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Surveillance of ticks parasitizing Tennessee beef cattle and investigations into the microbial communities of cattle associated and questing *Amblyomma maculatum*

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To the Graduate Council:

I am submitting herewith a thesis written by David Paul Theuret entitled "Surveillance of ticks parasitizing Tennessee beef cattle and investigations into the microbial communities of cattle associated and questing *Amblyomma maculatum*." 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 Trout Fryxell, Major Professor

We have read this thesis and recommend its acceptance:

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Surveillance of ticks parasitizing Tennessee beef cattle and investigations into the microbial communities of cattle associated and questing *Amblyomma maculatum*

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Abstract

Despite the risks that ticks and tick-borne disease pose to the beef cattle industry, many Tennessee producers are unaware of the dangers they represent. This mindset could facilitate the invasion and establishment of exotic ticks and pathogens that would devastate the cattle industry. Current control practices rely on chemical methods, which are not effective long-term; therefore, investigations into creating an integrated approach to control would create more sustainable methods. This study aims to address this through two objectives: The first is to determine the species composition, seasonal prevalence, geographic distribution and diversity of ticks on Tennessee cattle. The second is to elucidate the core microbial community of *Amblyomma maculatum* and determine differences associated with blood-feeding, collection location, and sex. Ticks were collected from cattle at University of Tennessee research and education centers (REC), through an extension agent survey, and livestock auctions. 25% of the herd or 10 animals were sampled (IACUC# 2192, IBC# 384-15) whichever was greater. The V3-V4 region of the 16S rRNA segment of bacterial genomes was amplified using the Illumina MiSeq platform. Mothur 1.33.2 was used in conjunction with the statistical software R (v3.3.0) to investigate the microbiome of *A. maculatum*. SAS software (9.4) was used to answer questions from objective one. Our results demonstrated that four tick species were parasites of beef cattle: *Amblyomma americanum, Amblyomma maculatum, Dermacentor variabilis,* and *Ixodes scapularis*. Seasonal impacts were not shown to have an impact on either infestation prevalence or burden of any tick species, although region of collection did have an effect on the infestation prevalence and burden of both the Total and *Amblyomma maculatum*. Co infestation was rare, although *D. variabilis* was commonly found feeding with *A. americanum*. Several core microbial inhabitants of *A. maculatum* matched previous investigations, with sex, feeding status significantly influencing alpha diversity. Results suggested that *A. americanum* is a widespread and abundant pest of cattle, and added further support that the bacteria *Francisella* is an endosymbiot of *A. maculatum.* Ultimately, investigating tick diversity and microbiome composition will improve existing control efforts and prepare cattle producers for invasive ticks and pathogens.

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Tick threats to cattle health: From a global perspective, ticks and tick-borne pathogens represent a serious threat to cattle and other livestock and hamper development of livestock production systems in many poor and developing countries. In fact, an estimated 80% of the world's cattle are at a substantial risk of morbidity and mortality due to ticks and tick-borne pathogens (Snelson 1975). Ticks are ectoparasitic arthropods that require blood to complete their life cycle, and create protein for spermatogensis (Males) and oogenesis (Females). Ticks use specialized mouthparts to attach themselves to a host and blood-feed. This attachment can directly damage the host, causing blood loss and anemia. A notable example is 'Gotch ear', where heavy infestations of *Amblyomma maculatum* can lead to necrosis of the ear tissue as well as damage to the cartilage in cattle (Edwards 2011). Additionally, tick attachment can indirectly damage a host via the saliva secreted during feeding, including allergic reactions, irritation, toxicosis, bacterial infection of the wound, and accidental introduction of pathogens (Jongejan and Uilenberg 2004). Tick paralysis is a unique form of toxicosis, wherein toxins are released into the host's bloodstream when the tick begins to feed (Goethe et al. 1979); for example, feeding by *Dermacentor andersoni* can cause paralysis and even death if not removed (Rich 1973). Tick-borne pathogens are responsible for serious morbidity and mortality in livestock, with anaplasmosis, babesiosis, heartwater, and theileriosis representing the greatest risk to cattle worldwide (McCosker 1979).

Economics: The resulting damage from ticks and tick-borne diseases can cause significant economic impacts, with a global estimate of $7 billion USDs, including losses and control costs (McCosker 1979). More specifically, the estimated cost of theileriosis control in several countries in Africa was $168 million USD in 1989 (Mukhebi et al. 1992). In India, theileriosis cost $383.4 million USD (Minjauw and McLeod 2003). In Zimbabwe, the cost of mortality, treatment and control of Heartwater is $6.45 million USD (1991) (Meltzer et al. 1996) with the annual costs projected at $5.6 million USD (1997) if the disease continued to spread to unaffected parts of the country (Mukhebi et al. 1999). The combined cost of control and production losses attributed to ticks and tick-borne diseases in Tanzania was estimated at $364.8 million USD, with theileriosis, anaplasmosis, babesiosis and heartwater accounting for $247.7, $48.13, $45.82, and $22.43 million USD respectively (Kivaria 2006). Although these figures are estimations, they do highlight the clear danger to animal health and food security that ticks represent and emphasize the importance of controlling these pests.

Challenges in the U.S.: The current threat of ticks and tick-borne diseases in the United States (U.S.) does not reflect the full breadth of risks and challenges that these pests pose upon cattle production in other countries. While many countries struggle to develop productive cattle industries, the U.S. cattle industry thrives, contributing significantly to the country's economy with sales of cattle and calves estimated at $76.4 billion USDs in 2012 (Vilsack and Clark 2014). However, ticks and tick-borne disease are still a threat to this industry due in part to direct damage to cattle through feeding attachment. Damage due to biting pressure has been noted for some tick species, including *Amblyomma americanum* where 15 feeding females is considered the injury threshold to pre-weaner beef cattle in Oklahoma (Barnard 1985) and *A. maculatum* where infestations of 25 – 30 adult ticks can result in decreases in weight gain observed in calves

(Williams et al. 1977, Williams et al. 1978). Additionally, ticks are still considered a threat because of their capacity to transmit the pathogen which causes Bovine Anaplasmosis.

Bovine anaplasmosis (BA): is caused by infection with *Anaplasma marginale*, and has been detected over a large geographical area of the U.S. including the southern, midwestern, and western states (McCallon 1973). Transmission of *A. marginale* can occur mechanically, wherein infected blood comes into contact with a naïve host through biting arthropods (Ewing 1981) or contaminated fomites. Additionally, biological transmission can occur in ticks (especially those in the genus *Dermacentor*) (Dikmans 1950, Kocan et al. 2004), where infected erythrocytes are ingested during blood-feeding and replication of the pathogen occurs within the tick gut. Once infected, cattle can experience symptoms including: fever, anorexia, lethargy, decreased milk production, abortions, and death (Ristic 1977). The pathogen evades the host immune system via antigenic variation by variable expression of surface proteins MSP-2 (French et al. 1998, Palmer et al. 2006) and MSP-3 (Futse et al. 2009). Therefore, animals that survive acute infection have permanent rickettsemia and are considered reservoirs for life (Richey 1981). Tetracycline antibiotics are used to treat infection, although there has been no clear evidence that this therapy can clear the carrier state (Franklin et al. 1965, Coetzee et al. 2005). Vaccines are available to mitigate symptoms of the disease, but cannot prevent cattle from becoming carriers (Kocan et al. 2003). Producers with BA infected cattle can incur financial losses due to treatment and control, which Goodger et al. (1979) estimated at $1.48 million USDs for California beef cattle. Therefore, although the damage incurred by ticks and tick-borne pathogens in the U.S. is comparatively less severe to other countries, these factors still represent a significant risk to the cattle industry; In addition to these endemic threats, there are several tick and tick-borne disease systems poised to invade the U.S., including bovine babesiosis and ehrlichiosis.

Bovine babesiosis (BB): more commonly known as Texas cattle fever, is caused by infection with protozoan parastites in the genus *Babesia*, namely *Babesia bovis*, and *Babesia bigemina*. These pathogens are transmitted primarily through tick species of *Rhipacephalus* (formerly *Boophilus*), with the most important species in North America being *R. microplus* and *R. annulatus*. Cattle infected with these pathogens typically experience fever, loss of appetite, depression, weakness, abortions, muscle wasting, tremors, and coma leading to death (De Vos and Potgieter 1994). The discovery by Smith and Kilborne (1893) that *R. annulatus* served as a vector of the causative agents of BB combined with the significant economic damage to the U.S. cattle industry (estimated at $40 – 60 million USD annually in 1906 (Temeyer et al. 2004)) spurred eradication efforts that were declared successful in 1943. Florida suffered infestations of *B. microplus* until 1960, and continued infestations within a quarantine buffer zone persist in Texas (Graham and Hourrigan 1977). Efforts to prevent reestablishment of the tick vectors in the U.S. have consisted primarily of strict regulation of animals being imported from Mexico. Current treatments with babesiacides such as imidocarb diproprionate and diminazene aceturate can be effective to control infections in affected cattle (Bock et al. 2004). Vaccine use is limited due to several factors including: antigenic variance of *Babesia* parasites (Palmer et al. 1991, Allred et al. 1994, O'Connor et al. 1997), the disadvantages of using live vaccines (strict cold chain requirements and potential failure to achieve long term immunity), and a lack of commercially available killed vaccines (De Waal and Combrink 2006).

Bovine ehrlichiosis (BE): Commonly known as Heartwater, BE is caused by infection with *Ehrlichia ruminantium* (formerly *Cowdria ruminantium*). Several species of ticks in the genus *Amblyomma* can act as vectors, primarily *A. variegatum* and *A. hebreum*. Symptoms in infected cattle range from subclinical infection to acute disease characterized by fever, loss of appetite, neurological signs, and death (Van de Pypekamp and Prozesky 1987). In the Carribean, an eradication program targeting *A. variegatum* was initiated due the widespread distribution of the tick throughout the islands (Burridge 1985), and the discovery of Heartwater on several islands (Perreau et al. 1979, Uilenberg et al. 1984, Birnie et al. 1985). Treatment with antibiotics must be administered quickly, as animals may not present symptoms before succumbing to the disease. Cattle that survive can have persistent infections, and remain as carriers of the pathogen for up to 246 days (Andrew and Norval 1989). The best method of protection is with vaccination, via infection with live bacteria and subsequent treatment with tetracycline antibiotics following onset of febrile illness (Allsopp 2015). Investigations into other vaccination methods are hampered by a lack of cross reactivity to strain variants of *E. ruminantium* and increased virulence of tick challenge compared to needle challenge used in testing (Collins et al. 2003).

Invasion potential of BB and BE: BB and BE are currently not found in the U.S., but do present a threat to animal health, food security, and the economic stability of the cattle industry. In general, there are several factors that can complicate prevention and control of foreign animal diseases, including "… free trade agreements, free trade blocks, regionalization, increased international passenger travel, intensification of animal production, the constant evolution of infectious agents, and the uncertain impact of biotechnology and bioterrorism" (Arnoldi 1998, p. 12). Indeed, many factors are increasing the likelihood of invasion of the U.S. by these ticks and tick-borne pathogens. For BB, maintenance of a quarantine zone along the Texas-Mexico border has been accomplished through use of chemicals used to dip imported cattle. Although this method has proven successful in the past, *R. microplus* from Mexico have been shown to be resistant to a broad range of acarcides, including pyrethroids, amitraz, and organophosphates (Li et al. 2003, 2004, Miller et al. 2005, Li et al. 2007, Miller et al. 2008, Busch et al. 2014). White-tailed deer (*Odocoileus virginianus*) serve as a complicating factor, as they are suitable hosts for both *R. annulatus* (Graham et al. 1972, Gray et al. 1979, Cooksey et al. 1989), and *R. microplus* (Kistner and Hayes 1970). Additionally, several of these authors note that deer may act as a vehicle for introduction of cattle fever ticks from infested into non-infested areas (Graham et al. 1972, Gray et al. 1979, Cooksey et al. 1989). Unregulated movement of deer across the Texas-Mexico border would allow for continual re-infestation of cattle, making possible eradication efforts near impossible.

Several routes of introduction are possible for BE entering the U.S., primarily through the movement of animals. Animals imported into the U.S. have been found to be viable hosts for *Amblyomma* ticks capable of transmitting *E. ruminantium*, or have been shown to serve as subclinical reservoirs for this pathogen. These include other wild ruminants (Peter et al. 1998, Wesonga et al. 2001) as well as reptiles (Allan et al. 1998, Burridge et al. 2000a, Burridge et al. 2000b). Unregulated movement of wildlife also poses a threat of introduction as evidence suggests that cattle egrets (*Bubulcus ibis*) may serve as suitable hosts for immature *A. variegatum* (Barré et al. 1987, Barré et al. 1988, Barré et al. 1991) and that these birds can migrate from the Carribean to Florida (Corn et al. 1993). Additionally, *E. ruminantium* has been shown to infect *O. virginianus* in laboratory settings (Dardiri et al. 1987). The risk of

introduction is further complicated by the ability of the endemic tick species *A. maculatum* to act as an experimental vector of *E. ruminantium* (Uilenberg 1982). In fact, Mahan et al. (2000) demonstrated that *A. maculatum* was equally efficient at transmission as the primary vectors *A. variegatum* and *A. hebraeum*. Therefore, invasion by BE could occur through blood feeding on infected animals without the presence of the primary vectors.

Tennessee beef cattle: The economic success of the beef cattle industry in the U.S. is mirrored in Tennessee, where sales from beef cattle produced primarily through cow-calf operations are estimated at over $735.5 million USDs in 2012 (Vilsack and Clark 2014). A large proportion of residents are involved in beef cattle production compared to other agricultural endeavors, and the cattle industry will likely remain a major source of revenue for the state (Neel 2013).

Tick threats to the Tennessee cattle industry: Surveys have revealed that multiple tick species inhabit Tennessee (Durden and Kollars Jr 1992, Reeves et al. 2007, Cohen et al. 2010), with the species of greatest concern to human and animal health being *Amblyomma americanum*, *Dermacentor variabilis*, and *Ixodes scapularis*. *Amblyomma maculatum*, the vector of the human pathogen *Rickettsia parkeri* (Paddock et al. 2004) is comparatively much less common in Tennessee (Bishopp and Trembley 1945, Durden and Kollars Jr 1992). The only investigation of the ticks that parasitize cattle was conducted by Pompo et al. (2016), who found that *A. americanum*, *D. variabilis*, and *A. maculatum* were common to both cattle and pastures. These tick species are members of the Ixodidae and exhibit a three-host life cycle. Table 1 (Appendix A) estimates the seasonal activity and hosts of the life-stages of these aforementioned tick species

Tick control methods: Cattle producers in Tennessee normally control tick pests incidentally when initiating control measures against other parasites (e.g. flies and worms), due in part to a lack of concern regarding ticks on cattle. In general, these control measures are almost solely reliant on chemical pesticides. This strategy is not uncommon, in fact past and current control methods for ticks and other ectoparasites of cattle in the U.S., have relied almost solely on chemical pesticides. From the discovery of the arsenical compounds to the most recent macrocyclic lactones and milbemycins, acaricides have offered an effective means of quickly controlling ticks at relatively low cost. Despite these short-term benefits, reliance on this single control method has several negative consequences that make effective control difficult to achieve in the long term. First, many pestiferous tick species have developed resistance to most if not all the acaricidal compounds (compiled by George et al. (2004)). Resistance is considered one of the primary reasons for creating new acaricidal compounds (Graf et al. 2004); However, new compounds are increasingly expensive to manufacture and market, resulting in a shift in the acaricide industry to chemical products for companion animals which require less rigorous testing (Graf et al. 2004). This leaves cattle producers with a dwindling number of acaricides at their disposal. Second, chemical pesticides have a long list of negative consequences including non-target toxicity, runoff, bioaccumulation, and biomagnification that have become serious issues as public ecological awareness has risen.

Due to these factors, alternative control strategies must be investigated and implemented to mitigate the consequences of acaricide use and promote sustainable control. A well-established practice that deserves more attention is integrated pest management (IPM) of ticks and other

veterinary pests, defined as "… the systemic application of two or more technologies, in an environmentally-compatible and cost-effective manner, to control arthropod pest populations which adversely affect livestock and poultry" (Bram 1994, p. 1358). In addition to acaricides, which would still be used judiciously to drop pest populations below economically damaging levels, several tactics can effectively control ticks and reduce losses to cattle productivity including: immunization against ticks and tick- borne disease, use of resistant cattle breeds, pasture and vegetation management, and biological control of ticks (Young et al. 1988, Barnard et al. 1994, de Castro 1997). An IPM program would be more sustainable compared to the methods currently employed in Tennessee, ultimately reducing the likelihood of ticks developing resistance to acaricides and extending the effective life of the products currently available to producers. Several key pieces of information must be discovered before such a strategy could be implemented in Tennessee, and includes the ecology of ticks, damage estimates, and control costs (Tatchell 1992). Furthermore, research into control tactics that could be employed in an IPM strategy will be vital to provide producers with a diversity of choices for achieving sound control. A potential technique to explore is Microbial Resource Management (MRM), which would harness the functions of microbes to overcome problems faced by humanity (Verstraete 2007). Application of this theory in arthropods has generally focused on the use of organisms within the microbiome to achieve control or modification.

Tick microbiome: The microbiome is a collection of all microorganisms that live on and within a host, and can include viruses, fungi, nematodes, protozoa, and bacteria. These microorganisms can serve various functions within the host, including acting in symbiotic (commensal, mutualistic) and parasitic (pathogenic) roles. Ticks are hosts for several genera of bacterial mammalian pathogens bacteria including *Borrelia*, *Anaplasma*, *Ehrlichia*, *Rickettsia*, *Francisella*, and *Coxiella* spp. (Nicholson et al. 2009). Investigations of the tick microbiome have focused primarily on the gut, as this is the first site of exposure to these pathogenic microbes following a blood meal. Several studies have investigated the microbiome of ticks, with a focus on those tick species that represent a risk to human and animal health, including: *A. americanum*, *I. ricinus*, *R. microplus*, and *A. maculatum*.

Clay et al. (2008) investigated the microbial communities of questing adults, and clutches of larvae and eggs of *A. americanum* in the southeastern U.S. PCR was used to target the 16s rRNA region of the bacterial genome, and resulting DNA products were ligated into plasmid vectors and grown in *E. coli*. Sequencing the *E. coli* colonies indicated that both adults and larvae were mostly dominated by three genera of bacteria: *Arsenophonas*, *Coxiella*, and *Rickettsia*, with *Coxiella* being the dominant organism.

Carpi et al. (2011) conducted a study to elucidate the microbiome of *I. ricinus*, as well as compare the microbiome composition of adults and nymphs collected in two distinct geographical locations in northern Italy. Using pyrosequing and Illumina technologies, they stated that the microbiome of *I. ricinus* was comprised of four bacterial genera (based on their ubiquitous detection): *Slenoltrophomonas*, *Pseudomonas*, *Rhodococcus*, and *Propionobacterium*.

Andreotti et al. (2011) sought to characterize the microbiome of *R. microplus* males and females, eggs, and tissues of the gut and ovaries using tag-encoded pyrosequencing. A wide diversity of bacteria was identified in all samples, including: *Arthrobacter*, *Bacillus*, *Enterobacter*,

Pseudomonas, and *Staphylococcus*. However, the authors did not surface sterilize samples and admitted to the possibly of bacteria from the environment skewing their results. They suggested that the three genera *Enterobacter*, *Pseudomonas*, and *Staphylococcus* are most likely components of the internal microbiome based on comparisons to a similar study that followed strict surface sterilization protocol and dissection of tissue (Rahman and Rahman 1980).

Budachetri et al. (2014) compared the microbiome of field caught and laboratory reared blood fed adult *A. maculatum* to elucidate the core microbiome. Using pyrosequencing of bacterial 16s rRNA, they found six bacterial genera were found in all wild caught tick tissues tested including *Francisella*, *Rickettsia*, *Pseudomonas*, and *Escherichia*, with *Francisella* being the most abundant and ubiquitous. Comparison to lab-reared ticks revealed that *Francisella* and *Propionibacterium* were common to both groups.

Tick endosymbionts: Ticks are host to several non-pathogenic endosymbiotic bacteria related to pathogenic microbes including *Francisella* (Noda et al. 1997, Sun et al. 2000, Scoles 2004, Budachetri et al. 2017), *Coxiella* (Noda et al. 1997, Duron et al. 2015, Machado-Ferreira et al. 2016) and *Rickettsia* (Noda et al. 1997). The exact function of these endosymbionts is not well understood, although it has been suggested that vertically transmitted bacteria [symbionts] must enhance their host (Fine 1975, Ewald 1987). Indeed, within *A. americanum* it has been demonstrated that the *Coxiella* endosymbiont likely functions in reproduction (Zhong et al. 2007) and vitamin production (Smith et al. 2015). Additionally, the advantage of being infected with a vertically transmitted parasite is the prevention of infection by horizontally transmitted parasites (Lively et al. 2005). An example of this concept was demonstrated in Tsetse flies (*Glossina morsitans*) which had increased susceptibility to infection by the causative agent of African sleeping sickness (*Trypanosoma brucei*) following antibiotic elimination of the bacterial endosymbiont *Wigglesworthia glossinidia* (Pais et al. 2008).

Interestingly, pathogens and endosymbionts often have close phylogenetic relationships that suggest evolution of one into another. For example, analysis of the genus *Rickettsia* suggested that endosymbiotic variants may be the source of pathogenic *Rickettsia* through outer surface protein alterations (Weller et al. 1998). Similarly, a *Coxiella* endosymbiont of ticks was the origin of the Q fever agent *Coxiella burnetii*, potentially through the acquisition of virulence genes (Duron et al. 2015). Conversely, *Francisella* endosymbionts were found to be a sister taxon to the mammalian pathogen *F. tularensis*, suggesting that loss of virulence genes from the pathogen gave rise to the endosymbiont (Gerhart et al. 2016). Close phylogenetic relationships could also mean there is potential for horizontal gene transfer between endosymbionts and pathogens. Two potential outcomes of this scenario are that endosymbionts could acquire virulence genes resulting in new emerging pathogens (Clay and Fuqua 2010), or existing pathogens could acquire antibiotic resistance genes from endosymbionts (Narasimhan and Fikrig 2015).

Microbiome variation: Changes in the structure and composition of the microbiome might further complicate the interactions between pathogenic and benign microbes. Several factors could lead to these changes including species, sex, life stage, environment, season, and geography (Narasimhan and Fikrig 2015). For instance, investigations into the microbial communities of *A. americanum* by Clay et al. (2008) found differential representation of

Arsenophonus and *Rickettsia* bacteria among collections in different geographic regions in the southeastern U.S. (AL, GA, NC, KY, IN, and MO) by analyzing 16s rRNA. The authors did not explain this effect, although geography could play a role. Similar findings were determined by Van Treuren et al. (2015) who investigated the effects of geography, species, and sex on the microbiome of *Ixodes* ticks in several locations along the eastern U.S. (NY, CT, NC, GA, VA). Using a combination of 454 pyrosequencing and Illumina, these authors found that the microbiomes of conspecific ticks from the same location had more similar microbes compared to ticks collected in different geographic locations, and that greater the distance between them the more distinct the microbiomes. These results support the idea that geography might impact the composition of tick microbiomes, and that this factor may cause differences among tick populations in different locations.

In the same investigation of *A. americanum*, Clay et al. (2008) found that larval clutches had a more diverse microbiome compared to adults. Although age may play a role in diversity, the authors state that a higher abundance of *Coxiella* spp. in adults can cause less represented bacterial groups to be harder to detect. A study by Menchaca et al. (2013) looking at the microbiome changes due to blood-feeding and age in *A. americanum* found that *Coxiella* and *Bradyhizobiaceae* changed in relative abundance from nymph to adult, although they noted that the differences between groups could be due to feeding and subsequent starvation rather than age alone. These two studies highlight the need to better understand factors that cause changes in tick microbiomes, and to elucidate whether a factor is really a combination of several factors.
An additional factor that would be important to consider is the feeding status of the tick. An investigation by Heise et al. (2010), which studied how blood feeding in *A. americanum* could change the microbiome using cloning of vectors containing 16s rRNA sequences, found that questing adult ticks collected from both OK and GA showed a higher diversity of bacteria following blood feeding.

Potential control methods: Understanding factors that change the structure and composition of the microbiome, and the interactions of the microbes within them, would provide a better understanding of regional and seasonal tick-borne disease dynamics; and could also provide novel methods for tick control. Paratransgenic transformation is a strategy where endosymbionts within the host are genetically altered. For blood-feeding arthropods, this could be used to create symbiotic bacteria that produce compounds that inhibit infection by pathogenic microbes. Durvasula et al. (1997) eliminated or greatly reduced *Trypanosoma cruzi* infection in the kissing bug (*Rhodnius prolixus*) through transformation of the symbiont *Rhodococcus rhodnii* to produce the antibacterial compound Cecropin A. Additionally, modulating the structure of the microbiome might alter the vectorial capacity of ticks by either eliminating microbes that facilitate pathogen invasion or by increasing the presence of microbes that competitively exclude pathogens. An example could be infecting ticks with non-pathogenic *Rickettsia*, as members of this genus have been found to competitively exclude each other (Macaluso et al. 2002). Simple elimination of endosymbionts is another possible strategy. This has been demonstrated by reducing reproductive fitness of *A. americanum* females and survival of offspring using antibiotics to eliminate a *Coxiella* symbiont (Zhong et al. 2007). Control methods using these techniques could be environmentally safe and have potentially no impacts on non- target organisms compared to acaricides, since targeted endosymbionts would be host specific.

Justification to Committee

The beef cattle industry in Tennessee is currently at risk from endemic tick-borne threats including direct feeding damage and morbidity and mortality caused by transmission of *A. marginale*. Control of these pests is complicated by several factors, including a general lack of awareness among cattle industry stake holders of the threats that ticks pose to cattle health. This lack of awareness means there is no push for research into ticks, creating a relative dearth of information regarding the basic ecology and phenology of these pests in Tennessee. This feed-back loop of non-information causes several cascading issues including producers misattributing biting damage from ticks to flies, further fueling the feed-back loop and leaving producers vulnerable to unseen losses. Producers that are aware of ticks and tick-borne pathogens face challenges primarily caused by decreasing numbers of acaricidal compounds available to them to control these pests. The reasons behind this decline are numerous, and include increased federal regulation, slow creation of new acaricides, loss of effectiveness of existing acaricides, and increasing public concern over pesticide use in agriculture. In addition to the endemic threats to the beef cattle industry, invasive ticks or pathogens could devastate the existing industry and due to the general attitude of producers to tick threats it is likely that a response to an invasive threat would lag behind initial establishment. Ultimately, eradication efforts would be made nearly impossible.

Current needs: Due to the challenges that endemic and invasive tick threats represent to the beef cattle industry in Tennessee, it is crucial that research must be conducted to determine which species are pests of cattle and to elucidate basic characteristics of the ecology and phenology to tick pests of cattle. Specifically, understanding of the regional and seasonal variation in tick populations could assist in creating 'risk assessments' that can provide producers with knowledge of when and where ticks are likely to infest cattle. Additionally, this information could act as a guideline for producers when deciding to enact control measures based on the location of the animal and the time of year. Within an IPM strategy, this would reduce acaricide use resulting in a decreased probability of ticks to develop resistance and an increased longevity of existing chemical control options. Concurrent investigation into new control methods would increase diversity of control methods available to producers, and increase the flexibility of the IPM strategy overall. Answers to questions regarding the composition and structure of the tick microbiome could open the door to a wide range of control techniques that would help cattle producers in Tennessee manage current tick issues, and potentially control invasive ticks and tick-borne pathogens that pose a threat to the Tennessee cattle industry. Lastly, information that could help to prepare the cattle industry for invasive ticks and pathogens will be an important tool for creating a collaborative network of industry stake holders necessary to enact effective eradication efforts and serve to defend cattle health and the state's economic interests. This includes not only investigations into which sources our best for monitoring for invasive ticks, but pathogens as well.

Hypothesis

This research project aims to tackle these challenges with the following objectives.

Objective 1- tick diversity survey: This study will test the hypothesis that there are multiple tick species that parasitize beef cattle in Tennessee, that tick activity will vary both seasonally and regionally, and that not all sources of collection will be suitable for monitoring for invasive tick species.

This objective will be accomplished using three primary sub-objectives:
1.) Identify the species that are commonly found parasitizing cattle
2.) Elucidate the seasonal and geographic variation in these tick populations
3.) Identify the most efficient means to achieve a state-wide collection program.

Objective 2- microbial community analysis of *A. maculatum*: This portion of the study will test the hypothesis that the microbial communities of Amblyomma maculatum will have a consistent presence of certain microbe taxa and will vary by factors such as region of collection, feeding status, and life-stage.

This objective will be accomplished using 2 primary sub-objectives:
1.) Determine the core microbiome of *A. maculatum*
2.) Investigate factors that lead to changes in the microbial community structure

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Appendix A

Table 1.1 Host preference and seasonal dynamics of common ticks in TN.

Species	Life Stage	Hosts	Peak Activity
Amblyomma americanum	Larvae	Birds, large mammals	Year round, except in midwinter
	Nymph	Birds, large mammals	Year round, except in midwinter
	Adult	Large mammals	Year round, except in midwinter
Dermacentor variabilis	Larvae	Small mammals	Spring
	Nymph	Small mammals	Spring
	Adult	Dogs, other medium sized mammals	Spring
Amblyomma maculatum	Larvae	Birds, small mammals	Year round, except in winter
	Nymph	Birds, small mammals	Year round, except in winter
	Adult	Large mammals	Late summer, early fall
Ixodes scapularis	Larvae	Birds, reptiles, mammals	Summer
	Nymph	Birds, lizards	Summer
	Adult	Medium and large mammals	Spring and fall

Information presented in this table was compiled from the following sources: (Bishopp and Trembley 1945, Durden and Kollars Jr 1992, Kollars et al. 1999, Kollars et al. 2000, Cohen et al. 2010, Teel et al. 2010). It is important to note that seasonal variation will differ geographically and as such these reported activities may not correspond to the seasonal dynamics of ticks in Tennessee.

Chapter 2: Tick Diversity Survey

Abstract

Ticks impact cattle health through attachment and transmission of pathogens. Bovine Anaplasmosis is currently a threat to Tennessee cattle, with Heartwater and Bovine Babesiosis poised to devastate the U.S. cattle industry. Research objectives were to investigate seasonal and regional impacts on infestation prevalence and burden of ticks on cattle and identify sources for invasive tick monitoring. 25% of the total herd size (or 10 animals) were sampled at 3 University of Tennessee Research and Education Centers (RECs), 6 livestock auctions, and 9 extension agents at 21 producer locations. SAS (9.4) was used to determine the effect of season, region, and collection source. SatScan™ (9.4.2) was used to detect high and low clusters of infestation. 740 ticks were captured from 1817 sampled cattle, including 573 *Amblyomma americanum* (77.4%), 125 *Amblyomma maculatum* (16.9%), 35 *Dermacentor variabilis* (4.7%), and 3 *Ixodes scapularis* (0.4%). Western and middle Tennessee were significantly different in infestation prevalence and burden of *A. maculatum*. For *A. maculatum* and the species total, infestation prevalence and burden were greater in spring than fall. Auctions and RECs had the greatest infestation prevalence of *A. maculatum*, and the greatest burden of *A. maculatum* and *D. variabilis*. High risk locations clustered in western and middle Tennessee, with low risk locations in middle and eastern Tennessee. Results from this study provide knowledge necessary to initiate control measures, including seasonal phenology and regional distribution of current tick threats. The RECs and livestock auctions should be used for monitoring invasive threats to Tennessee, and other southeastern states.

Introduction

Ticks are blood – feeding arthropods of significance to both human and animal health because they can damage a host via multiple mechanisms. Tick attachment can cause direct damage through dermatitis, allergies, introduction of toxic salivary compounds, and providing entry points for secondary infections (Jongejan and Uilenberg 2004). Additionally, ticks can indirectly damage their host via the transmission of pathogenic microbes. Ticks and tick- borne diseases are a serious threat to the cattle industry in the United States (U.S.). The U.S beef cattle industry significantly contributes to the country's economy, with a retail value estimated at $105 billion USDs in 2015 (USDA-ERS 2017). In Tennessee, cow - calf production for beef cattle is one of the state's top agricultural commodities at $735.5 million USDs (2012) (Vilsack and Clark 2014). The cattle industry's economic success is dependent upon proper management of factors that impact cattle production. Cattle heath is of major importance, with annual losses from health-related issues estimated at $20 - 25 million USDs in Tennessee (Neel 2013). Although ticks likely contribute to health losses in Tennessee, many producers are unconcerned or unaware of the consequences these pests can have on cattle health. This pervasive mindset makes the cattle industry vulnerable to endemic ticks and pathogens and creates conditions that allow for invasion of new threats.

Bovine Anaplasmosis (BA) is a serious disease of cattle that occurs in many parts of the U.S. (McCallon 1973), including Tennessee (Merriman et al. 1966, Whitlock et al. 2014). For California beef cattle, the estimated cost of direct losses from BA infection combined with treatment and control costs is $1.48 million USDs (Goodger et al. 1979). The etiological agent, *Anaplasma marginale*, can be transmitted mechanically by biting arthropods or fomites contaminated by blood, and biologically by *Dermacentor* ticks (Dikmans 1950, Kocan et al. 2004)). *Dermacentor variabilis* and *Dermacentor albipictus* have been documented in Tennessee (Durden and Kollars Jr 1992, Reeves et al. 2007, Cohen et al. 2010), but only *D. variabilis* has been shown to be a pest of cattle (Pompo et al. 2016). While BA infected ticks were not captured from cattle-associated ticks previously (Pompo et al. 2016), the pathogen is found in Tennessee cattle (Whitlock et al. 2014); Therefore, this tick-pathogen complex represents an issue that producers must monitor for.

Two tick and pathogen complexes are primed to invade the U.S. and pose a significant risk to the cattle industry. In Mexico, *Rhipicephalus microplus* and *Rhipicephalus annulatus* are vectors of *Babesia bigemina* and *B. bovis*, the pathogens that cause Bovine Babesiosis (BB). Unfortunately, factors including resistance of *R. microplus* to acaricides used to treat cattle moving across the border (Li et al. 2003,2004, Miller et al. 2005) and movement of suitable alternate wildlife hosts such as white-tailed deer (*Odocoileus virginianus*) (Busch et al. 2014) have made breaks in the quarantined zone a grim reality. In the Caribbean, the Tropical Bont Tick (*Amblyomma variegatum*) is a vector of *Ehrlichia ruminantium*, the agent of Heartwater (HW). The invasion of this tick is made possible via imported pets and livestock (Deem 1998), and movement of cattle egrets (*Bulbulcus ibis*) which serve as suitable hosts for immature bont ticks (Burridge et al. 1992). While BB and HW are not currently found in the U.S., they are of concern to the cattle industry because of the high estimated death loss (≥70%) (Wagner et al. 2002) and potential economic impact (Dietrich and Adams 2000) following introduction.

To prepare for these impending threats, it is vital that the cattle industry collect key information on the biology and activity of current tick threats to mitigate economic losses. Additionally, investigating monitoring techniques will create detection methods to prevent establishment and spread of invasive ticks. To protect the cattle industry from ticks we are testing the overarching hypothesis that the infestation prevalence and burden of ticks will vary by season and region and collection source. To test this hypothesis our objectives were to characterize the tick infestation prevalence and burdens to Tennessee cattle and determine the best strategies for monitoring for invasive ticks in Tennessee.

Materials & Methods

Collection sources: We used three collection sources to sample from a large number of cattle, to collect a variety of ticks, and to capture an accurate representation of tick species on cattle. In total, 30 collection locations used for this study consisting of three University of Tennessee (UT) Research and Education Centers, six livestock auctions, and nine extension agents collaborating with a total of 21 cattle producers (Fig 1). Additionally, twelve United States Department of Agriculture (USDA) approved livestock slaughter houses were contacted, of which zero were willing to participate in this study. Before sampling, we obtained approval to collect ticks from the cattle sources via signed documentation and from the UT Institutional Animal Care and Use Committee (IACUC) committee (IACUC #2192).

The UT Research and Education Centers (RECS) carry out field studies for the benefit of producers in the agricultural and natural resource industries. The RECS sites in this study were used previously in a project investigating tick-cattle associations in Tennessee (Pompo et al. 2016). Ames Plantation (~7,446 hectares) has approximately 200 head of Angus beef cattle and is located in Western Tennessee (35.114394, -89.211781) within the Mississippi Valley Loess Plains ecoregion (Griffith et al. 1997). The Middle Tennessee Research and Education Center (~511 hectares) has approximately 140 cows consisting of angus, charlois and black baldy [hereford x angus]) and is located in central Tennessee (35.718806, -86.965131) within the Interior Plateau ecoregion, (Griffith 1997). The Plateau Research and Education Center (~850 hectares) has approximately 200 head of Angus beef cattle and is located in Eastern Tennessee (36.105349, -85.132090) within the Southwestern Appalachians ecoregion (Griffith 1997).

UT employs approximately 400 extension agents working in 95 offices located in every county in Tennessee. Their objective is to serve as the primary means of disseminating academic research to the public in an effort to improve quality of life through education. These agents work closely with livestock producers, and were considered an asset for this project. Agents were contacted via email and/or phone to determine interest in participating in the study (n = 50 agents). Twenty-six agents (52%) were willing to participate and subsequently sent a training video demonstrating the sampling methods employed for this study (Theuret and Trout Fryxell 2016). Agents were sent collection kits with the following items: Thermo Scientific™ Nunc ™15ml tubes (ThermoFisher, Waltham MA) filled with approximately 7.5 ml of 80% ethanol, data sheets, and producer participation agreement forms. Additionally, agents were sent instructions and labels for shipping samples in ethanol. These were approved by the UT Institute of Agriculture's biological safety officer. Of the agents initially interested, nine (34.6% of interested investigators) reported collection data.

In Tennessee, there are 47 facilities used for livestock auctions. Of these, 27 (57%) were contacted, with 6 (22% of contacted) willing to participate. These included three locations in the Interior Plateau (Tradition Livestock Services [35.895403, -86.38175], Warren County Livestock [35.709283, -85.791516]) and Dickson County Livestock Auction [36.023889, -87.341512]), two locations in the Mississippi Plains (Somerville Livestock Auctions [35.289827, -89.36078], and Scott's Hill Livestock Auction [35.51478, -88.238319]), and one location in the Ridge and Valley (East Tennessee Livestock Center [35.633839, -84.437595]). These locations held weekly auctions of cattle and calves, in addition to other livestock including pigs, and goats.

Tick collections from cattle hosts: Ticks were collected directly from cattle run through a chute to maximize the efficiency of collections, and protect the safety of both the investigator and the animal. The greater of 25% of the total herd size or ten animals were sampled to capture ticks and avoid reducing the efficiency of the husbandry practices of the producer. For example, herds of less than 40 cattle sampled 10 animals, whereas a herd of 45 cattle sampled 12 cattle (11.25 rounded up). Collections were performed based on the schedule of the respective producer / herd manager, and were typically done concurrently with standard husbandry practices: vaccinations, pregnancy checks, ear tag insertions, and aging. Cattle were scratched (investigator used hands for tactile detection) and visually checked, with special attention to the ears, head, neck, tail, and underside of the tail, as these sites have been shown to be common attachment sites of ticks (Gladney et al. 1974, Barnard 1981, Barnard et al. 1982, Bloemer et al. 1988) and are safe for the inspector. Animals were sampled for a maximum of five minutes to minimize animal stress. Collected ticks were placed into a vial containing 80% ethanol, with one vial used per animal. Any ticks found on cattle that were not part of the sampled group were also collected and considered 'opportunistically collected'. At all collection sites, any cattle that posed a threat to the safety of themselves or the investigator were not sampled. Information about each animal was recorded, including the ear tag number, breed, and age.

Tick identification: All collected ticks were identified to species, life-stage, and sex using dichotomous keys (Sonenshine 1979). Following identification, ticks were placed into new labeled vials of 80% ethanol for storage. Two variables used for statistical analysis were infestation prevalence (defined as the percentage of cattle within a sampled group that were infested with ticks) and tick burden (the mean number of ticks found on infested animals). No opportunistically collected ticks were used for statistical analyses. These variables along with traditional descriptive variables (e.g., mean no. ticks) were calculated for each species and the total. Data were visualized using ArcGIS (v 10.3.1) (ESRI 2011) to map tick collection sites, infestation prevalence, and tick burden. For all tests conducted, *Ixodes scapularis* collections were excluded from analyses because this species was rarely captured. We also investigated co-feeding on an animal (when two species occur together on the same host). Co-feeding rates were compared using the Cole's index (C7) of ecological interaction (Cole 1949). Positive values indicate a mutualistic relationship, negative values indicate competition, and numbers near zero indicate no association (neutral). Analyses were conducted on the spring and summer cattle collections using a Chi-square analysis to determine significance of association ($\alpha = 0.05$).

Seasonal and regional effect: To determine seasonal and regional effects of tick prevalence and tick burden of cattle, we used a PROC GLM in SAS software 9.4 (Cary Institute, NC). This suite of tests included a MANOVA for multiple comparisons, ANOVA for species comparisons, and

LSM separation adjusted for multiple comparisons using Tukey-Kramer. Response variable data were ranked transformed to satisfy the assumption of normality and equal variance required by the model. Seasons were defined by calendar month as Spring (Mar, Apr, May), Summer (Jun, Jul, Aug), and Fall (Sep, Oct, Nov), for ease of interpretation by cattle producers. This included collections from 3 RECS centers (n = 798 animals; 266 ± 36.1 per season) at least once during these periods. Regions were defined according to the regions of the University of Tennessee Extension Service (western, middle, and eastern), and included collections from 3 RECS centers (n = 604 animals; 201.33 ± 72.47), 6 livestock auctions (n =419 animals; 69.83 ± 38.87), and 9 Extension agents at 21 collection sites (n =374; 17.81 ± 2.34). For this analysis, only spring and summer collections were used; fall and winter collections were excluded because only 4 ticks were collected in fall and winter combined. Significance for the PROC GLM was determined at $\alpha = 0.05$. Results are displayed in Figure 3, 4 (Appendix B).

Spatial analysis was performed using SatScan™ (v 9.4.2) (Kulldorff 2015) to detect both high and low rates of clustering of infestation. The parameters of this analysis require the size of the population at risk, the number of cases, and geographic coordinates. For this, the number of cattle sampled at a location was used as the population, with the number of cattle infested as the cases. A circular window with a radius equal to 50% of the cattle population size was used with no geographical overlap between windows. A Discrete Poisson model (Kulldorff 1997) was chosen because it is not sensitive to changing population sizes, a common occurrence in this study resulting from differences in the number of cattle sampled. Relative risk values are reported, with values < 1 indicating decreased risk compared to baseline and values > 1 indicating increased risk. For both analyses, the alpha level was $\alpha = 0.05$. Fall and winter collections were again excluded from analysis. Clustering results were displayed in ArcGIS (v10.3.1) (ESRI 2011) (Figure 5, Appendix B).

Sites for invasive monitoring: To determine which collection method (RECS, EXT, and/or auctions) would be best for future tick monitoring opportunities we compared infestation prevalence and tick burden from collections in the peak collection periods (spring and summer). This was used to make comparisons between collection sources due to greater temporal overlap in collections. Likewise, when investigating sex and age of animals as risk factors for tick parasitism animals were chosen from among regions that were not statistically different and from spring and summer. Significance for the PROC GLM was determined at $\alpha = 0.05$.

Results

Tick collections: A total of 740 ticks were collected from cattle consisting of four species (Table 1, Appendix B). A majority (77.2%) of the collection were *Amblyomma americanum* (573 specimens) of which 61.6% were females, 31.4% were males, and 6.6% were nymphs. *Amblyomma maculatum* comprised 16.8% of the collection (125 specimens) of which 84.8% were males and 15.2% were females. *D. variabilis* comprised 4.7% of the collection (35 specimens) of which 60.0% were female and 40.0% were male. The remaining 1.2% were 5 *I. scapularis* (adults) and 4 specimens missing key morphological features that made them unidentifiable using dichotomous keys. Due to our wide collection, some specimens were opportunistically collected and they included 53 *A. maculatum* (34 females and 19 males) and 35 *A. americanum* (24 females and 11 males); as mentioned, these were not used in any analyses.

The species total mean infestation prevalence was 17.8 % ± 3.2%, and the mean burden was 1.2 ± 0.2. Mean infestation prevalence was 14.6 % ± 3%, and mean burden was 1.0 ± 0.2. Mean infestation prevalence among from all sampling events was 2.5 % ± 1.3%, and mean burden was 0.3 ± 0.1. Mean infestation prevalence among the total sampled cattle was 1.6 % ± 0.6%, and mean burden was 0.29 ± 0.1.

Most cattle sampled during spring and summer were not infested with ticks (1094 cattle;78.3%) and if an animal was infested with ticks it was typically infested with only one species (285 cattle; 94.1%). Consequently, we rarely identified two different tick species co-feeding (simultaneously feeding) on the same animal (Table 2, Appendix B). Co-feeding occurred on 22 different animals (1.6% of sampled animals) and we never observed three different species on the same host. Cole's index of association for *A. americanum* and *D. variabilis* was 0.299 ± 0.079 (χ^2 = 14.09; P = 0.0002) indicating a significantly positive interspecific relationship. Whereas, Cole's index of association for *A. americanum* and *A. maculatum* was -0.437 ± 0.335 (χ^2 = 1.695; P = 0.193) and for *A. maculatum* and *D. variabilis* was 0.03946 ± 0.0329 (χ^2 = 1.242; P = 0.2650) indicating no significant relationship between the different co-feeding species.

Knowing these tick species mate on their hosts, we also compared intraspecific interactions. Cole's index of association for *A. americanum* adults and nymphs was 0.686 ± 0.083 (χ^2 = 69.17; P < 0.0001) and for males and females it was 0.351 ± 0.034 (χ^2 = 105.54; P < 0.0001) indicating all nymph, male, and female *A. americanum* were significantly associated together on cattle. This was also significant for *A. maculatum* males and females; their Cole's index of association was 0.606 ± 0.044 and positively associated with one another (χ^2 = 187.21; P < 0.0001).

Effects of season and region: Infestation prevalence (F = 9.54; df = 2; P = 0.0021) and burden (F = 11.16; df = 2; P = 0.0011) were different between fall and spring collections (P < 0.005). Both infestation prevalence (F = 0.16; df = 2; P = 0.8488) and burden (F = 0.30; df = 2; P = 0.7408) were found to be not significant between regions of Tennessee. 1 cluster encompassing 9 locations in Middle and Western Tennessee was significant for high rates of infestation (P < 0.0001) with a relative risk of 3.01. There were also 2 clusters encompassing 11 locations in middle and eastern, and 1 in western, Tennessee were significant for low rates of infestation (P < 0.001) with relative risk ranging from 0.19 – 0. Locations for both high and low rate clusters comprised all three collection source types (RECS, auctions, extension collections).

Amblyomma americanum: Neither infestation prevalence (F = 1.59; df = 2; P = 0.2361) or burden (F = 1.96; df = 2; P = 0.1756) were significantly impacted by season. The same pattern observed in the total category was seen in the infestation prevalence (F = 0.13; df = 2; P = 0.8811) and burden (F = 0.85; df = 2; P = 0.4375) in that they were not significant between regions. Further spatial analysis revealed 1 high rate cluster comprised of 4 locations in Middle Tennessee that had significant clusters of infestation for *A. americanum* (P < 0.001) with a relative risk of 3.82. This cluster included an auction and several extension collections. 4 significant low rate clusters (P < 0.05) with relative risk ranging from 0.092 to 0 were detected in neighboring locations comprised of RECS and extension locations.

Amblyomma maculatum: Season had a significant effect on infestation prevalence (F = 6.82; df = 2; P = 0.0078) and burden (F = 6.68; df = 2; P = 0.0084), with fall lower than spring ($P < 0.05$). Both infestation prevalence (F = 4.83; df = 2; P = 0.0161) and burden (F = 4.53; df = 2; P = 0.0201) were shown to be significant between regions. Least squared means demonstrated that western Tennessee was significantly different from middle Tennessee in both infestation prevalence (P = 0.0176) and burden (P = 0.0222) and both of these regions were not significantly different from eastern Tennessee for either variable. Cluster analysis showed 1 auction and 1 RECS along the border of middle and western Tennessee were a cluster of high infestation rates ($P = 1.0 \times 10^{-17}$) with a relative risk of 24.85. Several locations in middle and eastern Tennessee formed a significant cluster of low rates of infestation ($P = 7.6 \times 10^{-11}$) with a relative risk of 0, and were comprised of all three collection source types.

Dermacentor variabilis: Season did not significantly impact infestation prevalence (F = 3.54; df =2; P = 0.0550) or burden (F = 3.55; df = 2; P = 0.0546). Similar to the patterns seen in total and *A. americanum*, infestation prevalence (F = 2.10; df = 2; P = 0.1416) and burden (F = 2.68; df = 2; P = 0.0868) were not significant between regions. 1 location in western Tennessee was shown to be a significant high cluster for *D. variabilis* (P = 0.039) that had a relative risk of 6.25. There were no locations that were considered significant low clusters for *D. variabilis*.

Sites for invasive monitoring: We attempted to compare phenotypic traits of the animals including sex and age, but all comparisons were insignificant ($P > 0.05$). There was a significant effect due to site type (F = 6.68; df = 16; $P < 0.0001$), which was driven by differences observed in *A. maculatum* and *Dermacentor variabilis*. The infestation prevalence (F = 18.33; df = 2; $P <$ 0.0001) and burden (F =18.58; df = 2; $P < 0.0001$) of *A. maculatum* were greatest at the auctions and RECS ($P < 0.001$). For *D. variabilis*, burden (F = 11.13; df = 2; P = 0.0003) was significantly greater the auctions and RECS compared to extension collections ($P < 0.05$).

Discussion

The results of this study found that *A. americanum*, *A. maculatum*, and *D. variabilis* were primary pests of cattle, confirming findings by Pompo et al. (2016). One difference in these two studies is that in this survey *I. scapularis* was also identified as a parasite of cattle and was completely absent from the previous study. Adult *I. scapularis* have been previously documented as a pest of cattle with a seasonal activity ranging from October through March / April (Bishopp and Trembley 1945, Harris 1959, Drummond 1967, Barnard 1981). Our results corroborate these findings, in that *I. scapularis* were captured in low numbers (n = 5) in winter and early spring. Therefore, the absence of *I. scapularis* from Pompo et al. (2016) is likely due to the summer sampling employed in their survey which would have missed the window of activity for adult *I. scapularis*.

The most common tick species collected was *A. americanum*. This species is abundant, captured at 23 sites and all collection types, has high infestation prevalence and tick burden throughout the spring and summer and has a wide geographic range. These characteristics make *A. americanum* a primary pest of cattle in Tennessee. Previously, 15 female A. americanum per animal was the injury threshold for pre-weaned beef cattle (Barnard 1985). None of the animals sampled in this study had more than the threshold (maximum was 11 female *A. americanum* per single animal)

indicating these tick populations were not at damaging levels; however, we could only sample from a limited portion of the animal's body surface unlike Barnard (1985) who performed whole body inspections. Given this consideration, it is possible that infested herds had more ticks than we could capture, and thus producers in Tennessee may already be suffering economic losses due to *A. americanum* feeding damage.

Conversely, *D. variabilis* were collected from only 10 locations, both infestation prevalence and burden were low and not impacted by either season or region and had little geographic clustering, and were collected at all site types and regions. Previous survey results found *D. variabilis* in 40 of 49 sampled counties in Tennessee, suggesting that it has a wide geographic range (Cohen et al. 2010). Knowledge of the geographic range of this pest is important because *D. variabilis* is a biological vector of *A. marginale*, and its distribution may indicate geographic range of this pathogen. A high proportion of Tennessee beef cattle (56%) tested between 2002 and 2012 were shown to be infected with *A. marginale*, with 10.53% of samples positive in 2013 (Whitlock et al. 2014). Since this species is widespread, but has a low infestation prevalence and low tick burden, this may explain Tennessee's relatively low BA rates. Furthermore, knowledge of the phenology and regional distribution of *D. variabilis* is important for veterinarians to prescribe medication under new regulations outlined by the veterinary feed directive (VFD). The VFD dictates that the supervision of a veterinarian who has a veterinarian client patient relationship (VCPR) with the producer is necessary to administer medicated feeds to herds, with medications only given to treat or prevent disease; the latter case should only occur if the veterinarian is able to determine that contracting an illness is likely (FDA 2012, 2013, 2015). Future studies should determine the infection rates of *A. marginale* in *D. variabilis* to elucidate the risk to cattle and to assist veterinarians in making informed decisions about prescription of feed through antibiotics within the boundaries of the VFD.

A. maculatum had a restricted distribution to middle Tennessee and was collected from six sites; none in eastern Tennessee. Originally distributed along the Gulf Coast region of the United States, populations of *A. maculatum* have expanded via cattle movement into Oklahoma and Kansas (Teel et al. 2010) with only occasional collections of *A. maculatum* in western Tennessee (Bishopp & Trembly 1945, Durden & Kollars 1992). This tick has also been sporadically collected within the middle Tennessee region. A single *A. maculatum* was captured in Marhsall County Tennessee (Pompo et al. 2016), while a single tick from both Perry and Decatur Counties was found during a statewide tick survey (Cohen et al. 2010). In this study, we captured 160 adult *A. maculatum* in Maury County, which is within 100 miles of Perry, Decatur, and Marshall Counties. Previous collections of this tick within Tennessee were attributed to accidental introductions either through livestock importation (Bishopp & Trembly 1945) or movement of bird hosts (Durden & Kollars 1992). Our results, combined with recent findings by other authors, suggest that the range of *A. maculatum* is continuously expanding in western and middle Tennessee.

Given its recent expansion into the state, *A. maculatum* should be considered a 'model' invasive tick species. Results from our investigation into which sources would be best for invasive monitoring revealed that cattle infested with *A. maculatum* had high prevalence and burden at the RECS and auction collections. Within Tennessee the RECS and auctions should continue to be used as monitoring resources, with the RECS acting as 'sentinels' that can detect established

populations of invasive species and the auctions as a checkpoint for potential invasions. Livestock auctions should be the primary means of monitoring for invasive ticks. First, the number of new cattle moving into these locations is greater compared to the RECS, potentially increasing the likelihood of capturing invasive ticks. Especially useful would be auctions located at the borders of Tennessee, which would have a greater chance of sampling imported cattle crossing state lines. Second, the number of auction locations in the state is far greater (40+) compared to the number of RECS (7), which would allow for greater regional spread in collections. Lastly, although the RECS willingly cooperated with sampling efforts in this study, collections were scheduled to coincide with other husbandry practices (ear tagging, vaccinations, pregnancy checking, etc.) which are performed a limited number of times annually. The auctions, if they offer pregnancy checking at their facilities, have more regular inspections of cattle with many of the locations in this study conducting auctions once a week. This could therefore offer a weekly monitoring schedule for tick activity. These factors combined result in an effective means for monitoring for invasive ticks, and offers the opportunity for increased resolution of geographic tick distribution and seasonal phenology in future surveys.

Interestingly, *A. americanum* and *D. variabilis* were found co-feeding on 1.0% (n = 14) of sampled animals and have a significant positive co-infestation relationship, meaning that when *D. variabilis* is found on a host it is likely that *A. americanum* will also be present. There are several factors that can explain this relationship, including similar host use, overlapping geographic distribution, and matching temporal patterns of activity. Indeed, in a survey of ticks in Tennessee using drag and mammal trapping Cohen et al. (2010) noted that *D. variabilis* and *A. americanum* were common and collected across multiple locations in Tennessee. Additionally, these species have been known to parasitize cattle in Tennessee (Pompo et al. (2016). Lastly, several publications have shown that the seasonal activity of *A. americanum* (Davidson et al. 1994, Jackson et al. 1996) and *D. variabilis* (Burg 2001) occurs primarily in spring with adults disappearing by August. The finding that these two species co-infest cattle is important for two reasons. The first is the potential for *D. variabilis* to act as an indicator of infestation by *A. americanum*, which may be useful for determining if the economic threshold has been surpassed, although more research would be required to elucidate the relationship between *D. variabilis* and *A. americanum* densities on cattle. Second, although *A. americanum* is not considered a biological vector of *A. marginale*, it could nonetheless play an important role in pathogen transmission by suppressing the host immune response (Wikel and Whelen 1986, Wikel et al. 1994, Wikel 1999), allowing for infection via *D. variabilis* feeding.

Importantly, several pathogens and invasive ticks are threatening the US cattle industry. As mentioned, the distributions of *A. americanum* and *D. variabilis* may serve as predictors for *Anaplasma marginale* distributions and *A. maculatum's* distribution may serve as a predictor for *E. ruminantium* distribution. Several southern US states could be invaded by multiple threats, including the Texas Cattle fever ticks (*R. microplus* and *R. annulatus*) that transmit the agents of Texas Cattle fever (*Babesia bovis* and *B. bigemina*) and the Bont ticks (*A. variegatum* and *A. hebreum*) which can both transmit the agent of Heartwater (*E. ruminantium*). Future work should expand the surveillance strategy from Tennessee into other states at risk from these invasive threats to protect the U.S. cattle industry as a whole. This multi-state collaboration would be beneficial in that invaded states could serve as an early detection system for yet impacted states. Additionally, this strategy would make concerted eradication and quarantine efforts possible.

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Appendix B

Table 2.1 Tick species parasitizing cattle in Tennessee.

Species	Life-stage	No. Ticks	No. Animals	Mean (± SEM)	Infestation Prevalence (%)	Tick Burden
Amblyomma americanum	Nymph	40	32	0.02 ± 0.004	1.76	1.25
	Male	180	109	0.01 ± 0.01	5.99	1.65
	Female	353	185	0.19 ± 0.02	10.18	1.91
	Total	573	252	0.32 ± 0.03	13.87	2.27
Amblyomma maculatum	Nymph	0	0	0	0	0
	Male	106	35	0.06 ± 0.01	1.93	3.03
	Female	19	13	0.01 ± 0.003	0.72	1.46
	Total	125	40	0.07 ± 0.02	2.20	3.13
Dermacentor variabilis	Nymph	0	0	0	0	0
	Male	14	14	0.01 ± 0.002	0.77	1
	Female	21	21	0.01 ± 0.002	1.16	1
	Total	35	33	0.02 ±0.003	1.82	1.06

Amblyomma americanum, Amblyomma maculatum, and Dermacentor variabilis were found parasitizing cattle in Tennessee. Additional specimens collected from sampled cattle include 3 *Ixodes scapularis* and 4 tick specimens (0.5%) missing key morphological features which made them unidentifiable using dichotomous keys.

Table 2.2 Co-feeding relationships among tick species.

Dominant Species	Co-feeding Species	Number of Cattle: Both Present	Only Dominant	Only Co-feeding	Both Absent	Cole's Index (C_7 ± SE)
Interspecific Competition						
Amblyomma americanum	*Amblyomma maculatum*	4	244	36	1113	-0.437 ± 0.3350 (P = 0.1929)
Amblyomma americanum	*Dermacentor variabilis*	14	234	19	1130	0.300 ± 0.0799 **(P = 0.0002)**
Amblyomma maculatum	*Dermacentor variabilis*	2	38	31	1326	0.039 ± 0.0329 (P = 0.2650)
Intraspecific Competition						
Amblyomma americanum adults	*Amblyomma americanum* nymphs	23	225	8	1141	0.686 ± 0.0825 **(P < 0.0001)**
Amblyomma americanum females	*Amblyomma americanum* males	46	130	56	1165	0.351 ± 0.0341 **(P < 0.0001)**
Amblyomma maculatum females	*Amblyomma maculatum* males	8	27	5	1357	0.606 ± 0.0443 **(P < 0.0001)**

Ixodes scapularis was found co-feeding with *D. variabilis* and *A. maculatum*. These interactions occurred only once each.

Table 2.3 Infestation prevalence.

Variable of Interest (n = No. cattle)	*Amblyomma americanum*	*Amblyomma maculatum*	*Dermacentor variabilis*	Overall
		Seasonal Effect		
Spring (n = 297)	6.5 ± 3.9	16.2 ± 10.7 [a]	2.5 ± 1.2	23.6 ± 10 [a]
Summer (n = 307)	9.1 ± 9.1	0.9 ± 0.93 [ab]	1.9 ± 0.9	10.9 ± 8.5 [ab]
Fall (n = 194)	0.0 ± 0.0	0.0 ± 0.0 [b]	0.0 ± 0.0	0.0 ± 0.0 [b]
Statistic	1.59 (0.2361)	**6.82 (0.0078) ***	3.54 (0.0550)	**9.54 (0.0021) ***
		Regional Effect		
Western (n = 362)	20.0 ± 4.5	3.9 ± 1.9 [a]	3.6 ± 1.7	24.9 ± 5.5
Middle (n = 628)	26.2 ± 7.5	1.2 ± 1.2 [b]	1.6 ± 1.1	27.4 ± 7.3
Eastern (n = 407)	22.3 ± 18.9	0.0 ± 0.0 [ab]	1.1 ± 0.6	23.3 ± 18.4
Statistic	0.13 (0.8811)	**4.83 (0.0161) ***	2.10 (0.1416)	0.16 (0.8488)
		Site Effect		
REC (n = 604)	7.7 ± 7.0	9.5 ± 7.4 [a]	2.0 ± 0.3	17.6 ± 7.5
EXT (n = 374)	27.4 ± 7.2	0.0 ± 0.0 [b]	1.9 ± 1.2	27.9 ± 7.2
Auction (n = 419)	21.9 ± 6.5	3.9 ± 2.20 [a]	2.6 ± 1.0	25.6 ± 7.1
Statistic	0.52 (0.5985)	**18.3 (<0.0001) ***	**4.81 (0.0163) ***	0.19 (0.8271)

Statistics are reported as the F value and respective P value as $F(P)$. P values that are significant are bolded and denoted by (*). Mean values are calculated from raw data and do not reflect rank transformed data. Mean values within a column with different lower-case letters are significantly different at $P < 0.05$.

Table 2.4 Burden.

Variable of Interest (n = No. cattle)	*Amblyomma americanum*	*Amblyomma maculatum*	*Dermacentor variabilis*	Overall
		Seasonal Effect		
Spring (n = 297)	0.6 ± 0.3	1.7 ± 0.7 [a]	0.6 ± 0.23	2.0 ± 0.64 [a]
Summer (n = 307)	0.4 ± 0.4	0.3± 0.3 [ab]	0.5 ± 0.3	0.9 ± 0.3 [ab]
Fall (n = 194)	0.0 ± 0.0	0.0 ± 0.0 [b]	0.0 ± 0.0	0.0 ± 0.0 [b]
Statistic	1.96 (0.1756)	**6.68 (0.0084) ***	3.55 (0.0546)	**11.16 (0.0011) ***
		Regional Effect		
Western (n = 362)	2.1 ± 0.8	0.6 ± 0.2 [a]	0.6 ± 0.2	1.8 ± 0.5
Middle (n = 628)	1.5 ± 0.4	0.3 ± 0.2 [b]	0.2 ± 0.1	1.7 ± 0.4
Eastern (n = 407)	1.5 ± 0.2	0.0 ± 0.0 [ab]	0.7 ± 0.4	1.5 ± 0.2
Statistic	0.85 (0.4375)	**4.83 (0.0161) ***	2.68 (0.0868)	0.30 (0.7408)
		Site Effect		
REC (n = 604)	0.9 ± 0.5	1.8 ± 1.3 [a]	1.1 ± 0.1 [a]	2.4 ± 1.0
EXT (n = 374)	1.5 ± 0.4	0.0 ± 0.0 [b]	0.1 ± 0.1 [b]	1.5 ± 0.4
Auction (n = 419)	2.4 ± 0.8	0.7 ± 0.2 [a]	0.7 ± 0.2 [a]	2.1 ± 0.6
Statistic	1.62 (0.2165)	**18.58 (<0.0001)***	**11.13 (0.0003)***	1.81 (0.1829)

Statistics are reported as the F value and respective P value as $F(P)$. P values that are significant are bolded and denoted by (*). Mean values are calculated from raw data and do not reflect rank transformed data. Mean values within a column with different lower-case letters are significantly different at $P < 0.05$.

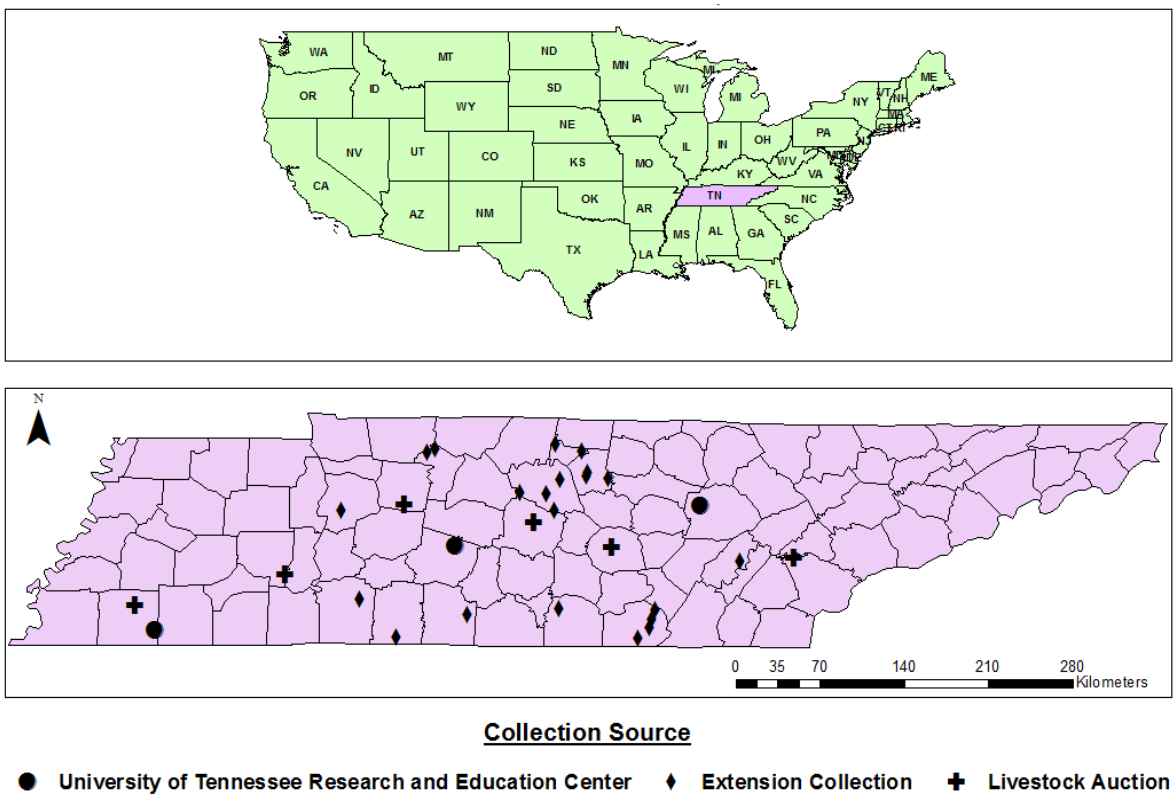

Figure 2.1 Collection sources across Tennessee 2015 – 2016.

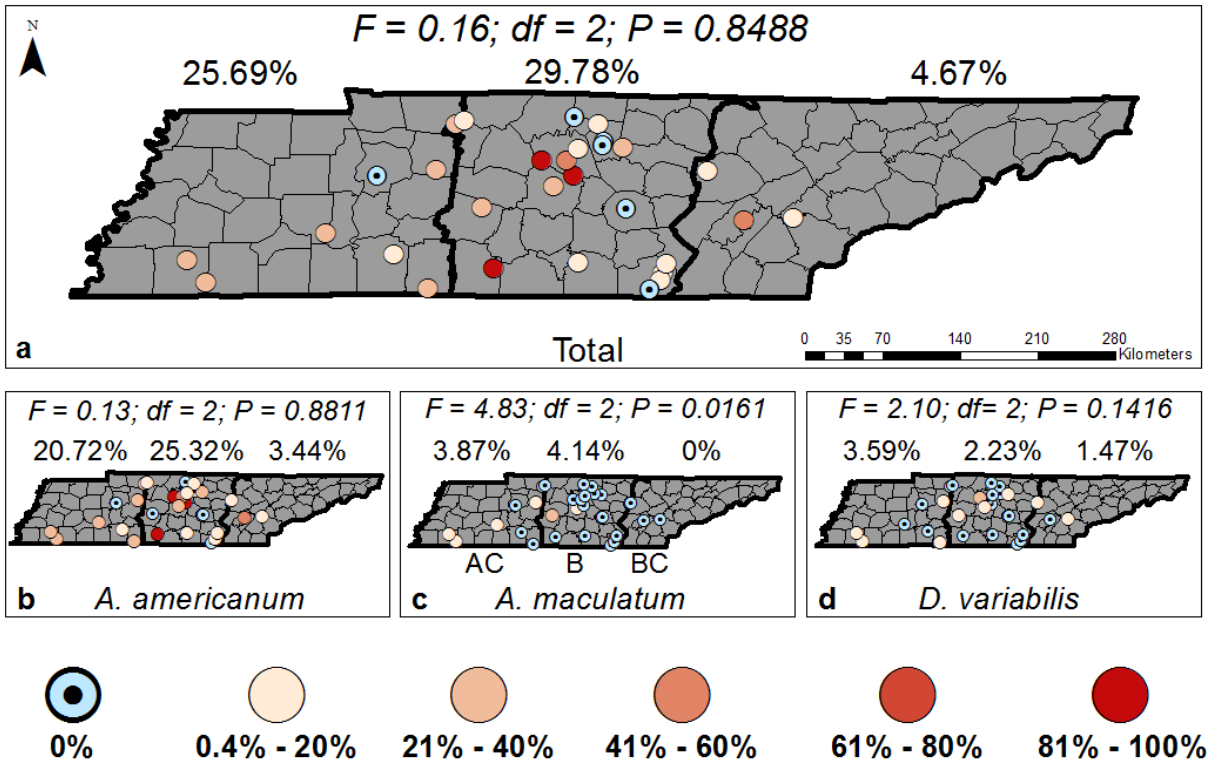

Figure 2.2 Infestation prevalence of cattle infested with ticks in Tennessee. Values shown are calculated from raw data and do not represent transformed data. Infestation prevalence varied by the species total (a) and each tick species; *Amblyomma americanum* (b), *Amblyomma maculatum* (c), and *Dermacentor variabilis* (d). Region was only significant for *A. maculatum* infestation prevalence, with regions with different upper-case letters being significantly different at $P < 0.05$.

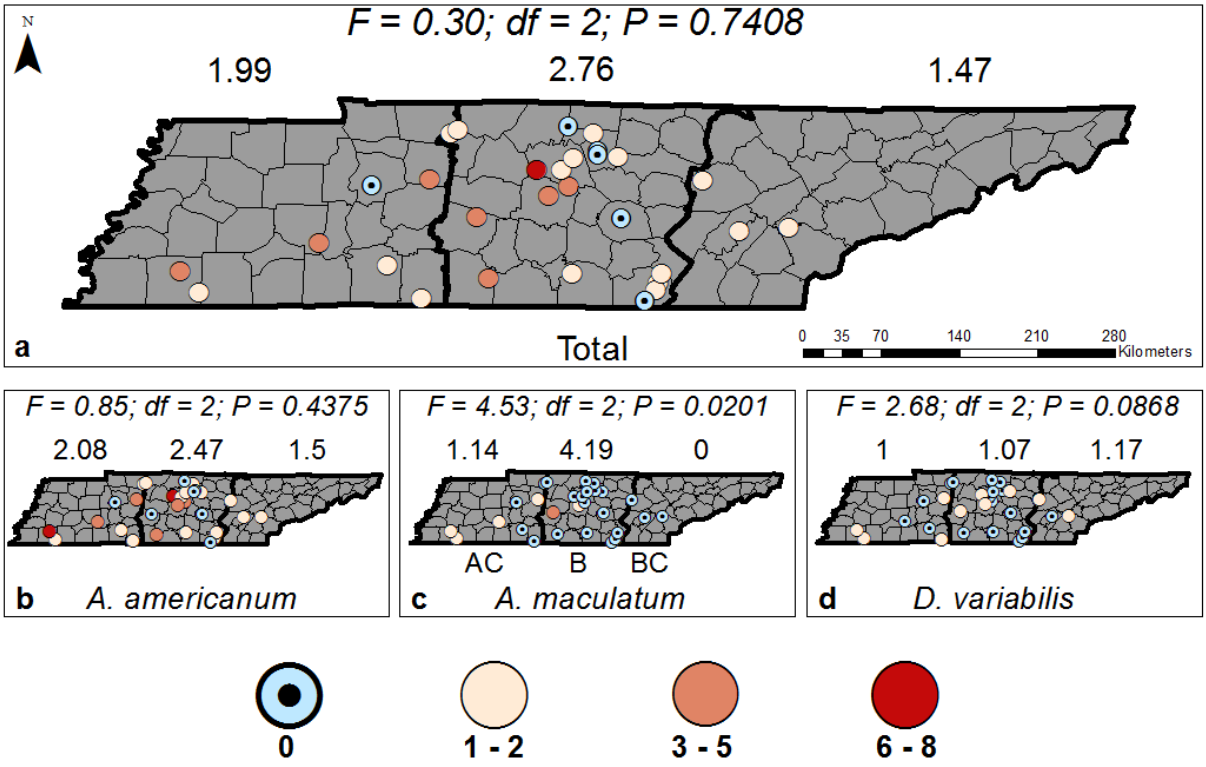

Figure 2.3 Burden of ticks on cattle in Tennessee. Values shown are calculated from raw data and do not represent transformed data. Burden varied by the species total (a) and each tick species; *Amblyomma americanum* (b), *Amblyomma maculatum* (c), and *Dermacentor variabilis* (d). Region was only significant for *A. maculatum* burden, with regions with different upper-case letters being significantly different at $P < 0.05$.

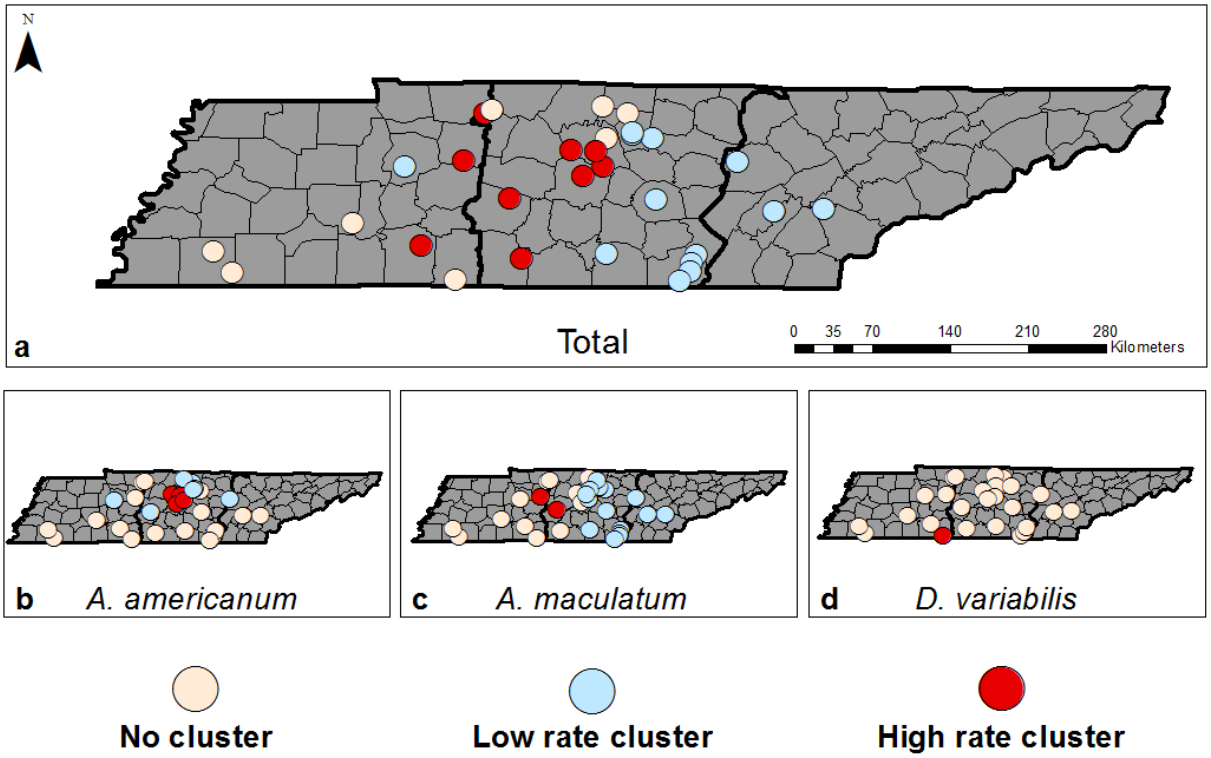

Figure 2.4 Spatial cluster analysis of tick infestation on cattle in Tennessee. High rate clusters were found for the species total (a) and each tick species; *Amblyomma americanum* (b), *Amblyomma maculatum* (c), and *Dermacentor variabilis* (d). Low rate clusters were found for each species except *D. variabilis*.

Chapter 3: Investigations into the Microbial Communities of Cattle-Associated and Questing Amblyomma maculatum

Abstract

The gulf coast tick *Amblyomma maculatum* is an emerging threat to both human and animal health capable of damaging hosts through direct attachment and transmission of pathogens such as *Rickettsia parkeri* and *Hepatozoon americanum*. The objectives of this study were to determine which microbes were consistently detected in the microbiome, and elucidate the potential impact of factors such as region of collection, sex, life-stage, and feeding status on the microbiome. A total of 182 *A. americanum* were collected from either hosts (n = 77), cattle pastures (n = 92), or laboratory reared (n = 5). We also had several egg batches (n = 5) included to investigate the role of vertical transmission of microbes. The Illumina MiSeq platform was used to sequence 300 bp paired end reads which were processed using Mothur 1.33.2. The R statistical package in R studio was used to compare differences in community structure with PERMANOVA and beta dispersion, Chao1 estimated richness, and Inverse Simpson estimated diversity. The bacterial phyla Actinobacteria, Bacteroidetes, and Proteobacteria were present in all samples (n = 182), with Proteobacteria comprising the largest proportional abundance (75%). At the genus level, *Francisella* was found in all samples. PERMANOVA results revealed significant differences in β-diversity for comparisons between environment (P = 0.003), sex of questing adults (P = 0.001), and feeding status (P = 0.001). Differences in α- diversity were shown for both richness (environment (P = 0.003), feeding status (P = 0.0002)) and diversity (sex of questing (P = 0.0001) and attached (P = 0.03) adults). Collection location and life stage had no significant impact on either α- or β-diversity measures. These results provide further evidence that *Francisella* may serve as an endosymbiote to *A. maculatum*, and demonstrate that differences in α- and β-diversity can be driven by tick associated factors. Exploring the microbiome of *A. maculatum* and determining factors that can lead to changes in the microbiome is an important first step in understanding heterogenous risk of pathogen transmission and identifying endosymbiotic bacteria for use in integrated pest management strategies against this pest.

Introduction

Amblyomma maculatum Koch, the Gulf Coast tick, is an important medical and veterinary pest. Direct attachment to a host causes damage through dermatitis, allergies, introduction of toxic salivary compounds, and by providing entry points for secondary infections (Jongejan and Uilenberg 2004). Additionally, this tick is a known vector of several pathogens important to human and animal health.

As a medical pest, *A. maculatum* transmits the pathogen *Ricekttsia parkeri* (Paddock et al. 2004). This pathogen can cause febrile illness in humans, and symptoms can be similar to other Spotted fever group *Rickettsia* including the etiological agent of Rocky Mountain Spotted Fever *R. rickettsii* (Paddock et al. 2008). Additionally, another rickettsial organism (Candidatus *R. andeanae*) has been discovered infecting *A. maculatum* in the U.S. (Paddock et al. 2010, Fornadel et al. 2011, Jiang et al. 2012). The pathogenicity of this *Rickettsia* is currently undetermined.

As a veterinary pest, *A. maculatum* is damaging to both companion animals and livestock especially cattle. Infestations of *A. maculatum* on cattle can cause damage to the cartilage in the ear, leading to a characteristic drooping known as Gotch ear (Edwards 2011). Biting pressure from this pest can be severe, and has been shown to cause decreases in weight gain in calves (Williams et al. 1977, Williams et al. 1978). Although this species does not currently act as a vector of pathogens important to the health of cattle in the U.S., it should be considered a serious threat given that it has been shown to be an efficient vector of *Ehrlichia ruminantium*, the causative agent of Heartwater (Uilenberg 1982, Mahan et al. 2000). In countries where this pathogen is endemic, economic losses can measure into the millions of USD (Meltzer et al. 1996, Mukhebi et al. 1999, Kivaria 2006). Given that cattle in the U.S. are susceptible to Heartwater, it is likely that the establishment of this invasive pathogen would devastate the cattle industry, with expected death losses estimated at ≥70% (Wagner et al. 2002). In addition to the food security and cattle health risk this tick species represents, *A. maculatum* is also a vector of *Hepatozoon americanum*, the causative agent of Hepatozoonosis in dogs in the southcentral and southeastern U.S. (Vincent-Johnson et al. 1997, Mathew et al. 1998). Although new treatments have improved prognosis, there is currently no medication to permanently clear infection and thus dogs are prone to relapse (Potter and Macintire 2010).

Measures to control ticks have typically relied on the use of chemical acaricides. Although this strategy can be efficacious, there are several important issues with sole reliance on acaricides including the development of resistance in pest tick populations. Therefore, the challenge facing livestock producers, pet owners, and citizens is to decrease the use of acaricides to control *A. maculatum* while simultaneously maintaining animal and human health and welfare. One option for striking a balance between these two goals is to explore new control methods that fit within an integrated pest management (IPM) strategy. The aim of this strategy is to combine control tactics to decrease impacts to the environment in a cost-effective manner (Bram 1994). This strategy is defined by using two or more control tactics in concert to reduce pest populations, and is advantageous because it reduces the likelihood of resistance development, and the negative impacts to the environment. A potential avenue that could lead to new control tactics lies within the tick microbiome, the collection of all microorganisms living on and within the tick host

including bacteria, fungi, nematodes, protozoa, and viruses. In this study, and others focused on tick microbiomes, bacteria are of special interest because ticks are hosts to several bacteria that are closely related to vertebrate pathogens including those in the genera *Francisella*, *Coxiella*, and *Rickettsia* (Bonnet et al. 2017). Additionally, there are a diverse set of bacteria whose relationship with their tick host is currently unknown.

Having insight into the structure and composition of the tick microbiome can be a source of potential control methods including competitive exclusion, paratransgenic control, and endosymbiont elimination. It has been suggested that in the ecology of the microbiome, vertically transmitted bacteria (mother to offspring) must enhance the host or be lost from the population (Fine 1975, Ewald 1987). In this scenario, the advantage to the host of being infected with a vertically transmitted parasite is the prevention of infection by horizontally transmitted parasites [pathogens] (Lively et al. 2005). Indeed, studies on Tsetse flies (*Glossinia morsitans*) found that the absence of the endosymbiont *Wigglesworthia glossinidia* through antibiotic elimination caused an increase in the host's susceptibility to infection by Trypanosoma brucei the causative agent of African sleeping sickness (Pais et al. 2008). [replace with burgdorfer paper]. Paratransgenic transformation is a strategy where endosymbionts within the host are targeted for genetic manipulation. For blood feeding arthropods, this could be used to create symbiotic bacteria that produce compounds that inhibit infection by pathogenic microbes. Durvasula et al. (1997) eliminated or greatly reduce infection of the kissing bug (*Rhodnius prolixus*) by *Trypanosoma cruzi*, the etiological agent of Chagas disease through transformation of the symbiont *Rhodococcus rhodnii* to produce the antibacterial compound Cecropin A. Alternatively, elimination of endosymbionts is another possible strategy. This has been demonstrated by reducing reproductive fitness of *A. americanum* females and survival of offspring using antibiotics to eliminate a *Coxiella* bacterial symbiont (Zhong et al. 2007). Before these potential control options can be explored and integrated into an IPM strategy, it is imperative to gather baseline information on what microorganisms reside within the microbiome and elucidate the identities of the organisms present. Furthermore, understanding the factors that can lead to changes in the structure and composition of the microbiome and how these may lead to subsequent increases or decreases in risk of pathogen transmission will be vital in implementing control strategies and monitoring protocols.

Extensive research has been dedicated to investigations of the lone star tick *Amblyomma americanum*, which has resulted in the identification of a *Coxiella* symbiont (Jasinskas et al. 2007, Klyachko et al. 2007, Clay et al. 2008) likely needed for vitamin production (Smith et al. 2015). Furthermore, factors such as sex, geographic location, and blood feeding are shown to impact the microbiome (Clay et al. 2008, Heise et al. 2010, Ponnusamy et al. 2014, Williams-Newkirk et al. 2014). Comparatively, few studies have focused solely on the description of the microbiome of *A. maculatum* (Budachetri et al. 2014), with no studies into factors that can cause differences in the microbiome. Therefore, the objectives of this study were to 1) determine the common microbiome inhabitants between laboratory and field collected samples to elucidate the 'core' *A. maculatum* microbiome and 2) identify if the factors of sex, collection location, and blood-feeding can cause significant changes to microbial community structure. Additionally, we were interested in the ability of female ticks to vertically transmit bacteria to their offspring. We test the hypothesis that the microbiome of *A. maculatum* will have common bacteria and that life history factors will lead to differences in the microbiome.

Materials & Methods

Collection sources: Collections of *A. maculatum* were conducted at several sites across Tennessee, including three University of Tennessee Research and Education Centers (Ames Plantation, Middle Tennessee, and Plateau), six livestock auctions, and nine extension agents collaborating with cattle producers (up to four). Ticks were also purchased from the Oklahoma State University Tick Rearing Facility and served as controls, because they had fed on the same animals, were genetically similar, and should have similar microbial communities. All ticks were identified to life stage and sex using dichotomous keys (Sonenshine 1979). Additionally, ticks were visually assessed for the presence of a blood meal and were separated into three primary categories: questing, attached, or engorged. Ticks that had taken a blood meal (partial to full repletion) were considered engorged. Ticks that had inserted mouthparts but no apparent blood meal were attached. Ticks that were collected from the host that were not attached by their mouthparts, or collected from vegetation, were considered questing. Following identification and blood meal assessment, ticks were placed into 80% ethanol for storage.

Host collection: Ticks were collected directly from cattle run through a chute to maximize collection efficiency and protect the safety of both the investigator and the animal. Cattle were scratched and visually checked in areas known to be common tick attachment sites (Gladney et al. 1974, Barnard 1981, Barnard et al. 1982, Bloemer et al. 1988) for a maximum of five minutes to minimize animal stress. Collected ticks were placed into a vial containing 80% ethanol, with one vial used per animal. All ticks from hosts were collected in 2016 under University of Tennessee approved IACUC #2192 and originated from 6 sites (Figure 3.1, Appendix C). A total of 82 host-collected A. maculatum were used in the analyses and specimens were selected to reduce differences based on factors such as age of the host animals, region of collection, and collection period. Some female ticks were shipped to the laboratory (n = 14) in non-transparent containers without ethanol, resulting in some of these laying egg batches. For 3 samples, one female laid one egg batch; however, the remaining specimens that exact identity of the mother tick could not be determined. Therefore, the specimens were considered 'oviposited', with all females in one container associated with the egg batch. Therefore, female specimens were comprised of (14 oviposited, 10 attached females, 10 engorged), males were comprised of (48 attached), and there were 7 egg batches.

Field sampling: Concurrently with host collections described above, drag collections and CO_2 -baited traps were conducted in the same pastures as sampled cattle. No *A. maculatum* were collected using these methods. Consequently, we used sequences from *A. maculatum* that had been previously collected in 2012 and 2013 from vegetation at Ames plantation (35.115366 N, -89.216735 W), located in western Tennessee. Briefly, these specimens were collected using different methods including: CO2-baited traps, drags and flags, traditional dragging and flagging, and sweep netting (Mays et al. 2016). Collected ticks were stored in vials containing 80% ethanol, and extracted DNA was processed at the same facility using the same procedures as host-collected ticks. These specimens comprised of 42 questing females, and 50 questing males.

Lab-reared specimens: Seven lab specimens reared on rabbit or sheep blood were purchased from the Oklahoma State University Tick Rearing Facility and included: a single unfed male,

one engorged male, an unfed female, one engorged female, one oviposited female, and her corresponding egg batch.

DNA extraction: All procedures were performed using sterilized instruments and surfaces to reduce contamination by foreign bacteria. Host sampled specimens were also surface sterilized by exposing both the dorsal and ventral surface of the ticks to U.V. light for five minutes each. This sterilization was not done on eggs, because of the concern that the chorion would not adequately protect the bacterial contents of the eggs. Bacterial DNA from the field-collected questing specimens (n = 92) were extracted using Qiagen DNA Extraction kits as previously described (Mays et al. 2016). DNA from ticks collected from cattle and OSU ticks (n = 95) were extracted using the QIAamp 96 DNA QIAcube HT kit (Venlo, Netherlands). Following sterilization, tick samples were bisected using a sterile scalpel and placed in 200 uL of sterile water overnight to remove ethanol. Water was removed the following day, and 20 uL of ProK, 180uL of Digestion Solution, and a sterilized metallic bead were added prior to mechanical lysis in the QIAgen Tissue Lyser II for 20 seconds at 15 hz. The plates were then flipped and lysed a second time. Samples were then centrifuged at 8,000 rpm for 2 minutes and placed overnight into the Max Q™ 4450 Benchtop Orbital Shaker (Thermo Fisher, Waltham MA) at 56°C and 75 rpm. Extracted materials were loaded into the QIAcube HT, which automated the extraction process to completion. PCR grade water was subjected to the same extraction procedures and used as a negative control. Controls contained a total of 82 bacterial genera, with commonly found genera including: *Acinetobacter*, *Stenotrophomonas*, *Rhizobium*, *Pseudomonas*, and *Burkholderia*.

Microbial sequencing: The V3-V4 region of bacterial 16S rRNA from extracted DNA were amplified with the 341F and 785R primers at the Hudson Alpha Institute for Biotechnology (Huntsville, AL USA). The Illumina MiSeq platform was used to obtain paired 300 bp reads from pooled amplified sequences. Additionally, we wanted to reduce potential batch effects between the field collected samples that had been previously sequenced using the same methods. The samples that were able to be sequenced and therefore used in further analysis are shown in Table 3.1 (Appendix C).

Bioinformatics: MOTHUR is an open-source bioinformatics software package consisting of tools for microbial ecologists (Schloss et al. 2009). All read processing was carried out using MOTHUR v1.37.6 available in Newton, a general purpose linux cluster maintained available to researchers at the University of Tennessee. The standard operating procedure for processing MOTHUR MiSeq data (Kozich et al. 2013) was followed in addition to the protocol used by Trout Fryxell and DeBruyn (2016). Sequences that contained ambiguous bases or homopolymers with 8 or more nucleotides were removed. Chimeric sequences were detected and removed using the UCHIME chimera algorithm. Potential contaminants, such as mitochondrial or eukaryotic sequences, were also removed. Sequences were trimmed to 445 bases following alignment to a SILVA reference library. These trimming and removal steps throughout this process served as quality control. To further reduce batch effects, the samples from 2016 were processed simultaneously with samples from 2012 and 2013. Taxonomy was defined using the Ribosomal Database Project Data using 80% or greater similarity (Cole et al. 2013). Reads were binned at the genus level into Operational Taxonomic Units (OTUs) based on taxonomy using the phylotype clustering method in MOTHUR.

Statistical analyses: Analysis of results was performed using R v 3.3.0 (R Core Team 2016) in R Studio v 1.0.143 (RStudio Team 2016) with the packages reshape2 (Wickham 2007) and dplyr (Wickham and Francois 2015) to structure and manage data, phyloseq (McMurdie and Holmes 2013) and vegan (Oksanen et al. 2013) to analyze data using diversity measures, analyses and ordination methods, and ggplot2 (Wickham 2009), scales (Wickham 2016), grid (R Core Team 2016), randomcoloR (Ammar 2016), and cowplot (Wilke 2016) to create graphs to visually represent data. PERMANOVA using Bray-Curtis distances of reads scaled to the lowest read depth was used to determine differences in distance matrices between groups. Beta dispersion was conducted to test variance of groups from PERMANOVA, and make conclusions regarding beta diversity. Data for each test was rarefied prior to estimation of both richness (Chao1) and diversity (Inverse Simpson), with Kruskal-Wallis used to make comparisons of richness and diversity between groups. Samples for each test were selected to eliminate confounding variables.

Results

Description of *A. maculatum* microbial communities: A total of 9,669,598 sequences were obtained from 182 samples, with 27 bacterial phyla consisting of 797 genera level OTUs detected. The mean number of OTUs per library was 107.3 (± 3.21), and ranged from 26 – 307, with 159 unclassifiable at the genus level. 7 OTUs had a mean proportional abundance >2%, including *Sphingomonas* (3.33 ± 0.43%), *Rickettsia* (22.24 ± 2.42%), *Pseudomonas* (3.64 ± 0.65%), *Methylobacterium* (3.15 ± 0.34%), an unclassified Flavobacteriaceae (2.67 ± 0.68%), an unclassified Chlamydiales (2.47 ± 1.04%), and *Francisella* (25.05 ± 2.16%) which was detected in 100% of samples. At the phylum level, Actinobacteria, Bacteroidetes, and Proteobacteria were found in 100% of samples. Proteobacteria was found in high mean abundance (75 ± 1.8%), while Acintobacteria (2.9 ± 0.4%) and Bacteroidetes (8.7 ± 1%) comprised a relatively small fraction in microbial communities.

Core microbial comparisons: Field collected adult specimens (n = 169) were compared with laboratory reared specimen (n = 5) to detect 'core' microbial inhabitants. Significant differences in richness were observed between field collected samples and laboratory reared specimens (Table 2), with laboratory reared samples having lower mean richness (44.21 ± 6.32) compared to field specimens (86.01 ± 2.88). Of the 794 total OTU's detected in these samples, 122 OTUs were shared between field and laboratory samples with the highest proportional abundance observed in *Francisella* (laboratory = 31.9 ± 13.7%, field = 23.3 ± 2.14%).

Collection location comparisons: Host attached males from the middle Tennessee REC (n = 36), Ames REC (n = 6), and one auction in Dickson County (n = 5) had a total of 547 genera with samples from Ames containing 24 unique genera [*Modestobacter* (0.01 ± 0.01%), *Polynucleobacter* (0.04 ± 0.04%), *Dermacoccus* (0.003 ± 0.002%)], samples from the Dickson County auction containing 32 unique genera [*Elusimicrobium* (0.02 ± 0.01%), *Algoriphagus* (0.02 ± 0.02%), *Cellulosimicrobium* (0.03 ± 0.03%)] and samples from the middle Tennessee REC containing 180 unique genera [*Adhaeribacter* (0.03 ± 0.01%), *Buttiauxella* (0.003 ± 0.002%), *Dokdonella* (0.01 ± 0.004%)]. No differences in either alpha or beta diversity were found (Table 2). Collection comparisons are shown in Figure 3.2, Appendix C.

Sex comparison: Questing males (n = 50) and females (n = 42) had a total of 571 genera. Males had 117 unique taxa including *Dietzia* (0.01 ± 0.01%), *Sporocarcina* (0.004 ± 0.003%) and *Tissieriella* (0.003 ± 0.002%). Females had 66 unique genera that included *Blastopirelulla* (0.003 ± 0.002%), *Porphyromonas* (0.002 ± 0.001%) and *Gemella* (0.001 ± 0.001%). Significant differences in diversity were observed between sexes (Table 2) with females having lower mean diversity (3.42 ± 0.52) compared to males (7.64 ± 0.92). Attached males (n = 37) and females (n = 10) contained a total of 622 genera. Males had 179 unique OTUs that included *Acetivibrio* (0.01 ± 0.004%), *Aquabacterium* (0.02 ± 0.01%) *Larkinella* (0.02 ± 0.01%). Females had 75 unique OTUs, including *Anaerovorax* (0.007 ± 0.006%), *Citrococcus* (0.0008 ± 0.0005%) and *Desulfosporosinus* (0.009 ±0.008%). Again, significant differences in diversity were observed between sexes (Table 2) with females having lower mean diversity (2.98 ± 0.76) compared to males (10.2 ± 1.32). Abundant OTUs (>10%), alpha and beta diversity comparisons of sexes are shown in Figures 3.3 & 3.4 (Appendix C).

Feeding comparisons: 641 OTUs were found in comparisons of feeding status, with questing females (n = 42) containing the greatest number of unique genera (138) [e.g. Curvibacter (0.02 ± 0.003%), Dechloromonas (0.2 ± 0.04%), Schlegelella (0.1 ± 0.03%)], followed by attached females (99) [Cellulomonas (0.05 ± 0.04%), Leadbetterella (0.02 ± 0.01%), Muricauda (0.002 ± 0.001%)], oviposited females (16) [Lampropedia (0.01 ± 0.005%), Ketogulonicigenium (0.0004 ± 0.0003%), Anaerobacter (0.001 ± 0.0003%)]and engorged females having the fewest unique genera (9) [Agromyas (0.02 ± 0.02%), Corallococcus (0.01 ± 0.01%), Pastuerella (0.1 ± 0.1%)]. Analysis revealed differences in both beta and alpha diversity (Table 2), with engorged females (55.06 ± 9.07) having the lowest mean richness compared to questing (100.12 ± 5.73), attached (130.07 ± 18.37), and oviposited (83.23 ± 11.70) females. Figure 3.5 (Appendix C) depicts abundant OTUs, as well as comparisons between feeding levels.

Horizontal similarities in mother and egg batches: Females that were oviposited (n = 10) and corresponding egg batches (n = 5) were compared to determine potential transovarial transmission of microbes. In total, there were 322 OTUs in these samples with 96 unique genera found in adults (Gp4 (0.03 ± 0.02%), unclassified Acetobacteraeceae (0.03 ± 0.02%), unclassified Betaproteobacteria (0.02 ± 0.01%)) and 41 unique genera found in eggs (Elusimicrobium (0.01 ± 0.01%), Brevibacterium (0.01 ± 0.01%), Filimonas (0.01 ± 0.01%)). There were no significant differences in either alpha or beta diversity measures demonstrated (Table 3.2, Appendix C). Principle coordinate ordination (Figure 3.6, Appendix C) demonstrated that the mothers are not necessarily closest to their own offspring.

Discussion

This study identified several taxa that were ubiquitous in all samples tested. At the phylum level Proteobacteria, Actinobactera, and Bacteroidetes were found in all samples. These results match closely to previous investigations of the microbiome of *A. maculatum* which found Proteobactera, Actinobacteria, Firmicutes, and Bacteroidetes, with Protebacteria acting as the most dominant phyla (Budachetri et al. 2014, Budachetri et al. 2017). At the genus level, *Francisella* was detected in all samples tested. *Francisella* like endosymbionts (FLEs) have been previously detected in *A. maculatum* (Scoles 2004, Budachetri et al. 2014, Gerhart et al. 2016, Budachetri et al. 2017) and have been extensively documented in other tick species including

Ornithodoros moubata, *Ornitodoros porcinus*, *Dermacentor andersoni*, *Dermacentor variabilis*, *Dermacentor albipictus*, *Dermacentor hunteri*, *Dermacentor nitens*, and *Dermacentor occidentalis* (Niebylski et al. 1997, Noda et al. 1997, Sun et al. 2000, Scoles 2004, Clayton et al. 2015). The exact functional role that FLEs fulfill within the host are currently unknown, although in *A. maculatum* it has been shown to be closely related to the mammalian pathogen *Francisella tularensis* (Gerhart et al. 2016). It is possible that FLEs are necessary to the development of their host, as evidenced by both transovarial and transtadial transmission in *D. albipictus* (Baldridge et al. 2009). Results from this study add evidence to this, as egg batches were dominated by *Francisella* (44.6 ± 17.3%), suggesting maternal transmission. Further studies into characterizing the functional role of the FLE present in *A. maculatum* are warranted to determine its suitability as a target of control within an integrated tick management program. Our results demonstrated that for both questing and host-associated adults, there was no difference in richness between sexes but a significant difference in diversity. Reduced diversity of female ticks compared to male ticks has not been previously studied in *A. maculatum*, but has been noted for other species of ticks including *Ixodes scapularis* (Van Treuren et al. 2015, Zolnik et al. 2016) and *Amblyomma americanum* (Ponnusamy et al. 2014, Williams-Newkirk et al. 2014), with dominance of *Rickettsia* found to be characteristic of females. In this study, both questing (♀ (20 ± 4.1%), ♂ (17.4 ± 4.1%)) and attached (♀ (48.9 ± 13.9%), ♂ (28.9 ± 5.9%)) ticks had relatively similar proportional abundance of *Rickettsia*; However, *Francisella* was markedly different between sexes for both questing (♀ (45.5 ± 4.8%), ♂ (16.6 ± 2.9%)) and attached (♀ (23.2 ± 8%), ♂ (8.2 ± 2.2%) adults. Dominance of *Francisella* within females may serve to increase the likelihood of transovarial transmission to offspring (Williams-Newkirk et al. 2014); Indeed, FLEs can invade malphigian tubules and ovaries of their host (Noda et al. 1997), a necessary precursor to transovarial transmission. The lack of significant differences in α- and β-diversity between mother ticks and egg batches in this study could be potentially attributed to this phenomenon. Similar findings demonstrated that field captured females and larvae had the lowest diversity compared to nymphs and males of *I. scapularis* (Zolnik et al. 2016). Further studies into the mechanisms by which sexually divergent microbial communities arise in adult ticks is warranted.

Our results indicated no differences between ticks collected from different locations in either α- or β-diversity measures. These results are counter to evidence that location can impact microbial communities in other tick species (Clay et al. 2008, Williams-Newkirk et al. 2014, Van Treuren et al. 2015). An important consideration is that these studies investigated ticks from different states in the U.S. separated by large geographical distances. In comparison, differences in microbial communities were not shown for ticks from a small geographic area within Indiana (Hawlena et al. 2013). In our current study, tick samples tested between locations were found attached to their host, which could have stabilized the differences between samples. Currently the impact of the host is unclear, with some evidence that differences in microbial community structure are not derived by blood feeding (Hawlena et al. 2013, Rynkiewicz et al. 2015, Zolnik et al. 2016). However, diversity of bacterial taxa was previously shown to differ between *I. ricinis* collected from three forests in the Netherlands with the potential explanatory variable being local distribution of available hosts (Van Overbeek et al. 2008). This is supported by the impact of host choice (mammalian or reptilian) on microbial community structure and richness of *I. pacificus* (Swei and Kwan 2017). It is possible that the abundance and presence of suitable hosts for *A. maculatum* are not different between collection locations investigated, resulting in

the observed findings of this study. Further research to clarify the small geographic scale effects on the microbiome of *A. maculatum* are necessary for understanding potentially heterogenous distribution of pathogens, which is important for identifying risk areas of pathogens that impact human and animal health.

This study revealed that diversity was not significantly different between stages of feeding engorgement, but that richness and β-diversity were affected. Similarly, blood feeding caused significant differences in community composition with no effect on the diversity of the microbiome of *I. persulcatus* (Zhang et al. 2014). A decrease in richness as engorgement increases is likely driven by the influx of proteins and toxic metabolites following a blood-meal, resulting in a bottleneck effect that alters microbiome composition, as seen in the malaria mosquito *Anopheles gambiae* (Wang et al. 2011). This could explain why engorged and oviposited ticks had the lowest mean richness; counter intuitively attached ticks had the highest mean richness (130.07 ± 18.37). Blood feeding in *A. americanum* resulted in increased diversity, proposed to be driven by both the reduction of dominant bacteria and the increased detection of less common taxa (Heise et al. 2010). These factors could help to explain the increased richness at attachment if these ticks had taken blood meals. Another potential factor that could cause the pattern observed in our current study is a greater number of contaminants resulting from host interaction compared to environmental contaminants from questing ticks. Ultimately, the cause of greatest richness at attachment is worth further exploration.

The controls used in this study were contaminated by several bacterial genera. Many of these genera, including: *Pseudomonas*, *Stenotrophomonas*, *Burkholderia*, *Acinetobacter*, and *Sphingomonas* are common soil or water contaminants that can be introduced from biological grade water, PCR reagents, or DNA extraction kits (McFeters et al. 1993, Nogami et al. 1998, Tanner et al. 1998, Corless et al. 2000, Kulakov et al. 2002, Grahn et al. 2003, Mohammadi et al. 2005, Mühl et al. 2010, Laurence et al. 2014, Salter et al. 2014). The number of studies on the tick microbiome that discuss the identity of contaminants are few, although some have reported genera including *Stenotrophomonas* (Clay et al. 2008), and *Acinetobacter* (Clayton et al. 2015). Samples for this study were stored in 80% ethanol and U.V. sterilized, therefore it is likely that contamination was introduced during subsequent sample processing although it cannot be determined with certainty the exact source. Contaminants are problematic because they can impact results and subsequent conclusions, especially in low bacterial biomass environments where contaminants become the majority of sequence reads (Salter et al. 2014, Clayton et al. 2015). Furthermore, the control run with the samples processed in 2016 had both *Francisella* and *Rickettsia*. If the control serves as an indicator of potential contamination of the samples from 2016 is unclear, as *Francisella* and *Rickettsia* were not detected in the control used for the 2012 and 2013 specimens, but were found in great abundance in the respective samples; Furthermore, we would expect to find these genera in high abundance based on previous research that explored the microbiome of *A. maculatum*. This current research should be added to the growing body of evidence that microbiome studies using amplified bacterial 16s rRNA should take precautions when collecting, storing, and processing samples to prevent contamination leading to potentially spurious results. Some precautions that future researchers could exercise are outlined in Salter et al. (2014) and include randomization of sample processing, maximizing the starting bacterial biomass of samples, and using multiple no template controls to identify contaminant sources.

The results from this study have demonstrated that the microbiome of *A. maculatum* has several bacterial taxa that were identified in all samples tested, which gives support to the idea that these bacteria are core components of the microbiome. To our knowledge, this research represents the first attempt to describe how factors such as sex, feeding status, and collection location impact the microbial communities of *A. maculatum*. Results from this research give important insights into the microbial communities of this important vector, which will be vital in tracking emerging pathogens important to human and animal health and gaining future understanding of risk factors for increased pathogen transmission. This will become paramount upon the introduction of the devastating cattle pathogen *Ehrlichia ruminantium*. Future studies should aim to clarify the role of *Francisella* in *A. maculatum*, and further explore interactions between microbes to determine the best means to incorporate these factors into an integrated control strategy.

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Appendix C

Table 3.1 Specimens of *A. maculatum* used for analysis.

Stage	Status	Region		Control	**Total**
		Middle	Western	Laboratory	
Male	Questing	0	50	0	50
	Attached	36	11	1	48
	Engorged	0	0	1	1
Female	Questing	0	42	0	42
	Attached	7	3	1	11
	Engorged	10	0	1	11
	Oviposited	10	0	1	11
Egg Batch	Developing	7	0	1	8
Total		70	106	6	**182**

Samples listed below produced viable libraries. Of the original number of submitted sampled, five samples did not produce libraries, including 1 attached male and 4 oviposited females from middle Tennessee.

Table 3.2 Summary of statistics from comparisons across multiple factors.

Comparison	α diversity H (*P*)		β diversity F (*P*)	
	Chao1	Inverse Simpson	PERMANOVA	Beta Dispersion
Environmental	**8.55 (0.003)***	0.07 (0.8)	**3.46 (0.003)***	**12.1 (0.003)***
Location	2.2 (0.33)	1.21 (0.55)	0.16 (0.09)	0.75 (0.47)
Sex				
Questing	2.66 (0.10)	**14.6 (0.0001)***	**8.96 (0.001)***	**9.66 (0.002)***
Attached	0.93 (0.33)	**4.67 (0.03)***	1.91 (0.08)	**6.57 (0.01)***
Engorgement	**19.7 (0.0002)***	4.68 (0.2)	**4.21 (0.001)***	0.85 (0.48)
Transovarial	0.38 (0.54)	0.96 (0.33)	0.68 (0.64)	0.81 (0.41)

Results in alpha diversity are reported as Kruskal-Wallis Chi^2 with respective *P* values [H (*P*)]. Beta diversity is reported by F value and *P* values [F(*P*)]. Significant values are bolded and marked with an asterisk (*). At the alpha diversity level, only sex caused differences in diversity, while engorgement and environment drove differences in richness. True differences in beta diversity (those without significant Beta Dispersion) were observed only for engorgement.

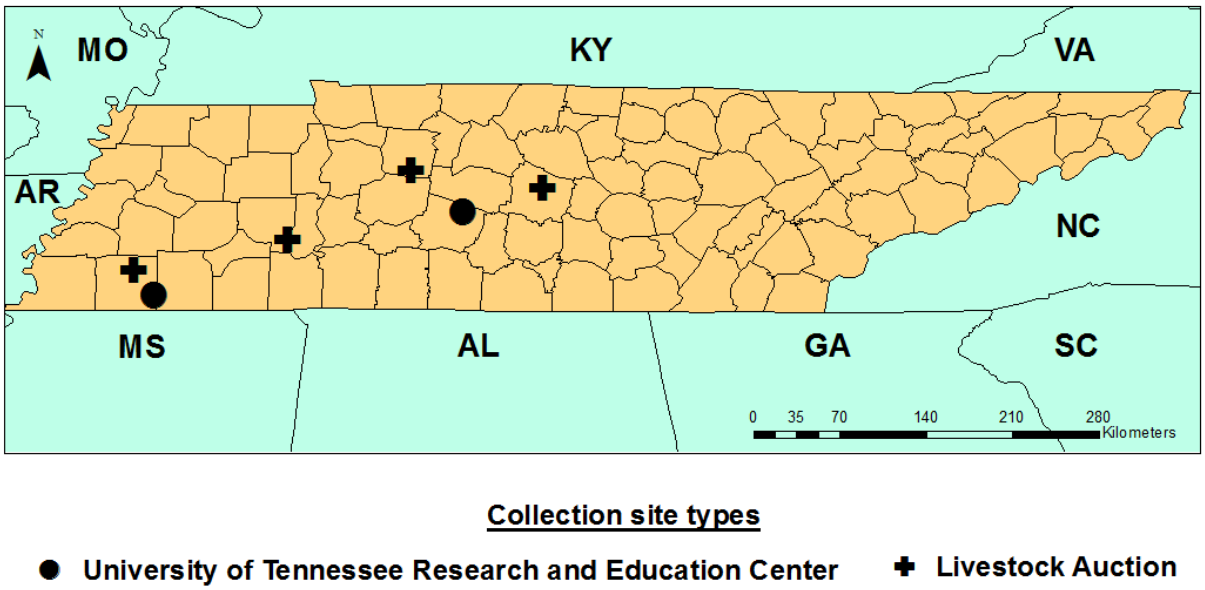

Figure 3.1 Collection sites where *A. maculatm* was captured. A. maculatum was never captured in eastern Tennessee, owed primarily to its recent expansion into the state

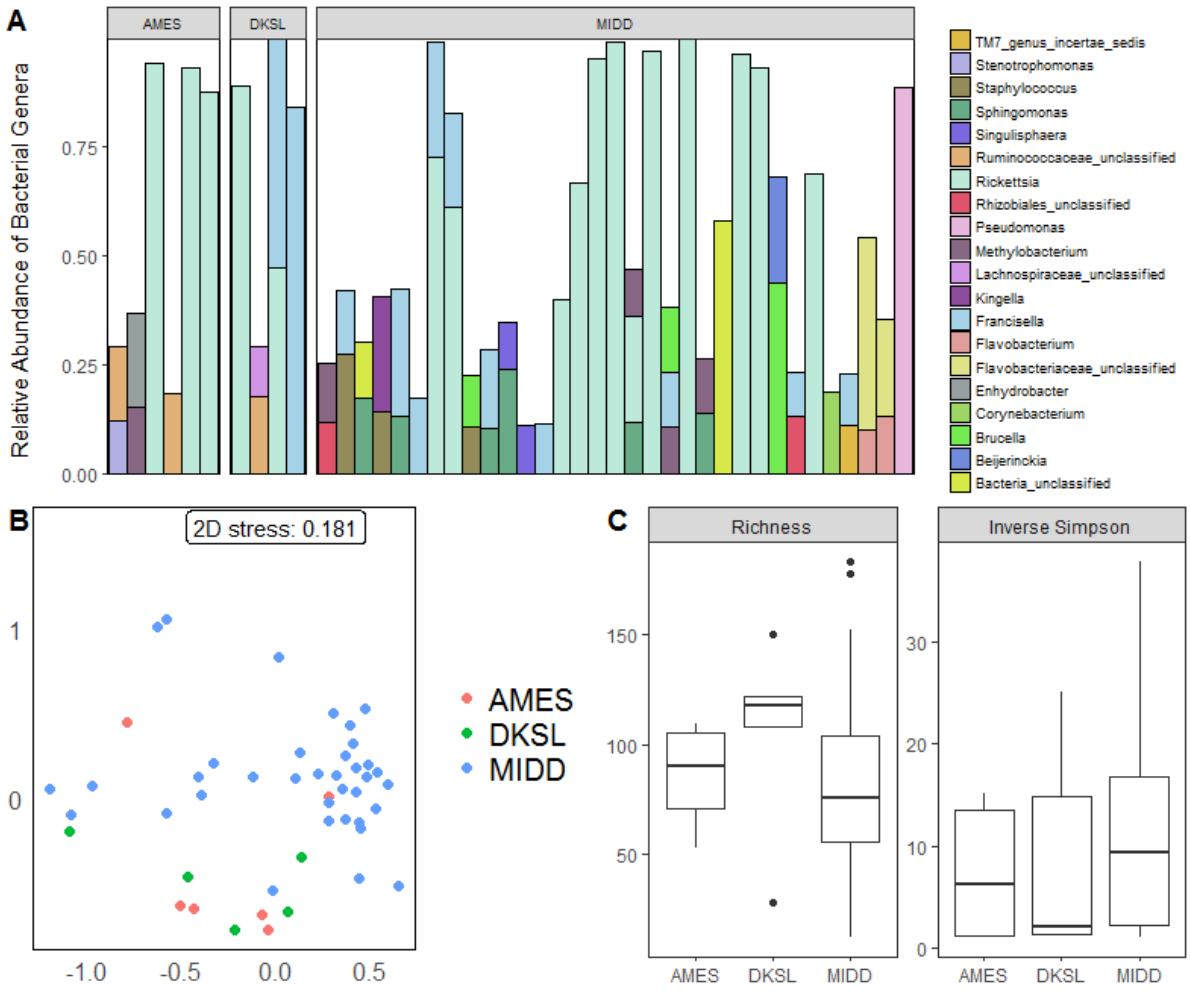

Figure 3.2 Graphs for collection location comparisons. Relative abundance of bacterial genera that comprise >10% of the total bacterial community is shown in (A), with each stack representing a sample and white space denoting bacterial genera that comprised < 10% relative abundance in the microbiome. 10% was used as the cutoff to reduce the number of unique colors required (98 colors at >2 %). Non-metric Multidimensional Scaling (NMDS) of Bray Curtis distances of samples from three collection locations (B) , PERMANOVA revealed no significant differences in β-diversity. Kruskal wallis revealed no significant differences in either richness (chao1) or diversity (inverse simpson) (C).

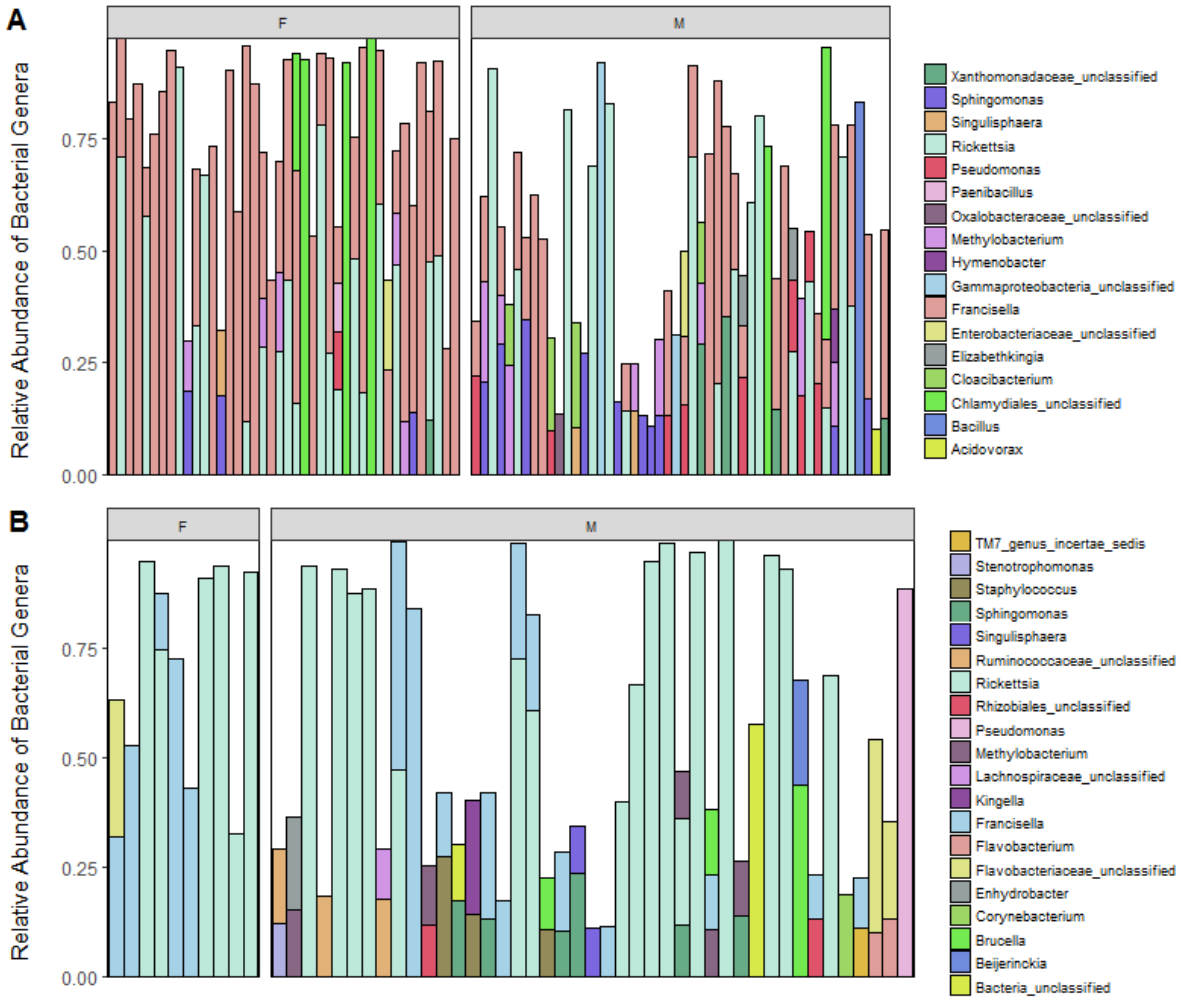

Figure 3.3 Bacterial genera that comprised >10% of the total taxa, for both questing (A) and attached (B) separated by sex (F= Female, M= Male). As in previous figures, each stack denotes a sample, with white space indicating bacteria that comprised < 10% of the relative abundance of the microbiome.

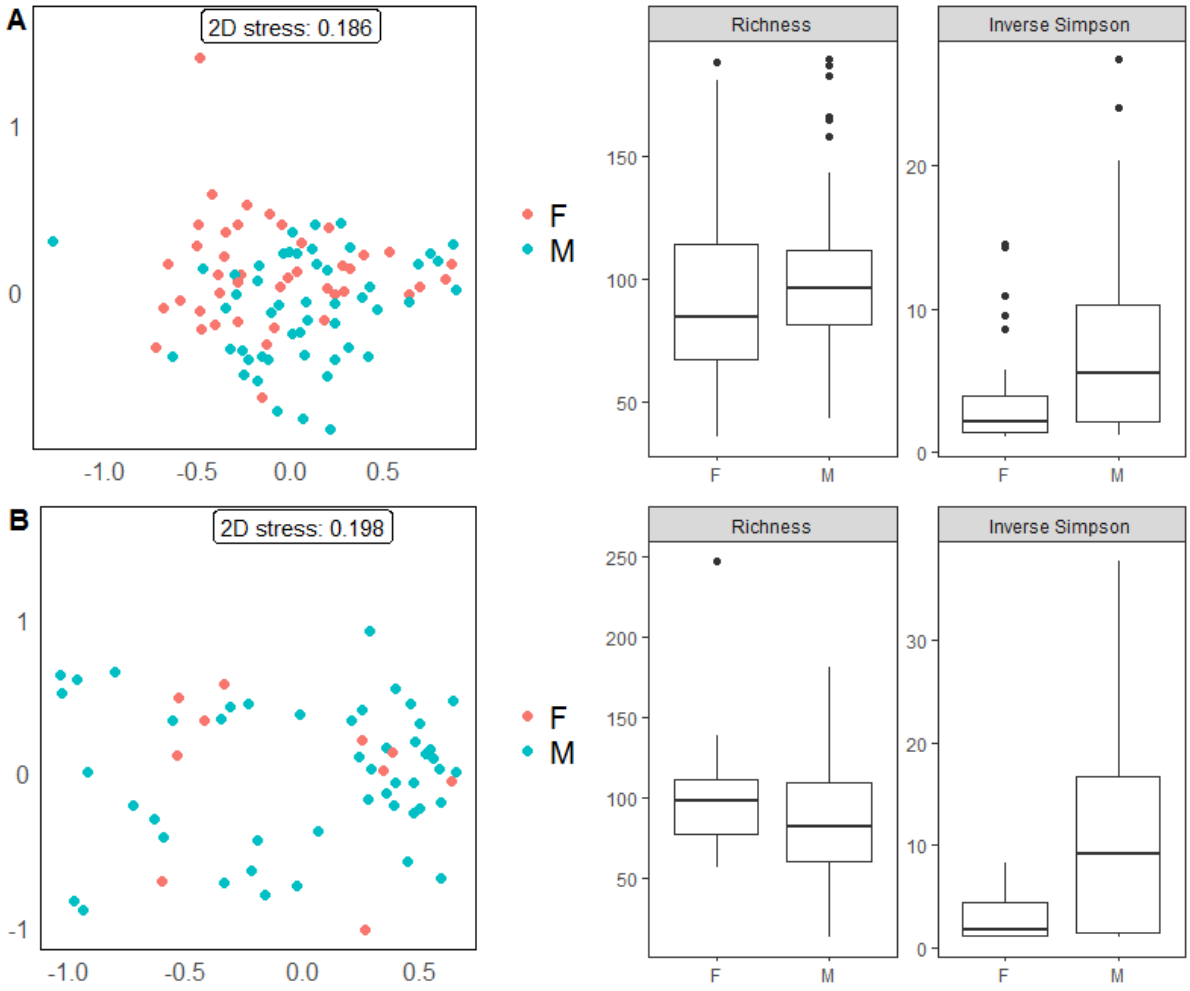

Figure 3.4 NMDS and box plot differences by sex (F = Female, M = Male) for questing (A,) and attached (B) ticks. Results from PERMANOVA showed a potential difference in β-diversity only for questing ticks, although beta dispersion was significant. In α-diversity richness was not significantly different between sexes of either feeding status, but diversity (inverse simpson) was significantly different for both comparisons.

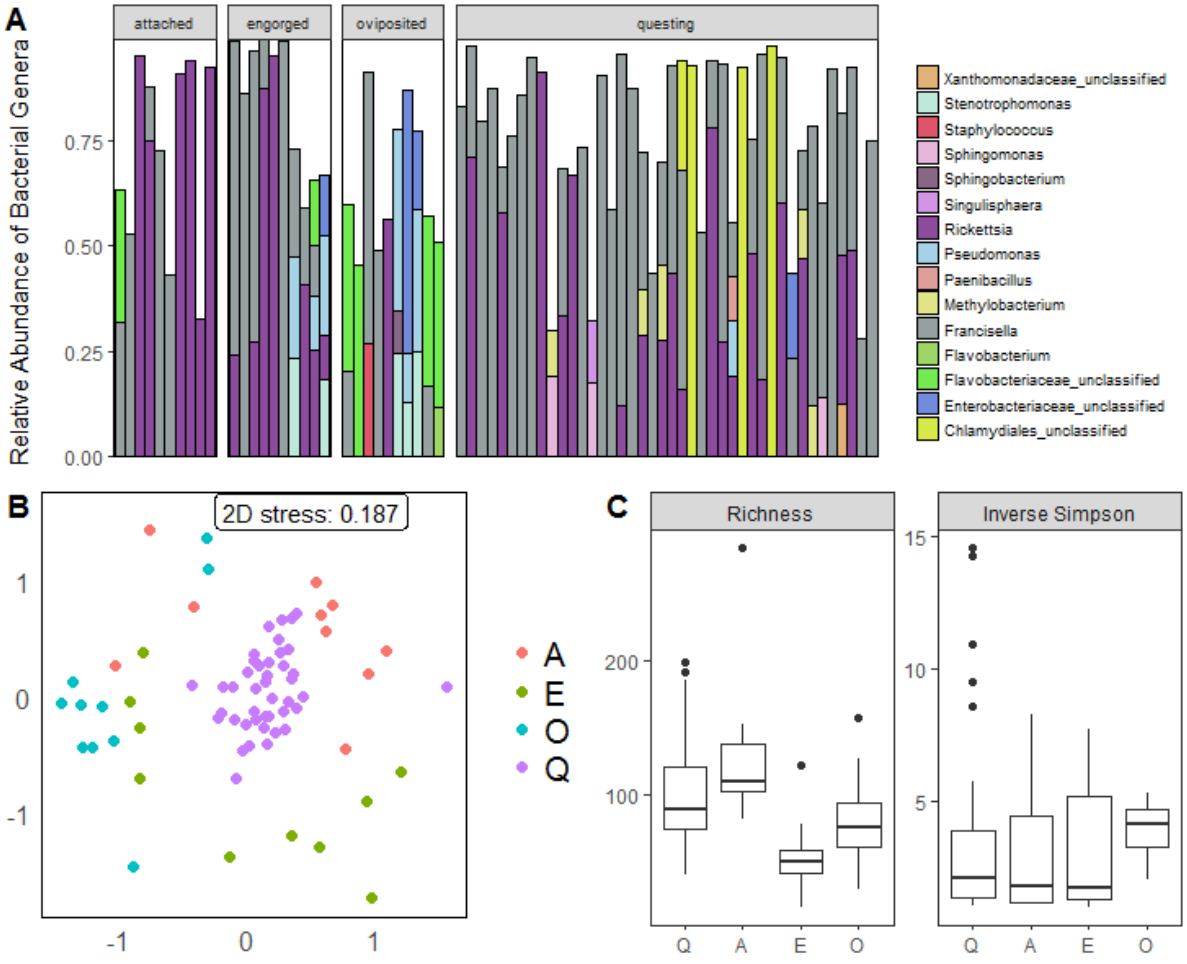

Figure 3.5 Comparisons of engorgement levels among female ticks. Relative abundance of bacterial taxa that comprise >10% of the total abundance are shown, with *Francisella* (Grey) and *Rickettsia* (Purple) being quite common (A). NMDS of engorgement levels, which demonstrates clustering of questing (purple) samples and host associated (blue, green and pink) (B). Boxplot of engorgement levels (C), shows that richness (Chao1) is lower for engorged females (E) compared to questing (Q), attached (A), and oviposited (O). No differences observed in diversity (Inverse Simpson) between engorgement levels.

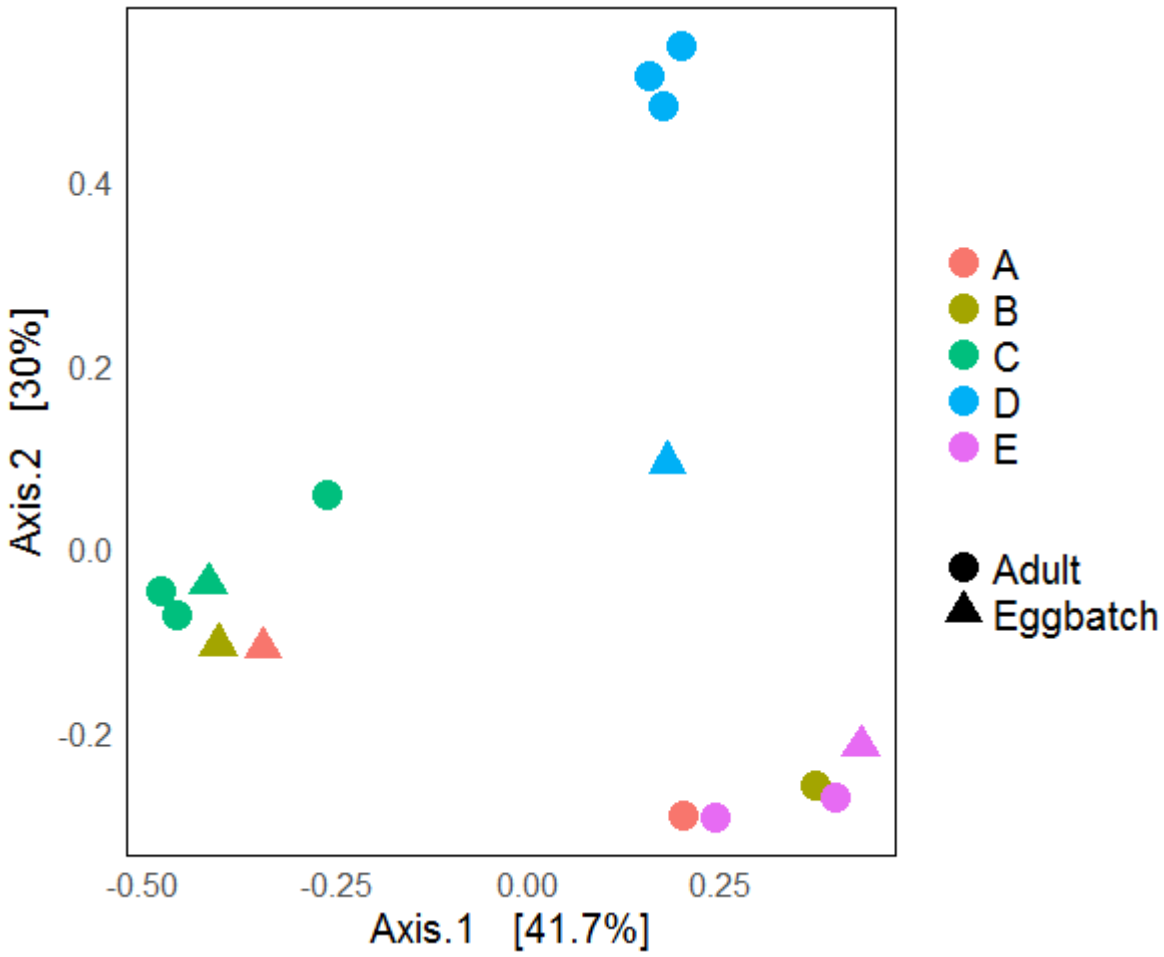

Figure 3.6 Principle Coordinate Ordination (PCoA) demonstrating the bray- curtis distance between mothers and egg batches. Colors represent the pairings of mothers and egg batch (A – E). PERMANOVA demonstrated no significant differences between groups. Interestingly, the distance between eggs batches and the respective mother was sometimes greater than the distance between egg batches and unrelated mothers.

Chapter 4: Conclusion

During my thesis research, I evaluated and identified information critical for developing an Integrated Pest Management (IPM) strategy for ticks infesting beet cattle in Tennessee. First, I determined that three species primarily infest cattle, and that seasonal and regional effects were only significant for the gulf coast tick *A. maculatum* with collections greatest in the spring and summer in western Tennessee. Cluster analysis revealed that the areas of the state at the highest risk of exposure to ticks were at the border of the middle and western region. Second, I found that the RECs and auctions were the best means to monitor for invasive ticks and proposed that the RECs would act as sentinels and the auctions would serve as an early detection system. Additionally, my investigation into the microbiome of *A. maculatum* revealed that *Francisella* and *Rickettsia* were both common and abundant and that the microbiome could change due to sex of the tick, and engorgement level.

Combined, my thesis serves as a foundation for building a strategy to combat endemic and invasive tick threats to the cattle industry; however, several additional steps need attention to put the results from this work into practice and to give producers and stakeholders the best chance of tackling the challenges that ticks and tick-borne disease pose. Several of these future steps, their rationale, and my opinions are discussed below and focus on: tick ecology, economic impacts, and education.

Tick Ecology: Organismal and Microbial

Although regions and time of year when ticks can be expected to parasitize cattle was documented in this study, several questions remain that would contribute to a deeper understanding of the ecology of these pests and ultimately provide the basis for an IPM program. One line of inquiry that deserves further investigation is questing activity in cattle pastures. In fact, I began to explore this research question by conducting drag and CO_2-baited trap collections at the RECS. Briefly, one CO_2-baited trap was placed for every 5 acres of pasture. Traps were set prior to host sampling, with three drag samples done following completion of host sampling and using the traps as a starting point. Drags were done in 100m long transects, with 20m between transects. The drag cloth was inspected for ticks every 20m, with any ticks collected placed into a vial of 80% ethanol. The original intention was to compare *Amblyomma americanum* ticks collected in pastures to those collected from hosts to investigate how region, trap-type and blood-feeding might drive differences in microbial community structure. Ultimately, this objective was altered due to few *A. americanum* captured in eastern Tennessee pastures (n=3) and because several publications have previously investigated microbial communities of this species (Clay et al. 2008, Heise et al. 2010, Williams-Newkirk et al. 2014). Further investigation into tick questing in cattle pastures is warranted, especially into increasing the efficiency of performing these collections. Pasture sampling in this study was typically conducted in the late morning and afternoon. It is possible that sampling did not coincide with the peak timing for tick questing activity, which may fluctuate depending on favorable environmental conditions such as temperature and humidity. Therefore, research into questing activity could help determine when cattle are at the greatest risk of exposure to ticks.

Another factor to consider is that the ticks found in this study undergo a three-host life cycle, with the immature stages preferring to feed on small mammals (*A. maculatum, D. variabilis*) and birds (*A. americanum, I. scapularis*) (Bishopp and Trembley 1945). These hosts can easily enter cattle pastures unimpeded by gates meant to contain cattle. A trapping study focused on these

alternate hosts could answer questions regarding how ticks invade pastures, and assist in devising future control tactics targeting these hosts.

In addition to further investigations into tick activity and dispersal, questions about the microbial ecology of tick microbiomes remain. Objective 2 of this research focused on a less studied species, *A. maculatum*, with results demonstrating differences in microbial community structure under varying conditions including sex and engorgement level. Importantly, *Francisella* was found in all tick samples tested, which corroborated results by previous research into the A. maculatum microbiome (Budachetri et al. 2014, Budachetri et al. 2017). Although recent investigations have discovered that the *Francisella* symbiont within *A. maculatum* evolved from closely related animal pathogens (Gerhart et al. 2016), no studies have determined the role of this endosymbiont. This information will be vital to gaining an understanding of the importance of this symbiont to the survival and physiology of *A. maculatum*. For example, in *A. americanum*, the symbiotic bacteria *Coxiella* was elucidated to contribute to reproductive fitness (Zhong et al. 2007) with sequencing revealing that it contained genes that coded for synthesis of several vitamins and cofactors necessary for feeding on a nutrient deficient source such as blood (Smith et al. 2015). Knowledge of the functional role of *Francisella* within *A. maculatum* could definitively determine if this bacterium should be the target of future control efforts within an IPM strategy. This will be especially vital to protect cattle health and the cattle industry as a whole from devastating losses following the introduction of *Ehrlichia ruminantium*. Investigations into the microbiome of *D. variabilis* are also warranted since it was collected in a wide geographic and temporal range in Tennessee and is a vector of the agent of Bovine Anaplasmosis, *Anaplasma marginale*. Studies have found that *D. variabilis* does contain a *Francisella* symbiont (Sun et al. 2000, Scoles 2004), but there have been no studies that have investigated the microbiome of this species in its entirety. Establishing its baseline microbial community and understanding factors that lead to changes in microbial community structure could provide critical information necessary to develop BA risk assessments in the future and modulate vectorial capacity.

Economic Impact

Objective 1 of this research was able to determine which ticks were pests of cattle and elucidate both the seasonal phenology and regional distribution of these species. These components are vital for understanding the life history of these pests and contribute to the baseline information needed to move towards an IPM program. The other key component required is the economic injury threshold (ET). The ET is a guiding principle in IPM which allows producers to estimate the pest density at which they will experience economic losses. It is at this point that chemical methods would be used to decrease the pest population back below damaging levels. Investigating this would translate damage the ticks inflict upon cattle into a monetary amount, which could increase producer interest and participation in future research endeavors. This would ultimately increase collaboration, leading to a more robust monitoring program. Each species should have a corresponding economic threshold which will vary depending upon factors such as location, vector status of the tick, its vectorial capacity and the disease severity (Black and Moore 2004). These last two factors will be important considerations when determining an economic threshold for *D. variabilis* given it is a vector of *A. marginale*. Some tick species in this study have previously undergone investigation into damage estimates for cattle. For *A. americanum*, it was found that 15 feeding females per one animal could lead to

damage to pre-weaner cattle (Barnard 1985). Steers infested with low levels of *A. maculatum* (n=25-30) would weigh 14 kg less on average compared to control animals (Williams et al. 1977). These established damage levels should be considered a starting point, but not used as current thresholds for an IPM strategy in Tennessee because these estimates were determined in Oklahoma over 40 years ago when producers, pest populations, and animal genetics were quite different from current conditions. Therefore, thresholds need to be developed to meet the current needs of Tennessee producers.

Education

This project has offered several opportunities for education and outreach, including a Youtube video (Theuret 2016) which demonstrated to producers and extension agents participating in the study the methods employed and how to remove ticks from an animal. Additionally, I shared my research findings directly with cattle producers at two meetings in 2017, including the UT Beef and Forage Center meeting and the Advanced Master Beef Producer class.

Although these steps increased awareness and knowledge of ticks to cattle producers, an overall lack of awareness and understanding of the threats that ticks pose to the cattle industry persists. In my opinion, this arises from a general focus on fly control driven by feeding differences between flies and ticks. Flies, especially the horn fly (*Heamatobia irritans*), often feed on cattle in prolific numbers in visible parts of the body (back, face). In these situations, producers can easily spot congregations of feeding flies even from a distance. Comparatively, ticks feed in protected regions of the body (ears, under the tail, legs) which makes their presence harder to detect; often ticks are noticed only when fully engorged females are found the few times cattle are worked in a chute. This combination of factors creates a 'blind spot' for tick threats.
This 'blind spot' is supported by several sources of information that are either currently lacking or are not used to their full potential. The University of Tennessee (UT) Department of Entomology & Plant Pathology (EPP) currently lacks an extension veterinary entomologist. This vacancy may create cascading effects that ultimately contribute to the lack of information found on ticks. For instance, EPP compiles control recommendations for pests into the Insect and Plant Diseases Control Manual and has no recommendations currently for pests of livestock (UT-EPP 2017) due to a lack of expertise regarding current pesticide registration and husbandry practices. This is further reflected in two documents published by the UT extension service that directly pertain to beef cattle production: the master beef producer manual and the beef production calendar. Within the master beef producer manual, ticks are mentioned as one of the means by which cattle can become infected with BA, but are not listed as ectoparasites of concern (flies, lice, and grubs are discussed) (Daugherty et al. 2013). The management calendar provides producers with a monthly checklist for cattle production, and does mention flies and their control but does not mention ticks (UT-AES 2017).

It is for these reasons that further steps need to be taken to address the issue of lack of information which impedes the development of control strategies. As mentioned previously, studies on the economic thresholds of the ticks that infest cattle could serve to shift ticks from the 'blind spot' and into the general discourse regarding serious health threats to cattle. The best potential solution towards resolving the issue of a lack of information would be for UT to consider creating a position for an extension veterinary entomologist to help protect the health of livestock and producer livelihood in the state. This position would play a pivotal role in

disseminating information to producers, and would provide much needed expertise that could eventually allow for more in-depth recommendations in UT extension documents pertaining to tick threats to livestock production.

Conclusion

This current research should not be considered self-contained, but rather as a spring board for future studies that will build upon my research findings to protect the health of cattle in Tennessee and surrounding areas. Research into the topics described above will provide greater insight into aspects of tick life history, which allows for future researchers to identify the best means of controlling them. The greatest impediment to an IPM strategy is a lack of concern for tick threats among cattle industry stakeholders; therefore, increased education and outreach needs to be a priority in order to effectively collaborate on research endeavors.

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Vita

David Theuret was born in Brawley CA to the parents of David and Theresa Theuret. He graduated from Brawley Union High School in 2008 before pursing an undergraduate degree in physiological sciences at the University of California Los Angeles. During his sophomore year, he decided that instead of pursuing a career in the medical field, he should instead follow his childhood dream of being an entomologist. He applied and was accepted to the College of Natural and Agricultural Sciences (C.N.A.S.) Summer Bridge to Research Program, targeted at Hispanic and low-income transfer students at the University of California Riverside. Through this program, he was able to conduct research in a veterinary entomology laboratory under the supervision of Dr. Alec Gerry, where he continued to do research on nuisance flies of veterinary importance until his graduation in August of 2013. This experience sparked his interest in medical and veterinary entomology, which he was able to professionally explore following graduation as a laboratory assistant at the Northwest Mosquito and Vector Control District in Riverside County. He is currently finishing his Masters in Entomology & Plant Pathology at the University of Tennessee Knoxville and serving as the first public health entomologist at the Arkansas Department of Health. He will graduate in December of 2017.