Identification and Characterization of Peak Activity, Environmental Variables, and Bacterial Pathogens in *A. americanum* L. at Ames Plantation, West Tennessee

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Abstract

The status of tick-borne diseases (TBD) in the southeastern United States is uncertain due to a number of factors including, but not limited to emerging pathogens, misdiagnoses, and modifications to landscapes. Ehrlichiosis and rickettiosis are two of the most common TBDs; these are caused by *Ehrlichia* and *Rickettsia* bacteria that can be transmitted by a number of different tick species. The objectives of this study were to identify *Amblyomma americanum* (the Lone Star tick) peak activity and habitat preferences and characterize the potential role of *A. americanum* in tick-borne disease cycles in southwestern Tennessee. Using vegetation drags and CO_2-baited traps, ticks were collected monthly from May to September 2012 from 100 sites on the Ames Plantation Research and Education Center (Ames). Using a one-way analysis of variance, we identified the peak activity of *A. americanum* for adults as being in May or June and of nymphs as being bimodal with a peak in June and again in August. Trapping data were analyzed in a contingency table; results indicated significant trapping differences in the number of nymphs and adults collected by the two trapping methods. Environmental and trapping data were correlated using an ANCOVA to evaluate trapping efficacy under different environmental stressors and to identify landscapes in which *A. americanum* adults and nymphs are notably more abundant. Of 925 adult *A. americanum* screened for *Ehrlichia* and *Rickettsia* bacteria, 1.8% (n = 17) and 38% (n = 353) were PCR positive, of which 8 ticks (0.8%) were positive with both pathogens. Using ArcGIS we displayed pathogen positive *A. americanum* locations; calculating Moran's I for each pathogen indicated there was no significant clustering among pathogen positive locations. The identification of pathogens and co-infections within *A. americanum* from western Tennessee warrants further investigations to understand the role ticks and their environment have in the distribution of TBD.

Keywords: Tennessee, *Amblyomma americanum*, Environment, *Rickettsia*, and *Ehrlichia*

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1. Introduction

1.1 Abstract

Amblyomma americanum L. 1754 (the Lone Star tick) was the first human-biting tick described and has since been regarded as an economically important pest of humans and livestock. Although extremely abundant, serious attention was not given to *A. americanum* as a vector of human pathogens until the identification of zoonotic *Ehrlichia* species in the early 1990's. The extent to which *A. americanum* plays a role in many tick-borne disease cycles in the Southeast remains uncertain; perhaps more clear, yet frightening, is the increase in human tick-borne disease cases in the southeastern United States (including Tennessee) and the increasing abundance of *A. americanum* along with its associated pathogens. This review aims to summarize what is currently known about *A. americanum* development, hosts, distribution, ecology, and associated pathogens to provide a platform for research conducted to help clarify the uncertainty surrounding tick-borne disease in the southeastern United States.

1.2 Background and Significance

1.2.1 Tick Vectors of Concern in Tennessee

Ticks are ideal vectors of disease and transmit a wider variety of zoonotic pathogens than any other known arthropod vector [1]. One explanation for this is that ticks are more efficient vectors of disease than most insects because their internal tissues shed gradually, promoting maintenance of biological agents and allowing for pathogen transmission throughout different life stages [2]. Their involvement in human and animal disease cycles comes from the various adaptations ticks have undergone, including long lifespans that extend across multiple seasons, high reproductive potential, highly sclerotized bodies that provide protection from the environment, and relative freedom from natural enemies [3]. During their relatively long life cycles hard ticks like *A.*

americanum must feed three separate times on three potentially different hosts (including humans). The feeding process is slow, which allows time for pathogen dissemination and an increased likelihood of pathogen transmission [1], [3]. Feeding ticks actively secrete saliva with pharmacologically active biological agents that are believed to promote feeding success, and also aid in the transmission of pathogens [3].

Currently there are five tick species in Tennessee that are commonly encountered by humans and pets: Black-legged tick (*Ixodes scapularis* Koch), American Dog tick (*Dermacenter variabilis* Say), Lone Star tick (*Amblyomma americanum* L.), Gulf Coast tick (*Amblyomma maculatum* Koch), and Brown Dog tick (*Rhipicephalus sanguineus* Latreille) [4]. While all of these ticks are capable of transmitting one or more pathogens, *A. americanum* is the tick species most frequently encountered by humans in the southeastern United States. It is of particular public health concern because its bites cause significant discomfort and it has been implicated in the transmission of pathogens to humans and other animals [5]. It is an aggressive, generalist feeder, actively seeking out potential hosts (including humans). This aggressive nature is evident in the study by Hair (1979), who documented *A. americanum* abundance on white-tailed deer fawns (*Odocoileus virginianus* Zimmerman) so extreme that it resulted in exsanguination and death. Additionally, *A. americanum* will feed on humans opportunistically at every life stage with such aggression that larval *A. americanum* (commonly referred to as 'seed ticks') have been found parasitizing sensitive regions, such as the conjunctiva of the human eye, making simple tick removal hazardous [7].

1.2.2 Lone Star Tick Development and Control

Ticks are in the class Arachinda and have pronounced physiological differences when compared to that of their insect vector counterparts, such as the lack of body segmentation and four pairs of walking legs in the mature stage. In addition, ticks do not have a brain or ventral nerve chord, but rather their central nervous system is concentrated into a singular nerve mass called the synganglion [1]. Hard ticks have three morphologically different life stages: larva, nymph, and adult. Larvae have three pairs of walking legs while nymphs and adults have four. Only adult ticks display sexual features such as the genital aperture located on the opisthosoma. Adult ticks are sexually dimorphic (Figure 1.1) and exhibit varying feeding behaviors. Female ticks can feed for longer periods of time than can males. Male hard ticks have the scutum extended along the entire dorsal length of the body, whereas in females the scutum has become shortened to enable the opithosoma to be more elastic to facilitate engorgement and egg development [1].

Feeding behavior differs not only between sexes, but between life stages as well. For most ixodid ticks, like *A. americanum*, larvae tend to feed for the shortest amount of time while adult females feed for the most prolonged amount of time [1]. Tick feeding duration is not heavily influenced by environmental factors such as temperature and humidity, but is strongly influenced by variables related to the host, such as immunity [1]. Tick species like *A. americanum* feed on a variety of host species, including white-tailed-deer (*Odocoileus virginianus*) and wild turkey (*Meleagris gallopavo*) [8], [9]. These significantly different hosts have differing levels of sensitivity to the biological agents in tick saliva depending on the number of previous tick bites [1]. It is this steadily developing sensitivity that will dictate the amount of time future ticks feed on a host in order to secure a blood meal large enough to successfully molt. Differences in

feeding duration have been demonstrated among ticks within the same life stage under identical environmental situations [1].

In addition to the energy supplemented by hosts during tick development, hosts also serve as a mechanism for dispersal. In acquiring a host, ticks must employ ambush and hunter host seeking strategies [3]. The ambush strategy is the technique employed by *A. americanum* during host seeking [3]. This tactic (termed questing) involves crawling up vegetation and waiting (potentially hours) for an unsuspecting host to pass by and brush up against the vegetation where the tick is waiting [3]. Questing ticks in temperate regions such as the United States are strongly influenced by host cues such as CO_2, NH_3, and body heat [1]. The time of year and duration of questing behavior is reliant upon environmental conditions such as ambient temperature. As ticks quest they are vulnerable to desiccation; as conditions worsen ticks must descend the vegetation every so often to reacquire moisture from the cooler humid ground layer [10], [11]. Once *A. americanum* has latched onto one of the many transient hosts available they will feed for several days after which time they will drop off of the host into a naïve environment. It is this exhibition of generalist and aggressive behavior that has resulted in the continuing expansion of the *A. americanum* zoogeographic range in the United States. Currently, they are found in west-central Texas, north to the lower Midwest, east to the Atlantic coast, and northward to Maine [8], [12] (Figure 1.2).

The range of *A. americanum* is so extensive that seasonal variation occurs within its geographic distribution in the United States. The seasonal activity of *A. americanum* in the northern part of its distribution occurs later than in the southeast portion [13]. The longevity of the hard tick life cycle is one of the most notable characteristics about tick development. Typically, it is measured in years and trans-seasonally, rather than in days or months like their insect vector counterparts [1]. For example, mosquitoes can complete their entire life cycle from egg to adult in less than ten days during the warm summer months [14], and unlike mosquitoes ticks are parasitic at every life stage [15]. In the southeast, *A. americanum* are active from spring until early fall, but typically overwinter as nymphs and emerge in the spring to actively seek a host, and molt [12]. Adult activity peaks in May or June and diminishes in July; during this peak time adults will seek a large mammalian host to feed and mate upon [12]. Engorged female *A. americanum* lay eggs in the environment after detaching from the host; these eggs will hatch into larvae as early as June in most cases [12], [16]. Larval activity typically peaks in August and diminishes by October [12].

Larvae that successfully feed in August will molt into nymphs and undergo a dynamic state of decreased metabolic and locomotive activity typically onset by unfavorable environmental conditions known as diapause [11]. Diapause may persist for several weeks or months depending on the tick species and the region, but is typically terminated by the onset of favorable environmental conditions [11]. It is believed that ixodid ticks like *A. americanum* developed this ability in response to dynamic changes in climate during the transition from the Mesozoic to Cenozoic era to survive harsh dynamically changing environmental conditions [11]. In addition to the increased survivability ixodid ticks gain from this adaptation, it also enables them to

synchronize their development and reproduction during times of maximum food availability [11]. Each year there are two generations of *A. americanum* nymphs: one emerges in spring from diapauses to feed and become adults and a second that will molt into nymphs by late summer and then undergo a behavioral diapause until more favorable conditions are present [16].

1.2.3 *Ehrlichia* and *Rickettsia* in *A. americanum*

Lyme disease has gained much recognition as the most prevalent vector borne disease in the nation [5]. However recent studies have indicated that the risk of contracting Lyme disease or any tick-borne disease correlates with regional variation in the abundance of human biting tick species [5]. In the Southeast, *A. americanum* is the most frequently encountered human biting tick species and is known to transmit *Ehrlichia* and *Rickettsia* species of varying pathogenicity to humans throughout their geographic range [5], [12], [17]. Of serious concern, the majority of tick-borne human illness cases are from patients who cannot even recall being bitten, presumably because the bite was in an obscure place or too small to easily recognize [17]. Clinical manifestations of human ehrlichiosis or rickettsiosis are similar in nature and often result in sudden onset of fever, chills, and headaches [17]. It is because of these relatively vague and uniform clinical manifestations that patients suffering from tick-borne illness are misdiagnosed, and often the true cause of infection goes undocumented [18].

The majority of ehrlichiosis and rickettiosis cases are reported from the southeastern United States including Missouri, Oklahoma, Tennessee, Arkansas, and Maryland [17]. In Tennessee, human ehrlichiosis is the second most frequently reported tick-borne disease just behind Rocky Mountain spotted fever (RMSF) [18]. Currently three species of *Ehrlichia* have been identified as etiological agents of human ehrlichiosis: *E. ewingii*, *E. chaffeensis*, and Panola Mountain

Ehrlichia [5], [12], [19]. *Rickettsia rickettsii*-associated human rickettiosis is the number one tick-borne disease reported in the state of Tennessee, yet little is known about the presence of this pathogen in ticks collected in Tennessee [20]. While Tennessee only accounts for 2.4% of the total cases of human Rickettiosis in the United States, these cases represent a staggering 26% of severe illness or deaths reported in the country; suggesting that Tennessee is a potential "hotspot" for severe human rickettiosis infections [21]. Consequently, there is a critical need for increased surveillance of the human biting ticks and their associated pathogens in Tennessee.

1.2.4 Management of Ticks and Tick-Borne Disease

Self-protection is by far the most universally applicable method for control of ticks on humans or animals [22]. For the personal protection method to be effective in the prevention of tick-borne disease the ticks must be removed from the host as soon as possible to prevent the transmission of the disease organism or paralytic toxin from the tick [23]. Proper technique is an additional factor in the prevention of disease transmission [23]. Careless handling of ticks during removal can result in foreign matter left in the skin that can cause chronic or severe irritation for several weeks or accidental inoculation of disease organisms from the tick's bodily fluid through a tear in the skin [23]. For the safe mechanical removal of ticks from humans or animals it is highly recommended to grasp the tick as close to the skin as possible with forceps or protected hands and pull straight up applying a constant pressure [23]. Patients should exercise caution when using other tick removal strategies like application of petroleum jelly, finger nail polish, or lit matches directly on the tick as these methods are considered folklore and have little or no scientific backing [23]. In fact, several of these methods may increase the risk of exposure to disease causing organisms in the tick by preventing detachment or stimulating regurgitation [23].

Although self-inspection and rapid removal of ticks is the most economic means of control for ticks, a substantial conscientious effort is required and in areas of extreme tick density this method may require such considerable diligence that self-protection is impractical [22].

For residential areas or parks where tick density can be exceptionally high, particularly in forest and lawn edge eco-tones, vegetation management is a practical option for management of ticks [22]. Removing the excessive vegetation from an area increases the relative temperature of the ground and reduces the humidity and soil moisture all of which are necessary for increased abundance of ticks [24]. Examples of landscape modifications in residential areas include mowing and clearing brush, leaf litter, or other vegetative cover [22], [25]. Alternative approaches to vegetation management are host exclusion or application of acaricides [22], [25]. Typically the focus of host exclusion for *A. americanum* is white-tailed deer populations [22], [24], [25]; no doubt due to the fact that the white-tailed deer is considered the primary host for all *A. americanum* life-stages [26]. The primary variable which influences efficiency of this management type is the size of the fenced tract [22]. Previous studies have shown as much as 98% reduction in larvae and 53% reduction in nymphs [24], but there seems to be little or no effect on control of *A. americanum* adults by deer exclusion [24], [27]. Special interest has been given to acaricides (pesticides that kill mites and ticks) like Cyfluthrin, Amitraz, and Permethrin for rapid decimation of *A. americanum* populations, since its recognition as a vector of concern in the United States [28]. This is not an uncommon practice and is already widely accepted for the short-term control of *I. scapularis* in high-risk Lyme disease residential areas [29]. However environmental and public safety concerns restrict the availability and wide spread usage in additional tick management programs [22].

In 1984 the Tennessee Valley Authority (TVA) investigated the effectiveness of vegetation management in combination with host exclusion and application of acaricides at The Land between the Lakes Kentucky-Tennessee family campgrounds for control of the *A. americanum* [24]. They found that the use of vegetation management, acaricides, or deer exclusion alone was not enough to suppress areas of high tick density for more than a year, and after one year these areas were subject to re-infestation [24]. However, when used together a 92-98% mean reduction of free-living *A. americanum* larvae, nymphs, and adults was achieved at the campgrounds [24]. Current tick control practices focus on the short-term suppression of the vector over small geographic areas, primarily through the use of acaricides, however sustainable management of risk of human tick related illnesses like Lyme disease or *Ehrlichia chaffeensis* infection will require a more integrated approach, with an ecological foundation [22]. In short, no singular management technique can control the vector life cycle, vertebrate reservoirs, various pathogens, and the risk of exposure to these pathogens [22].

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Appendices



Figure 1.1: Sexual dimorphism among male (Left) and female (Right) adult *Amblyomma americanum*



Figure 1.2: Distribution of *A. americanum* as of 2010 courtesy of the Centers for Disease Control and Prevention (2011).

2. Effect of Habitat type and Environmental Variables on the Distribution and Peak Activity of *Amblyomma americanum* L. at Ames Plantation

Abstract

Background: The Lone Star tick, *Amblyomma americanum* L. is the most abundant human-biting tick species in Tennessee and continues to be implicated in human disease cases throughout the southeast region. Previous studies evaluating the influence of external environmental variables on *A. americanum* strongly suggest changing current surveillance practices to include more observations over smaller study areas to reduce within site variations in environmental variables. Consequently, the objectives of this study were to investigate seasonal activity, trapping efficacy, and habitat preferences of *A. americanum* nymphs and adults in small (0.12 km^2) transects at 100 sites spread across 75.584 km^2 (18,430 acres) of mixed landscapes in southwestern Tennessee using drag and CO_2-baited collections.

Results: Monthly collections of *A. americanum* nymphs and adults were significantly higher in May and June for drags, and only in June for CO_2-baited collections. Significantly more nymphs (28.47±0.37) were collected by vegetation drags than CO_2-baited collections (18.57± 3.43), while greater numbers of adults were collected by CO_2-baited trapping (5.35± 0.71) than drag collections (1.91± 0.28). Ground cover and habitat type were positively associated with vegetation drag-collected nymph abundance; whereas, for CO_2-baited collections ground cover and soil type were significantly correlated with increased nymph abundance. Adult collections were significantly associated with ground cover with both collection methods and by temperature for vegetation drags only.

Conclusions: The identification of peak activity, trapping efficacy, and habitat preferences of *A. americanum* within southwestern Tennessee will lead to improved surveillance and management of ticks and their associated pathogens in the Southeast. Further research is warranted to establish a connection between environmental variables effecting tick (and pathogen) abundance

and the possible implementation of a habitat modification driven control program for *A. americanum* in the southeastern United States.

Keywords: *Amblyomma americanum*, Vegetation Drags, CO_2-baited traps, Tennessee, and Environment

Introduction

The Lone Star tick, *Amblyomma americanum* L., is the most frequently encountered tick species in the southeastern United States and is considered both a nuisance and an important vector of various *Ehrlichia* and *Rickettsia* species that differ in their pathogenicity to humans and other animals [1]. Of serious concern *A. americanum* is the primary vector of *Ehrlichia chaffeensis*, the causative agent of human monocytic ehrlichiosis (HME), in the southeast [2], and has been implicated in the transmission of Spotted fever group Rickettsiae to humans [3]. In Tennessee, human ehrlichiosis is second only to RMSF [4] as the most frequently reported tick-borne disease, but reasons for this are unknown [4], [5]. One possible explanation is that the increase in case numbers is the result of better-informed physicians and improved surveillance tools to identify human infections [4], [5]. There is speculation that the cases of human illness will continue to increase over the next 25 years as a direct result of increased reservoir host populations and suburbanization [5].

Amblyomma americanum is considered aggressive and generalist ectoparasites because they will parasitize numerous mammalian and non-mammalian hosts such as small rodents, opossums, squirrels, canids, ground-dwelling birds, and ruminants [5]. White-tailed deer (*Odocoileus virginianus*, Zimmermann) have played the largest role in the dynamic geographic expansion of *A. americanum* [5]. In the early 1900's, white-tailed deer populations had drastically diminished because of excessive commercial hunting, but reintroduction efforts resulted in a 50-fold increase in white-tailed deer populations and by the mid-1930's white-tailed deer populations had increased from 350,000 to 17 million animals [6]. Although white-tailed deer are considered principal hosts of *A. americanum*, other non-mammalian hosts such as the wild turkey

(*Meleagris gallopavo* L.) have also contributed to the expansion of *A. americanum* [7], [8] and eventual spread of *Ehrlichia* spp. into naïve landscapes [5]. Currently the distribution of *A. americanum* ranges from the southeastern United States to south central United States, with a few reported cases as far north as Maine [9].

In the southeast, A. *americanum* are active from spring until early fall and are known to feed on humans at every life stage [2]. Concurrently, 90-93% of *Ehrlichia* and *Rickettsia* human infections occurs during the spring and summer months, coinciding with outdoor recreational activity in those months [10], [11], [12], [13]. Questing of *A. americanum* occurs in a variety of different landscapes. Habitats with young second-growth oak, hickory, or elm forests with a dense understory that fosters high relative humidity and low temperature [14] have notably more *A. americanum* and extended seasonal activity of these ticks [15]. Questing behavior can also be observed in scrub, meadow margins, hedgerows, and riparian vegetation along streams and rivers [16], [17].

Several studies have investigated the efficacy of sampling methods (e.g., vegetation drags) for the collection of questing *A. americanum* and determined that further research is warranted to examine the influence that external environmental variables (e.g., habitat type) have on the number of *A. americanum* collected [18], [19]. A previous studies suggested re-designing current tick sampling measures to include decreased plot size and increased number of sampling sites to account for variations in tick abundance resulting from external environmental factors [19]. Consequently, the purpose of this study was to investigate the influence different habitat types and environmental variables have on *A. americanum* collections using two conventional

sampling methods in small (~0.12 km) transects at an extensive number of sampling sites (n = 100) in an area of southwestern Tennessee where *A. americanum* has reached nuisance status. Our objectives were to (1) investigate monthly activity of *A. americanum*, (2) compare the efficacy of trapping methods for the collection of nymphal and adult *A. americanum*, and (3) identify preferred nymphal and adult habitats. Results from this study will support future investigations regarding tick-borne disease management, such as specific habitat modifications (e.g., application of acaricides) for control of the most notorious human biting tick throughout the southeastern United States.

Materials and Methods

Site Selection. Our study area was Ames Plantation Research and Education Center (AMES), a 74.5 km^2 (~18,400 acre) University of Tennessee-managed facility in western Tennessee (35.12 N, -89.21 W). Habitat types at AMES include commodity row crops (i.e., cotton, soybean, wheat, and corn), forests (i.e., loblolly pine, upland hardwoods, and bottomland hardwoods), and pastures (i.e., horse and beef cattle pastures). Current and past research at AMES has focused on a wide range of wildlife species and silvacultural treatments [20], [21]. Each habitat type is home to different animals and plants including several human-biting tick species, of which *A. americanum* is most abundant. Tick sampling effort was spread across AMES using a previously established white-tailed deer hunting grid system, whereby each grid square comprised 0.40 km^2 (100 acres). A total of 100 grids, each containing a single transect approximately 0.12 km in length, were selected at random; and each transect was assumed to represent a single habitat type. Grids randomly selected in inaccessible areas such as rivers were replaced with the nearest accessible grid square.

Tick Collection. Ticks were collected monthly from each site from May through September 2012 using vegetation drags [22]. The efficacy of dry ice-baited trapping (CO_2-trapping; [22], [23]) was initially evaluated at ten of the 100 chosen sites in May, and then implemented afterwards (June through September) at each of the 100 chosen sites. Vegetation drags were constructed from a 1 m^2 piece of corduroy cloth attached to a 0.025 m diameter wooden dowel rod with a 0.96 m rope handle. To collect passively questing ticks (defined here as ticks questing on vegetation rather than actively moving through the vegetation), six 30-second vegetation drags were conducted along each transect within the predefined grid. To collect actively questing ticks (defined here as ticks actively moving through the vegetation while seeking a host), CO_2 traps were constructed from a 1.9 L cylindrical blue cooler (Igloo Corporation Katy, TX) with seven holes drilled around the circumference of the cooler to allow for CO_2 sublimation [24]. Each cooler was filled with ca. 2.3 kg of dry ice and placed on a 1.37 m^2 piece of light colored duck cloth in the field. The duck cloth and cooler with dry ice remained at each site for a minimum of 12 hours to attract host-seeking ticks. All ticks attached to the drag cloth or found on the duct cloth were stored in individually labeled vials containing 80% ethanol, for later identification to species, life stage, and sex using morphological keys [25] at the Medical and Veterinary Entomology lab at the University of Tennessee Institute of Agriculture, Knoxville.

Environmental Data Collection. Environmental variables were classified as discrete or continuous for consideration of appropriate statistical analysis (Table 2.1). Continuous variables including temperature (^{o}C), relative humidity (% RH), and barometric pressure (hPa) were collected during each monthly visit at the beginning of each site visit using a Kestrel 3500

(Nielson Kellerman, Boothwyn, PA). Tree basal area and percent vegetation ground cover data at each site were measured in July 2012 while sampling. A 10x factor wedge prism (Forestry Suppliers, Jackson, MS) was used to estimate the basal area (ft^2/acre) for each transect [26] and ground cover (%) was estimated at the end of each 30-sec transect by classifying ground coverage within a 5 m^2 plot around the end point of each transect [27], [28]. The six basal area and ground cover measurements per site were averaged to minimize observer error [27]. Hunter deer observation data for the 2012 AMES hunting season were obtained to investigate the presence of deer and evaluate the impact that deer populations have on free-living *A. americanum* nymph and adult collections. Discrete variables such a soil type and aspect were classified in ArcGIS 10.0 (ERSI, Redlands, CA) using data obtained from USDA Geospatial Gateway [29] and classified further into groups by drainage capabilities, texture, and tree suitability using data obtained from the Tennessee Spatial Server [32] and the Hardeman and Fayette county soils survey [30], [31] (Table 2.2). The resulting habitat types were bottomland hardwood (n = 17), upland hardwood (n = 43), grass consisting of both pastures and tall grasses (n = 21), and pine stands (n = 19).

Statistical Analysis. Monthly relative activity of *A. americanum* was calculated by dividing the number of each life stage in a given sample by the total count of each life stage collected over the six sampling periods. Tick count data were log transformed to standardize the variance for subsequent statistical analysis. Monthly activity and trapping differences among *A. americanum* adults and nymphs collected from May-September 2012 were analyzed in Statistix 8.0 (Analytical Software, Tallahassee, FL) using a one-way analysis of variance (ANOVA), followed by a least significant difference (LSD) mean separation procedure. Collections in May

were excluded from this analysis because during this sampling period CO_2 trapping was not conducted across all 100 sites. Habitat preferences of adult and nymph *A. americanum* collected by vegetation drags were analyzed in an analysis of covariance (ANCOVA) that enabled continuous and discrete data recorded in the field could be used in the model. Habitat associations of *A. americanum* collected by CO_2-trapping were analyzed using an ANCOVA without climate data because data were not recorded throughout the duration of the 12-hour sampling period. As with the vegetation drag ANCOVA, an LSD procedure was used to investigate significant interactions between CO_2 trap-collected *A. americanum* adults and nymphs and the environmental variables recorded in the field. To identify the optimal level of each continuous environmental variable for *A. americanum*, sites were ranked by the number of ticks collected per visit; the mean (± 95% confidence interval) of each continuous variable was then calculated for the top 10% of sites in that ranking and presented graphically on scatterplots of the variation in tick abundance among the 100 sites. In addition to the LSD mean separations, 95% confidence intervals were also calculated for discrete variables after first calculating the mean and standard error for each variable.

Results

***A. americanum* Collection.** A total of 9450 adult and nymph ticks were collected, comprising 6908 *A. americanum* L. (73%), 2522 *Dermacenter variabilis* Say (26.7%), and 20 *A. maculatum* L. (0.3%). Vegetation drags (n = 4745) consisted of 4008 *A. americanum* nymphs (84.5%), 366 *D. variabilis* adults (7.7%), 361 A. *americanum* adults (7.6%), and 10 *A. maculatum* adults (0.2%). CO_2-trapping (n = 4705) attracted and collected 2151 *D. variabilis* adults (45.8%), 1954 *A. americanum* nymphs (41.5%), 585 *A. americanum* adults (12.4%), 10 *A. maculatum* adults

(0.2%), and 5 *D. variabilis* nymphs (0.1%). No *Ixodes scapularis* specimens were collected with either sampling method during the 6 month sampling scheme.

Relative activity of adult *A. americanum* peaked in May for drag samples and due to sampling methods (CO_2 traps were not employed in May) peaked in June for CO_2-trapping. Nymphal activity was bimodal, with peaks in June and August for both methods (Figure 1a, 1b, 1c). Using vegetation drags, the mean number of *A. americanum* nymphs collected per site in May (11.61 ± 1.34) and June (9.7 ± 1.16) were significantly greater ($F = 10.9$; df = 5; $P < 0.001$) than collections in July (4.81 ± 0.819), early August (5.75 ± 1.15), late August (8.18 ± 2.03) or September (0.03 ± 0.02). The mean number of vegetation drag collected adult *A. americanum* collected in May (1.7 ± 0.191) and June (1.14 ± 0.18) were significantly greater than collections in July (0.51± 0.13), early August (0.24 ± 0.05), late August (0.02 ± 0.01), or September (0 ± 0) ($F = 28.1$; df = 5; $P < 0.001$). No adult *A. americanum* were collected in September. Dry ice baited collections of nymph *A. americanum* was significantly lowest in September (0.05 ± 0.03) ($F = 2.89$; df = 4; $P = 0.0221$) compared to June (5.09 ± 1.14), July (3.49 ± 0.74), early August (4.65 ± 1.44), late August (5.29 ± 0.02) . Adult collections were significantly greater ($F = 22.1$; df = 4; $P < 0.001$) in June (2.73 ±0.42) than in July (2 ± 0.37), early August (0.57 ± 0.11) late August (0.05 ± 0.02), or September (0 ± 0). Significantly more *A. americanum* nymphs ($X^2 = 7.65$; df = 1; $P = 0.006$) were collected per site using vegetation drags (5.69 ± 0.56) than with CO_2 trapping (3.71 ± 0.57). Conversely, CO_2 trapping collected significantly more ($X^2 = 77.93$; df = 1; $P < 0.001$) adult *A. americanum* per site (1.07 ± 0.12) than did vegetation drags (0.38 ± 0.05).

***A. americanum* Habitat Characterization.** Pearson's correlation results indicated that ground cover and basal area possess a strong inverse relationship (r = - 0.79) with one another. Multivariable models were analyzed with these two terms separately and together; based on the consistently lower *P* values produced by the ground cover term when analyzed without basal area. Ground cover was chosen to represent overall vegetation density in the following analyses.

Drag Collection Discrete Variables. Significantly fewer nymphs ($F = 2.78$; df = 3; $P = 0.0472$) were collected via drags from grasslands (2.62 ± 0.44) compared to bottomland hardwood (29.5 ± 13.07), pine stands (19.6 ± 5.72), or upland hardwood sites (21.6 ± 5.30) (Figure 2.2). Neither soil type ($F = 1.37$; df = 7; $P = 0.23$) nor aspect ($F = 0.39$; df = 7; $P = 0.90$) were significantly associated with the number of nymphs collected via drags per site (Fig 3a, b). Adult abundance varied significantly in relation to overall site aspect ($F = 2.25$; df = 7; $P = 0.0422$); however, when analyzed using a least significant difference comparison of means procedure no significant differences between specific aspects were identified (Figure 2.2). There was no significant difference in adult abundance in relation to habitat type ($F = 0.35$; df = 3; $P = 0.7905$) or soil group ($F = 0.47$; df =7; $P = 0.8499$) (Figure 2.2).

Drag Collection Continuous Variables. Results of the overall ANCOVA model investigating habitat factors affecting drag-counts are shown in Table 2.3. Nymph abundance did not vary significantly in relation to differences in temperature ($F = 2.08$; df = 1; $P = 0.1537$), relative humidity ($F = 0.19$; df = 1; $P = 0.6333$), barometric pressure ($F = 0.44$; df = 1; $P = 0.5115$), or hunter observation data ($F = 1.73$; df = 1; $P = 0.1929$) (Figure 2.3). Nymphs were most frequently collected with drags at sites with a mean of 28.59% ground cover (± 5.71; 95% CI:

17.17% to 40.01%) (F = 4.44; df = 1; P = 0.039) (Figure 2.3). Adults were most frequently collected with drags at sites with 31.69% ground cover (± 9.42; 95% CI: 12.85 to 50.53) (F = 5.67; df = 1; P = 0.021) (Figure 2.4). Adult *A. americanum* were also most frequently collected with drags at temperatures ranging from 27.81°C to 30.10°C; the mean temperature was 28.96°C (± 0.575) (F = 9.30; df = 1; P = 0.0034) (Figure 2.4). No significant difference in adult collections with drags in relation to relative humidity (F = 0.22; df = 1; P = 0.6422), barometric pressure (F = 2.47; df = 1; P = 0.1212), or hunter observation data (F = 0.03; df = 1; P = 0.8541) (Figure 2.4).

CO_2-Baited Trap Discrete Variables. Nymph abundance in CO_2 traps was significantly higher (F = 2.31; df = 7; P = 0.0358) at sites with soil groups 2 (73.8 ± 37.0) and 8 (57.5 ± 27.6), which were deep, moderately drained soils with medium to sandy texture, and located on upland or gullied landscapes (Figure 2.2). No significant difference in nymphal CO_2 collections was evident in relation to habitat type (F = 1.15; df = 3; P = 0.3343) or aspect (F = 0.90; df = 7; P = 0.5154) (Figure 2.2). Similarly there was no significant difference adult tick CO_2 collections in relation to soil group (F = 0.63; df =7; P =0.7321), habitat type (F = 1.27; df = 3; P = 0.2913), or aspect (F = 0.56; df =7; P = 0.7849) at our sites (Figure 2.2).

CO_2-Baited Collection Continuous Variables. Results of the overall ANCOVA model investigating habitat factors affecting CO_2 trapping counts are given in Table 3. Significantly more nymphs (F = 6.88; df = 99; P = 0.010) and adults (F = 6.22; df = 99; P = 0.0153) were collected by CO_2 trapping at sites with a mean ground cover of 36.49 (± 9.31; 95% CI: 17.87% to 55.11%) for nymph abundance (Figure 2.3) and a mean ground cover of 21.5(±5.52; 95% CI:

10.46% to 32.54%) for adult abundance (Figure 2.4). There was no significant effect of deer abundance data (measured by hunter observations) on either nymph (F = 0.29; df =1; P = 0.59) (Figure 2.3) or adult (F = 0.48; df = 1; P = 0.49) CO_2 collections (Figure 2.4).

Discussion

The results of our five month 2012 field season indicated that *A. americanum* is the most abundant tick species at AMES in southwestern Tennessee from May through September. This finding is consistent with previous tick studies conducted in Tennessee, all of which identified *A. americanum* as the most abundant [4], [34], [35]. In our study, seasonal activity of *A. americanum* varied by life stage such that nymphal activity was bimodal with peak activity in June and late August. Adult activity peaked in May using vegetation drags and June when using CO_2-baited collections. Previous work in the southeast investigating the seasonal activity of *Amblyomma* species corroborate our findings that *A. americanum* nymph activity was bimodal with initial peaks in May/June and second peaks in August, and that adult activity peaked from April through July [2], [36]. June was the first month which CO_2 trapping was conducted at all 100 sites, so a comparison between sampling methods could only be made for the period June-September.

Over this period, CO_2-baited trapping was the more efficient means of collecting adult *A. americanum*, but vegetation drags were the more efficient means of collecting nymphs. These findings are consistent with previous studies evaluating efficacy of dry ice-baited traps using mark and recapture techniques that found adult *A. americanum* travelling further towards (and in higher numbers) a dry ice attractant than nymphs, regardless of habitat type [23].

Nine environmental variables were investigated in relation to nymph and adult count data, but two of these — "percent ground cover" and "basal area" — were highly correlated so they could not both be included in our ANCOVA model. Of the two, percent ground cover alone contributed the greatest strength to our predictive model and so the basal area factor was not analyzed further. Tick count data for vegetation drags and CO_2-baited collections were analyzed separately to investigate life stage differences and to gain a broader understanding of environmental variables that influence each sampling method's efficacy. Percent ground cover was found to be a significant predictor for both nymphal and adult tick collections regardless of sampling method. The greatest numbers of ticks were collected at sites with 10% to 55% ground cover; this level of ground cover was indicative of forest type habitats sampled during our study. Previous studies conducted on tick rates from drag cloths over time and distance reported that the number of ticks able to remain attached to the vegetation drags in lower ground cover is three times as high as those which remain on drags in high ground cover situations [18]. This trend is consistent with our findings, lower ground cover (indicative of forested sites in our study) have higher overall collections of ticks. For CO_2-baited collections, previous studies have revealed a bias towards forested habitat types, which is consistent with our findings that forested habitats (indicative of 10-55% ground cover in our study) yield significantly more *A. americanum* than open field habitat types (indicative of >95% cover in our study) [18], [33].

Temperature was found to have a significant effect on adult tick presence, with 95% of tick collections occurring at temperatures from 27.81 °C to 30.10 °C. Our results are consistent with previous studies implicating micro-scale variables (such as temperature as stressors) influencing

vegetation drag sampling through the restriction of free-living tick questing behavior and availability [19], [33], [37]. Dragging success for nymphs was significantly lower at grassland sites than in any other habitat type sampled during our study. There are numerous studies that have evaluated the efficacy of sampling methods by habitat type and found results consistent with ours: i.e., that low numbers of ticks are collected from open field, hay meadow, and grassland sites and this was confirmed in Oklahoma [15], New Jersey [19], and Michigan, Illinois, and Wisconsin [38]. Specifically for CO_2-baited collections, soil groups that are deep and moderately drained with medium silty or sandy textured soil and located on upland or gullied landscapes collected significantly more nymphs. Previous studies investigating the effect of soil moisture and texture on the number of ticks collected found soils with good moisture content and a sandy or silty soil texture to have significantly higher tick abundance in Michigan, Illinois, and Wisconsin [38], Mississippi [39].

Conclusions

We documented peak activity of different free-living life stages of ticks collected by various conventional tick-sampling methods and found that the peak activity for nymph and adult *A. americanum* did not differ between collection methods. Sampling methodologies were evaluated to determine most efficient trapping method for nymph and adult free-living *A. americanum* and to investigate the precise nature of each sampling method. Considerations in our analysis were made for environmental variables like habitat type that have a direct relationship to tick behavior and have a possible confounding effect on sampling protocols. Results from this study indicate that sampling method, site locations, and seasonality may alter the relative numbers and life stages of *A. americanum* that are collected. This finding is consistent with previous work that

suggests using multiple sampling methods when estimating the relative abundance of wild-caught *A. americanum* [19]. It is critical that we take into account these variables with future tick and tick-borne pathogen studies because they will lead to the improved efficacy of trapping methodologies and development of more cost effective pathogen surveillance in the fight to prevent future human and animal tick-borne illness cases.

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Appendices

Table 2.1: Environmental variables included in the statistical analysis of *A. americanum* adult and nymphal habitat preferences at Ames Plantation in southwestern Tennessee.

Variable	Attributes	Agency	Means of Attainment	Effect on *A. americanum* Abundance
		Discrete or Categorical Variables		
Aspect	Aspect Dataset	USDA	Geospatial Gateway: http://datagateway.nrcs.usda.gov	No effect on nymphs or adults
Habitat Type	Tennessee Land Cover Database	Tennessee GIS	Tennessee Spatial Server: http://www.tngis.org/	Drag: nymphs fewest in grassland sites adults no effect
Soils	Hardeman and Fayette County Soil	U.S. Geological Survey	Personal Communication with Ames Plantation Flower's 1960, Thomas 1987	CO_2: nymphs abundant in soil types 2 & 8 adults no effect
		Continuous Variables		
Temperature	Temperature (^{o}C)	Nielson Kellerman Boothwyn, PA	Field collected with 3500 Kestrel Unit	Drag: adults associated with 28.96 ± 0.58^{o}C
Relative Humidity	Humidity (%)	Nielson Kellerman Boothwyn, PA	Field collected with 3500 Kestrel Unit	No effect on nymphs or adults
Barometric Pressure	Barometric Pressure (hPa)	Nielson Kellerman Boothwyn, PA	Field collected with 3500 Kestrel Unit	No effect on nymphs or adults
2012 Hunter-deer Observation	Deer observed per site (#)	Not Applicable	Personal Communication with Ames Plantation	No effect on nymphs or adults
Ground Cover	Ocular estimation of vegetation density (%)	Not Applicable	Field collected with ocular estimation	Drags: nymphs associated with 28.59 ± 5.71 and adults 31.69 ± 9.42 CO_2: nymphs associated with 36.49 ± 9.31 and adults 21.5 ± 5.52

Table 2.2. Soil grouping system proposed by Flowers (1960) and Thomas (1987) for Hardeman and Fayette County soils. This system groups soil types with respect to their drainage, texture, and tree suitability.

Soil Type	Attributes	Plant Growth	Example
Group 1	Well drained and moderately well drained, medium textured soils that are permeable in the subsoil	Woodland tree suitability: Upland oaks, Yellow poplar, Black Walnut, Loblolly pine, and shortleaf pine	Memphis silt loam
Group 2:	Deep moderately well drained, medium textured soils that have a fragipan, and occur on uplands and terraces;	Woodland tree suitability: Loblolly pine, shortleaf pine, and upland oaks	Lexington silt loam
Group 3:	Deep somewhat poor to poorly drained, silty soils on upland flats and on terraces	Woodland tree suitability: Red oak and loblolly pine	Calloway silt loam
Group 4:	Deep well drained to moderately well drained, medium texture soils on bottomlands;	Woodland tree suitability: yellow poplar, black walnut, loblolly pine, and cottonwood.	Henry silt loam
Group 5:	Deep to moderately deep, excessively drained, medium to coarse textured soil on bottomlands over silty alluvium sandy alluvial land	Woodland tree suitability: Cottonwood	Gullied land complex
Group 6:	Deep somewhat poorly drained to poorly drained soils of varied texture on bottomlands	Woodland tree suitability: Cypress	Waverly Silt loam
Group 7:	Deep sandy soils on uplands	Woodland tree suitability: loblolly pine and shortleaf pine.	Grenada-Gullied land complex
Group 8:	Gullied sand and silty soils	Woodland tree suitability: loblolly pine and shortleaf pine.	Gullied Land

Table 2.3: The effect of biotic and abiotic factors on the mean abundance per site of nymphal and adult *A. americanum* collected by vegetation drags and CO_2-baited traps at Ames Plantation Research and Education Center in southwestern Tennessee. Continuous variables were tested together using ANCOVA. Categorical variables were tested independently of each other using one-way ANOVA. Raw data were log-transformed before analysis. Factors were considered significant at alpha <0.05 (*) and <0.02 (**).

	Nymph		**Adult**	
Factor	**Drag**	**CO_2-baited**	**Drag**	**CO_2-baited**
Continuous Variables (Co-efficient)				
Ground Cover	0.0041*	0.0065**	0.0038*	0.0062**
Deer Observed	0.0016	-0.0008	0.0001	0.0009
Atmospheric Pressure	-0.0139	-0.0327	-0.0282	-0.0028
Relative Humidity	0.0021	-0.0003	-0.0020	-0.0018
Temperature	-0.0207	-0.0181	-0.0384*	-0.0029
Categorical Variables (*P-value*)				
Aspect	0.9034	0.7117	0.0422*	0.8089
Habitat	0.0472*	0.3990	0.3598	0.3245
Soils	0.2296	0.0428*	0.7380	0.8224

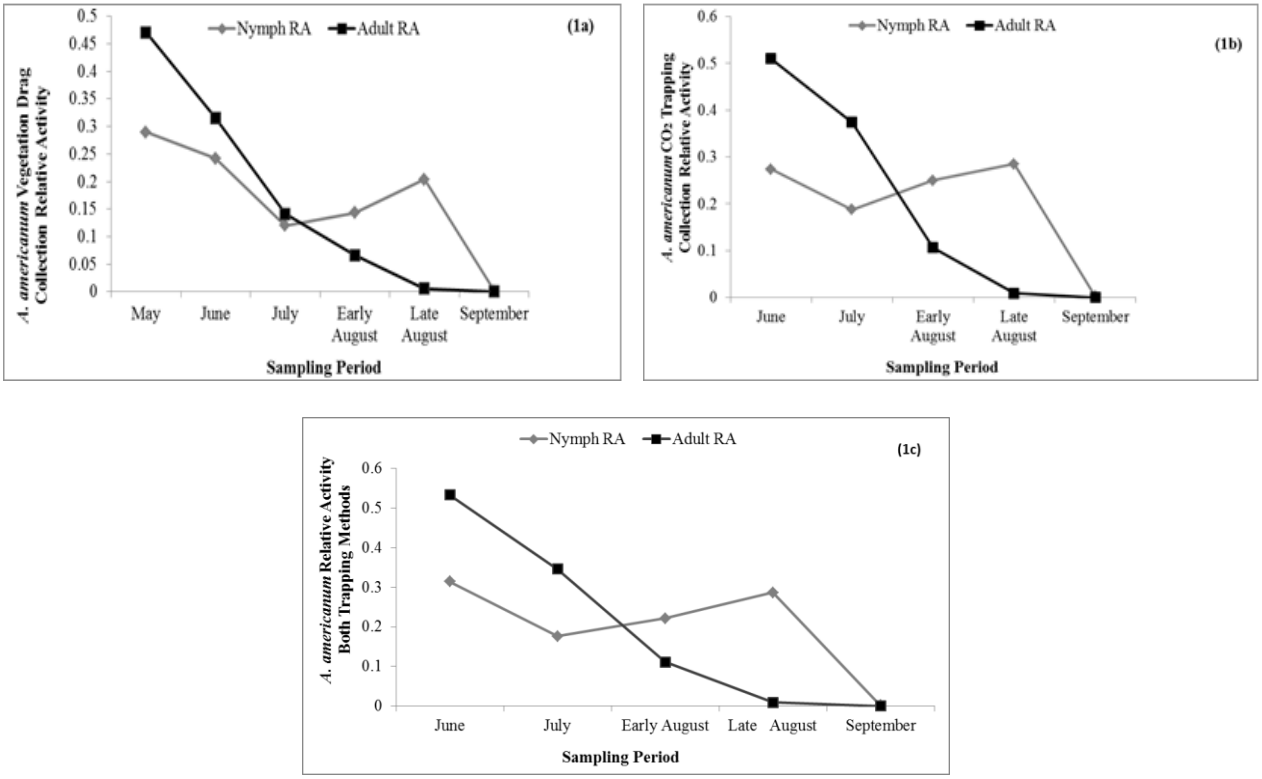

Figure 2.1: Relative activity of *A. americanum* collected at Ames Plantation Research and Education Center May-September 2012 using vegetation drags (1a), June-September using CO_2 trapping (1b) and combined (1c).

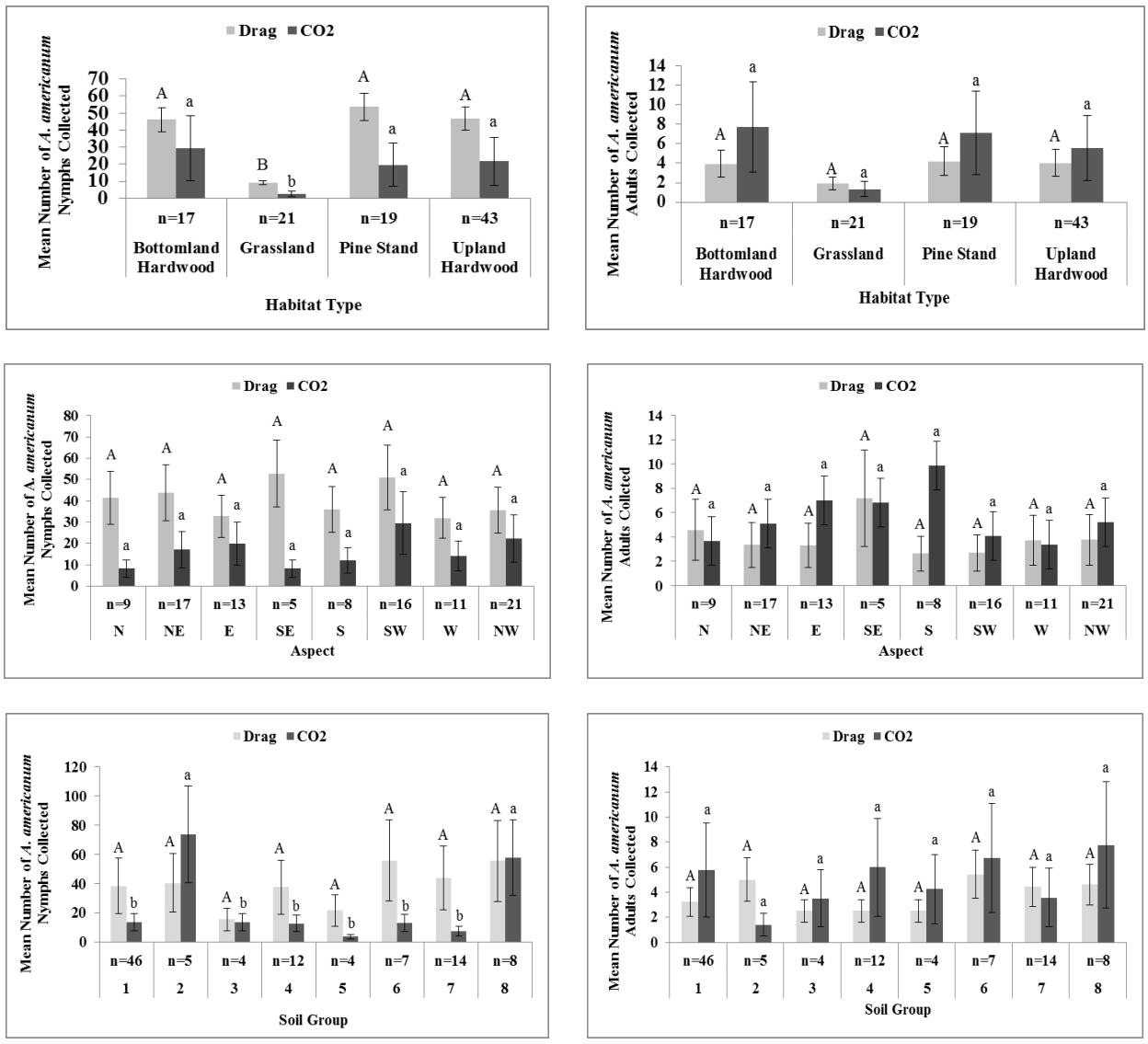

Figure 2.2: Variation by habitat type, aspect, and soil group in the mean number of nymphs (Left) and adults (Right) drag and CO_2-sampled per site from May –September 2012 at Ames Plantation. Means that differ significantly, using a LSD comparison procedure with an alpha of 0.05, are indicated with different letters. The number of sites (n) in each attribute is listed above the attribute

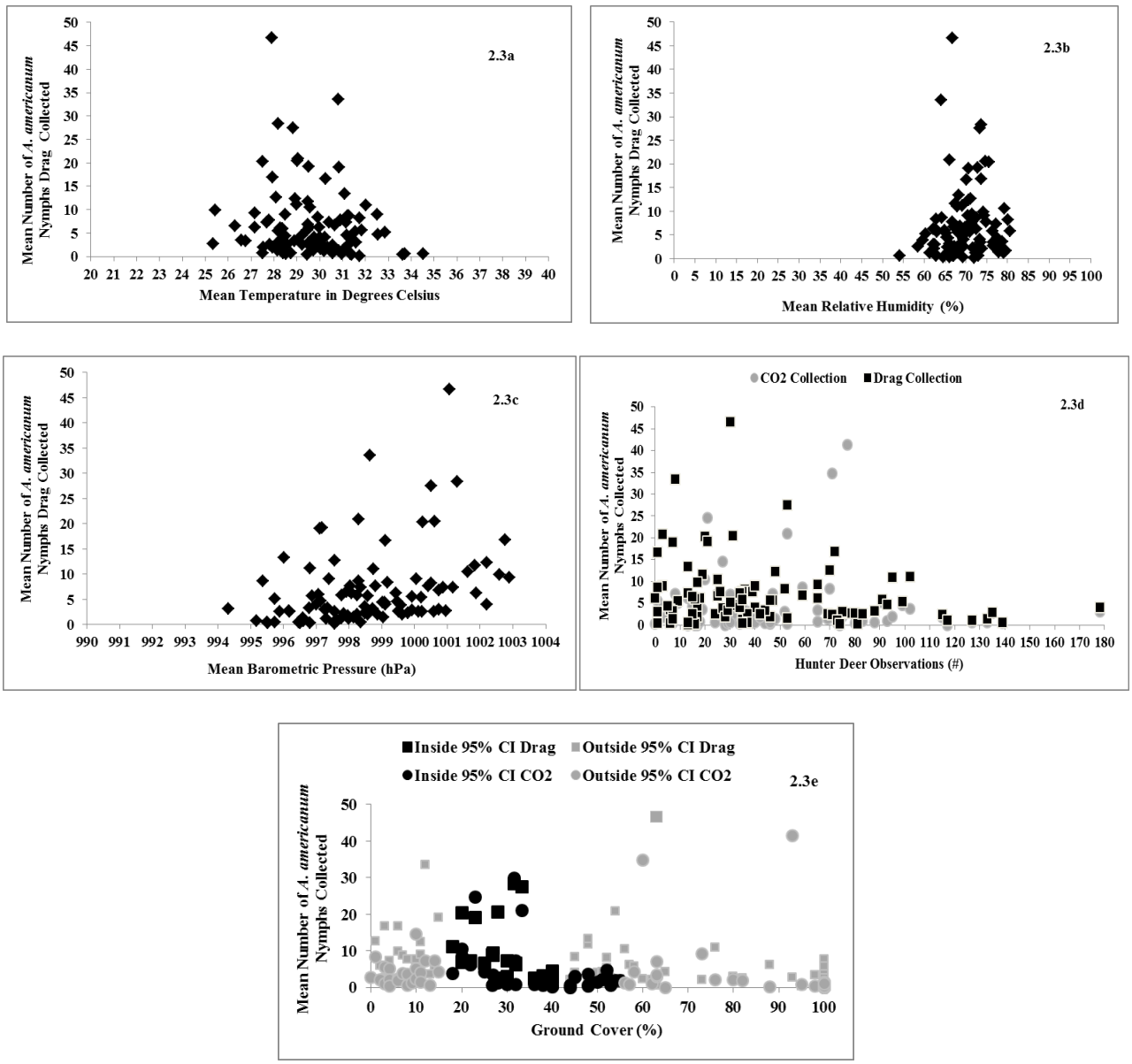

Figure 2.3: Scatterplots of nymphal *A. americanum* drag-counts in relation to a) temperature, b) relative humidity, c) barometric pressure. Both trapping methods drags (black squares) and CO_2collections (light gray circles) are shown in scatterplots for d) deer observations, and e) 95% CI were calculated for the significant interaction between nymphs collected for both trapping methods in relation to ground cover from May-September 2012 ($P >> 0.05$).

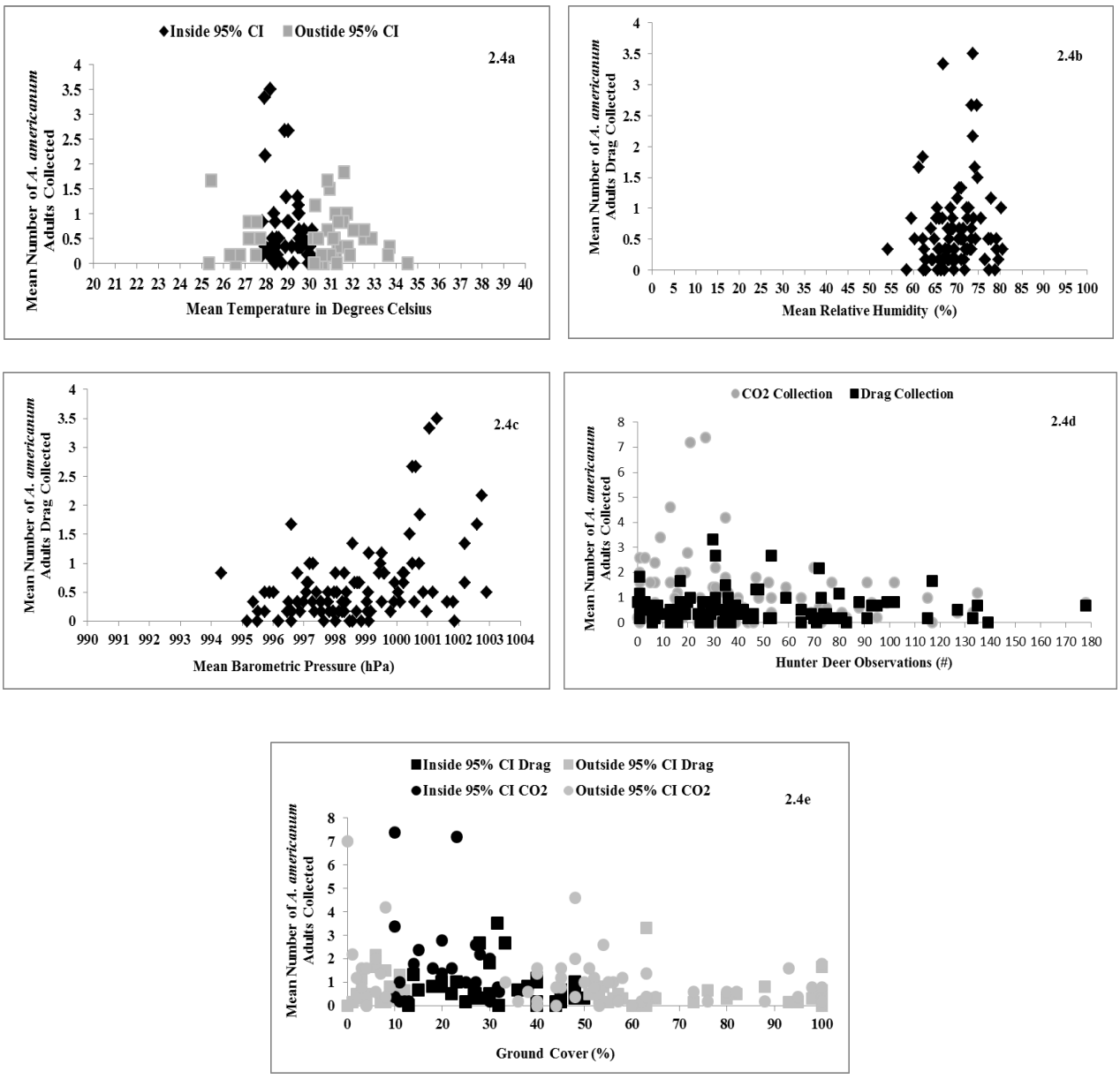

Figure 2.4: Scatterplots of adult *A. americanum* drag-counts in relation to a) 95% CI were calculated for the significant interaction between adults collected by drags in relation to temperature. Scatterplots of non-significant weather variables and drag collected adult *A. americanum* b) relative humidity, c) barometric pressure. Both trapping methods drags (black squares) and CO_2collections (light gray circles) are shown in scatterplots for d) deer observations, and e) 95% CI were calculated for the significant interaction between adults collected for both trapping methods in relation to ground cover from May-September 2012 ($P >> 0.05$).

3 Identity and Prevalence of Bacterial Pathogens Associated with *Amblyomma americanum* L. at Ames Plantation in Southwestern Tennessee

Abstract

The reported incidence of tick-borne disease in the southeastern U.S. has increased in the last decade and continues to cause serious human illness or death when left untreated. Previous work in eastern Tennessee implicated Lone Star ticks, *Amblyomma americanum* in the transmission of *E. chaffeensis* Ehrlichiosis (formerly Human Monocytic Ehrlichiosis; HME) as well as Spotted Fever Group Rickettsiosis (SFGR). Consequently, the objective of this study was to identify the different species of *Ehrlichia* and *Rickettsia* associated with *A. americanum* and each pathogen's potential temporal and spatial distribution at Ames Plantation Research and Education Center in western Tennessee. Ticks were collected monthly from May to September 2012, using vegetation drags and CO_2 traps. Associations between expected and observed prevalence, habitat type, collection month, and site differences were evaluated using a chi square analysis and *P* values were adjusted with Bonferroni's correction. Moran's I values were calculated to test for spatial autocorrelation of pathogen-positive ticks in specific habitats. Of the 925 adult *A. americanum* screened for *Ehrlichia* and *Rickettsia* bacteria, 1.8% (n = 17) and 38% (n = 353) were PCR positive respectively, of which eight ticks (0.08%) were positive with both pathogens. All sequenced *Rickettsia* were identified as being *R. amblyommii*. The 17 *Ehrlichia* positives were identified as 12 *E. ewingii* (GenBank AF195273), one *E. chaffeensis* (GenBank L10917), two Panola Mountain *Ehrlichia* (GenBank HQ658904), and two *Anaplasma odocoilei* (GenBank JX876642). The identification of pathogens and co-infections within *A. americanum* from western Tennessee warrants further investigations to understand the role ticks and their environment have in the distribution of tick-borne diseases (TBD). Such research can provide insights into improving best management practices and inform the public on how to better protect themselves from ticks and tick-associated pathogens.

Key Words: *Ehrlichia*, *Rickettsia*, *Amblyomma americanum*, and Tennessee

Introduction

Tick-borne diseases (TBD's) have increased in incidence in the last decade and continue to cause serious human illness and death when left untreated [1],[2],[3]. While residents of the southeastern United States have minimal risk of exposure to *Borrelia burgdorferi*,the causative agent of Lyme disease [4],[5], other pathogens present in the region result in significant numbers of TBD cases being reported each year [4],[5],[6]. In the southeastern United States, the two TBDs of most concern are ehrlichiosis (*Ehrlichia* species) and rickettiosis (*Rickettsia* species) [5],[6],[7],[8]. Although distinctly different in their etiology and epidemiology, the bacteria *Ehrlichia* and *Rickettsia* are morphologically and genetically similar and invoke diagnostically similar symptoms in infected humans [3]. These non-*Borrelia* bacteria are often unidentified because many clinicians are unfamiliar with ehrlichiosis and rickettiosis [7],[9]. The most characteristic symptom of Lyme disease is an erythema migrans (bulls-eye) rash; however, for ehrlichiosis a skin rash is not considered diagnostic and is only found in 30% of human cases and for rickettiosis 80% of patients will present with non-itchy "spotted" rash [10].

The majority of ehrlichiosis and rickettiosis cases are reported from the southeast and encompass Missouri, Oklahoma, Tennessee, Arkansas, and Maryland [3]. The bacterial agents that cause ehrlichiosis and rickettiosis are transmitted by a variety of tick species including the Lone Star tick, *Amblyomma americanum* L. [3]. Prior to the recognition of human ehrlichiosis in 1986, *A. americanum* was regarded primarily as an economically important nuisance species [7], [11]. The tick also transmits E. *ewingii*, Panola Mountain *Ehrlichia,* and *E. chaffeensis,* all of which have been recently implicated in human ehrlichiosis cases [11],[12],[13]. While *A. americanum*

is not an efficient vector of *R. rickettsii* (the causative agent of Rocky Mountain Spotted Fever) [14], other Spotted Fever Group Rickettsiae (SFGR) may be transmitted by *A. americanum* [5].

While Tennessee accounts for only 2.4% of reported Rocky Mountain Spotted Fever (RMSF) cases in the United States, these cases represent 26% of fatal or severe cases nationwide; suggesting by extension that Tennessee appears to be a hotspot for clinically severe cases [15]. Human exposure to tick-borne rickettsial agents is positively correlated with the presence of canines living in close proximity to humans [16], [17]. In addition to canines directly introducing infected ticks into the home, handlers can also encounter tick-borne rickettsial agents by removing engorged ticks from their pets [18]. Studies investigating the seroprevalence of *Ehrlichia* and *Rickettsia* species in dogs found prevalence's as high as 20.6% from South Carolina [19] and 83% from North Carolina [20].

In 1993, an outbreak of human ehrlichiosis in an east Tennessee retirement community led to an investigation of *A. americanum*, the main vector of *E. chaffeensis.* [21]. Results from the study investigated the two fold increase (10 cases per 100,000 people) over what is typically reported in Tennessee (3-5 cases per 100,000 people) for human ehrlichiosis cases and determined that the reason for the increase in case numbers was due to the close proximity of the retirement village to a wildlife reserve [21]. In 2009, a follow up study conducted at this same east Tennessee retirement community reported *E. ewingii* (6.03%), Panola Mountain *Ehrlichia* (1.72%), and *E. chaffeensis* (0.86%) in the screened *A. americanum* [9]. Vegetation drags were the primary means of collection for that particular study. Additional studies conducted in Tennessee identified *E. ewingii* (0.8%) and *E. chaffeensis* (2.6%) from 309 *A. americanum* [22]

and detected 40% *R. amblyommii* and 0.3% *R. montana* from 655 *A. americanum* collected [8] using vegetation drags and/or mammalian hosts. Dry ice-baited traps have yet to be evaluated as a potentially more cost-efficient method for TBD surveillance in this state. This study attempts to improve monitoring protocols and further our understanding of the presence of tick-borne disease in Tennessee. To address this need our objectives were: (1) to screen questing *A. americanum* for *Ehrlichia* and *Rickettsia* species, (2) to investigate the seasonal occurrence of *Ehrlichia* and *Rickettsia* species, (3) to characterize pathogen-positive tick habitat type, and (4) to compare collection methods for *Rickettsia*- and *Ehrlichia*-positive *A. americanum* collected from a large land holding in southwestern Tennessee to test the hypothesis that *A. americanum* is intimately involved in the maintenance of tick-borne *Ehrlichia* and *Rickettsia* agents in southwestern Tennessee.

Materials and Methods

Tick Collection and Identification. Ticks were collected from the 74.5 km^2 Ames Plantation Research and Education Center (AMES) (35.11 N, -89.21 W) in southwestern Tennessee. A total of 100 widely dispersed, randomly chosen sites were sampled six times (May-September 2012) between the hours of 700-1900. Collections were conducted using vegetation drags for ticks questing on vegetation [23] and dry ice-baited traps for ticks questing at ground level [23],[24]. Vegetation drags were constructed using a 1 m^2 piece of corduroy cloth and consisted of six 30 second drags in an approximate 0.12 km transects representing a singular habitat type. Dry ice-baited traps consisted of a 1.9 L cylindrical cooler (Igloo Corporation Katy, TX) with seven holes drilled around the circumference and filled with approximately 2.3kg of dry ice. The dry ice then sublimated from the holes as CO_2, a known tick attractant [23]. Dry ice-baited traps

were placed on a 1 m^2 piece of light-colored duck cloth at each site for a minimum of 12 hrs. All ticks attached to vegetation drags and attracted to the CO_2 were stored in vials with 80% ethanol and later identified to species, sex, and life stage using morphological keys [25] at the Medical and Veterinary Entomology lab at the University of Tennessee Institute of Agriculture, Knoxville.

DNA Extraction and Pathogen Identification. A total of 925 adult *A. americanum* representing 98% of the total adult *A. americanum* collected were screened for *Ehrlichia* and *Rickettsia* pathogens. Prior to DNA extraction, each tick was rehydrated in Milli-Q Water (EMD Millipore, Billerica, MA) for a minimum of 4 hours to prevent ethanol from disrupting the extraction process. Each specimen was then longitudinally dissected using a scalpel with blades sterilized using a Hot Glass Dry Bead Sterilizer (Fisher Scientific, Pittsburgh, PA). The left midsagittal plane was stored as a voucher specimen and the right midsagittal plane underwent total DNA extraction using the Fermentas Gene Jet Genomic DNA Purification Kit and protocol (Thermo-Fisher Scientific, Pittsburgh, PA) which yielded a 200 ul final product of genomic DNA. Once extracted the total genomic DNA (tick and potential pathogen/host) was stored at -20°C until pathogen detection via polymerase chain reaction (PCR). Extraction, PCR, and gel electrophoresis were each conducted in different labs with equipment specific to each procedure to prevent contamination. To screen for each pathogen, separate pathogen specific master mixes were made under a laminar flow hood (LabConco, Kansas City, MO) using Maxima Hot start (Thermo-Fisher Scientific, Pittsburgh, PA) with *Ehrlichia* specific primers [26] and *Rickettsia* specific primers [27]. PCR reactions used the previously published *Ehrlichia* [26] and SFGR [27] protocols. Within each 96 well plate, eight positive and eight negative controls (nuclease free water) were used as indicators for optimal PCR conditions as well as indicators for potential

contamination. Positive controls were obtained from the Vector-Borne Disease Section Tennessee Department of Health and consisted of DNA extractions from ticks having previously tested positive for *R. amblyommii* and *E. chaffeensis*. To save time and resources, 5 ul of 10 tick DNA extraction final products were pooled into a single tube (~50 ul pooled DNA), and 5 ul of that pooled DNA sample was used for primary screening for each pathogen. If the pool was positive, each tick from that positive pool was then individually screened for each pathogen. Following each PCR reaction, 8 ul of PCR product were loaded onto a 1.5% agarose gel (1x TAE buffer with ethidium bromide) and subjected to gel electrophoresis for two hours at 120 V. To verify the PCR amplicon size a 100 bp molecular ladder (Life Technologies, Carlsbad, CA) was with the samples. The gel was visualized using ChemiDoc^{TM} Universal Hood (Bio-rad, Hercules, CA). *Ehrlichia* PCR products that produced an amplicon for *Ehrlichia* at ~ 350 bp were bidirectional sequenced at the University of Tennessee Division of Biology Sequencing Facility (MBRF) to confirm positivity and identity. *Rickettsia* PCR products that produced the ~510 bp amplicon were subjected to a species specific restriction digest using *PST I* and *RSA I* restriction enzymes [8],[27]. Digested samples with different amplicon patterns from the positive control (*R. amblyommii*) were also bi-directionally sequenced at MBRF for *Rickettsia* species identity (n =54) and 54 that were identified as *R. amblyommii* from the digest. Resulting sequence reads were aligned in Sequencher 5.1 (Gene Codes Corporation Ann Arbor, MI) and compared with homologous GenBank sequences in Bio-edit 7.2.0 (IBS Biosciences Carlsbad,CA). Phylogenetic trees were assembled using BEAST v1.0 software package and the consensus tree was generated in FigTree 1.4.0 [28].

Statistical Analysis. Pathogen prevalence was calculated by dividing the number of PCR positive-samples by the total number of adult *A. americanum* screened. Contingency tests (chi-square) were used to compare pathogen prevalence, monthly prevalence, habitat prevalence, number of positive sites, and efficacy of trapping methods for *Ehrlichia*- and *Rickettsia*-positive adult *A. americanum* collected. Bonferroni's correction was used to adjust calculated *P* values to prevent type I error caused by the various comparisons. Lastly Moran's I z values were calculated for pathogen positive sites to test for spatial autocorrelation. A k-nearest neighbors spatial weight (n= 4) was used for our pathogen positive site evaluation after examining the spatial weight histogram and determining no islands existed in the connectivity of our data.

Results

Pathogen Identification. Of the 925 adult *A. americanum* screened 1.8% (17/925) were PCR positive for *Ehrlichia,* 38% (353/925) were PCR positive for SFGR, and 0.08% (8/925) were PCR positive for both bacteria. Four different *Ehrlichia* species were identified in adult *A. americanum* at 13% (13/100) of sites sampled (Figure 3.1). Of the 17 *Ehrlichia* positives, 12 were 97-100% homologous to *E. ewingii* (GenBank AF19527), 1 was 99% homologous to *E. chaffeensis* (GenBank L10917), 2 were 100% homologous to Panola Mountain *Ehrlichia* (GenBank HQ658904), and 2 were 99% homologous to *Anaplasma odocoilei* (GenBank JX876642) (Table 3.1). All 353 positive SFGR were identified as *R. amblyommii* via restriction digest (n = 353) and confirmation sequencing (n = 54). Of the 54 sequenced SFGR, seven different sequence variants were characterized (Table 3.2). These seven variants were homologous with previously published GenBank sequences 97-100% EF450696 and 96-100% HM446484 (Figure 2.2), and were identified at 79% (79/100) of the sites sampled (Figure 2.3).

Only 8 of the 925 (0.08%) adult *A. americanum* were PCR positive for both *Ehrlichia* and *Rickettsia;* 4 tested positive for *E. ewingii* and *R. amblyommii,* 2 tested positive for Panola Mountain *Ehrlichia* and *R. amblyommii,* and the other two co-infections were not sequenced due to time constraints.

Pathogen Characterization. We first calculated the expected prevalence as 8.6% for *Ehrlichia* [9] and 40% for *Rickettsia* [8]. The number of expected positive ticks was then calculated using the given prevalence [29]. The 1.8% observed prevalence of *Ehrlichia* in adult *A. americanum* was significantly lower (X^2 = 42.23; df = 1; $P < 0.001$) than the 8.6% expected prevalence reported previously for eastern Tennessee [9]. However, there was no significant difference (X^2 = 0.07; df = 1; $P = 0.2463$) between the observed prevalence of *R. amblyommii* (38%) in field collected *A. americanum* from southwestern Tennessee and the 40% expected prevalence of *R. amblyommii* in *A. americanum* for Tennessee [8]. We observed eight *Ehrlichia* and *Rickettsia* co-infections in adult *A. americanum* tested, which is close to the six that would be expected if the two pathogens occur independently of one another in these ticks (i.e., 925*0.383*0.018).

Based on Bonferroni's correction, seasonal comparisons that yielded a *P* less than 0.012 were considered statistically significant. Significantly more *Ehrlichia*-positive *A. americanum* adults were collected in July (X^2 = 14.64; df = 3; $P = 0.002$) than any other month, but there was no significant relationship (X^2 = 5.58; df = 3; $P = 0.134$) between collection month and number of *R. amblyommii*-positive ticks collected (Table 3.3). No significant difference was detected between the number of positive *A. americanum for Ehrlichia*- (X^2 = 6.45; df = 3; $P = 0.09$) or *R. amblyommii*- (X^2 = 1.80; df = 3; $P = 0.6149$) and habitat type sampled (Table 3.4). Based on Moran's I, a test that identifies how attributes are distributed in space and whether they are

clustered or randomly dispersed, sites that were positive for *E. ewingii* (Z = -0.0111), *E. chaffeensis* (Z = -0.0076), Panola Mountain *Ehrlichia* (Z = 0.2398), and *A. odocoilei* (Z = -0.0230) were dispersed randomly throughout our chosen sampling sites. The *R. amblyommii*-positive sites (Z = 0.0306), and those sites that were positive for both pathogens (Z = -0.15), were also dispersed randomly. Additionally, there was no significant difference by trapping method in the proportions of adult *A. americanum* positive for *Ehrlichia* (X^2 = 0.15; df = 1; P = 0.7028) or *Rickettsia* (X^2 = 0.01; df = 1; P = 0.916) (Table 3.5).

Discussion

This study evaluated the prevalence of *Ehrlichia* and *Rickettsia* on a large land-holding with a range of habitats representative of southwestern Tennessee, with a specific focus on adult *Amblyomma americanum*. Post-PCR sequencing of 925 adult *A. americanum* confirmed the presence of three *Ehrlichia* (*E. ewingii*, *E. chaffeensis*, Panola Mountain *Ehrlichia*), one *Anaplasma* (*A. odocoilei*), and one *Rickettsia* (*R. amblyommii*) species. Despite the lower prevalence of *Ehrlichia* species found in *A. americanum* collected from western Tennessee, the detection of these pathogens implicates them as likely suspects in human disease cases in the area [22]. In addition to the pathology related to human and animal disease, a greater diversity of pathogenic *Ehrlichia*-related organisms was confirmed in western Tennessee than in previous studies investigating *groEL* identified *Ehrlichia* like organisms in eastern Tennessee [9], which may complicate accurate diagnosis of human and animal disease and stresses the need for proper and consistent surveillance efforts.

Although *A. americanum* has been implicated in the maintenance of Rocky Mountain Spotted Fever [5],[30],[31], no *R. rickettsii* was detected in 925 field-collected *A. americanum*. The only member of the SFG *Rickettsiae* detected was *R. amblyommii,* an SFGR considered to have little to no human pathogenicity [32]. The high (38%) prevalence of this non-pathogenic bacterium in field-collected *A. americanum* adults does not implicate this tick in the transmission of pathogenic SFGR at this time, but it does complicate accurate diagnosis of human rickettsiosis because it may be triggering false-positives in serological tests for suspected *R. rickettsii*. Tennessee is an epicenter for clinically severe or fatal cases of human rickettsiosis [15]. Finding co-infections of *Ehrlichia*- and *Rickettsia*-related organisms in field-collected *A. americanum* is of serious health concern. Aside from pathological risk that tick-borne co-infections have in human health concerns; they may also complicate proper reporting of tick-borne infections in the state [32]. Due to their similar clinical manifestations, it is likely that a patient may be diagnosed with RMSF, but actually be infected with a pathogenic *Ehrlichia*-related organism such as *E. chaffeensis* [32].

After identifying the *Ehrlichia* and *Rickettsia* species present in adult *A. americanum,* we focused on characterizing seasonal, habitat, and trapping preferences for the positive ticks. *Ehrlichia*-positive ticks had no trapping or habitat preferences, but significantly more ticks tested positive for *Ehrlichia* species in July than in other months sampled. The increase in *Ehrlichia*-positive ticks in our study is consistent with previous studies which correlate peak activity of *A. americanum* nymphs and adults in June or July to increased cases of human ehrlichiosis in the southeast [7]. The identification of pathogenic *Ehrlichia* species at AMES is cause for concern and warrants further study of the complex interactions between pathogens, ticks, hosts, and their

shared environments. Of serious concern, national award-winning canines participate in the National Bird Dog trials hosted at AMES every year from all over the United States and may acquire *Ehrlichia* and then introduce the pathogens to their home environment [33].

Conclusions

In this study, we documented the various *Ehrlichia* and *Rickettsia* species associated with *A. americanum* collected from western Tennessee. A lower prevalence of *Ehrlichia* was detected than expected, but interestingly a greater diversity was found. Habitat and seasonal considerations were applied to positive-tick collections, which led to the indication that *Ehrlichia* species are more prevalent in *A. americanum* during July than any other month sampled. Although the sample size for *Ehrlichia* positive *A. americanum* adults was low (n = 17), further research is warranted to investigate the potential contributions of other native human-biting tick species at Ames Plantation. Distributions of pathogens were investigated to describe temporal activity of tick-borne pathogens and identify locations of pathogen-positive tick clustering. Results from these analyses indicated that there was no spatial clustering among pathogen-positive sites. Additional research at AMES is necessary to explore the diversity of tick-borne bacteria present, and to investigate the extent of possible combinations of these bacteria co-infecting the human-biting tick species. The results of this study provide preliminary insight into improving surveillance protocols for pathogen-positive ticks.

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Appendices

Table 3.1. Seventeen *Amblyomma americanum* were PCR positive for the *groEL* gene and GenBank BLAST comparisons of 300 base pairs confirmed the presence of three *Ehrlichia* and one *Anaplasma* species in southwestern Tennessee.

Sequence	**% Homologous**	**No. Seq.**	**Single nucleotide variations**													
Ehrlichia ewingii																
GenBank (AF195273) (% homologous)		**N**	**4A**	**17 -**	**18 -**	**38 -**	**50 G**	**97 -**	**247 -**	**259 T**	**275 G**	**289 T**	**292 T**	**293 A**	**294 A**	**296 G**
Variant 0806		**5**	**A**	**.**	**.**	**.**	**G**	**.**	**.**	**T**	**G**	**T**	**T**	**A**	**A**	**G**
Variant 0618		**2**	**C**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**
Variant 0478		**1**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**C**	**A**	**A**	**A**	**T**	**T**	**-**
Variant 0885		**1**	**.**	**T**	**T**	**C**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**
Variant 0808		**1**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**A**	**.**	**T**	**.**	**A**
Variant 0810		**1**	**.**	**.**	**.**	**.**	**A**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**
Variant 0821		**1**	**.**	**.**	**.**	**.**	**A**	**A**	**A**	**.**	**.**	**.**	**.**	**.**	**.**	**.**
Ehrlichia chaffeensis																
GenBank (L10917)		**N**	**31 -**	**52 -**												
Variant 0822		**1**	**T**	**T**												

Anaplasma odocoilei															
GenBank (JX876642)	**N**	**13-**	**21-**	**40-**											
Variant 0820	**1**	**T**	**C**	**A**											
Variant 0893	**1**	**.**	**C**	**.**											

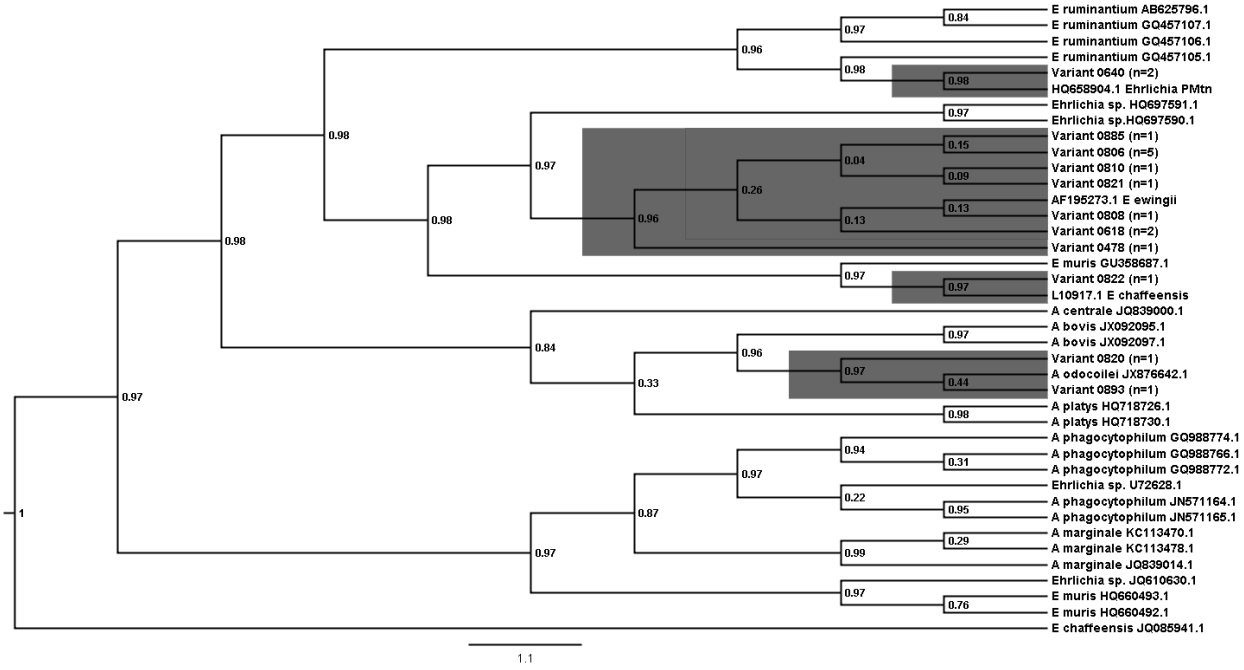

Figure 3.1: Four different pathogens were amplified from *groEL* (Takano et al. 2009, Harmon et al. 2010) within adult *Amblyomma americanum* collected at Ames Plantation Research and Education Center. Phylogenetic inferences suggest our sequence variants were homologous to previously published Panola Mountain *Ehrlichia*, *E. ewingii*, *E. chaffeensis*, and *Anaplasma odocoilei.* The dark gray highlights areas on the tree where inferences were made between our sequence variants and previously published sequences from Genebank.

Table 3.2: Seven sequence variants (510 base pairs) were identified within the 54 *Rickettsia amblyommii* amplified and sequenced from adult *A. americanum* after Spotted Fever Group *Rickettsiae* pathogen screening targeting the *ompA* gene (Eremeeva et al 1994, Harmon et al 2010).

Sequence	Accession (% homologous)	N	Single Nucleotide Variations															
			31	35	36	37	39	45	46	59	71	177	337	440	461	468	472	475
GenBank	EF450696 (100%)	NA	T	G	-	-	C	-	A	A	A	C	T	-	-	-	T	A
Variant 0009	EF450696 (100%)	48	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Variant 0206	EF450696 (99%)	1	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
Variant 0269	EF450696 (96%)	1	T	C	G	C	T	A	C	.	.	.	.	.	.	.	C	T
GenBank	HM446484 (100%)	NA	T	G	-	-	C	-	A	A	A	C	T	-	-	-	T	A
Variant 0050	HM446484 (99%)	1	.	.	.	.	.	.	.	.	.	.	.	A	.	.	A	.
Variant 0203	HM446484 (99%)	1	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
Variant 0799	HM446484 (98%)	1	A	.	.	.	.	T	.	T	.	.	.	.	A	.	.	.
Variant 0803	HM446484 (97%)	1	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.

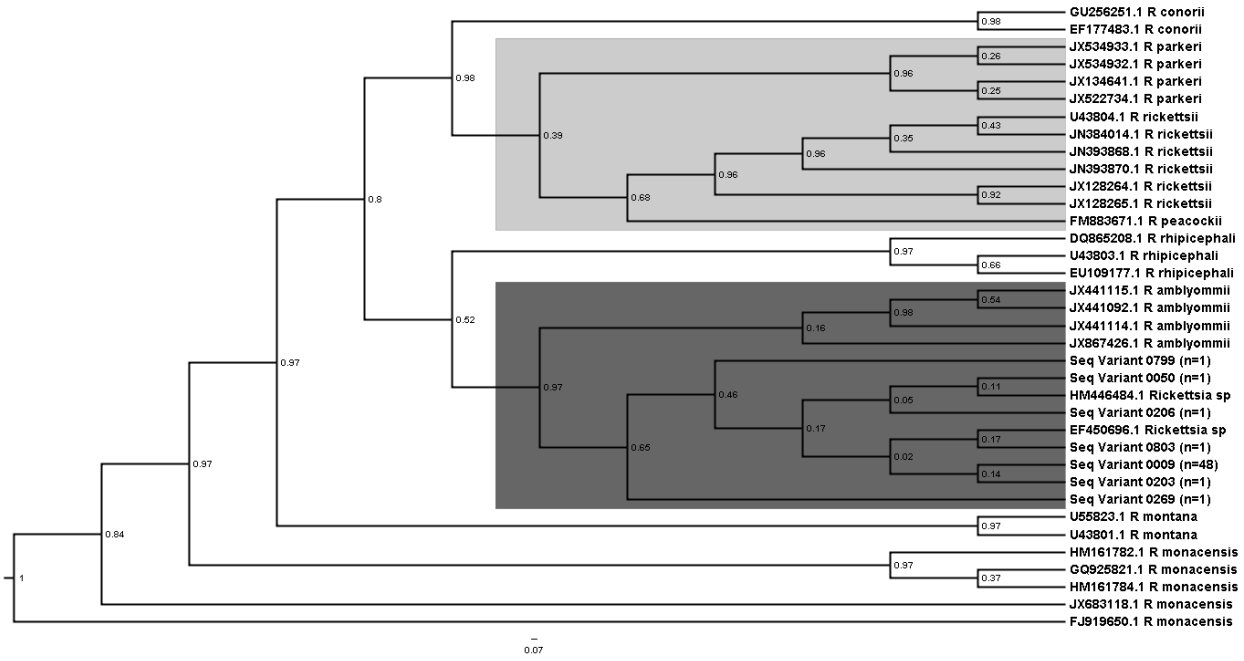

Figure 3.2: One currently non-pathogenic organism was amplified from *ompA* (Eremeeva et al 1994, Moncayo 2010) within adult *A. americanum* collected (n = 54) at Ames Plantation Research and Education Center. Phylogenetic inference suggests our sequence variants were homologous to previously published *R. amblyommii*. The light grey highlights the location of the causative agent of Rocky Mountain Spotted Fever (*R. rickettsii*) as well as an additional pathogenic SFGR member (*R. parkeri*). The dark grey highlights the area on the tree where inferences were made between our sequence variants (n = 7) and previously published sequences from Genebank.

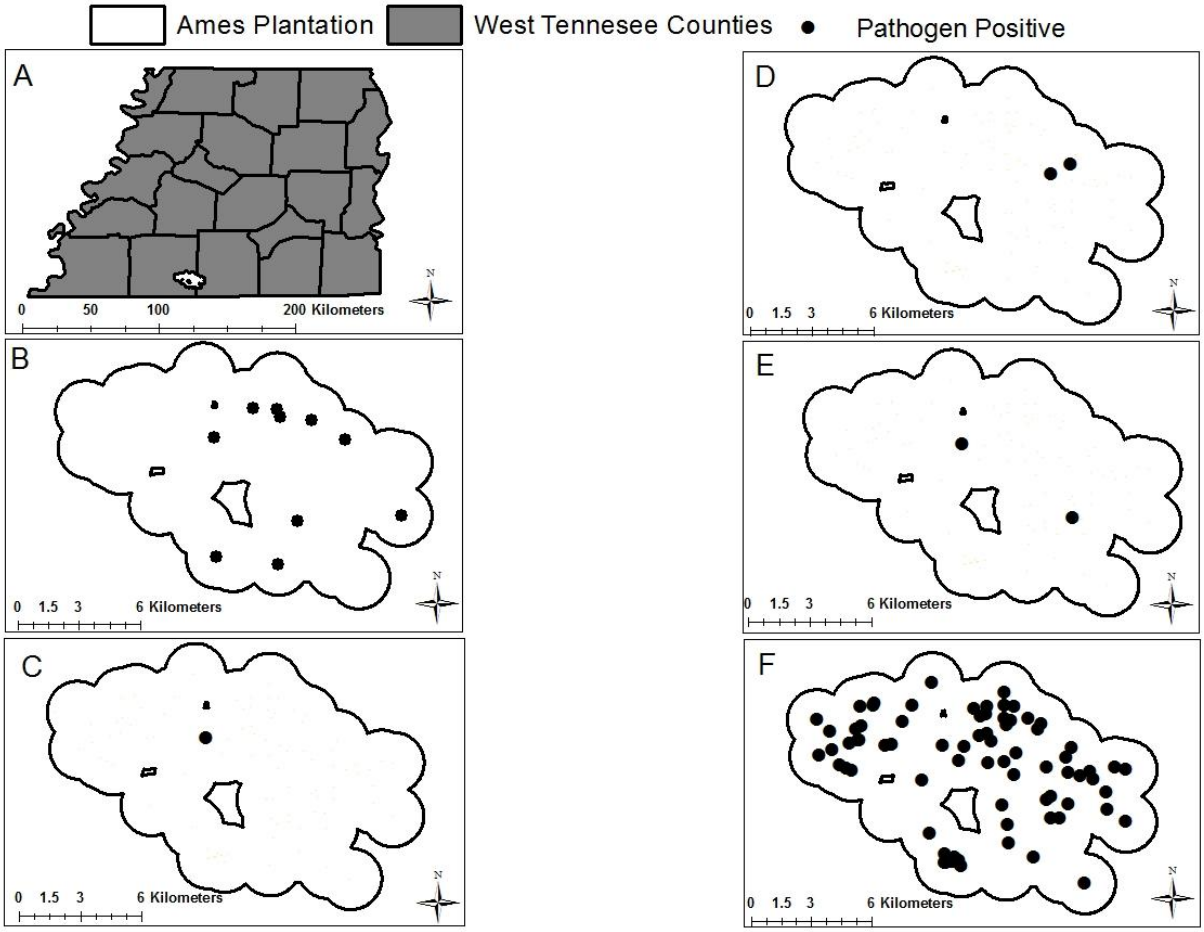

Figure 3.3: (A) Map of the counties of western Tennessee and location of Ames Plantation, (B) distribution of *Ehrlichia ewingii* positive sites (*n* = 10), (C) distribution of *Ehrlichia chaffeensis* positive sites (*n* = 1), (D) distribution of Panola Mountain *Ehrlichia* positive sites (*n* = 2), (E) distribution of *Anaplasma odocoilei* positive sites (*n* = 2), and (F) distribution of *R. amblyommii* positive sites (n=79).

Table 3.3: Seasonality and frequency of tick-borne bacteria in adult *A. americanum* tested using *ompA* and *groEL* primers for identification of *Rickettsia* and *Ehrlichia* species at Ames Plantation in southwestern Tennessee

Month	**No. Adults Collected**	**No. Adults Screened**	**No. *ompA* Positive (% Pos.)**	**No. *groEL* Positive (% Pos.)**	**No. Co-infections (% Pos.)**
May	220	214	67 (31%)	5 (2.3%)	2 (0.9%)
June	387	384	154 (40%)	0 (0%)	0 (0%)
July	251	242	97 (40%)	10 (4.1%)	5 (2.0%)
Aug	88	85	35 (41%)	2 (2.4%)	1 (1.1%)
Sept	0	0	0	0	0
Total	**946**	**925**	**353**	**17**	**8**

Table 3.4: Habitat type and frequency of tick-borne bacteria in adult *A. americanum* tested using *ompA* and *groEL* primers for identification of *Rickettsia* and *Ehrlichia* species at Ames Plantation in southwestern Tennessee

Habitat	**No. Adults Collected**	**No. Adults Screened**	**No. *ompA* Positive (% Pos.)**	**No. *groEL* Positive (% Pos.)**	**No. Co-infections (% Pos.)**
Bottomland Hardwood	203	191	79 (41%)	1 (0.5%)	0 (0%)
Pine	253	248	96 (39%)	7 (2.8%)	3 (1.2%)
Grass/Pasture	68	64	21 (33%)	3 (4.6%)	0 (0%)
Upland Hardwood	422	422	157 (37%)	6 (1.4%)	5 (2.0%)
Total	**946**	**925**	**353**	**17**	**8**

Table 3.5: Trapping method and frequency of tick-borne bacteria in adult *A. americanum* tested using *ompA* and *groEL* primers for identification of *Rickettsia* and *Ehrlichia* species at Ames Plantation in southwestern Tennessee

Trap Type	**No. Adults Collected**	**No. Adults Screened**	**No. *ompA* Positive (% Pos.)**	**No. *groEL* Positive (% Pos.)**	**No. Co-infections (% Pos.)**
Vegetation Drag	361	340	129 (38%)	7 (2.1%)	2 (0.5%)
Dry Ice Baited	585	585	224 (38%)	10 (1.0%)	6 (1.0%)
Total	**946**	**925**	**353**	**17**	**8**

4. Project Conclusions

4.1 Project Conclusions

The risk of tick-borne disease exposure in the southeastern United States is uncertain given the changing distributions of human biting tick species and their closely associated pathogens [1]. In Tennessee, *Amblyomma americanum* are the most frequently collected tick species and *Ehrlichia* are the most commonly identified zoonotic pathogens within ticks assayed in eastern Tennessee [2]. Yet the majority (>50%) of healthcare providers in the southeast describe their knowledge of human ehrlichiosis infections as "weak or none" [3]. Older physicians are aware of tick-borne illnesses such as Rocky Mountain Spotted Fever and Lyme disease both of which have effected humans for decades; however, a majority lack the formal training needed to properly diagnose and test for new or emerging pathogens such as ehrlichiosis infections [1]. Consequently, there is a dire need for increased education efforts such as distributing up-to-date outreach materials with information concerning local human biting ticks and their associated pathogens to aid physicians in the diagnosis of human tick-borne illness in the southeast region.

Like most of the southeast, patrons of Ames Plantation were reporting frequent tick bites some of which resulted in probable cases of tick-borne illness. Many of these reports were rumored to be Lyme disease a tick-borne illness closely associated with the black-legged tick (*Ixodes scapularis*). To investigate these claims and evaluate the presence of tick-borne disease at Ames Plantation we surveyed 100 sites spread throughout AMES using conventional trapping methodologies to collect human biting tick species present. We did not collect any *I. scapularis* during our 2012 summer field season, but they were present in low numbers on deer killed during hunting season. During our field season we collected *Amblyomma americanum*, *Dermacenter variabilis*, and *Amblyomma maculatum* all of which are known human biting tick

species in Tennessee [4]. Of the numerous tick species collected *A. americanum* was the most frequent; this finding is consistent with other tick trapping studies conducted in Tennessee which also found *A. americanum* as the most frequently collected human biting tick species [2],[5]. Due to the frequency of contact with humans and generalist feeding behavior *A. americanum* were used in pathogen assays to investigate the presence of pathogens at AMES. We were able to identify several known pathogenic *Ehrlichia* species from adult *A. americanum* tested and implicate this tick as a vector of concern and potential contributor to the cases of tick-borne illness occurring at the Ames Plantation in southwestern Tennessee.

Future research is warranted to investigate the role that different host and tick species play in the maintenance of tick-borne disease at Ames Plantation. AMES is home to a variety of tick vectors some of which were collected during our summer field season. However other tick vectors of concern such as *Ixodes scapularis* were absent in our field season collection, but present on deer harvested during deer season at the Ames Plantation. The fact that we were able to collect different tick vectors of concern using a variety of different trapping methods (e.g. deer checks, vegetation drags, and CO_2-baited collections) stresses that using a variety of collection methods is critical when surveying tick species present and investigating claims of tick-borne illness. Future research efforts at AMES should focus on integrating host trapping with tick and pathogen identification. Just as *Ixodes scapularis* (primary vector of *B. burgdorferi* causative agent of Lyme disease) was verified at AMES only through deer collections it is possible that other tick vectors of concern may be present, but not accounted for in this study because the methods used in our study did not take advantage of the behavioral differences and as such were not the most effective means of collecting these different tick vectors.

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Project Appendices

A.1 DNA Extraction Protocol: Fermentas Gene Jet Genomic DNA Clean Up Kit

Day 1: Ethanol Wash

a. Remove the ethanol from the sample tube * use a clean tip each time
b. Add 200ul of Millipore water to each sample tube to wash the ethanol away
c. Place the samples back in the refrigerator *for a minimum of 4 hrs

Day 1.5: Cell Disruption

a. Remove the 200ul of Millipore water from each sample tube *use a clean tip each time
b. Add 20ul of Pro-K and 180ul of Digestion solution to each sample
c. Cut each tick with a scalpel blade making sure to sterilize blade between uses
d. Leave one half of the tick in the tube with the digestive solution and put the other in a tube with 80% ethanol to serve as a voucher specimen and catalog away
e. Vortex the tube with the digestive solution and halved tick
f. Place in incubator overnight at 56°C

Day 2: DNA Extraction

a. Remove the samples from the incubator, switch flipper and take samples to the clean lab
b. Sterilize the counter tops with 70% ethanol
c. Add 200ul of Lysis solution, vortex thoroughly, and let sit for 5 min
d. Add 400ul of 50% ethanol to each sample and vortex thoroughly
e. Transfer all of the solution (~800ul) to the spin columns *Make sure they're labeled
f. Centrifuge for one minute at 6,000rpm and discard the collection
g. Place spin column in fresh collection tube provided by the kit and add 500ul of Wash Buffer 1 *Make sure ethanol is added
h. Centrifuge for 1 min at 8,000 rpm, empty the collection and reuse for Wash Buffer 2
i. Add 500 ul of Wash Buffer 2 and centrifuge for 3 min at 12,000rpm
j. Place the spin column in a 1.5ml microcentrifuge tube not provided by the kit * make sure its labeled with yr, location, genus, and species
k. Add 200ul of Elution Buffer and spin for 2 min at 8,000rpm
l. Place DNA extraction products in the -20°C freezer to await further processing and clean the counter with 70% ethanol

A.2 Amplification of *Ehrlichia* spp. *groEL* operon Takano et al. 2009 and Harmon et al. 2010

Primer Sequence:
groEL primary Forward: GAA GAT GCW GTW GGW TGT ACK GC
groEL Primary Reverse: AGM GCT TCW CCT TCW ACR TCY TC
groEL Nested Forward: ATT ACT CAG AGT GCT TCT CAR TG
groEL Nested Reverse: TGC ATA CCR TCA GTY TTT TCA AC

Maxima Hot Start Green PCR Master Mix for Primary and Nested PCR per sample (N + Error):

a. 25ul Hot Start
b. 1ul Forward primer *make sure diluted
c. 1 ul Reverse primer *make sure diluted
d. 5ul DNA Template
e. 18ul Nuclease Free Water

Total Volume: 50ul

Amplification Conditions:

Denaturation 15 min @ 95C
Denaturation 30 sec @ 95C
Annealing 30 sec @ 58C
Elongation 30 sec @ 72C
} 40 Cycles for primary PCR; 35 Cycles for Nested PCR
Final Elongation 3 min @ 72C

Ehrlichia species expected amplicon size: 300-350 base pairs

A.3 Amplification of Spotted Fever Group *ompA* gene Eremeeva et al. 1994 and Moncayo et al. 2010

Primer Sequences:

Rr190.70p *R. rickettsii* 190-kDa antigen Forward: ATG GCG AAT ATT TCT CCA AAA
Rr190.602n *R. rickettsii* 190-kDa antigen Reverse: AGT GCA GCA TTC GCT CCC CCT

Maxima Hot Start Green PCR Master Mix per PCR sample (N + Error):

a. 25ul Hot Start
b. 1ul Forward primer *make sure diluted
c. 1 ul Reverse primer *make sure diluted
d. 5ul DNAE Template
e. 18ul Nuclease Free Water or until total volume is 50ul

Total Volume: 50ul

Amplification Conditions:

Denaturation 12 min @ 95C
Denaturation 20 sec @ 92C
Annealing 30 sec @ 48C } 35 Cycles
Elongation 2 min @ 60C
Hold @ 4C Forever

R. amblyommii expected amplicon size: 510 base pairs

A.3 Spotted Fever Group Rickettsia Restriction Digest Eremeeva et al. 1994 and Moncayo et al. 2010

PSTI Restriction Digest

Nucleotide Sequence: CTGCAG

Restriction Enzyme Digest Master Mix per Sample (N + Error):

a. 18ul Nuclease Free Water
b. 1-2ul PSTI Restriction Enzyme
c. 10ul PCR Reaction
d. 2 ul 10x Optizyme Buffer

Total Volume: 32 ul
*** Keep cold until the reaction mixture enters the thermocycler**

Incubation Conditions:

Incubate for 1 hr. @ 37C

RSAI Restriction Digest

Nucleotide Sequence: GTAC

Restriction Enzyme Digest Master Mix per Samples (N + Error):

a. 16.5 ul Nuclease Free Water
b. 1ul RSAI Restriction Enzyme
c. 0.2 ul BSA
d. 2ul Buffer C
e. 10ul PCR Reaction

Total Volume: 29.7ul
*** Keep cold until the reaction mixture enters the thermocycler**

Incubation Conditions:
Incubate for 1 hr. @ 37C

VITA

Brian Hendricks is originally from Akron, OH. He earned his Bachelor of Science degree in biology with a minor in business administration from the University of Charleston in Charleston, WV. He was first introduced to entomology when he participated in a 6 month study where he collected native insect fauna from the Charleston State Park using a variety of different trapping methods. Prior to beginning graduate school Brian worked as a technician in a microbiology lab at his undergraduate alma mater. Currently, Brian is finishing his Master's degree in entomology and plant pathology at the University of Tennessee, Knoxville and plans to graduate in August 2013.