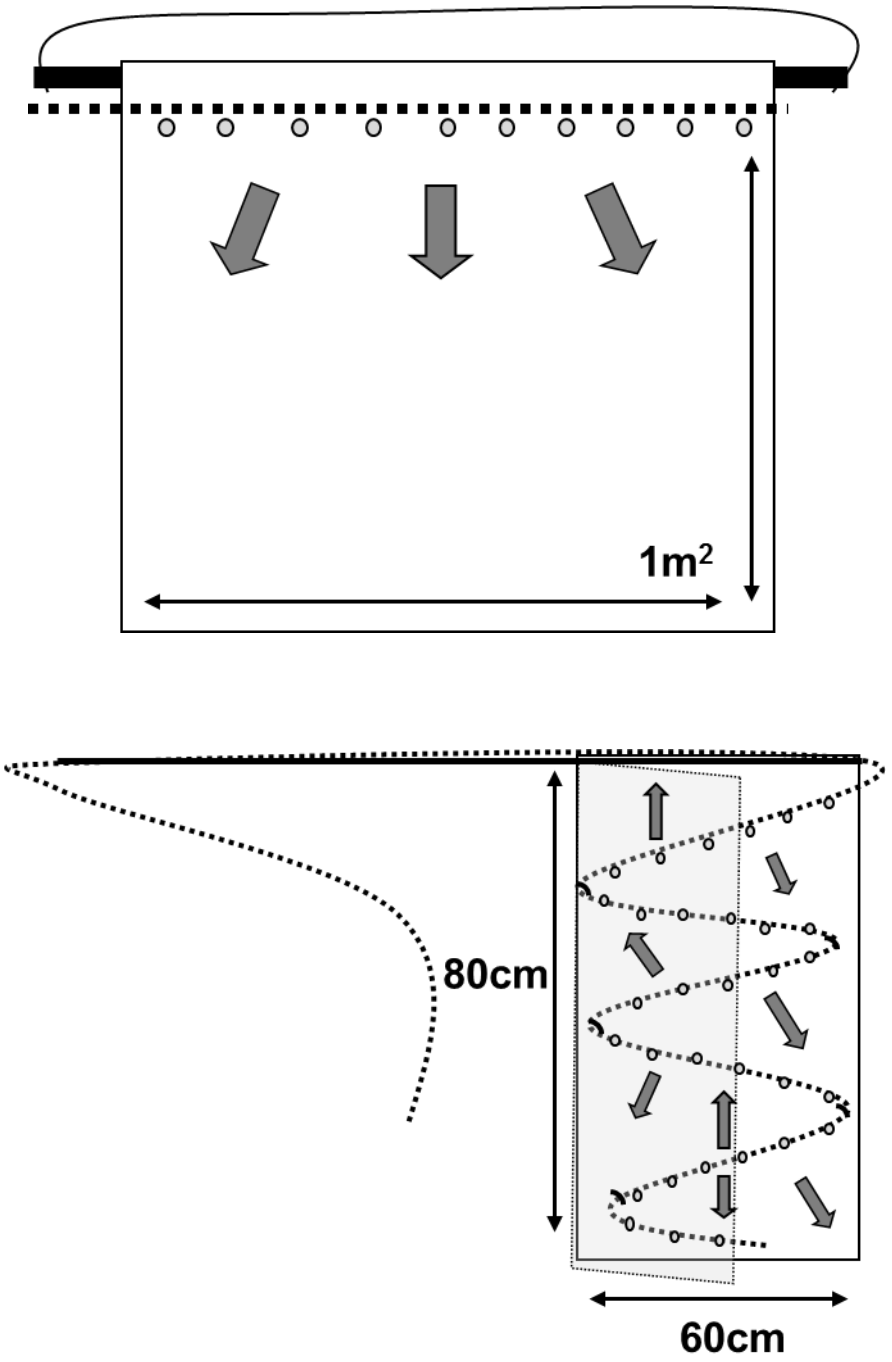
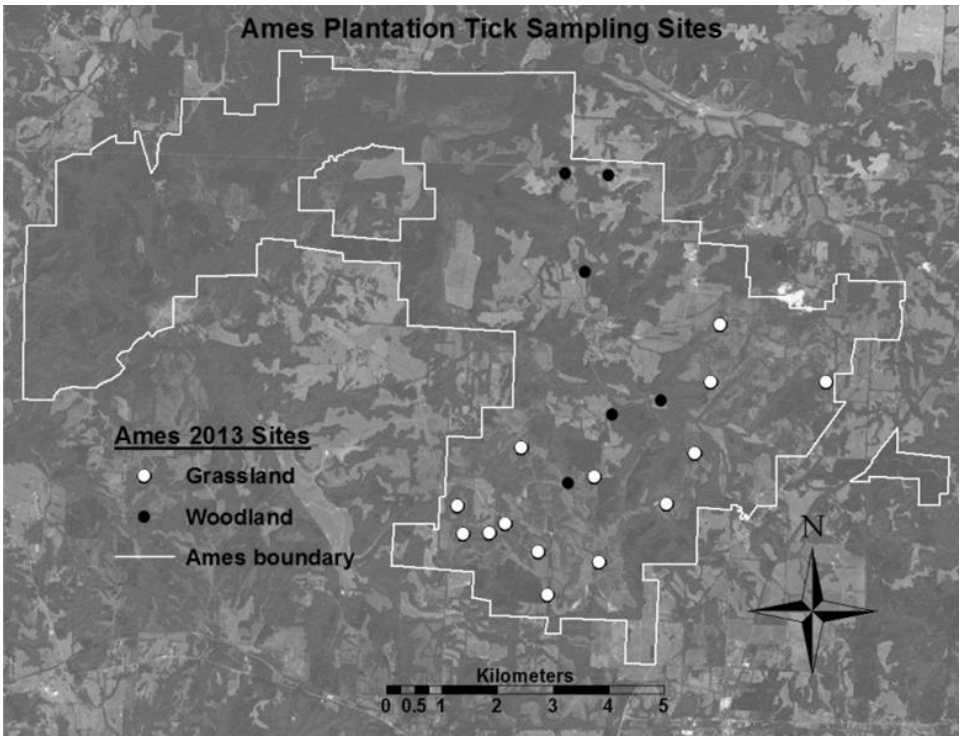


Figure 2.1: Diagrams of CO_2-reinforced drag (top) and CO_2-reinforced flag (bottom). Dotted lines represent hose, small dots represent holes for gas flow. Arrows represent release of CO_2. Diagrams not to scale.



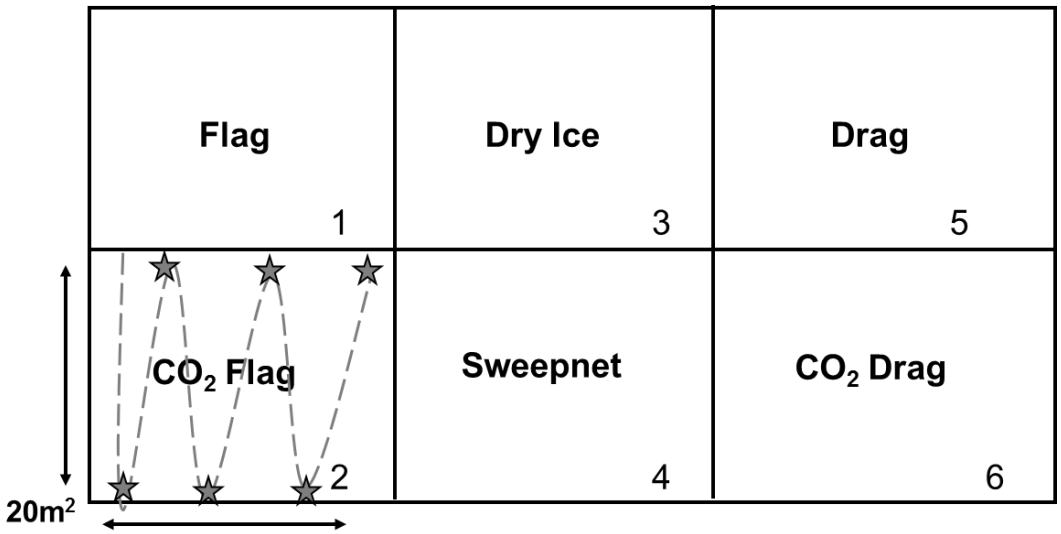


Figure 2.2: Map of sites at AMES (top) and plot design example (bottom) for 2013 temporal trapping methods comparison. Dotted line indicates transect within each plot. Stars indicate each 20-m segment.

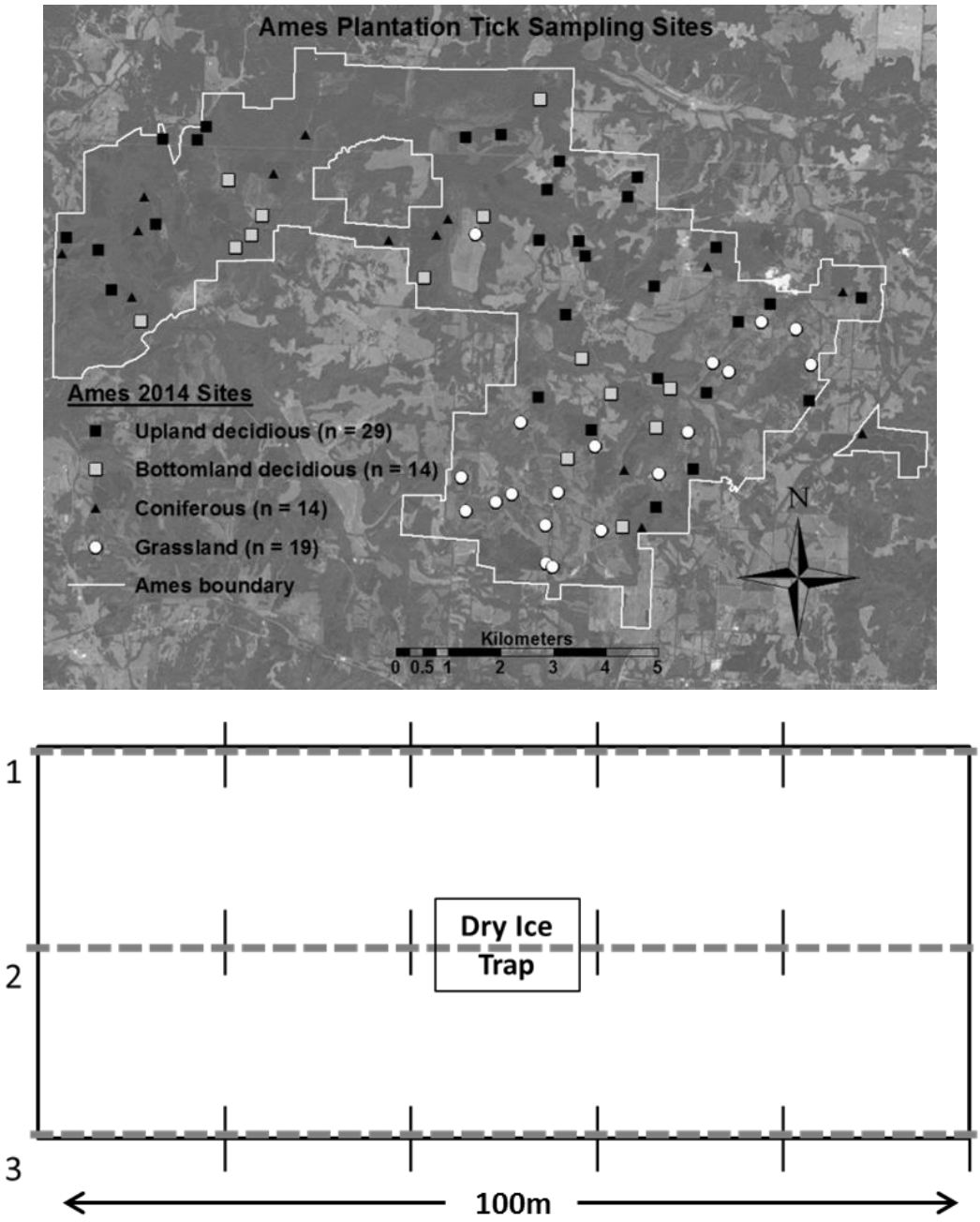
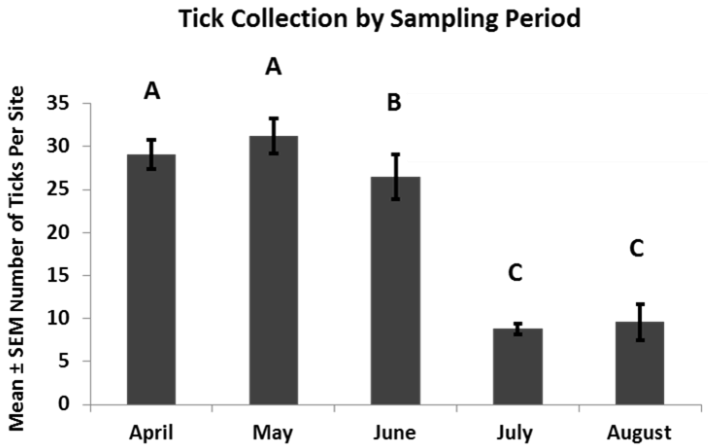


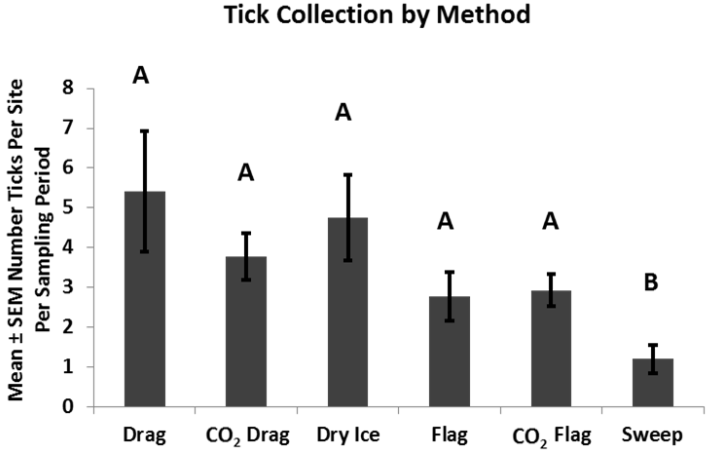
Figure 2.3: Map of sites at AMES (top) and plot design example (bottom) for 2014 trapping methods by habitat comparison. Dotted lines indicate parallel transects. Dashes indicate 20-m segments.

Figure 2.4: Means for 2013 temporal comparison of trapping methods for tick collection at Ames Plantation for tick collection by sampling period (a), by method (b), tick collection by method by month (c) *Amblyomma americanum* by method by month (d), *A. americanum* adults by method by month (e), *A. americanum* nymphs by method by month (f), *Dermacentor variabilis* by method by month (g), and *A. maculatum* by method (h). Letters indicate significant differences. For all method by month graphs, means were compared within month.

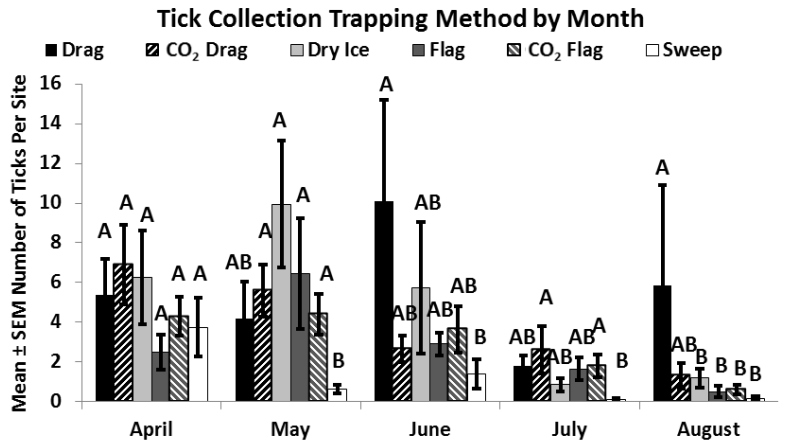
(a)



(b)



(c)



(d)

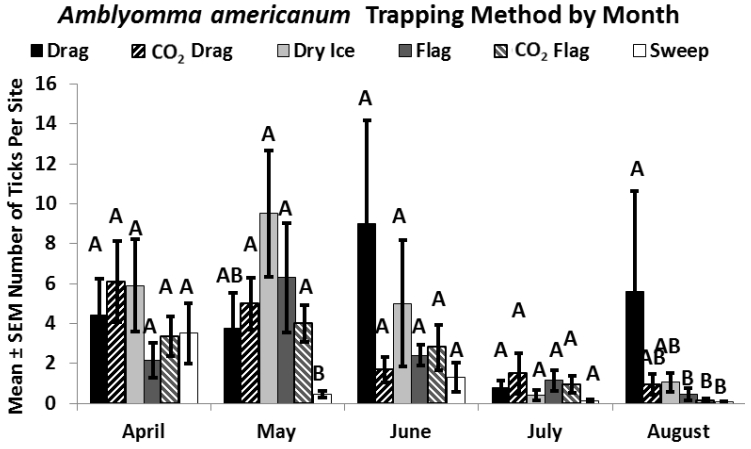
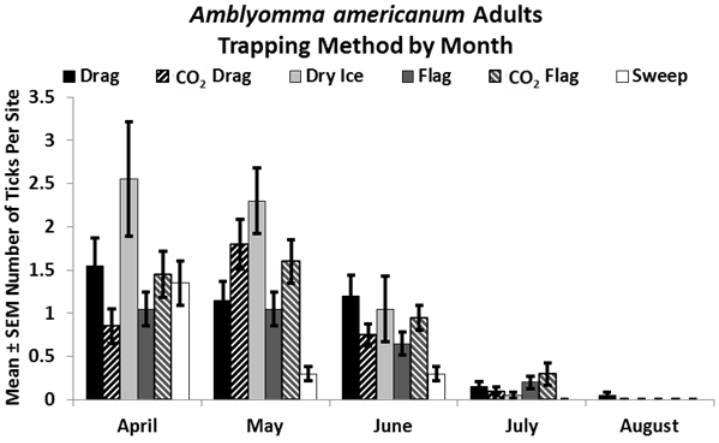
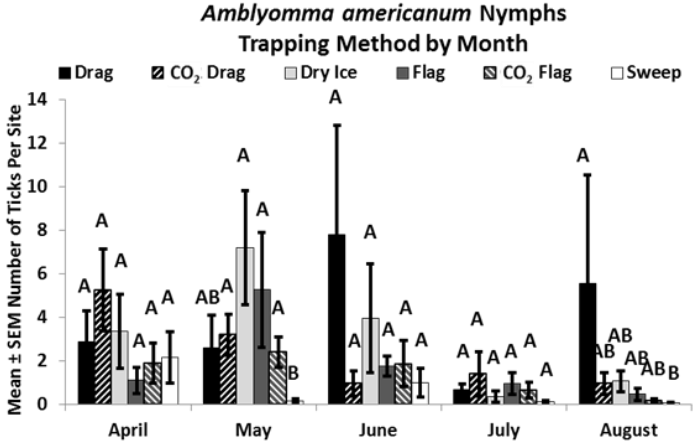


Figure 2.4. Continued

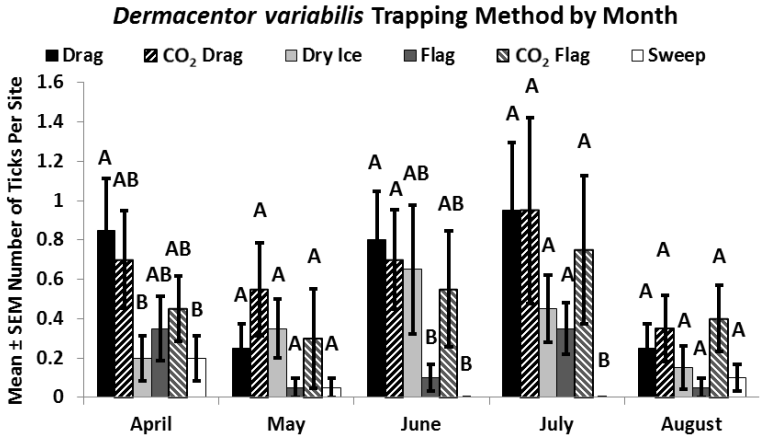
(e)



(f)



(g)



(h)

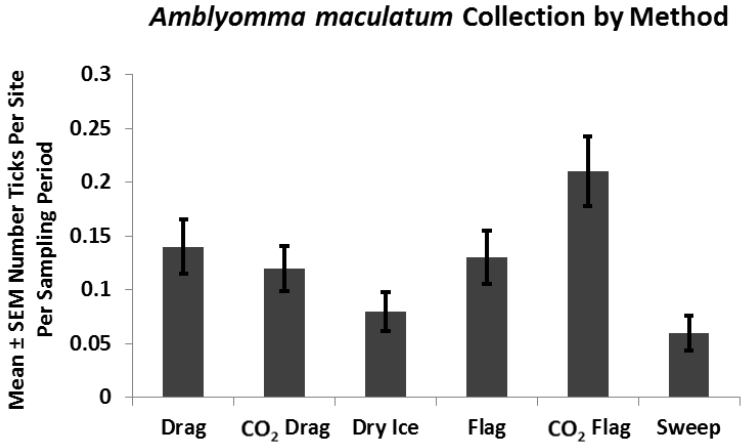
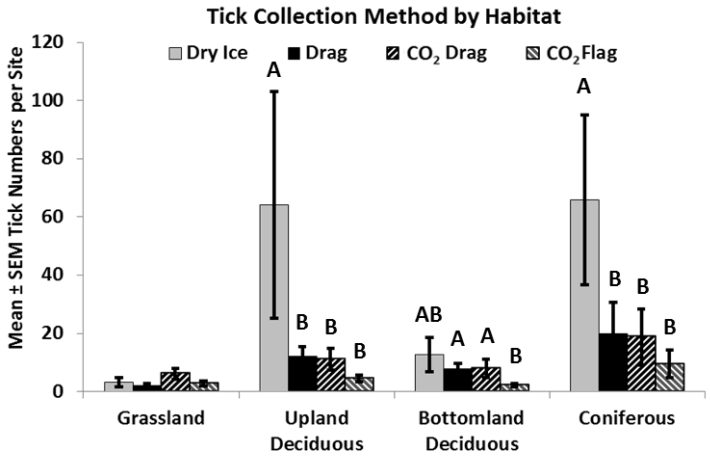


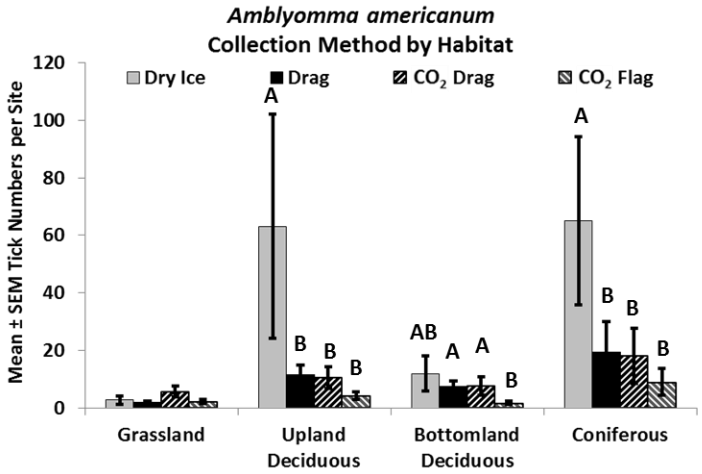
Figure 2.4. Continued

Figure 2.5: Means for 2014 habitat comparison of trapping methods for tick collection at Ames Plantation for total tick collection method by habitat (a), *Amblyomma americanum* total method by habitat (b), *A. americanum* adults method by habitat (c), *A. americanum* nymphs by method by habitat (d), *Dermacentor variabilis* method by habitat (e), *A. maculatum* method by habitat (f), and *Ixodes scapularis* method by habitat (g). Letters indicate significant differences. For all method by habitat graphs, means were compared within habitat

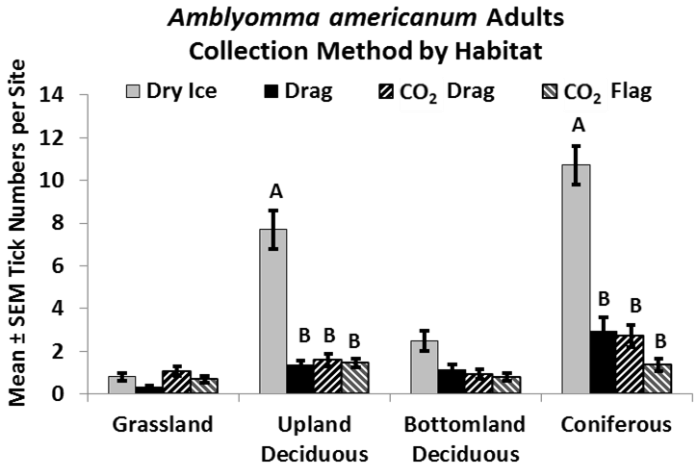
(a)



(b)



(c)



(d)

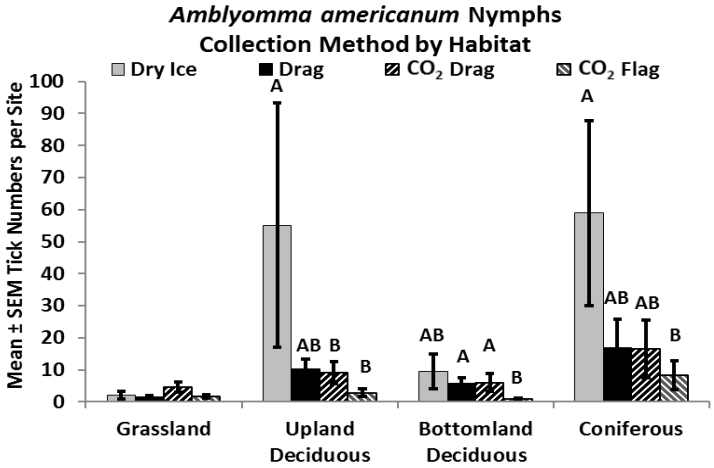
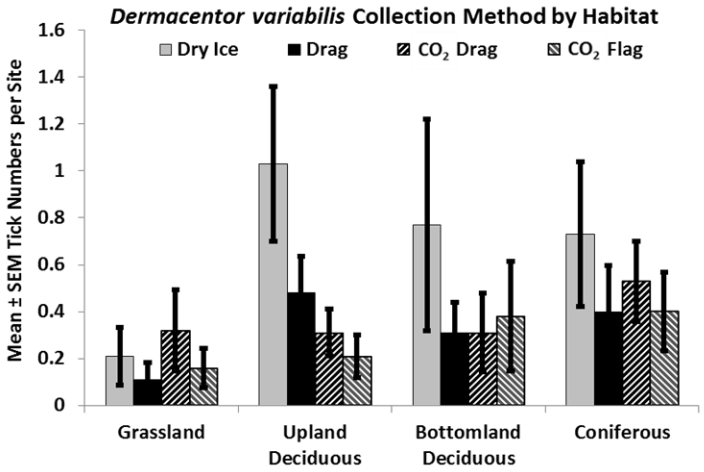
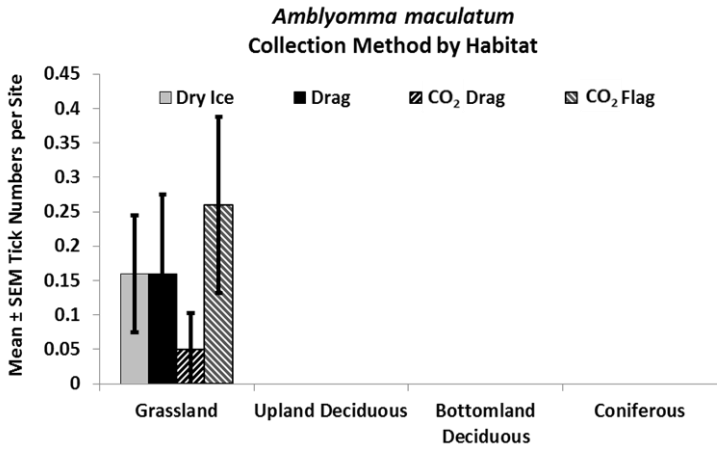


Figure 2.5. Continued

(e)



(f)



(g)

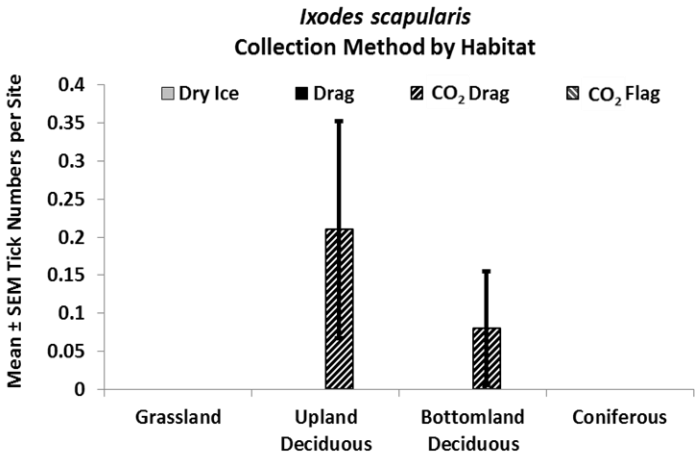


Figure 2.5. Continued

3. Specifying pathogen associations of *Amblyomma maculatum* in western Tennessee

Abstract

Amblyomma maculatum is established in western Tennessee, a region with increased risk for Rocky Mountain spotted fever and ehrlichiosis. This tick transmits *Rickettsia parkeri* to humans, contributing to cases of rickettsiosis. The objective was to determine pathogen associations within questing and host-collected *A. maculatum*, and identify ecological factors associated with pathogen infection that may increase the effectiveness of surveillance methods. Of 265 ticks tested, 60 (22.6%) were infected with *R. parkeri*, and 15 (5.7%) with *Candidatus* R. andeanae, a *Rickettsia* of unknown pathogenicity. Two deer-collected ticks tested positive for *Ehrlichia ewingii*. No ticks were positive for *Anaplasma* or *Borrelia* species. None of the ecological factors tested (collection month, collection source, sex, and habitat type) were associated with *R. parkeri* infection. This project developed baseline prevalence and incidence data for monitoring pathogen prevalence in *A. maculatum* populations, and identified an inexpensive method for distinguishing *R. parkeri* from *Ca.* R. andeanae.

Keywords: *Amblyomma maculatum*, *Borrelia*, *Ca.* Rickettsia andeanae, *Ehrlichia*, *Rickettsia parkeri*, Tennessee, tick-borne pathogens

Introduction

The range of *Amblyomma maculatum* Koch (Acari: Ixodidae), the Gulf Coast tick, is expanding both northward and east of the Appalachian mountains from its historical range within approximately 100 miles of the Gulf Coast (Bishopp & Trembly, 1945). Early expansion was preceded by the establishment of populations in Oklahoma and Kansas, likely transported there on livestock (Semtner & Hair, 1973). More recently, the range of *A. maculatum* has continued expanding, with collection of questing and host-feeding ticks in northern Mississippi and Kentucky, Arkansas, Tennessee, North Carolina, Delaware, and Maryland (Goddard and Norment, 1983; Trout *et al.,* 2010; Wright *et al.,* 2011; Varela-Stokes *et al.,* 2011; Jiang *et al.,* 2012; Florin *et al.,* 2014; Pagac *et al.,* 2014; Hendricks, 2013; Mays *et al., in preparation*). This species has a wide host range, feeding on livestock, avian and mammalian wildlife species, pets, and humans (Teel *et al.,* 2010).

Amblyomma maculatum is associated with several pathogens of concern. This species has long been associated with the bacteria *Rickettsia parkeri* (Parker *et al.,* 1939), which has been established as a human pathogen (Sumner *et al.,* 2007). *Rickettsia parkeri* is a spotted fever group, gram negative bacterium that causes a similar disease to Rocky Mountain spotted fever, known as American Boutonneuse fever; this illness is typically less severe than that caused by *R. rickettsii*, the causative agent of Rocky Mountain spotted fever (RMSF) (Paddock *et al.,* 2008). Although *R. parkeri* sometimes presents with a rash, it usually includes an eschar at the site of tick attachment (Paddock *et al.,* 2008). The initial symptoms of *R. parkeri* infection can be difficult to distinguish from RMSF on a case-by-case basis and diagnostic tests are cross-reactive; however, *R. parkeri* symptoms often include fever, myalgia, malaise, headache, and rash (Paddock 2008; Goddard *et al.,* 2009). To date, *R. parkeri* has been isolated from *A.*

maculatum in Oklahoma (Sumner *et al.*, 2007), Mississippi (Sumner *et al.*, 2007; Goddard *et al.*, 2009; Paddock *et al.*, 2010; Ferrari *et al.*, 2012), Arkansas (Trout *et al.*, 2010), Georgia (Sumner *et al.*, 2007), Florida (Sumner *et al.*, 2007; Paddock *et al.*, 2010), Kentucky and Tennessee (Sumner *et al.*, 2007; Pagac *et al.*, 2014), South Carolina (Sumner *et al.*, 2007), North Carolina (Varela-Stokes *et al.*, 2011), Virginia (Fornadel *et al.*, 2011; Wright *et al.*, 2011), Delaware, and Maryland (Florin *et al.*, 2014).

In addition to these pathogens, *Borrelia* species have been identified from *A. maculatum*, including low levels of infection with the pathogen *Borrelia burgdorferi*, as well as *Borrelia lonestari* and an unrecognized *Borrelia* species; however, *A. maculatum* is not known to transmit any of these *Borrelia* species (Trout Fryxell *et al.*, 2012; Lee *et al.*, 2014).

In Tennessee, cases of rickettsiosis have recently been on the rise (TNDOH 2015); however, the causative agent for Rocky Mountain spotted fever has not been identified in ticks in Tennessee (Moncayo *et al.*, 2010). *Amblyomma maculatum* may be involved in the transmission of pathogens, particularly *R. parkeri*, to humans, and may contribute to the rising number of rickettsiosis diagnoses in the state. The purpose of this study was to examine the pathogen associations of *A. maculatum* collected from AMES and southwestern Tennessee, to determine what threat they may pose to human health. This study also attempted to identify collection sources or ecological factors associated with pathogen infection to increase the efficiency of surveillance efforts. The identification of such factors, such as a habitat type effect on the proportion of ticks infected with a pathogen, would allow the development of monitoring and surveillance protocols that maximize the probability of detecting a pathogen if present, and improve the ability to determine pathogen presence/absence.

Materials and Methods

Study Site: This study was carried out at Ames Plantation (AMES), located in southwestern Tennessee. AMES, an 18,400-acre University of Tennessee Research and Education Center owned by the Hobart Ames Foundation, is a site for forestry, wildlife, ecological, and historical research, as well as crop, timber and livestock production. AMES also hosts the annual bird dog National Championship Field Trials, and a hunting club for deer and turkey. Information from the Tennessee Department of Health indicates that rising numbers of various tick-borne disease cases have been diagnosed in the area (TNDOH 2015). Additional human-collected ticks (n = 5) were submitted from a golf course and the University of Tennessee West Tennessee Research and Education Center in Jackson, TN.

Tick collections: Questing and host-feeding *A. maculatum* were collected from AMES during 2012-2014 with a variety of collection methods (Table 1). In 2012, questing *A. maculatum* were collected as part of an ongoing project (Hendricks 2013), and in 2013, 20 sites of both woody and grasslands habitat types were targeted for the collection of questing *A. maculatum* (Mays *et al., in prep.*). In the summer of 2014, 76 sites classified as one of four habitat types (native grasslands (n = 19), coniferous (n = 14), bottomland deciduous (n = 14), or upland deciduous (n = 29)) were sampled for questing *A. maculatum*. In the spring of 2013 and 2014, ~500 cattle were sampled for ticks (Pompo *et al., in prep.*), and in 2013 *A. maculatum* were collected from small mammals (five cotton rats and two white-footed mice) during an ectoparasite survey (Long *et al., in prep.*). Throughout the duration of the studies (2012-2014), ticks were collected opportunistically from field investigators and from personnel at Ames Plantation, and from individuals at a golf course and the University of Tennessee West Tennessee Research and Education Center in Jackson, TN. Ticks were stored in vials containing

80% ethanol, and all specimens were identified to life stage, sex, and species (Cooley & Kohls 1944; Keirans & Litwak 1989; Keirans & Durden 1998).

DNA Extraction and Pathogen Screening: Before DNA extraction, specimens were placed in sterile water overnight to precipitate ethanol from the specimens; each specimen was then bisected longitudinally with a sterile razor blade. Half of the tick was retained as a voucher specimen in a tube of 80% ethanol and stored at -20°C. Total genomic DNA was extracted from the remaining half of each tick with a Fermentas DNA extraction kit (Thermo Scientific, Pittsburg, PA), and stored in elution buffer at -20°C until PCR screening. Extracted DNA was screened for infection with pathogens using genus-specific primers in PCR reactions with positive and negative (no added template) controls. For initial screening for *Borrelia* and *Ehrlichia* spp., samples were pooled in groups of 5 or 10, and samples from positive pools were subsequently run individually.

Samples were screened for *Borrelia* spp. with real-time PCR targeting the *23S* gene, with B31-strain *B. burgdorferi* DNA as a positive control (Courtney *et al.*, 2004, Mays *et al.*, 2014). Real-time PCR reactions consisted of a 20 µL reaction containing 2 µL extracted DNA, 10 µL Taq Polymerase (Applied Biosystems, Grand Island, NY), 1 µL primer/probe mix, 0.4 µL ROX dye (Applied Biosystems, Grand Island, NY), and 6.6 µL of nuclease-free water.

Samples were screened for *Ehrlichia* and *Anaplasma* spp. using a conventional nested PCR with primers targeting the *groEL* gene (Takano *et al.*, 2009, Mays *et al.*, 2014), and Panola Mountain Ehrlichia amplified from a tick (100% homologous to GenBank HQ658904) as a positive control. Nested conventional PCR pooled reactions consisted of a 50 µL reaction containing 5 µL pooled DNA (2 µL of initial reaction for nested reaction), 25 µL of Maxima Hot Start Green PCR Master Mix (Thermo Scientific, Pittsburg, PA), 1µL each of forward and

reverse primers (Eurofins, Huntsville, AL), and 18 µL nuclease free water. If a pool was positive then individual sample reactions were conducted and consisted of a 25 µL reaction containing 2 µL extracted DNA, 12 µL Hot Start Master Mix, 0.5 µL each of the forward and reverse primers, and 10 µL nuclease free water.

Samples were screened for *Rickettsia* spp. using a conventional PCR with primers targeting the *ompA* gene (Eremeeva *et al.*, 1994, Mays *et al.*, 2014) with *Rickettsia* spp. extracted from *A. maculatum* used as a positive control (>99% homologous to GenBank KJ657736). Initially these samples were first screened in pools as described above for *Borrelia* and *Ehrlichia* spp., but due to the high number of positive pools, all subsequent samples were screened individually. Individual sample reactions consisted of a 25 µL reaction containing 2 µL extracted DNA, 12 µL Hot Start Master Mix, 0.5 µL each of the forward and reverse primers, and 10 µL nuclease free water. All conventional PCR products (*Ehrlichia* and *Rickettsia* reactions) were run on a 1.5% agarose gel stained with ethidium bromide to visualize positive results. DNA extractions, PCR amplification, and gel electrophoresis were carried out in different locations with specifically dedicated equipment and reagents to prevent contamination.

All positive samples were sequenced for species confirmation. Positive samples were cleaned with ExoSAP-IT (Affymatrix, Inc., Cleveland, OH) to remove excess primers and nucleotides smaller than 200bp, and sent to the University of Tennessee Molecular Biology Resource Facility for bi-directional sequencing. Sequence results were cleaned with Sequencher (Gene Codes Corporation, Ann Arbor, MI) and aligned with ClustalW in BioEdit (Ibis Biosciences, Carlsbad, CA). Resulting sequences were compared to sequences published in GenBank (Benson *et al.*, 2005) and used to generate phylogenetic trees (cladograms) for genetic comparison to determine species identity. Phylogenetic trees based on Bayesian analyses were

created using Bayesian Evolutionary Analysis Sampling Trees (BEAST) 1.7.5 and Fig Tree software (Drummond *et al.*, 2012) to display the associations between the amplified sequences and GenBank-published sequences.

Statistical Analysis: Contingency tables were used to compare the percentage of *Rickettsia*-infected and *R. parkeri*-infected ticks by ecological factors (collection month, collection method, sex, and habitat type) using an α- value of 0.05 to determine significance. Since multiple comparisons (n = 6) were conducted using the same dataset, P values ≤ 0.008 were considered significant after Bonferonni's correction.

Results

Pathogen Infection: A total of 265 *A. maculatum* were collected and screened for each pathogen. This number included 111 questing *A. maculatum* (110 adults, 1 nymph), 110 *A. maculatum* from cattle (all adults), 2 *A. maculatum* from white-tailed deer (adults), 7 *A. maculatum* from rodents (6 nymphs, 1 larva), and 35 *A. maculatum* from humans (adults) (Table 3.1). The samples from humans were opportunistically collected off of the investigators and collaborators listed in the acknowledgements. All 265 were negative for *Borrelia* species, 2 (0.75%) were positive for *Ehrlichia*, and 75 (27.9%) were positive for *Rickettsia* species. All questing *A. maculatum* were negative for *Anaplasma* and *Ehrlichia* species; the two *Ehrlichia*-positive ticks were collected from deer. Both of the resulting sequences were 100% homologous to *E. ewingii* amplified from a human patient (GenBank AF195273) (Fig. 3.1a).

Seventy-five (27.9%) *A. maculatum* were positive for *Rickettsia* species (Table 3.2); 60 were infected with *R. parkeri*, a human pathogen, ≥99% homologous to *R. parkeri* previously amplified from *A. maculatum* (GenBank KC003476) (Fig. 3.2). An additional 15 were 100%

homologous to *Ca.* R. andeanae previously isolated from *A. parvum* (GenBank KF179352) (Fig. 3.1b). Of the *R. parkeri*-infected ticks collected from cattle, 3 were engorged females. The *R. parkeri*-positive tick from a rodent (a cotton rat) was an engorged nymph. The one questing nymph that was collected was not infected with any of the bacterial genera for which the ticks were tested.

The percentage of *Rickettsia* infected ticks was not influenced by collection month for questing ticks (X^2 = 7.38; df = 4; P = 0.117), or by collection source (X^2 = 2.88; df = 4; P = 0.581). Neither collection month (X^2 = 9.51; df = 4; P = 0.05) nor collection source (X^2 = 1.06; df = 4; P = 0.901) had any influence on the percentage of *R. parkeri*-positive ticks collected (Table 2). Additionally, habitat and sex had no effect on the collection of *Rickettsia*-positive ticks (habitat: X^2 = 4.03; df = 3; P = 0.258; sex: X^2 = 0.66; df = 1; P = 0.4166) or *R. parkeri*-positive ticks (habitat: X^2 = 2.07; df = 3; P = 0.558; sex: X^2 = 0; df = 1; P = 1) (Table 3.3).

***Rickettsia* identification via gel electrophoresis:** The *R. parkeri* samples consistently showed bands on the gel of ~500bp, while *Ca.* R. andeanae had a larger band size of ~900bp, and were easily distinguishable from the *R. parkeri* samples on the gel (Fig. 3.3). All of these samples were confirmed with sequencing, but the location (size) of the bands on the gel consistently reflected the sequencing results. PCR primers and conditions, *Rickettsia* spp. genotype variants, and sequences are provided in the supplementary material (Tables 3.4 & 3.5).

Discussion / Conclusions

This project was designed for investigation of pathogen prevalence in questing and host-collected *A. maculatum*, including individuals collected from humans, to help determine what risk this tick may pose to human health. The results of this study establish presence of a human

pathogen (*R. parkeri*) associated with *A. maculatum*, and indicate that this tick is a vector of human health concern in western Tennessee. This study also demonstrates the need for continued monitoring and surveillance of *A. maculatum* to identify continued changes in its distribution and associated pathogens.

Although no ticks were infected with *Anaplasma* or *Borrelia* species, the two ticks collected from white-tailed deer were both positive for *Ehrlichia ewingii*. Because the infected ticks were engorged females collected from a natural reservoir of some *Ehrlichia* species (Yabsley *et al.*, 2002), it is likely that these ticks were infected by their host blood meal. In this situation, the infected ticks had fed upon their final host; subsequent feeding on a new host and transmission of infection would be unlikely. It is thought that *Ehrlichia* does not undergo transovarial transmission, though studies have only focused on *E. chaffeensis* (Long *et al.*, 2003; Stromdahl, 2008); therefore these specimens likely pose little threat to human health. Infection of human-collected *A. maculatum* with *E. chaffeensis* and field-collected *A. maculatum* with Panola Mountain *Ehrlichia* has been reported (Blount, 2007; Williamson *et al.*, 2010; Paddock and Goddard, 2015), but to our knowledge this is the first report of *E. ewingii* in *A. maculatum*. Further investigation is necessary to determine if this tick is a competent vector of this *Ehrlichia* species.

Rickettsia parkeri was prominent in these collections, followed by *Ca.* R. andeanae, a *Rickettsia* of currently undetermined pathogenicity (Ferrari *et al.*, 2012; Jiang *et al.*, 2012). The *Rickettsia* species identified in this study consistently demonstrated obvious differences when run on a gel, allowing them to be distinguished from one another based on band size; *R. parkeri* was consistently at ~500bp, and *Ca.* R. andeanae was consistently at ~900bp. This difference can be a valuable tool for future monitoring and surveillance of rickettsial infections in *A. maculatum*

as it reduces the need for sequencing to confirm species identity. Similarly, Eremeeva *et al.* (1994) found that the use of *RsaI* or *PstI* digestion following amplification with the primers used in this study facilitated differentiation between many species of *Rickettsia*. *Rickettsia parkeri* and *R. rickettsii* were included in their study, though *Ca.* R. andeanae was not. For the species identified in this study (*R. parkeri* and *Ca.* R. andeanae), the additional restriction fragment length polymorphism step was not necessary for species differentiation. *Rickettsia rickettsii* amplified with these primers is expected to have a fragment size of 532bp when run on a gel, and *R. amblyommii* a fragment size of 510bp (Regnery *et al.*, 1991). These species may be difficult to distinguish from *R. parkeri* using this primer set, without confirmatory sequencing.

Amblyomma maculatum is likely responsible for some human diagnosed cases of RMSF, and may be a contributing factor in the increasing number of human rickettsiosis in Tennessee. Because of the cross-reactivity of serological tests for RMSF, a number of other Rickettsiosis cases, such as *R. parkeri*, may be misdiagnosed as RMSF (Paddock *et al.*, 2004; Raoult, 2004; Cohen *et al.*, 2009; Jiang *et al.*, 2012). Of the 265 ticks collected, 60 (22.6%) were infected with *R. parkeri*, the human pathogen. A total of 27.9% of the questing ticks collected were infected with *Rickettsia* species, of which more than 80% was *R. parkeri*. Of the ticks collected from humans, a total of 37.1% were infected with *Rickettsia* species, of which more than 61% was *R. parkeri* (Table 3.2). The pathogenicity of *Ca.* R. andeanae is currently unknown (Ferrari *et al.*, 2012; Jiang *et al.*, 2012). It is important to note that the pathogenicity of *R. parkeri* was not established until more than 60 years after it was first described in *A. maculatum*.

These rates of infection with *R. parkeri* pose a risk to humans who come into contact with these ticks, and indicate that these ticks may be contributing to human rickettsiosis cases. Awareness and prompt removal is important for individuals who may be exposed to these ticks

to decrease the risk of pathogen transmission. The close association between *A. maculatum* and *R. parkeri* may be the cause of the relatively high prevalence of infection compared to other tick species and associated pathogens such as *D. variabilis* and *R. rickettsii* (Sumner *et al.*, 2007). Although many areas of the southeast report a high prevalence of *R. parkeri* in *A. maculatum*, Paddock *et al.* (2015) found 73% of specimens collected in Oklahoma and 47% of specimens collected in Kansas from 2011-2014 were infected with *Ca.* R. andeanae, while *R. parkeri* was absent. This may indicate that infection with one species may prevent infection with the other species, and a high prevalence of *Ca.* R. andeanae in a population may exclude *R. parkeri*; but further research is necessary to confirm this speculation. If true, this could result in reduced transmission of *R. parkeri* in regions where *Ca.* R. andeanae is prevalent; however, the pathogenicity of this species and its potential as a disease agent in humans and animals is uncertain. Paddock *et al.* (2015) also hypothesize that the high prevalence of infection with *Ca.* R. andeanae may be indicative of patterns of establishment of *A. maculatum* populations, and that the populations in Kansas and Oklahoma may represent remnants of larger historic populations, or result from host distributions.

The rates of infection with *R. parkeri* found here are similar to or slightly higher than those observed in questing ticks collected in other southern and eastern states: 11-15.2% in Mississippi (Sumner *et al.*, 2007; Ferrari *et al.*, 2012), 11-22% in Florida (Sumner *et al.*, 2007; Paddock *et al.*, 2010), 29% in North Carolina (Varela-Stokes *et al.*, 2011), and 14.7% in Ft. Campbell (Kentucky and northern Tennessee) (Pagac *et al.*, 2014), though rates as high as ~40% have been observed in both Mississippi and Virginia (Goddard *et al.*, 2009; Paddock *et al.*, 2010; Fornadel *et al.*, 2011; Wright *et al.*, 2011). In this study, 22.6% of all *A. maculatum* collected were infected with *R. parkeri* (60/265).

Of almost 1,000 ticks collected from humans over the study period (2012-2014), a total of 35 *A. maculatum* were collected, along with 796 *A. americanum*, 168 *Dermacentor variabilis*, and 1 *Ixodes scapularis* (unpublished). Of the 35 *A. maculatum* collected, 8 (22.9%) were infected with *Rickettsia parkeri*. A total of 0.8% of the total collection of human ticks was infected with *R. parkeri*. All five of the *A. maculatum* collected from humans in Jackson, TN, were negative for any *Rickettsia* species. Although *A. maculatum* made up a small percentage of the total collection of ticks from humans (3.5%), a high percentage of human-collected *A. maculatum* were infected with *R. parkeri*. Additionally, human-collected *A. maculatum* accounted for 13% of the total number of *A. maculatum* collected in this study. This high prevalence of infection in human-collected *A. maculatum* is a cause for concern, as an increasing number of *A. maculatum* in an area may increase the risk of human contact with a pathogen-infected tick.

Continued research should be focused on identifying pathogens associated with *A. maculatum*, particularly in areas of recent establishment. It is important to continue to monitor the expansion of *A. maculatum*, especially with regard to its potential to contribute to the diagnosis of cases of spotted fever illnesses. Public health education efforts should be made to increase awareness of this tick species, especially in areas where it has not historically been encountered. Because of the relatively high rates of pathogen infection in this tick in many areas, prompt removal from an individual will help to decrease the likelihood of pathogen transmission. As many tick-borne disease symptoms are non-specific and difficult to distinguish (Chapman, 2006), accurate identification of the tick will be important in the event that an individual develops disease symptoms. Continued monitoring and surveillance, as well as increased

awareness will help to reduce the impacts of this tick and its associated pathogens on human health.

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Appendices

Table 3.1: *Amblyomma maculatum* from western Tennessee collection sources by year and total numbers screened for pathogens, and collection numbers by life stage (male, ♂; female, ♀; nymph, N; and larva, L) and number of engorged female and immature ticks collected from hosts

	Collection Year					
Source	**2012**	**2013**	**2014**	**Total**	**Life Stage**	**# Engorged**
Questing	20	74	17	111	61♂, 49♀, 1N	-
Cattle	-	83	27	110	91♂, 19♀	9♀
Deer	-	2	0	2	2♀	2♀
Rodents	-	7	0	7	6N, 1L	2N
Human	0	30	5	35	17♂, 18♀	0
Total	**20**	**196**	**49**	**265**	**169♂, 88♀, 7N, 1L**	**11♀, 2N**

Table 3.2: Number (percent) *Rickettsia* species infection in *Amblyomma maculatum* from western Tennessee by collection year and collection source.

Source	*Rickettsia* spp.	Collection Year (No. positive / No. collected)			Total
		2012	2013	2014	
Questing	*R. parkeri*	4/20 (20%)	17/74 (23%)	4/17 (23.5%)	25/111 (22.5%)
	Ca. R. andeanae	1/20 (5%)	3/74 (4%)	2/17 (11.8%)	7/111 (6.3%)
	Total	5/20 (25%)	20/74 (27%)	6/17 (35.3%)	31/111 (27.9%)
Cattle	*R. parkeri*	-	18/83 (21.7%)	8/27 (29.6%)	26/110 (23.6%)
	Ca. R. andeanae	-	2/83 (2.4%)	2/27 (7.4%)	4/110 (3.6%)
	Total	-	20/83 (24.1%)	10/27 (37%)	30/110 (27.3%)
White-tailed deer	*R. parkeri*	-	0/2 (0%)	-	0/2 (0%)
	Ca. R. andeanae	-	0/2 (0%)	-	0/2 (0%)
	Total	-	0/2 (0%)	-	0/2 (0%)
Rodents	*R. parkeri*	-	1/7 (14.3%)	-	1/7 (14.3%)
	Ca. R. andeanae	-	0/7 (0%)	-	0/7 (0%)
	Total	-	1/7 (14.3%)	-	1/7 (14.3%)
Human	*R. parkeri*	-	7/30 (23.3%)	1/5 (20%)	8/35 (22.9%)
	Ca. R. andeanae	-	4/30 (13.3%)	1/5 (20%)	5/35 (14.3%)
	Total	-	11/30 (36.7%)	2/5 (40%)	13/35 (37.1%)
Total	***R. parkeri***	**4/20 (20%)**	**43/196 (21.9%)**	**13/49 (26.5%)**	**60/265 (22.6%)**
	***Ca.* R. andeanae**	**1/20 (5%)**	**9/196 (4.6%)**	**5/49 (10.2%)**	**15/265 (5.7%)**
	Total	**5/20 (25%)**	**52/196 (26.5%)**	**18/49 (36.7%)**	**75/265 (28.3%)**

Table 3.3: Habitat types for 2012 and 2014 collections of *Amblyomma maculatum* infected with *Rickettsia* species infection and total number of *A. maculatum* collected.

Habitat Classification (# 2012 / 2014 sites)	***R. parkeri* Pos. (%)**	***Ca.* R. andeanae Pos. (%)**	**Total *Rickettsia* spp. Pos. (%)**	**Neg.**	**Total # Ticks**
Grasslands (21 / 18)	7 (22.6%)	2 (29%)	9 (29%)	22 (70.9%)	**31**
Upland Deciduous (43 / 28)	0 (0%)	0 (0%)	0 (0%)	3 (100%)	**3**
Bottomland Deciduous (17 / 15)	1 (50%)	0 (0%)	1 (50%)	1 (50%)	**2**
Coniferous (19 / 15)	0 (0%)	1 (100%)	1 (100%)	0 (0%)	**1**
Total (100 / 76)	**8 (21.6 %)**	**3 (8.1 %)**	**11 (29.7 %)**	**26 (70.3 %)**	**37**

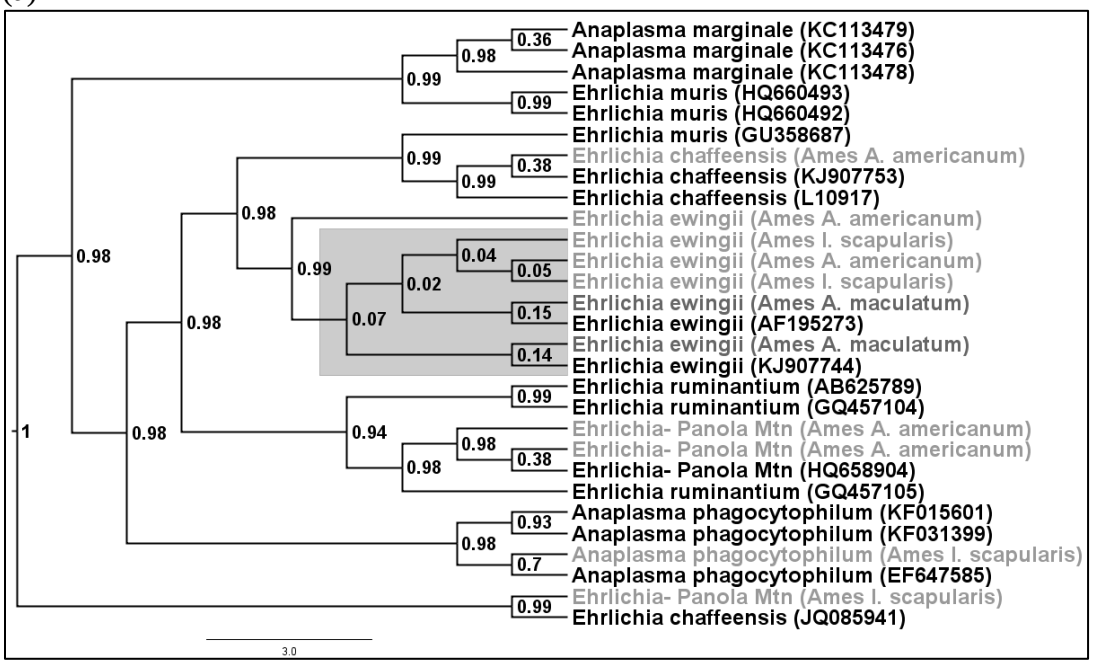
Table 3.4: PCR Primers and Conditions

Pathogen	PCR	Reference	Primers	Thermal cycling conditions	Expected amplicon size (bp)
Anaplasma & *Ehrlichia* species	Conventional	Takano *et al.*, 2009	GroEL Primary For- 5' GAA GAT GCW GTW GGW TGT ACK GC GroEL Primary Rev- 5' AGM GCT TCW CCT TCW ACR TCY TC GroEL Nested For- 5' ATT ACT CAG AGT GCT TCT CAR TG GroEL Nested Rev- 5' TGC ATA CCR TCA GTY TTT TCA AC	Initial Denaturation- 15 min, 95° C 40 cycles for primary, 35 for nested: Denaturation- 30 sec, 95° C Annealing- 30 sec, 58° C Elongation- 30 sec, 72° C Final elongation- 3 min, 72° C	365
Borrelia species	Quantitative	Courtney *et al.*, 2004	For- 5' CGA GTC TTA AAA GGG CGA TTT AGT Rev- 5' GCT TCA GCC TGG CCA TAA ATA G	Initial Step- 2 min, 50° C Initial Denaturation- 10 min, 95° C 40 cycles: Denaturation- 15 sec, 95° C Annealing/Elongation- 1 min, 60° C	75
Rickettsia species	Conventional	Eremeeva *et al.*, 1994	OmpA For- 5' ATG GCG AAT ATT TCT CAA AAA OmpA Rev- 5' AGT GCA GCA TTC GCT CCC CCT	Initial Denaturation- 15 min, 95° C 35 cycles: Denaturation- 20 sec, 95° C Annealing- 30 sec, 48° C Elongation- 2 min, 60° C	532

Table 3.5: *Ehrlichia* and *Rickettsia* species and genotype variants with the number of sequences of each variant amplified from *Amblyomma maculatum* and GenBank homologues with accession numbers.

Species (Variant)	**Number of Sequences**	**Collection Source (# sequences)**	**% homologous (GenBank Accession #)**
		GroEL amplicons for *Ehrlichia* & *Anaplasma*	
Ehrlichia ewingii (Variant 1)	2	white-tailed deer (2)	100% *Ehrlichia* ewingii (AF195273)
		OmpA amplicons for *Rickettsia*	
Rickettsia parkeri (Variant 1)	59	questing (24), cattle (26), humans (8), rodent (1)	100% *Rickettsia parkeri* (KC003476)
Rickettsia parkeri (Variant 2)	1	questing (1)	99% *Rickettsia parkeri* (KC003476)
Ca. Rickettsia andeanae (Variant 1)	8	questing (4), cattle (2), humans (2)	100% *Ca.* Rickettsia andeanae (KF179352)
Ca. Rickettsia andeanae (Variant 2)	4	cattle (1), humans (3)	100% *Ca.* Rickettsia andeanae (KF179352)
Ca. Rickettsia andeanae (Variant 3)	1	questing (1)	99% *Ca.* Rickettsia andeanae (KF179352)
Ca. Rickettsia andeanae (Variant 4)	1	cattle (1)	100% *Ca.* Rickettsia andeanae (KF179352)
Ca. Rickettsia andeanae (Variant 5)	1	questing (1)	99% *Ca.* Rickettsia andeanae (KF179352)

(a)



(b)

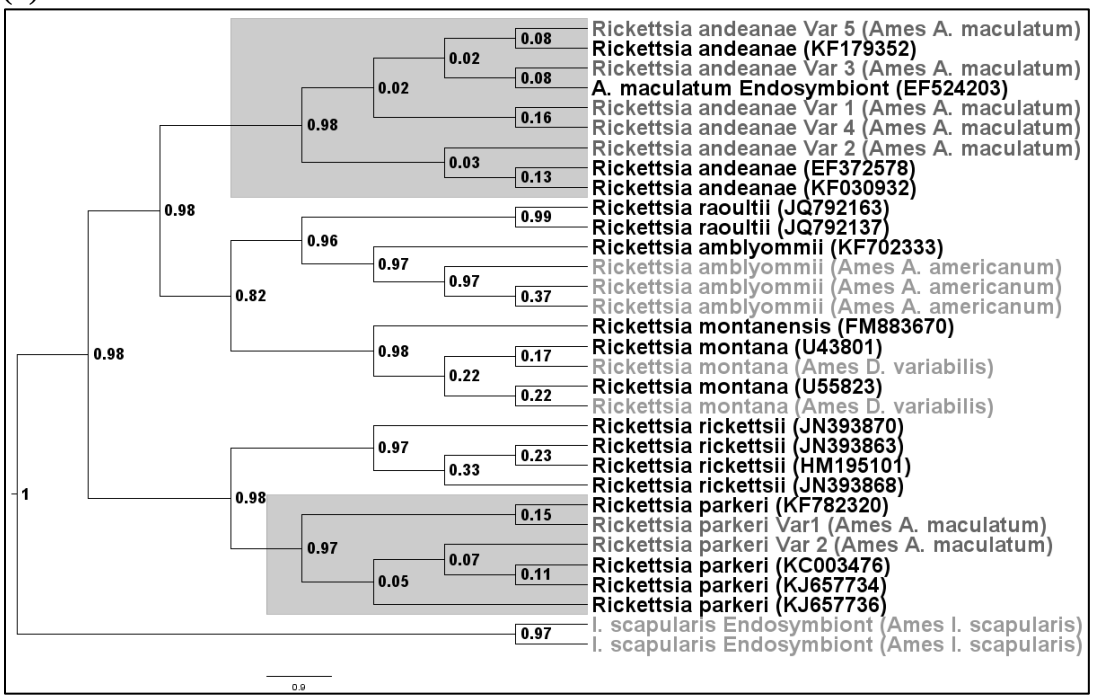


Figure 3.1. Phylogenetic tree of *Ehrlichia species* (a) and *Rickettsia* species (b) with *Amblyomma maculatum* sequences in gray, compared to GenBank sequences (black) and additional sequences isolated from ticks at Ames Plantation (light gray). *Ehrlichia ewingii* grouping and *R. parkeri* grouping of *A. maculatum* samples highlighted in gray.

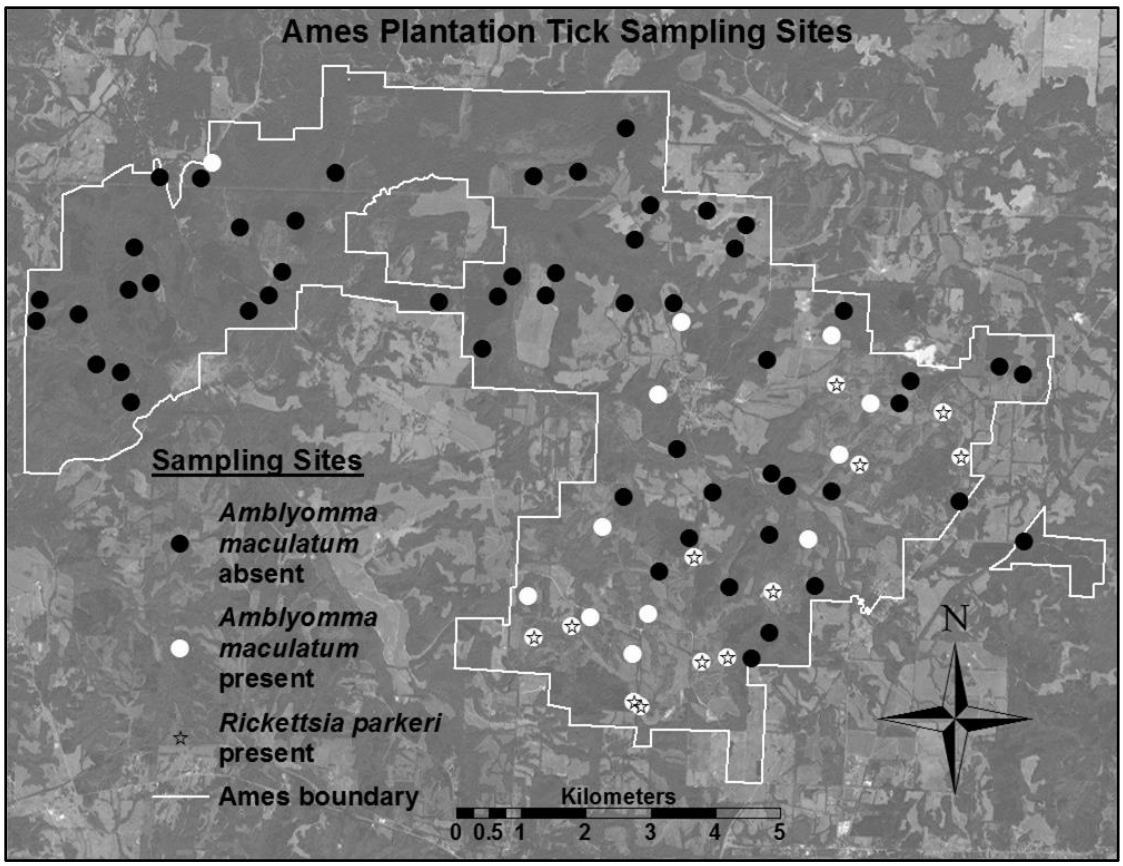


Figure 3.2. Map of Ames Plantation showing collection sites of questing *Amblyomma maculatum*, and collection sites of *Rickettsia parkeri*-infected questing *A. maculatum*.

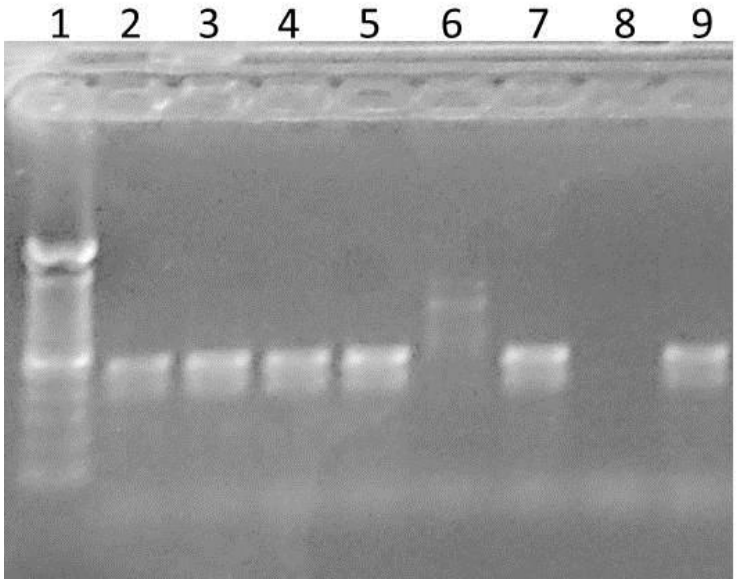


Figure 3.3: Gel demonstrating differences in fragment size between *Rickettsia parkeri* (~500bp) and *Ca.* R. andeanae (~900bp). Lane 1 is the DNA ladder, lane 2-5 and lane 7 are *R. parkeri*-infected samples. Lane 6 is a *Ca.* R. andeanae -infected sample. Lane 8 is a negative control, and lane 9 is a positive control.

Sequences:

***Ehrlichia ewingii* Variant 1**
ATAAAGTTGGTGATGGAACAACTACATGTTCTATCTTAACCGCAAAAGTTATAGAAG
AAGTATCTAAAGCTAAAGCTGCTGGAGCAGATATTGTTTGTATTAAGGAAGGTGTAC
TTAAAGCTAAAGAAGCTGTATTAGAAGCTTTGATGTCTATGAAACGTGAAATACTAT
CTGAAGAAGAAATTGCTCAAGTTGCTACTATTTCTGCTAATGGTGATAAGAATATAG
GTAGTAGGATTGCACAATGTGTTCAAGAAGTTGGTAGAGATGGAGTTATTA

***Rickettsia parkeri* Variant 1**
TAAAGCTGCTTTATTCACCACCT-
CAACCGCAGCGATAATGCTGAGTAGTAGCGGGGCACTCGGTGTTGCTGCAGGTGTT
ATTGCTACTAATAATAATGCAGCATTTAGTGATGATGTTAACAATAATAATTGGAGT
GAGATAACGGCTGCAGGGGTAGCTAATGGTGTTCCTGCTGGCAGTCCTCAAAACAA
TTGGGCATTTACTTACGGTGGTGATTATACTATCACTGCAGATGCAGCCGATCGTAT
TATTACGGCTATAAATGTTGCGGGTACTACTCCCGTAGGTCTAAATATTGCTCAAAA
TACCGTTGTTGGTTCGATTATAACGGGAGGTAACTTGTTGCCTGTTACTATTACTGCC
GGCAAAAGCTTAACTTTAAACGGTAATAATGCTGTTGCTGCAAATCATGGTTTTGAT
GCT-CCTGCCGATAAT

***Rickettsia parkeri* Variant 2**
TAAAGCTGCTTTATTCACCACCT-
CAACCGCAGCGATAATGCTGAGTAGTAGCGGGGCACTCGGTGTTGCTGCAGGTGTT
ATTGCTACTAATAATAATGCAGCATTTAGTGATGATGTTAACAATAATAATTGGAGT
GAGATAACGGCTGCAGGGGTAGCTAATGGTGTTCCTGCTGGCAGTCCTCAAAACAA
TTGGGCATTTACTTACGGTGGTGATTATACTATCACTGCAGATGCAGCCGATCGTAT
TATTACGGCTATAAATGTTGCGGGTACTACTCCCGTAGGTCTAAATATTGCTCAAAA
TACCGTTGTTGGTTCGATTATAACGGGAGGTAACTTGTTGCCTGTTACTATTACTGCC
GGCAAAAGCTTAACTTTAAACGGTAATAATGCTGTTGCTGCAAATCATGGTTTTGAT
GCTTCCTGCCGATAAT

***Candidatus* Rickettsia andeanae Variant 1**
TAAAGCCGCTTTATTCACCACCT-
CAACCGCAGCGATAATGCTAAGTAGTAGCGGGGCACTCGGTGTTGCTGCAGGTGTT
ATTTCTACTAATAA---
TGCAGCATTTAGTGATGTTGCTGAGTTCGGTCATTGGAATAAAATAGCGGCTGGAGG
GGTAGCTAATGGTATTCTTGCTAACGGTCCTCAAGACAATGAGGCATTTACTTACGG
TGGTGATCATACTATCACTGCAAATAAAGCCGGTCGTATTATTGTGGCTATAAATGT
TGCGGGTACTACTCCTGTAGGTCTAAATATTACTCAAAATACCGTTGTTGGTTCGATT
GTGACGGGAGGTAACTTGTTGCCTGTTACTATTACTGCCGGCAAAAGCTTAACTTTA
AACGGTACTAATGCTGTTGCTGCAAATCATGGTTTTGATGCT-CCTGCCGATAAT-----
TATAC---AGGTTTAGG-
AAATATAACTTTGGGGAAAGCGAATGCTGCACTAATTATACAATCTGTAACCCCGGC
AAAGATAACACTTGCAGGAAATATAGATGGAAGAGGTATAATAACTGTCAAGACAG
ATGCTGCCATTAACGGAATAATAGGTAATGTTATCCCAGCAGCTCAAATAAGAGTTG
GGGCAAGCACCCTTTCTCTTGGGGGAGCGGTTATTAAAGCTACTACGACTAAATTAA

CAGATGCTGCGTCGGTATTAACCCTTACAAATGCAAATGCAGTATTAACAGGTGCGA
TTGATAACACCACAGGTGGTGATAATGTAGGTGTCTTAAATTTAAATGGTGCATTGA
GTCAAGTGACCGGGAATATAGG-TAATACAAA-TTCATTAGCCACGAT

***Candidatus* Rickettsia andeanae Variant 2**
TAAAGCCGCTTTATTCACCACCT-
CAACCGCAGCGATAATGCTAAGTAGTAGCGGGGCACTCGGTGTTGCTGCAGGTGTT
ATTTCTACTAATAA---
TGCAGCATTTAGTGATGTTGCTGAGTTCGGTCATTGGAATAAAATAGCGGCTGGAGG
GGTAGCTAATGGTATTCTTGCTAACGGTCCTCAAGACAATGAGGCATTTACTTACGG
TGGTGATCATACTATCACTGCAAATAAAGCCGGTCGTATTATTGTGGCTATAAATGT
TGCGGGTACTACTCCTGTAGGTCTAAATATTACTCAAAATACCGTTGTTGGTTCGATT
GTGACGGGAGGTAACTTGTTGCCTGTTACTATTACTGCCGGCAAAAGCTTAACTTTA
AACGGTACTAATGCTGTTGCTGCAAATCATGGTTTTGATGCT-CCTGCCGATAAT-----
TATAC---AGGTTTAGG-
AAATATAACTTTGGGGAAAGCGAATGCTGCACTAATTATACAATCTGTAACCCCGGC
AAAGATAACACTTGCAGGAAATATAGATGGAAGAGGTATAATAACTGTCAAGACAG
ATGCTGCCATTAACGGAATAATAGGTAATGTTATCCCAGCAGCTCAAATAAGAGTTG
GGGCAAGCACCCTTTCTCTTGGGGGAGCGGTTATTAAAGCTACTACGACTAAATTAA
CAGATGCTGCGTCGGTATTAACCCTTACAAATGCAAATGCAGTATTAACAGGTGCGA
TTGATAACACCACAGGTGGTGATAATGTAGGTGTCTTAAATTTAAATGGTGCATTGA
GTCAAGTAACCGGGAATATAGG-TAATACAAA-TTCATTAGCCACGAT

***Candidatus* Rickettsia andeanae Variant 3**
TAAAGCCGCTTTATTCACCACCTACAACCGCAGCGATAATGCTAAGTAGTAGCGGGG
CACTCGGTGTTGCTGCAGGTGTTATTTCTACTAATAA---
TGCAGCATTTAGTGATGTTGCTGAGTTCGGTCATTGGAATAAAATAGCGGCTGGAGG
GGTAGCTAATGGTATTCTTGCTAACGGTCCTCAAGACAATGAGGCATTTACTTACGG
TGGTGATCATACTATCACTGCAAATAAAGCCGGTCGTATTATTGTGGCTATAAATGT
TGCGGGTACTACTCCTGTAGGTCTAAATATTACTCAAAATACCGTTGTTGGTTCGATT
GTGACGGGAGGTAACTTGTTGCCTGTTACTATTACTGCCGGCAAAAGCTTAACTTTA
AACGGTACTAATGCTGTTGCTGCAAATCATGGTTTTGATGCT-CCTGCCGATAAT-----
TATAC---AGGTTTAGG-
AAATATAACTTTGGGGAAAGCGAATGCTGCACTAATTATACAATCTGTAACCCCGGC
AAAGATAACACTTGCAGGAAATATAGATGGAAGAGGTATAATAACTGTCAAGACAG
ATGCTGCCATTAACGGAATAATAGGTAATGTTATCCCAGCAGCTCAAATAAGAGTTG
GGGCAAGCACCCTTTCTCTTGGGGGAGCGGTTATTAAAGCTACTACGACTAAATTAA
CAGATGCTGCGTCGGTATTAACCCTTACAAATGCAAATGCAGTATTAACAGGTGCGA
TTGATAACACCACAGGTGGTGATAATGTAGGTGTCTTAAATTTAAATGGTGCATTGA
GTCAAGTAACCGGGAATATAGG-TAATACAAAATTCATTAGCCACGAT

***Candidatus* Rickettsia andeanae Variant 4**
TAAAGCCGCTTTATTCACCACCT-
CAACCGCAGCGATAATGCTAAGTAGTAGCGGGGCACTCGGTGTTGCTGCAGGTGTT
ATTTCTACTAATAA---
TGCAGCATTTAGTGATGTTGCTGAGTTCGGTCATTGGAATAAAATAGCGGCTGGAGG

GGTAGCTAATGGTATTCTTGCTAACGGTCCTCAAGACAATGAGGCATTTACTTACGG
TGGTGATCATACTATCACTGCAAATAAAGCCGGTCGTATTATTGTGGCTATAAATGT
TGCGGGTACTACTCCTGTAGGTCTAAATATTACTCAAAATACCGTTGTTGGTTCGATT
GTGACGGGAGGTAACTTGTTGCCTGTTACTATTACTGCCGGCAAAAGCTTAACTTTA
AACGGTACTAATGCTGTTGCTGCAAATCATGGTTTTGATGCT-CCTGCCGATAAT-----
TATAC---AGGTTTAGG-
AAATATAACTTTGGGGAAAGCGAATGCTGCACTAATTATACAATCTGTAACCCCGGC
AAAGATAACACTTGCAGGAAATATAGATGGAAGAGGTATAATAACTGTCAAGACAG
ATGCTGCCATTAACGGAATAATAGGTAATGTTATCCCAGCAGCTCAAATAAGAGTTG
GGGCAAGCACCCTTTCTCTTGGGGGAGCGGTTATTAAAGCTACTACGACTAAATTAA
CAGATGCTGCGTCGGTATTAACCCTTACAAATGCAAATGCAGTATTAACAGGTGCGA
TTGATAACACCACAGGTGGTGATAATGTAGGTGTCTTAAATTTAAATGGTGCATTGA
GTCAAGTGACCGGGAATATAGGGTAATACAAA-TTCATTAGCCACGAT

***Candidatus* Rickettsia andeanae Variant 5**

TAAAGCCGCTTTATTCACCACCT-CAACCGCAGCGATA-TGCTA-
GTAGTAGCGGGGCACTCGGTGTTGCTGCAGGTGTTATTTCTACTAATAA---
TGCAGCATTTAGTGATGTTGCTGAGTTCGGTCATTGGAATAAAATAGCGGCTGGAGG
GGTAGCTAATGGTATTCTTGCTAACGGTCCTCAAGACAATGAGGCATTTACTTACGG
TGGTGATCATACTATCACTGCAAATAAAGCCGGTCGTATTATTGTGGCTATAAATGT
TGCGGGTACTACTCCTGTAGGTCTAAATATTACTCAAAATACCGTTGTTGGTTCGATT
GTGACGGGAGGTAACTTGTTGCCTGTTACTATTACTGCCGGCAAAAGCTTAACTTTA
AACGGTACTAATGCTGTTGCTGCAAATCATGGTTTTGATGCT-CCTGCCGATAAT-----
TATAC---AGGTTTAGG-
AAATATAACTTTGGGGAAAGCGAATGCTGCACTAATTATACAATCTGTAACCCCGGC
AAAGATAACACTTGCAGGAAATATAGATGGAAGAGGTATAATAACTGTCAAGACAG
ATGCTGCCATTAACGGAATAATAGGTAATGTTATCCCAGCAGCTCAAATAAGAGTTG
GGGCAAGCACCCTTTCTCTTGGGGGAGCGGTTATTAAAGCTACTACGACTAAATTAA
CAGATGCTGCGTCGGTATTAACCCTTACAAATGCAAATGCAGTATTAACAGGTGCGA
TTGATAACACCACAGGTGGTGATAATGTAGGTGTCTTAAATTTAAATGGTGCATTGA
GTCAAGTGACCGGGAATATAGG-TAATACAAA-TTCATTAGCCACGAT

4. Conclusions

In this project several traditional and novel methods of tick collection were compared and temporal and habitat variations in the efficiency of tick collection methods were identified. This study also demonstrated that *A. maculatum* in Tennessee are associated with *R. parkeri,* a human pathogen that may contribute to the high number of rickettsiosis cases diagnosed in Tennessee.

Tick Trapping Comparison: In the temporal trapping comparison, all three tick species (*A. americanum, A. maculatum,* and *D. variabilis*) were collected with all the evaluated methods. Sweep-netting was significantly less effective than the other methods for tick collection. Changes in the efficiency of methods over the sampling periods could be the result of changes in the vegetation structure over time, or the result of changes in the questing activity or behavior of the ticks later in the season. In this study, the two novel methods performed comparably to their conventional counterparts (CO_2 dragging and traditional dragging, and CO_2 flagging and traditional flagging). There was no significant difference between a novel method and its conventional counterpart for any of the sampling periods for any of the tick species, or for total tick collections.

In the habitat trapping comparison three of the tick species, *A. americanum, A. maculatum*, and *D. variabilis,* were collected with all methods used, though *A. maculatum* was only collected in grassland habitats. Only seven *I. scapularis* were collected in this study; all were nymphs collected with CO_2 dragging. The variability in method across habitat was consistent for total tick collections, total *A. americanum* collections, and *A. americanum* adult and nymph collections. No significant differences in method in any of the habitats could be detected for *D. variabilis* collection. There was no significant difference detected in any of the methods in the grassland habitat, likely due to the small number of ticks collected in the grassland habitat when compared to the other habitats. In this study, CO_2 dragging and

traditional dragging were comparable in all instances where significant differences in methods were identified. In bottomland deciduous habitat, CO_2 flagging was significantly less effective than all other methods for total tick collection, and for the total and nymphal *A. americanum* collection.

In both years, CO_2 dragging was comparable in efficiency to conventional dragging. There seemed to be a trend evident in both the temporal and habitat comparisons demonstrating greater trapping success with CO_2 flagging for the collection of *A. maculatum*, but no significant differences in trapping method were detected. This trend was not consistent with the trends seen in overall tick collection, or collections of any of the other tick species. The novel methods were each comparable with their conventional counterparts, but they did not demonstrate a significant advantage over the conventional methods and were associated with additional cost (e.g., the CO_2 tank), and additional maintenance, such as the time required to connect the hoses to the tanks at each site and the maintenance of the hoses, which could be damaged if tangled in vegetation. The tanks were also cumbersome to carry in areas of dense vegetation or rough terrain. The addition of CO_2 to these methods did not result in a significant improvement over the traditional methods, and was associated with several drawbacks that decreased the effectiveness of the method in terms of time and cost.

It is important to consider the temporal and habitat variability in trapping methods when designing collection protocols, and to choose a method that will be consistent across time and across habitat types. Both dry ice trapping and dragging were consistent across time and habitat, but the dry ice trap was significantly more effective than dragging in upland and coniferous habitats for overall tick collection and collection of *A. americanum*. Overall, dry ice was an effective trapping method, collecting significantly higher numbers of ticks in some habitats in the

habitat comparison study, and was among the most effective trapping methods in almost all sampling periods in the temporal study. However, dry ice is not always available, and can be cost-prohibitive. When large quantities are needed, it can be difficult to acquire the necessary amounts, and to transport it to where it is needed. When dry ice trapping is not an option, dragging is a simple alternative that is less costly. Depending upon the type of study that is being carried out, it may be necessary to use multiple methods to avoid selection bias.

Pathogen Surveillance. This study demonstrated that *A. maculatum* is associated with a human pathogen in Tennessee. Of the 265 Gulf Coast ticks screened, 60 (~23%) were infected with *R. parkeri*. The rates identified here are similar to, or slightly higher than, the infection prevalence found in many other areas of the southeast (Sumner *et al.*, 2007; Paddock *et al.*, 2010; Varela-Stokes *et al.*, 2011; Ferrari *et al.*, 2012; Pagac *et al.*, 2014). An additional 15 ticks from the collection were infected with *Ca.* R. andeanae, a species of undetermined pathogenicity (Jiang *et al.*, 2012). The relatively high prevalence of *R. parkeri* in *A. maculatum* in Tennessee and other areas of the southeast in comparison to other tick species and their associated pathogens, such as *D. variabilis* and *R. rickettsii*, seems to suggest a close relationship between *A. maculatum* and *R. parkeri* (Sumner *et al.*, 2007), and indicate a need for continued monitoring and surveillance for this tick species, particularly in areas with high incidences of rickettsiosis (Fig 4.1). The lack of *Borrelia* species in the collection was expected, due to the low number of diagnosed cases of borreliosis in Tennessee (TNDOH, 2015), and the identification of few *Borrelia*-infected ticks in the state (Rosen *et al.*, 2012). The ability of *A. maculatum* to transmit *Ehrlichia* species other than *E. ruminantium* is uncertain, and the *Ehrlichia*-infected specimens identified in this study likely pose little threat of pathogen transmission as these specimens were engorged adults collected from a known reservoir of *E. ewingii*. These individuals would not

likely encounter another host in a situation where vertical transmission through feeding would be a concern, and it is not thought that this species is transovarially transmitted (Long *et al.,* 2003; Stromdahl, 2008). However, because the expanding range of *A. maculatum* includes states with a high number of human ehrlichiosis cases (CDC, 2015; Fig. 4.1), its potential role in the transmission of *Ehrlichia* species merits further research.

Due to the risk of introduction, continued monitoring for *E. ruminantium* in the U.S. is merited. Of particular concern is the Gulf Coast tick's lab-demonstrated ability to serve as a competent vector of *E. ruminantium*, the agent of heartwater, which is a devastating and often fatal disease of livestock (Mahan *et al.,* 2000). This disease is characterized by a buildup of fluid in the pericardium of the heart, and around other internal organs. Symptoms in ungulates include fever, rapidly-progressing weakness, disorientation, and staggering, as well as hemorrhagic diarrhea in cattle. In some instances death may occur rapidly, without preceding signs (Deem, 1998). Though not passed directly between infected animals, it is easily spread by the bite of an infected tick (Kasari *et al.,* 2010). Heartwater is endemic to sub-Saharan Africa and has been spread to other areas, including the Caribbean Islands, along with its primary vector the Tropical Bont tick (*A. variegatum*) (Burridge *et al.,* 2002). This tick has an affinity for birds, and is occasionally found on migrating birds (Kasari *et al.,* 2010), or on livestock or wildlife imported into the U.S. (Burridge *et al.,* 2000); a serious concern because domestic livestock are highly susceptible to the disease (Deem, 1998; Burridge *et al.,* 2002). Although the pathogen has not yet been established in the continental U.S., it is possible that an infected tick could be introduced to the U.S. mainland through one of these avenues. If the pathogen is introduced, *A. maculatum* is a competent vector that could aid in its establishment and spread (Mahan *et al.,* 2000). The potential for its introduction and its high fatality rate makes this a pathogen of concern, as it may

affect a variety of domestic livestock species as well as native wild ruminants (Dardiri *et al.*, 1987; Deem, 1998; Burridge *et al.*, 2002; Kasari *et al.*, 2010). Some non-native cervids that are associated with the captive cervid industry may also serve as carriers of this disease (Deem, 1998; Kasari *et al.*, 2010). Of note, the retail value of the U.S. beef cattle industry in 2013 was approx. $88 billion (USDA-ERS, 2014), while in the U.S. in 2011, big game hunting expenditures totaled $16,853,654,000 (USFWS, 2014). In Tennessee alone in 2011, overall big game hunting expenditures totaled almost $300,000,000 (USFWS, 2014). In a 2007 survey by the Agricultural and Food Policy Center (AFPC) at Texas A&M University, the total economic impact of the captive cervid industry was estimated at $3 billion per year (AFPC, 2007). The introduction of heartwater to the continental U.S. could reduce the economic impact of both the livestock industry (Mahan *et al.*, 2000) and result in a decrease in big-game hunting expenditures.

Future Directions: *Amblyomma maculatum* is considered the primary vector of the protozoan *Hepatozoon americanum* which causes American Canine Hepatozoonosis, a disease in dogs acquired by the ingestion of an infected tick (Baneth *et al.*, 2003). This agent causes a more severe and more often fatal disease than the Old World *H. canis* (Old World hepatozoonosis), which is prevalent in parts of Europe, Africa, and Asia (Baneth *et al.*, 2003). This increased severity may be due in part to the different organs targeted (haemolymphatic tissue is the typical site of development for *H. canis*, while *H. americanum* more often encysts in skeletal muscle); the increased virulence of *H. americanum* may also indicate a more recent movement from a wild host to domestic canids, in comparison to a better-adapted *H. canis* (Baneth *et al.*, 2003). Both species have been found in canids in the U.S. (Allen *et al.*, 2008; Starkey *et al.*, 2013). Symptoms of *H. americanum* infection include fever, anemia, and muscle atrophy (Li *et al.*,

2008). *Hepatozoon americanum* encysts primarily in skeletal and cardiac muscle, merozoites invade leucocytes, and gamonts circulate in the blood and can be ingested by feeding ticks. Reproduction and the development of oocysts occur in the tick (Baneth *et al.*, 2003). Coyotes can spread and maintain this disease in the southern U.S. (Kocan *et al.*, 1999, Teel *et al.*, 2010). Because of the proximity of the *A. maculatum* collection sites at Ames Plantation to the course for the National Championship bird dog trails (Fig. 4.2), it is possible that both dogs and humans may come into contact with these ticks. Future research directions should include testing the ticks collected at Ames for *Hepatozoon* species, to determine what threat they may pose to the health of dogs that may come into contact with these ticks, in addition to the known human pathogen.

Other future research directions should include the determination of habitat associations of *A. maculatum* to aid in monitoring this tick and in predicting future range expansion. The determination of host associations of this tick in areas where it is more recently established could indicate how the tick is being maintained in new areas, and may indicate mechanisms of dispersal. This information would help to develop more comprehensive monitoring and management strategies for this species.

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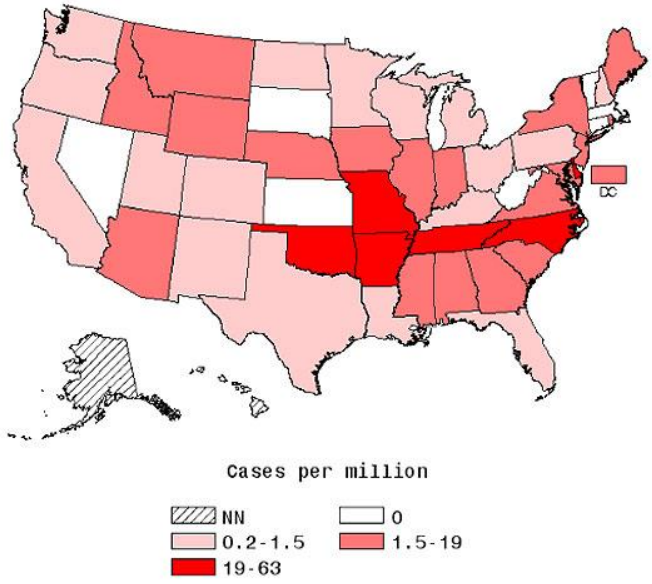
Appendices

a)

Rickettsiosis Incidence, 2010



b)

Ehrlichiosis Incidence, 2010

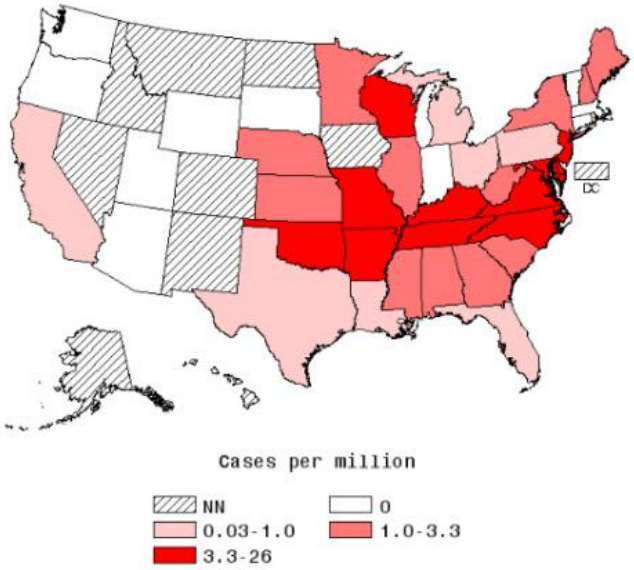


Figure 4.1: Distribution of rickettiosis (a) and ehrlichiosis (b) in the United States in 2010 (CDC, 2015).

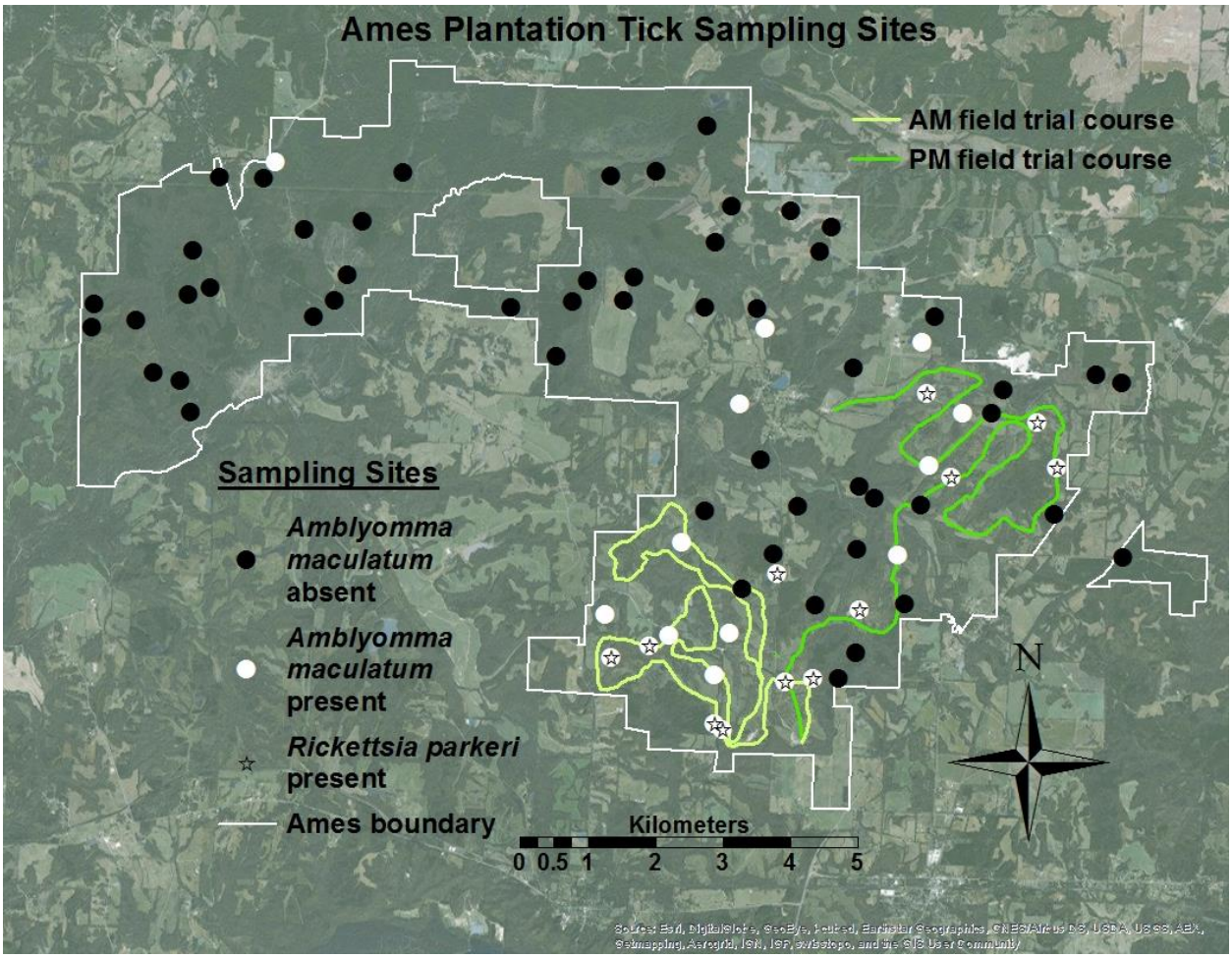


Figure 4.2: Location of collection of *Amblyomma maculatum* (white dots) and *Rickettsia parkeri*-infected *A. maculatum* (white dots with stars) at Ames Plantation in southwestern Tennessee from 2012-2014 in relation to National Championship field trial courses.

Vita

Sarah Mays is originally from Ardmore, Oklahoma. She attended Tarleton State University in Stephenville, Texas, where she earned her Bachelor's Degree in Wildlife Management with a minor in Biology. Sarah was involved in tick research as an undergraduate, which developed her interest in tick-borne disease and disease ecology. Sarah is currently completing her Master's degree in Entomology and Plant Pathology at the University of Tennessee, Knoxville, and will graduate in May 2015.