To the Graduate Council:

I am submitting herewith a thesis written by Jessica Rose Harmon entitled "Management of ticks and tick-borne disease in a Tennessee retirement community." 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.

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Management of ticks and tick-borne disease in a Tennessee retirement community

A Thesis Presented for the

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ABSTRACT

Human Monocytic Ehrlichiosis (HME) is an emerging disease first described in 1987 and is transmitted by the bite of *Amblyomma americanum*. Over the past 10 years, the CDC has documented increasing ehrlichiosis case reports nationwide. Our study site is a golf-oriented retirement community located in the Cumberland Plateau of Tennessee. In 1993, four men at the study site had symptoms consistent with HME which prompted a CDC outbreak investigation and led community managers to mitigate ticks feeding on deer. The objectives of this study were to measure the efficacy of current tick mitigation attempts, to determine the level of infection and composition of tick-borne disease in the study area, and to assess which wildlife species are potentially acting as reservoirs for disease.

Ticks were sampled in the community at eight sites of '4-poster' acaricide applicator utilization and at seven untreated sites. Close to the '4-poster' devices, larval, nymphal, and adult tick abundances were reduced by 90%, 68% and 49% respectively (larval $p<0.001$, nymphal $p<0.001$, adult $p=0.005$) relative to the untreated areas. We extracted DNA from *A. americanum* ticks collected at the treatment and non-treatment sites and tested for *Ehrlichia* spp. infections. Of 253 adult and nymphal *A. americanum* tested, we found 1.2% to be positive for *Ehrlichia chaffeensis*, 4.7% positive for *Ehrlichia ewingii*, and 1.6% positive for Panola Mountain *Ehrlichia*; in combination this prevalence is similar to that reported in other *Ehrlichia*-endemic areas of the eastern U.S.. We also performed blood meal analysis on DNA from *A. americanum* ticks and the results suggest that the most significant reservoir hosts for *Ehrlichia* spp. are white-tailed deer, turkeys, grey squirrels, and Passeriformes.

We conclude that while the '4-poster' acaricide applicators reduce the number of ticks close to treatment, at the density at which they are currently being used (8 applicators per 52.6 km^2, average distance between applicators = 6.6km) they will have no large-scale effect on the community's tick

population. In order to accomplish area-wide reduction of *A.americanum* and *Ehrlichia* spp. in this locale, community managers should develop an integrated management strategy that utilizes other techniques in addition to ‘4-poster’ devices.

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1 INTRODUCTION

1.1 Abstract

The current status of tick-borne disease (TBD) in the southeastern U.S. is challenging to define due to the presence of emerging pathogens, uncertain tick/host relationships, and changing TBD case definitions. In recent years, reports of TBDs such as Lyme Disease, Rocky Mountain Spotted Fever and Ehrichiosis have been on the rise in Tennessee (TDH 2008). In an attempt to lessen the human risk of TBD, management officials have begun trying to decrease the number of ticks to reduce transmission of pathogens. This literature review aims to clarify the current status of ticks and tick-borne disease in Tennessee and compare the various techniques for managing those tick species and the pathogens they can transmit.

1.2 Background and Significance

1.2.1 Tick-borne disease and associated ticks of relevance for the Tennessee Cumberland Plateau study site

Four tick species are commonly encountered by humans in Tennessee: blacklegged/deer ticks (*Ixodes scapularis* Say), lone star ticks (*Amblyomma americanum* L.), gulf coast ticks (*Amblyomma maculatum* Koch) and American dog ticks (*Dermacentor variabilis* Say). In Tennessee and nearby in Kentucky, three military bases send all ticks that bite personnel for identification and pathogen testing. From 2004 to 2008, 885 ticks were submitted for identification; of these 86.6% were *A. americanum,* 11.2% were *D. variabilis*, 1.8% were *A. maculatum* and only 0.3% were *I. scapularis* (E. Stromdahl, Entomologist in the Tick-borne Disease Laboratory with US Army Public Health Command, pers. comm., July 2009). Each of these species of tick is responsible for carrying different pathogen(s) that can lead to infection and disease in humans.

1.2.1.1 Blacklegged ticks and associated pathogens

Blacklegged ticks feed on various hosts, including mammals, birds, and reptiles, with primary blood meal hosts being white-footed mice and deer (Anderson et al., 2006). Blacklegged ticks are vectors for *Borrelia burgdorferi*, the causative agent for Lyme Disease (LD). LD, also known as Lyme borreliosis, is the most commonly reported vector-borne disease in the United States, with around 20,000 new cases reported each year (CDC, 2007). Early signs of infection include fever, headache, fatigue, and erythema migrans. Without treatment, patients can experience symptoms involving the joints, heart, and nervous system. In the past, Tennessee has had very few reported cases of LD (Apperson et al., 1993) but these have recently increased to an average of 30 case reports a year from 2003-2005 (CDC, 2007). *Borrelia burgdorferi* has been identified in ticks and small mammals in some southern states but transmission to humans has not yet been documented as reported cases are often a result of travelling or misdiagnosis (Barbour, 1996). Surveys from the University of Tennessee have failed to find *Borrelia burgdorferi* in ticks from the state, but have found *Borrelia myamotoi*, which is of unknown health significance (Rosen, 2009). Blacklegged ticks are also known to carry *Anaplasma phagocytophilum*, the pathogen responsible for Human Granulocytic Anaplasmosis, although as yet, no cases of this illness have been reported in Tennessee.

1.2.1.2 Lone star ticks and associated pathogens

In a recent study from North Carolina, the lone star tick made up 99.6% of over 6,000 collected specimens from suburban landscapes, making it the most widely distributed tick in the state (Apperson et al., 2008). At Henry Horton State Park in middle Tennessee, similar results have been shown with the lone star tick comprising 92% of ticks collected by dragging (Rosen,

2009). Lone star ticks are vectors for *Ehrlichia chaffeensis*, which is the causative agent of Human Monocytic Ehrlichiosis (HME) (Landaas et al., 1988), *Ehrlichia ewingii* which is the causative agent of *Ehrlichia ewingii* Ehrlichiosis (Buller et al., 1999), and Panola Mountain *Ehrlichia* which has been shown to cause mild infection in humans (Reeves, 2008). *E. ewingii* is generally thought to produce a milder form of disease than *E. chaffeensis*, however anemia, thrombocytopenia, polyarthritis, and neurological sequelae have been reported (Nicholson, 2010). Lone star ticks have also been shown to carry *Borrelia lonestari* and *Rickettsia amblyommii* but the human health implications of these bacterial agents are unclear (Apperson et al., 2008; Bacon et al., 2003; Billeter et al., 2007; Burkot et al., 2001; James et al., 2001; Mixson et al., 2006; Moore et al., 2003; Paddock and Yabsley, 2007; Schulze et al., 2005; Stegall-Faulk et al., 2003; Stromdahl, 2008; Varela et al., 2004). From 1998-2005, a study conducted in several southern states found overall prevalence for *E. chaffeensis*, *R. amblyommii*, and *B. lonestari* in ticks to be 4.7%, 41.2%, and 2.5%, respectively (Mixson et al., 2006). Panola Mountain *Ehrlichia* (PME), which is similar to *Ehrlichia ruminatum*, was recently discovered in Panola Mountain State Park, GA, USA (Loftis et al., 2006). Further research determined that this species of *Ehrlichia* is distributed throughout the range of *Amblyomma americanum*, suggesting that PME is not a newly introduced pathogen in the United States (Loftis et al., 2008). White-tailed deer and goats act as reservoir hosts for PME (Loftis et al., 2006; Yabsley et al., 2008) and human illness has also been associated with PME (Reeves, 2008). All life stages of lone star ticks feed on humans and animals such as deer, cattle, horses, and dogs (Burgdorfer, 1969). Clinical symptoms of HME in humans rarely involve a rash and commonly involve fever, headache, malaise, and muscle aches. Tennessee is considered endemic for ehrlichiosis, with

annual incidence rates of 4+ cases per 100,000 population (Figure 1.1)(Brady et al., 1988). The risk of HME appears to be greater in older people, and those with HME tend to be older than patients that have other tick-borne diseases (Eng et al., 1990).

Southern Tick Associated Rash Illness (STARI) is linked to the bite of lone star ticks but the causative agent is currently unknown; *Borrelia lonestari* and *Rickettsia amblyommii* have been suggested as causative agents for STARI, but definitive results have yet to emerge (Apperson et al., 2008; Billeter et al., 2007; Burkot et al., 2001; James et al., 2001; Moore et al., 2003; Varela et al., 2004). *Borrelia lonestari* was not detected in a study of skin biopsies and serum samples from patients presenting with erythema migrans after the bite of a lone star tick, leading researchers to look to other agents as the cause of STARI (Stromdahl, 2008).

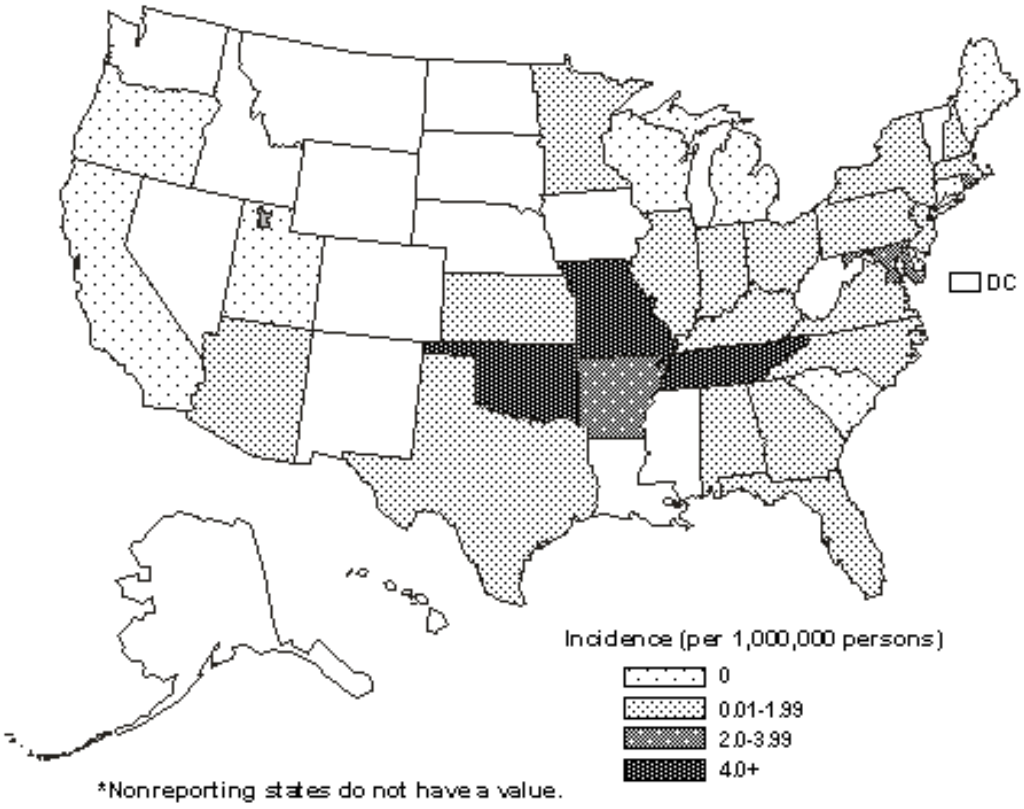

Figure 1.1 Average annual incidence of ehrlichiosis (caused by *Ehrlichia chaffeensis*) by state, as reported to CDC, 2001-2002 (Brady et al., 1988).

An additional complication of diagnosis is that antibodies to *R. amblyomii* have been found in sera from patients diagnosed with probable Rocky Mountain Spotted Fever (RMSF) cases (Apperson et al., 2008), suggesting that RMSF tests are cross-reactive with *R. amblyomii*. Furthermore, the presence of *R. amblyommii* in humans could simply be a consequence of high prevalence of the agent in ticks and not necessarily pathogenicity in humans. Clinicians and researchers continue to be plagued by the question of what role, if any, *Borrelia lonestari* and *Rickettsia amblyommii* play in rashes associated with the bite of the lone star tick. The rash associated with STARI is similar to that of LD; the rash presents as a lesion around the site of a tick bite and usually occurs within seven days of the bite. STARI can be associated with fatigue,

fever, headache, muscle and joint pains, but is quickly resolved with oral antibiotics (CDC, 2008). Currently, no tests are available to detect STARI in patients with tick bite associated illnesses.

1.2.1.3 American dog ticks and associated pathogens

American dog ticks most commonly parasitize dogs and medium-sized mammals but will also feed readily on birds and large mammals including humans (Kollars et al., 2000). Unlike in other TBD systems where a blood meal must occur for infection, American dog ticks are reservoirs as well as vectors for *Rickettsia rickettsii* and have shown 100% transmission to oocytes (Kollars and Kengluecha, 2001). However, the infection results in negative effects on the tick such as decreased production of eggs by an affected female and few of the infected larvae mature through the adult stage (Dumler and Walker, 2005). These harmful effects may explain the very low (1%) infection rate generally found in American dog ticks (Kollars and Kengluecha, 2001). Within the past decade, incidence of reported Rocky Mountain Spotted Fever (RMSF) in the south has been on the rise, with the rate of RMSF in Tennessee increasing by 42% from 2004 to 2005 and by 63% from 2005 to 2006 (Dumler and Walker, 2005). RMSF symptoms include fever, headache, myalgia, and a petechial rash. Early diagnosis is crucial, as a delayed diagnosis often results in severe illness or death (Dumler and Walker, 2005).

1.2.1.4 Gulf coast ticks and associated pathogens

Rickettsia parkeri belongs to the spotted fever group rickettsiae and was isolated from gulf coast ticks in 1939. *Rickettsia parkeri* has been detected in ticks from Florida, Georgia, Kentucky, Mississippi, Oklahoma, and South Carolina (Sumner JW, 2007). In 2004, clinical disease caused by *R. parkeri* was confirmed in a 40-year old man from southeast Virginia; the

disease manifested as a mild, febrile illness accompanied by scabs or sloughs on the skin and rash (Paddock et al., 2004). *R. parkeri* is cross-reactive to most available RMSF tests, so the occurrence of infection due to this and other spotted fever group rickettsiae may be greater than is currently perceived (Ono et al., 1988). However, while gulf coast ticks are considered prevalent in Gulf Coast states, they are not considered to be established within Tennessee; sporadic records of these ticks in the state are likely the result of immature life stages being transported into the state on migrating birds (Durden, 1992).

1.2.1.5 Ticks and tick-borne disease history at the Cumberland Plateau study site

In 1993, four men at our study site were hospitalized with an illness matching the symptoms of HME. Serum specimens from two men showed the presence of elevated IgG antibody to *E. chaffeensis* and positive polymerase chain reaction (PCR) tests of blood specimens from all four men confirmed the diagnosis of acute HME. Additionally, 12.5 percent of surveyed residents had serologic evidence of past *E. chaffeensis* infection (Standaert et al., 1995). The Tennessee Department of Health has reported 13 cases of Rocky Mountain Spotted Fever, 11 cases of ehrlichiosis, and 8 cases of Lyme Disease in Cumberland County from 1995-2006 (http://health.state.tn.us/Ceds/WebAim/WEBAim_criteria.aspx). However, these reports should be taken cautiously, as they do not include information on patient travel history or test specificity. Consequently, little is known about the pathogen prevalence and TBD risk in this retirement community and in the state of Tennessee.

The high prevalence of lone star ticks at the study site suggests that ehrlichiosis and STARI are the TBDs that are most likely to be a risk to residents in the area. Unlike other TBDs, incidence of ehrlichiosis has been shown to increase with age, with the highest reported

incidence and severity seen among those 60+ years of age (Figure 1.2), making this TBD a significant concern within a retirement community such as our study site (Brady et al., 1988). At the beginning of this assessment the presence of *Ehrlichia spp.* in Tennessee ticks was unconfirmed, however *E. chaffeensis* and *E. ewingii* have been recently found in several *A. americanum* ticks from the state (Meyers et al., 1988).

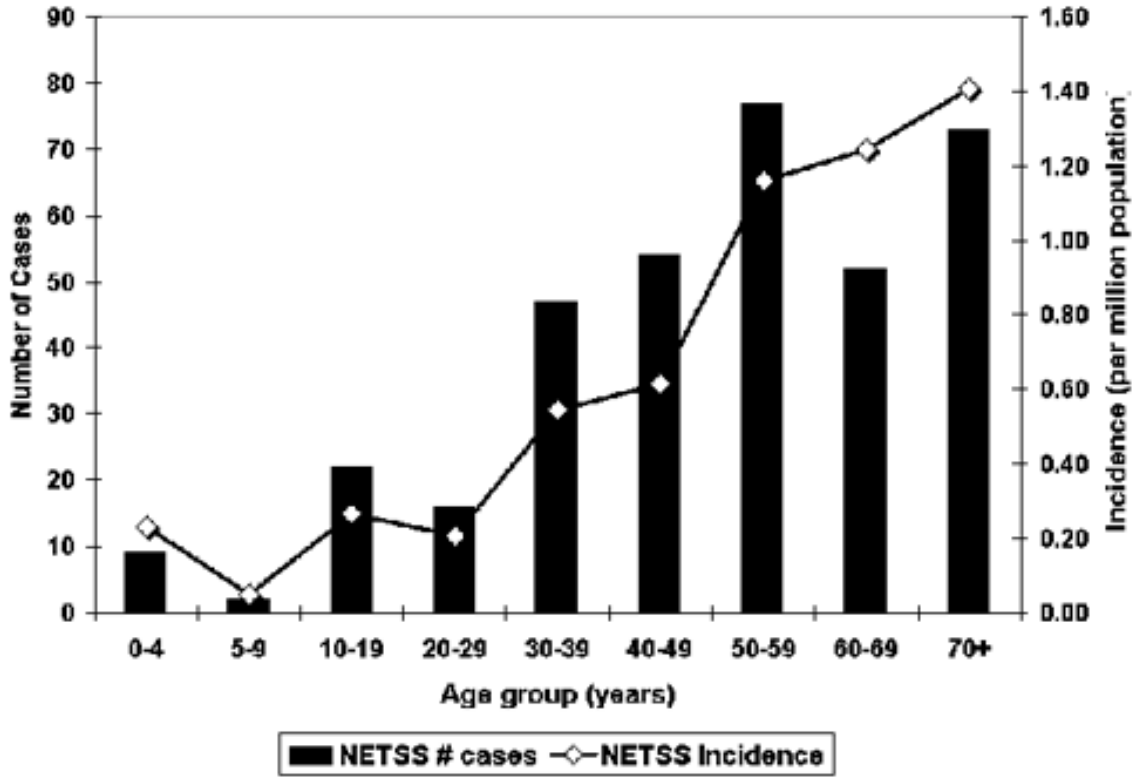

Figure 1.2 Age-specific incidence of ehrlichiosis (caused by *Ehrlichia chaffeensis*), reported to CDC 2001-2002 through NETSS (Brady et al., 1988).

1.2.2 Options for managing tick-borne disease

There are several available options for managing ticks and therefore tick-borne disease. These mitigation methods include managing ticks on host wildlife species and domestic animals, biological control using fungi, altering or treating the landscape and vegetation, managing hosts through exclusion or hunting, and prevention of tick-human contact. The following section considers these options in more detail.

1.2.2.1 Managing Ticks

1.2.2.2 Managing Ticks on Hosts

Blacklegged, lone star, and dog ticks are all known to feed on deer, making deer a way to target the tick species that are of most concern to human health. '4-poster' feeders (developed by USDA-ARS researchers) are devices that pinpoint deer in an attempt to alter the population of ticks feeding on those deer (Pound et al., 2000b). Whole kernel corn is used to attract deer to the devices where, as they feed, they rub their head, neck, and ears against paint rollers soaked with an acaracide. Each device has two feeding and application stations with bait and rollers. Control of *A. americanum* exceeded 91-96% under trial conditions with the use of one '4-poster' device per every 20 ha. (Pound et al., 2000a). The devices appear to be slightly less effective for *Ixodes scapularis*, with control estimates of 82-85% (Schulze et al., 2008). Several researchers have shown effective and increasing control of ticks over time using these devices (Bloemer et al., 1990; Brei et al., 2009; Carroll and Kramer, 2003; Carroll et al., 2009; Hoen et al., 2009; Miller et al., 2009; Pound et al., 2000a; Pound et al., 2000b; Schulze et al., 2008; Schulze et al., 2007; Solberg et al., 2003; Stafford et al., 2009). However, using '4-posters' in a large scale community is expensive and time-consuming. Devices are required to be >100 meters from the

nearest residence and nearly 200 '4-posters' would be required to meet the recommended application density in an area the size of our study site. It is important to recognize that the majority of studies documenting significant reductions in ticks have been on deer populations within fenced-in areas, on islands, or in other small areas (Bloemer et al., 1990; Carroll and Kramer, 2003; Pound et al., 2000b; Pound et al., 1996; Solberg et al., 2003).

In addition to the four-posters used for deer, host-based tick control methods have been developed for other wildlife, including small rodents and birds. Rodents are an important part of tick management because they are preferred hosts for nymphal and larval life stages of several different tick species. A three year study in Connecticut utilized commercial bait boxes to deliver acaracide to white-footed mice in an effort to control immature stages of blacklegged ticks (Dolan et al., 2004); infestations by nymphal and larval ticks were reduced by 68% and 84%, respectively. After three years of treatment, the number of questing adults and infection rates of *Borrelia burgdorferi* in ticks also decreased. Another study took advantage of nesting behavior by distributing permethrin-impregnated cotton for white-footed mice (Deblinger and Rimmer, 1991). Significant decreases in nymphal ticks were seen, although these results were not repeatable for all studies (Wilson, 1993). An adult female tick can deposit several thousand eggs in a month, so management strategies that target immature stages rather than adult stages are retroactive and may not be the best type of control as a stand-alone method.

Norcross looked for a solution to colony abandonment by brown pelicans as a result of excessive tick infestations (Norcross, 2002). Treated nests were sprayed three times with Permectrin dilutions during the nesting season. Fewer immature tick stages were observed in treated nests and no colony abandonment was observed in those nests. This study is especially

important for tick management consideration because birds have the ability through migration to disperse ticks and their respective pathogens across hundreds of miles (Morshed et al., 2005). By adapting tick control for birds, researchers can improve the fitness of a wild bird population while also improving public health.

Perhaps the most well-known tick control methods are those used for the protection of companion animals. Topical acaracides and collars that can be applied to the necks of dogs and cats are easily accessible, fast acting, and relatively inexpensive. Preventic® Tick Collars (active ingredient Amitraz) prevent ticks from attaching to dogs, kill existing ticks within 48 hours, and last for 3 months (Kranzfelder et al., 1988). Frontline Plus® is a topical product produced by Merial (active ingredients Fipronil and S-methoprene) that kills fleas within 12 hours and ticks within 48 hours of coming into contact with a dog or cat (Kranzfelder et al., 1988). In a study comparing the efficacy of Frontline®, Scalibor®, Advantix®, and Preventic®, the lowest tick counts were reported with the Preventic® Amitraz collar (Estrada-Pena and Bianchi, 2006). Revolution® (Active ingredient Selamectin) is also a topical acaracide, is manufactured by Pfizer, and controls the American Dog Tick, fleas, mites, and heartworms. Preventic® Collars, Frontline Plus®, and Revolution® are all waterproof and available for purchase from a veterinarian. These products should be used year-round as certain species of ticks are more active during colder months (Blagburn and Dryden, 2009). Flea and tick shampoos for dogs and cats vary in price and kill fleas and ticks for one to four weeks. Shampoos can be bought over-the-counter at any pet store. It is important to note that the use of collars, topical treatments, and shampoos do not replace the need for thorough tick checks, especially for hunting dogs and those pets that spend a lot of time outdoors. Additonally, a Lyme Disease prevention vaccine has been

available for dogs since 1990 (LymeVax, Fort Dodge Animal Health) and has shown a high efficacy level when administered to young at-risk dogs before they have come into contact with potentially infected ticks (Beier, 1988b). As is the case in humans, the most economical way to prevent disease in pets is to check for ticks after the animal has been outdoors. This can be accomplished easily in indoor/outdoor pets by brushing and grooming once a day.

1.2.2.3 Biological control

Biological control of ticks involves the use of an entomopathogenic fungus, *Metarhizum anisopliae*. *M. anisopliae* is lethal to engorged blacklegged tick larvae and adult female blacklegged ticks (Zhioua et al., 1997). This fungus has been shown to reduce engorgement weight as well as egg mass weight in ovipositing females and causes 52% mortality in questing females (Hornbostel et al., 2004). American dog tick nymphs have been shown to be more susceptible to entomopathogenic fungi than their corresponding adults, but other species do not have variations in susceptibility between life stages (Kirkland et al., 2004). With all species, in order for effective penetration of the tick's cuticle and subsequent death, spore density must reach a certain threshold (Zhioua et al., 1997). Unfortunately, fungal spores applied with a spray tower also cause significant non-target effects leading to mortality in beetles and crickets (Ginsberg, 2002).

1.2.3 Managing Hosts

1.2.3.1 Exclusion and Hunting

White-tailed deer are the species most commonly targeted for management by exclusion and hunting. Exclusion has been used as a solo method as well as in combination with additional

procedures in an effort to manage tick numbers and reduce disease risk (Bloemer et al., 1990; Ginsberg and Zhioua, 1999; Zhang et al., 2006). Ginsberg et al. (2002) utilized a fencing type which allowed small and medium-sized mammals to pass through the fence while excluding adult deer. The researchers observed a 48% reduction in ticks and concluded that movement of ticks on birds and small and medium-sized mammals diminishes the effects of fencing and therefore the potential for management of ticks using that method. To remedy this problem, fencing that also restricts medium-sized mammals or exclusion in combination with other techniques could be used for greater reduction and control of ticks. Bloemer et. al (1990) determined that overall deer exclusion is more economical than techniques such as acaricide treatment or management of vegetation, because this method requires minimal annual maintenance and lasts for 20+ years.

Hunting deer in an effort to manage ticks in residential areas has historically been a very controversial method which causes conflicts between animal welfare advocates, hunters, the general public, and wildlife agencies. This conflict is due to the necessity for deer to be nearly eradicated before significant reductions in the numbers of ticks are observed (Jordan et al., 2007). Reducing tick populations using deer control methods are most effective in geographically isolated areas such as islands and peninsulas where deer from elsewhere cannot re-inhabit the area easily. As is the case with exclusion fencing, this method is best used in combination with other mitigation techniques as small and medium-sized mammals and birds are capable of maintaining the tick population if deer are not reduced below a certain level.

1.2.4 Landscape alteration

Prescribed burning has become a common management practice throughout the United States to enhance pine timber production, facilitate turnover of nutrients, and influence plant community structure, all factors that benefit various wildlife species (Jacobson and Hurst, 1979). This method has also been investigated as a control technique for *A. americanum* (Allan, 2009; Cully, 1999; Davidson et al., 1994; Hoch et al., 1972; Jacobson and Hurst, 1979). These studies demonstrated high initial reduction of tick abundance using controlled burns, however Allan (2009) found >6 times higher abundance of larval *A. americanum* 2 years after burning which the author believed to be an effect of the attraction of wildlife hosts to recently burned habitats. Hoch et al. (1972) concluded that *A. americanum* descend into the forest floor underlitter during burns which may allow large proportions to survive, as only 1% of the underlitter is destroyed compared to 70% of leaf litter. Overwintering larvae are the most vulnerable life stage to prescribed burns, however in years between burns, larval abundance can increase to levels equal to or greater than the preborn abundance (Davidson et al., 1994). It has therefore been concluded that consistent prescribed burns can lead to suppression in tick abundance, given that burns are considered a yearly component of tick and wildlife management and are not used sporadically throughout years.

An additional technique for landscape alteration is mechanical removal of vegetation in areas where risk of coming into contact with ticks is high. Clearing of both undergrowth and over story cover in combination with pesticide or herbicide has been shown to effectively reduce the number of ticks within a 1-acre plot at a much greater rate than vegetation clearing alone (Mountz et al., 1988). Significant reductions have also been seen with combinations of various

types of vegetative management using over story reduction, understory reduction, and regular mowing of grasses to <15cm in height (Beier, 1988a). At Land Between the Lakes in Tennessee, 96% reduction in the number of ticks was reported with the use of acaricide applications, vegetation management and host management together, with lower levels of reduction seen with the use of any other combination of these methods (Bloemer et al., 1990). Therefore it is ideal to use either several different types of vegetation clearing in an area or use vegetation clearing in conjunction with additional tick mitigation techniques, such as acaricide treatment or host management.

1.2.5 Managing Human Exposure

The easiest way to protect oneself from exposure to TBD is to avoid recreational activities in tick infested areas during times of peak activity. The ecology and spatiotemporal patterns vary among tick species, but some basic principles for personal protection still apply. Recreational exposure usually occurs in densely wooded areas with ground cover predominately consisting of leaves with little to no surface vegetation. Avoiding brush and staying in the central part of a path while hiking can reduce risk of tick contact. Golf handicap has also been identified as a risk factor for ehrlichiosis, as retrieving golf balls from the rough brought golfers into contact with increased numbers of ticks (Standaert et al., 1995).

There are several other techniques that can be used if it is not possible to avoid recreational areas when ticks are present. Whenever feasible, people participating in outdoor activities should wear light colored long sleeves tucked into long pants tucked into tall socks. Light colors increase the visibility of ticks once on the clothing and the time needed for a tick to come into contact with the skin, which increases the potential for finding the tick before

attachment. Many commercial tick repellants are available for use on clothing and skin. The most commonly used repellants use either DEET or Permethrin as active ingredients. DEET has been shown to have very little efficacy against *A. americanum* nymphs, with only 25% of *A. americanum* repelled (Carroll et al., 2004). In comparison, Permethrin-impregnated clothing showed 100% knockdown of hard ticks, even after laundering (Faulde et al., 2003). Regardless of repellant use, thorough "tick checks" of both the clothing and skin should be performed often. Any attached ticks should be promptly removed using forceps and should be pulled straight up to keep the head intact. Ticks should be kept for submission to a doctor in the event that a rash or illness develops in the weeks following the bite. By correctly identifying the tick species, health care professionals will be better able to determine what illness the patient may be infected with and therefore the best method of treatment.

Proponents of disease prevention vaccines state decreased likelihood for both antibiotic resistance development and overuse, ease of application, and decreased costs as advantages. However, developing a vaccine for humans in the US has proven difficult. GlaxoSmithKline manufactured a recombinant vaccine for Lyme Disease known as LYMErix. The vaccine was found to protect 76% of adults and 100% of children from infection with *Borrelia burgdorferi* (Hoch et al., 1972*)*. However, many recipients of the vaccine began reporting autoimmune illness as a side effect. Class action suits were filed prompting an investigation by CDC and FDA which concluded that no evidence existed to substantiate these claims (Cully, 1999). The negative publicity resulted in decreased sales leading ultimately to the vaccine being removed from the market. Currently, there are no human vaccines available to prevent tick-borne disease in the United States.

The most important aspect of managing human exposure is educating the public on risk of infection and the most efficient ways of protecting themselves. It is essential that members of the community know what to do in the event of a tick bite, and especially if a subsequent rash or illness develops. The easiest way to prevent transmission of tick-borne illnesses is working proactively to prevent tick bites.

1.3 History of management strategies used at the study site

The 1993 outbreak of ehrlichiosis at our study site prompted collaboration between the retirement community and The Medical Entomology Laboratory at the University of Tennessee Entomology and Plant Pathology Department. Initial research at the area of the outbreak involved an experimental permit to supplement deer with Ivermectin treated corn to affect the reproductive capacity of the lone star tick (Marsland, 1997). Corn was treated at a rate of 50.0 ml pour on insecticide (5mg/ml, Merck) per 22.7 kg of whole kernel cleaned corn. The study resulted in reduction in the reproductive viability of females, determined by fewer larval masses being found in the treated versus untreated areas. A follow up study found that the number of lone star ticks increased over time after removing treated corn (Morris, 1999). Unfortunately, the United States Department of Agriculture subsequently reached a determination that feeding acaracide to deer carries too much risk for residues in hunter harvested deer and the method was not approved for widespread use.

The retirement community that was the focus of our study has been using ‘four-poster’ feeders to manage the tick population within the area. In 2009, four ‘4-posters were located in the northern half of the community and four were located in the southern half. The north is perceived to have higher tick abundance and disease risk due to the community’s northern border

with a wildlife management area and the resulting higher presence of wildlife in that area. We aimed to clarify the efficacy of the retirement community's current tick and tick-borne disease mitigation efforts as well as provide possible options for improvement of existing methods.

2 EVALUATION OF ‘4-POSTER’ ACARACIDE APPLICATORS TO MANAGE TICKS AND TICK-BORNE DISEASES IN A TENNESSEE RETIREMENT COMMUNITY

2.1 Abstract

In 1993, four residents of a retirement community in a forested area of middle Tennessee were hospitalized with symptoms of ehrlichiosis. This case cluster triggered a CDC outbreak investigation and led community managers to implement mitigation methods to reduce tick numbers. For the past four years, the community has utilized '4-poster' acaricide applicators that aim to reduce disease risk to residents by killing ticks that feed on deer in the periphery of the community. To determine the efficacy of this technique, we assessed *Amblyomma americanum* abundance in the vicinity of the feeders by dragging a series of 400m vegetation transects once per month while ticks were active. In 2009, adult tick activity peaked in May, nymphal tick activity peaked slightly later in June, and larval activity peaked in September. Close to the '4-poster' acaricide applicators, larval, nymphal and adult tick abundances were reduced by 91%, 68% and 49% respectively (larval $p<.001$, nymphal $p<.001$, adult $p=0.005$) relative to nearby untreated areas. No significant reduction in nymphal or adult *A. americanum* ticks was evident >300m from the '4-poster' acaricide applicators, however a ~90% reduction in larvae was observed out to the limit of our sampling (400m from the applicators). The effect of the applicators is likely to increase after consecutive years of utilization, nevertheless we conclude that at the density at which these feeders are currently being used (8 per 52.6 km^2, average distance between feeders = 6.6km) they will have no large-scale effect on the tick population. A much higher density of acaricide applicators would be necessary to have a community-scale effect on tick abundance. This study calls into question the feasibility and affordability of the '4-poster' acaricide applicator as a stand-alone strategy for tick management in a large residential area.

Introduction

We investigated the process of managing *Amblyomma americanum* with '4-poster' acaricide applicators in a golf-oriented retirement community of roughly 6,000 residents located in the Cumberland Plateau of Tennessee. The heavily forested area contains abundant wildlife that support large tick populations. In 1993, an outbreak of ehrlichiosis occurred in the community (Standaert et al., 1995) and managers consequently implemented measures to attempt to control ticks and reduce human disease risk. Acaricide treatment of white-tailed deer was suggested as a viable method to reduce tick populations and therefore lower the risk of tick-borne disease in the treatment area (Pound et al., 1996). Initial research at the site involved an experimental permit to supplement deer with Ivermectin treated corn to affect the reproductive capacity of *A. americanum* (Marsland, 1997). Corn was treated at a rate of 50.0 ml pour-on insecticide (5mg/mL) per 22.7 kg of whole kernel cleaned corn. The study resulted in reduction in the reproductive success of *A. americanum*, determined by fewer larval masses being found in the treated versus untreated areas. In a follow-up study, the number of lone star ticks increased over time after removal of Ivermectin treated corn from the treatment area (Morris, 1999). Unfortunately, the potential risk of chemical residues in hunter harvested deer was deemed to be too high with this method and it was therefore not approved by the USDA for widespread use beyond the initial experimental permit.

In recent years, residents and local health professionals have voiced increasing concerns that these tick populations may be continuing to transmit zoonotic pathogens to the local human population. Currently, '4-poster' devices (developed by USDA-ARS) are being utilized to manage the tick population within the area. The '4-poster' acts by attracting deer to a corn bait

source where the head, neck, and ears come into contact with paint rollers treated with acaricide (Pound et al., 2000). Significant reductions in ticks have been achieved using this technique; however studies documenting such reductions have focused primarily on deer populations within fenced-in areas or on a small community scale (Bloemer et al., 1990; Carroll and Kramer, 2003; Pound et al., 2000a). The Northeast Area-wide Tick Control Project evaluated the '4-poster' acaricide applicator for reducing the abundance of *Ixodes scapularis* ticks in Rhode Island, Connecticut, New York, New Jersey, and Maryland (Brei et al., 2009; Carroll et al., 2009; Hoen et al., 2009; Miller et al., 2009; Solberg et al., 2003). These studies found high variation in level of control in the first year of treatment with nymphal tick numbers decreasing in subsequent years by as much as 71% in 5.14km^2 treatment sites. For this study we focused on the application of '4-poster' devices in a large 52.6km^2 community where heavily human populated areas border heavily wooded areas and the density of devices is limited by financial considerations, available manpower, and regulations constraining the use of '4-poster' devices in close proximity to residences.

This study aimed to clarify the efficacy of the retirement community's current tick mitigation efforts as well as provide data for the design of improved integrated management options. We evaluated the percent reduction of tick populations at set distances from the '4-poster' devices through comparison with non-treatment sites where '4-poster' devices have never been used. Finally, we sought to determine the gradient of control of '4-poster' acaricide applicators in this area by assessing the level of tick reduction at increasing distances from the devices.

2.2 Methods

2.2.1 Study area and selection of sampling sites

Our study site is a golf-oriented retirement community of roughly 6,000 residents which encompasses 5,260 ha. of heavily wooded land on the Cumberland Plateau in Tennessee. The community's attractions consist of championship golf courses, tennis courts, swimming, lakes for boating and fishing, horseback riding, sightseeing, trails, and shopping. The border along the north end of the community is adjacent to a 32,370 ha wildlife management area and as a result, white-tailed deer and other wildlife are common throughout the community. The northern part of the community has a higher tick abundance and disease risk, presumably because of this shared border (R.Gerhardt, pers. comm., October 2010). The fragmentation of the community as a result of interspersed fairways, woodlands, and residences provides ample wildlife habitat, and therefore the opportunity for tick populations to thrive.

Community Services Management at the retirement community selected eight sites for deployment of '4-poster' acaricide applicators based on previous treatment locations, proximity to inhabited areas, and areas of known high tick abundance. Four '4-posters' are located in the northern half of the community and four are located in the southern half. '4-poster' usage regulations prohibit use of these devices within 100 yards of any residence or area where unsupervised children may be present, leading to difficulty of utilization within a residential community such as our study site. This was the first year of treatment using '4-poster' acaricide applicators at transects 1, 7, 8, and 9 whereas '4-poster' treatment has been used for at least two years at transects 4, 5, 12, and 13. Mitigation techniques had never been attempted at the six non-treatment sites (2, 3, 6, 10, 11, and 14) (Fig. 2.1).

Sampling method

Ticks were collected by 'dragging' vegetation (Falco and Fish, 1992) at approximately 4-week intervals during the ticks' active season to determine seasonal changes in population density and distribution in the community. Researchers dragged a white 1x1 meter corduroy cloth along 400m transects at the eight '4-poster' acaricide applicator sites and at six additional non-treatment sites where no applicator was present. At '4-poster' sites, the transect began at the applicator and nymphal and adult ticks that attached to the drag cloth were accumulated and placed into separate vials of 70% ethanol at 40m, 100m, 200m, 300m, and 400m distance from the applicator. Larval ticks were collected from drag cloths using lint roller sheets and labeled by transect and distance, matching the corresponding ethanol vials. This allowed for subsequent analysis of tick abundance versus distance from the feeder. Sampling at non-treatment sites consisted of two 200m transects through equivalent habitat with all adults and nymphs collected per transect accumulated into a single vial and larval ticks collected on a single lint roller sheet. To avoid any effect of tick removal on consecutive abundance estimates, transects were adjusted 5-10 meters to the left or right of the previous transect each subsequent month. Ticks were brought to the University of Tennessee's Medical and Veterinary Entomology laboratory, where they were identified to species, life stage, and sex. Larval tick lint roller sheets were analyzed by overlaying a 7x9 grid on each used sheet. A random number generator allowed for assignment of half of the grids for subsequent tick identification. The counts were then doubled to estimate the number of larvae collected on each sheet.

2.2.2 Trail Camera Monitoring

Bushnell trail cameras (Bushnell Corporation, Overland Park, KS) were utilized for 1-week intervals on three occasions in May-July 2010 at sites where '4-poster' acaricide applicators were located. These motion-triggered cameras took a picture after each 10 seconds of animal activity. Analysis of trail camera photos involved counting individuals of each species present in each photograph. Due to difficulty in determining one individual from another, every animal was counted in every photo regardless of whether or not it was present in previous photographs. Our counts of wildlife therefore represent the level of activity of each wildlife species at the sites rather than abundance of each species at the sites (Jennelle et al., 2002; Oliveira-Santos et al., 2010).

2.2.3 Statistical analysis

To correct for differences in sampling effort, all larval, nymphal, and adult counts were converted to counts per 100m of dragging. For statistical analysis, these corrected counts were then double-log transformed to normalize their variance structure and reduce the influence of outliers. When reporting results, means and standard errors for these transformed data were back-transformed so that plots and tables could be presented in units of tick counts per $100m^2$ dragged.

As a measure of the abundance of questing nymphal and adult ticks that community residents are exposed to during the summer, we constructed a map showing average counts for the three peak months of nymphs and adults (April through June for adults; May through July for nymphs) at each of our sampling sites. Seasonal phenology was determined for adult, nymphal, and larval *A. americanum* ticks at treated and untreated sites from March 2009-May 2010 by

calculating the mean number of collected ticks by visit for each transect and plotting these means *versus* week of visit. Differences in the abundance of nymphs and adults at treatment sites *versus* control sites were analyzed using separate General AOV models in Statistix 8 (Analytical Software, Tallahassee, FL) to assess TREATMENT, VISIT, and TREATMENT*VISIT effects. Interaction terms were non-significant, so they were removed and the models re-run.

Counts of ticks at specific distance intervals from the 4-poster applicators were compared using a General AOV model to assess DISTANCE, VISIT, and DISTANCE*VISIT effects, with the non-treatment area counts treated as a dummy distance category. Non-significant interactions were removed, and a post hoc Hsu's Multiple Comparisons test was run using Statistix 8 to assess the significance of differences of the tick counts at each distance interval versus tick counts at the non-treatment sites.

Treatment transect 5 was excluded from the phenology and distance analysis described above because it had abnormally high adult *A. americanum* densities that were a significant outlier from densities observed at all other sites (See Fig. 2.1). Transect 5 is also the site of a growing feral pig population and distance flagging was removed several times, presumably by residents living in the area. These complications resulted in difficulty collecting samples from the transect, analyzing transect data, and comparing that transect to other sites.

2.3 Results and Discussion

The great majority of collected ticks (99.43%) were *A. americanum* followed by *Dermacentor variabilis* (0.47%) and *Ixodes scapularis* (0.10%). We excluded *D. variabilis* and *I. scapularis* from further analysis as their low abundance suggests they present minimal risk to

humans in this community. In contrast, we found the *A. americanum* population to be widespread throughout the community, with all life stages of *A. americanum* ticks collected from all 14 sampling sites (Fig. 2.1). Our data confirmed the perception of community managers that *A. americanum* numbers are highest in the northern part of the community. Sites in the northern half had an estimated 91% higher nymphal *A. americanum* population density and 35% higher adult *A. americanum* population density than sites in the southern half of the community (Fig.2.1; $p<0.001$ for both comparisons). During the period of peak larval questing (August - October) the average abundance of larvae was 2.4 times higher on the northern transects than on the southern transects ($p=0.0011$). A strong seasonal effect was detected with adults peaking slightly earlier in the year than nymphs. The observed seasonality is the same in the treatment and non-treatment areas, although there are fewer ticks overall at the treatment sites than at the non-treatment sites (Fig. 2.2).

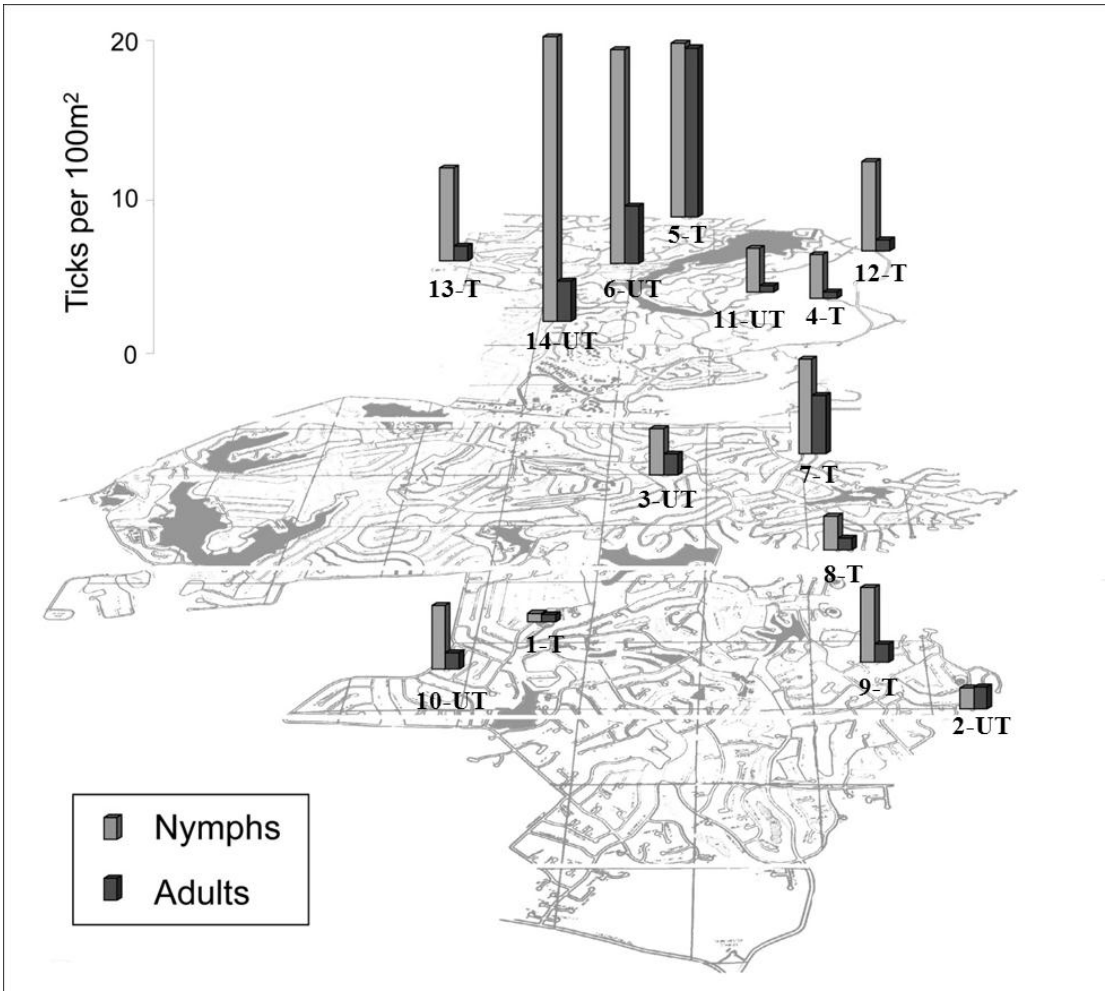

Figure 2.1 Mean counts of nymphal and adult *A. americanum* at 14 sites within the study area. Adult means are for the period March 24 – June 16, 2009; nymphal means are for the period May 11 – July 27, 2009. (T=treatment site, UT=untreated site)

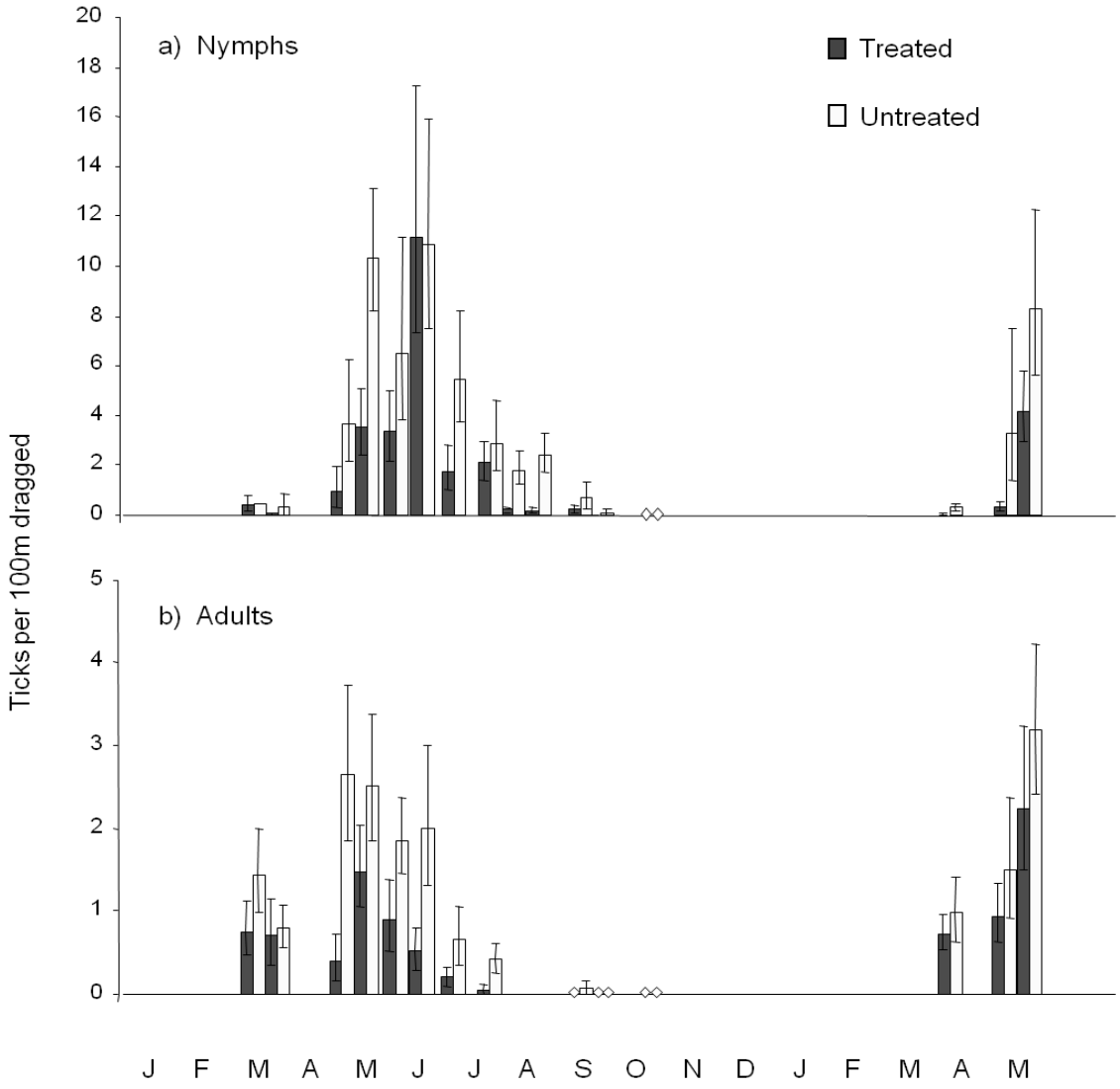

Figure 2.2 Seasonal variation in nymphal and adult tick abundance. Nymphs peak slightly before adult ticks and tick abundance at treated sites is less than at untreated sites in almost every month.

We observed a proximity effect of the '4-poster' treatment on tick populations, with the treatment effect becoming non-significant for nymphs and adults at >300m from the '4-poster'. Therefore the diameter of measurable effect around the '4-poster' acaricide applicators is 600m (Figure 2.3). Treatment effects were more evident for nymphs than for adults, with an observed 68% reduction of nymphs and 49% reduction of adults within $40m^2$ of the '4-poster' devices (nymphal $p<0.001$, adult $p=0.005$). A 90.1% percent reduction of larval *A. americanum* ticks was detected at treatment transects and is highly significantly different from non-treatment sites for the entire sampled distance (Fig. 2.4). These treatment effects exist at sites in the first year of treatment as well as sites in the second year of treatment, and the difference in effect for the two treatment classes is not significant. Time constraints and community set-up hindered our ability to test farther than the original 400m transect distance, therefore the extent of '4-poster' device distance effect on *A. americanum* larvae is unclear.

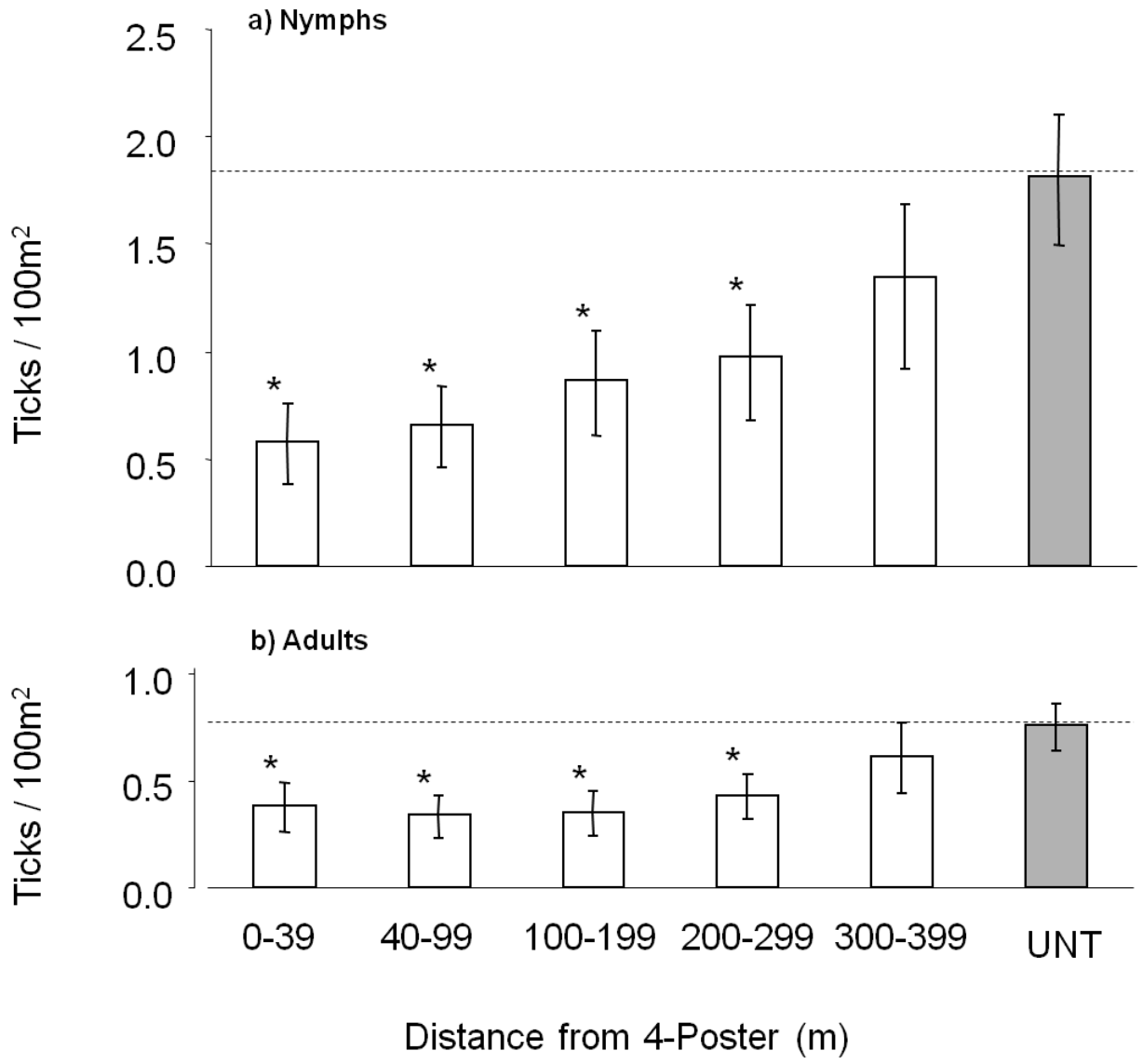

Figure 2.3 Distance effect of '4-poster' acaricide applicators on adult and nymphal tick abundance per 100m^2; at alpha = 0.05, starred bars indicate tick abundance that significantly differs from untreated sites (UNT; nymphal p<0.001, adult p=0.005).

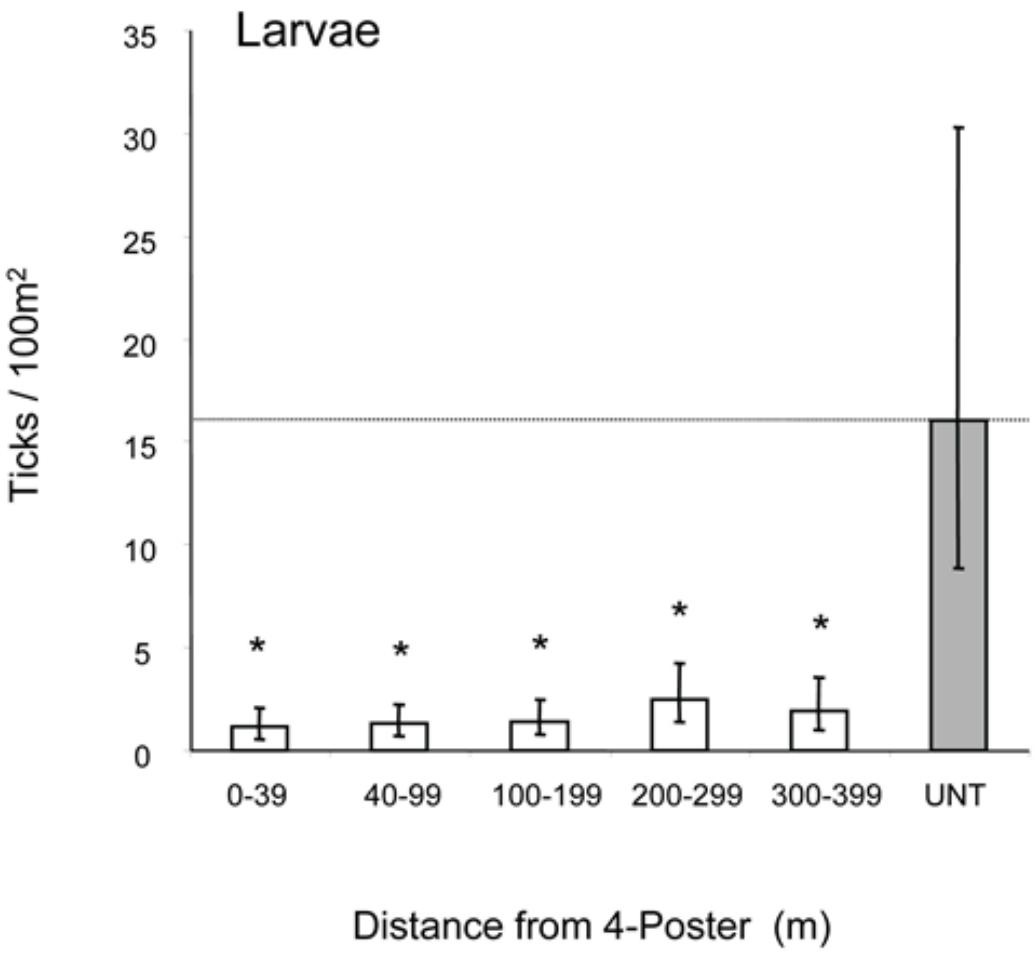

Figure 2.4 Distance effect of '4-poster' acaricide applicators on larval tick abundance per $100m^2$. At alpha = 0.05, tick abundance significantly differs from untreated sites up to 400m from '4-poster' acaricide applicators. Starred bars indicate statistical significance ($p<0.001$).

We obtained a total of 4,070 photographs from trail cams consisting of the following image counts for each species: 4,787 of deer, 1,694 of squirrels, 438 of raccoons, 285 of turkeys, 94 of crows, 54 of woodchucks, 50 of wild hogs, and one of a grey fox. Variation was seen with as few as 56 photos taken during a session at one site and as many as 997 photographs taken during the same sampling period at another site.

The relative abundance of collected tick species was highly skewed, so focusing tick mitigation strategies in this area specifically on management of *A. americanum* is appropriate. With limited resources for tick management, concentrating mitigation in the northern portion of the community may be favorable for better overall tick control. For long term mitigation of ticks, management officials would likely benefit from investing in exclusion techniques to keep wildlife species from the bordering wildlife management area from coming into the community. In a similar area of Tennessee, this method in combination with vegetation management and acaricide application led to significant overall reduction of *A. americanum* compared to each mitigation method used alone (Bloemer et al., 1990).

The lower observed reduction in adults is likely a result of how long the devices have been used at each site; half have been in use for only one year and thus have not had adequate time to impact the number of questing adults (i.e. adult ticks *before* they feed on deer). For that reason, we expect to see fewer nymphs in the second season of '4-poster' utilization. It is also expected that a third year of treatment with '4-poster' acaricide applicators will yield a larger percent reduction in the adult ticks. However, the lack of significance between the two treatment classes suggests that the decrease in ticks may be a result of a high density of non-target wildlife hosts near the '4-poster' devices rather than the acaricide treatment itself. Where high abundance

of wildlife hosts exists, greater proportions of ticks are able to find hosts, decreasing the ability of drag sampling to accurately assess tick population density (Ginsberg and Zhioua, 1999). Trail cams demonstrated that the '4-poster' acaricide applicators were routinely used by non-target wildlife species often without those species coming into contact with the acaricide treated paint rollers (Figure 2.4). The high number of photographed non-target wildlife species at '4-poster' acaricide applicator sites supports the hypothesis that the observed decrease in ticks is a result of questing ticks having readily available hosts and therefore not being draggable near acaricide applicators. This observed high wildlife activity in close proximity to '4-poster' devices also results in increased corn consumption and therefore higher costs of '4-poster' maintenance, while also increasing the risk for potential wildlife disease outbreaks. By baiting individuals of several different species to a centralized feeding site, there is an increased capacity for a '4-poster' to become a fomite for any number of wildlife diseases. This is especially disconcerting in this area because of the community's proximity to a wildlife management area where the target species (deer) and many of the non-target species (turkey, hog, raccoon, and squirrel) are hunter harvested. In several photographic series, certain species (primarily raccoons and hogs) also chased deer away from the acaricide applicators and therefore prevented the target species from feeding and self-treating with acaricide. Future studies should assess whether being chased from '4-poster' acaricide applicators has detrimental effects on deer self-treatment or whether deer simply return to the devices at a later time.

Figure 2.5 Trail camera picture of a squirrel, a woodchuck, and a deer utilizing a '4-poster' acaricide applicator.

Given the very small treatment area of the devices and the relative overall size of the study area, it is clear that eight devices are not sufficient to reduce the risk of tick-borne disease in the community as a whole. Additionally, because the majority of '4-poster' acaricide applicators in this area are located on the perimeter of the community, a high likelihood exists that deer in the interior part of the community may never come into contact with the devices. Again, these results emphasize the necessity for the community managers to consider integrated techniques rather than solely using acaricide self-treatment of deer for tick mitigation efforts. Considerable uncertainty exists among the community managers about the mode of action of the '4-poster' acaricide applicators. One '4-poster' was utilized in close proximity to a golf course

and had to be moved elsewhere due to resident complaints of deer-vehicle collisions on a nearby road. Half of the '4-poster' devices were moved to new sites at the beginning of our survey and almost all had been moved in the previous year. At the start of the 2010 treatment season, seven of the eight '4-poster' acaricide applicators were moved to new sites due to concern of poachers potentially using the devices to illegally hunt deer in the community. The high significance in reduction of *A. americanum* larvae in treatment sites in contrast to the significant, but less extensive reduction seen in nymphs and adults, demonstrates the importance of leaving '4-poster' devices at a site long enough to affect the tick population as a whole rather than just affecting one life stage. This result raises the question of whether a 90% percent reduction of *A. americanum* larvae in one year necessarily means the same reduction will be seen in nymphs in the following year. Small mammals have been shown to replenish early stage ticks at deer-focused tick management sites (Ginsberg and Zhioua, 1999), raising the possibility that the attraction of non-target species to '4-poster' acaricide applicators could lead to re-infestation of nymphal or adult ticks, despite removal of the local larvae.

Carroll et al. (2003) estimated the cost of maintaining a '4-poster' to be $20 per device per week including costs of labor, corn, and acaricide. Using this estimate, the cost of maintaining the eight '4-poster' devices currently utilized at our study site is $640/month, or $3,840 for the six-month period in which the devices are deployed each year. Estimations of the cost of treatment for *Ehrlichia* infections are unavailable, so Lyme Disease estimates are used here for cost comparison. The estimated median total cost of diagnosis and treatment for Lyme Disease patients in the early stage is approximately $397, increasing to approximately $923 for clinically defined late-stage Lyme Disease (Zhang et al., 2006). It is important to assess whether

the decrease in tick numbers seen in this community is worth the amount of money necessary for maintenance of the ‘4-poster’ devices and the potential increased risk to wildlife health.

Recommendations of one ’4-poster’ for every 20 hectares (Schulze et al., 2007; Solberg et al., 2003) suggest that in order to manage ticks in an area of this extent, roughly 200 ‘4-poster’ acaricide applicators would need to be employed. Suitable sites for ‘4-poster’ acaricide applicators and necessary funding are the limiting factors for complete management of ticks in this community by utilization of ‘4-poster’ acaricide applicators alone. Because of regulations on ‘4-poster’ devices, most being used in the community are in areas with a low likelihood of human presence. Given the history of tick-borne disease in the community, the ideal mitigation technique would be to create a buffer zone around golf courses where most residents are exposed to ticks. Spraying around golf courses with acaricide would likely work well to accomplish this goal, but further investigation into affordability of this technique should be investigated. Vegetation management such as overstory and understory reduction one to two times per year in high human populated areas in combination with ‘4-poster’ utilization in the more heavily wooded areas and exclusion fencing along the wildlife management area border is one option for controlling the tick population within this community. However, managing the tick population in the community does not automatically equate to mitigating tick-borne disease and while it is important not to make people paranoid or scare them away from participating in outdoor activities, investing money into resident education may be the most economical and efficient way to reduce disease risk for this residential area.

3 MOLECULAR IDENTIFICATION OF *EHRLICHIA SPP.* AND HOST BLOODMEAL SOURCE IN *AMBLYOMMA AMERICANUM*

3.1 Abstract

The current status of tick-borne disease (TBD) in the southeastern United States is challenging to define due to emerging pathogens, uncertain tick/host relationships, and changing disease case definitions. A golf-oriented retirement community on the Cumberland Plateau in Tennessee experienced an ehrlichiosis outbreak in 1993 that triggered a CDC outbreak investigation (Standaert et al., 1995). Anecdotal reports indicate that residents of the outbreak community have perceived resurgence in tick-related infections in recent years. *Amblyomma americanum* is by far the most abundant tick species in the study area; of 253 adult and nymphal *A. americanum* tested, we found two positive for *Ehrlichia chaffeensis* (0.86%), 14 positive for *Ehrlichia ewingii* (6.03%), and four positive for Panola Mountain *Ehrlichia* (1.72%; this is the first confirmation of Panola Mountain *Ehrlichia* in the state of Tennessee). The rate of *Ehrlichia* spp. infection in ticks from this community is broadly similar to recently reported rates in other *Ehrlichia*-endemic areas. Blood meal analysis (BMA) was used to determine the wildlife hosts on which ticks in this community feed. Our results suggest that the most significant reservoir hosts for *Ehrlichia* spp. are deer, wild turkeys, squirrels, and Passeriformes. Clarification of the species that act as reservoirs for pathogens in the community is the first step toward targeted management strategies to mitigate the disease risk for residents.

3.2 Introduction

In 1993, an ehrlichiosis outbreak occurred among residents of a golf oriented retirement community located in the Cumberland Plateau of Tennessee. The Centers for Disease Control conducted an outbreak investigation using patient history, serology, and PCR testing for *Ehrlichia chaffeensis*. From this study, 10 cases of ehrlichiosis were reported from the retirement

community, indicating an attack rate of 330 per 100,000 and 12.5 percent of surveyed residents had serologic evidence of past *E. chaffeensis* infection (Standaert et al., 1995). The researchers concluded that the high rate of *E. chaffeensis* was due to a bordering wildlife management area and human risk factors for infection included tick bites, exposure to wildlife, golfing, and lack of insect repellant use. The community is heavily forested and fragmentation due to residential development and golf courses provides ideal habitat for certain wildlife species and therefore ticks. Since the time of this outbreak, knowledge about *Ehrlichia* species has greatly improved, primarily with the understanding that *E. chaffeensis* is not the only *Ehrlichia* species that is capable of causing disease in humans and other animals. *Ehrlichia ewingii*, originally identified as the causative agent of canine granulocytic ehrlichiosis (Anderson et al., 1992a) was later recognized as an agent of human ehrlichiosis as well (Buller et al., 1999). In 2006, Panola Mountain *Ehrlichia* (PME), similar to *Ehrlichia ruminatum*, was discovered in a goat from Panola Mountain State Park in Atlanta, GA (Loftis et al., 2006). Subsequent studies determined that PME is widely distributed along the range of *Amblyomma americanum* (Loftis et al., 2008) and that it may cause tick-borne illness in humans (Reeves, 2008). With this increased knowledge, it is important to revisit the site of the previous outbreak investigation and determine whether *Ehrlichia* species other than *E. chaffeensis* could be contributing to the pathogen status of this community.

Historically, the primary methods for determining wildlife hosts and reservoirs for ticks and tick-borne disease have been field trapping and xenodiagnosis. These methods require extensive field research, extra laboratory and field technicians, and approval from the Institutional Animal Care and Use Committee (IACUC), yet are very limited in the breadth of

species that can be covered. Most published tick xenodiagnosis papers are based on small mammal and rodent species that can easily be reared in a laboratory setting. Published field ecology papers are limited by which wildlife species can feasibly be trapped and handled, primarily birds, mice and other small mammals, raccoons, and opossums. Studies assessing ticks on hunter harvested species (i.e. turkey and deer) are restricted to the hunting season which does not correspond to the active questing season of certain tick species (i.e. *A. americanum*) and therefore cannot give a complete picture of what wildlife hosts those tick species prefer. Blood meal analysis allows for questing ticks to be collected and analyzed in the lab to determine host bloodmeal source. Molecular methods using immunological techniques, multiplex PCR, and sequencing to determine host bloodmeal have been extensively used for mosquitoes, black flies, and tsetse flies which have a large amount of available fresh bloodmeal (Beier et al., 1988; Boakye et al., 1999; Hunter and Bayly, 1991; Kent and Norris, 2005; Ngo and Kramer, 2003; Tempelis, 1975). In contrast, free-living ticks have molted since taking a bloodmeal and could be questing for months to find a new host. As a result, the remaining testable bloodmeal in ticks is very low in quantity and of poor quality; highly sensitive methods are imperative for detection. In 2007, a molecular technique known as the Reverse Line Blot (RLB) method was developed in Switzerland to detect host bloodmeal source of questing ticks (Humair et al., 2007).

The goals of this project were to develop an assay to detect host bloodmeal for ticks from the southeastern United States and assess the wildlife species that are acting as hosts for ticks collected from the site of the original 1993 outbreak investigation in the Cumberland Plateau of Tennessee. Additionally, we sought to determine the profile of *Ehrlichia* species infecting ticks from this site and by matching up results of both assays, assess the wildlife hosts that are most

likely to be reservoirs for *Ehrlichia* spp. in this area. To assess whether this community may be a hot spot for *Ehrlichia* species, *Ehrlichia* and bloodmeal determinations for ticks from the retirement community were compared with ticks tested from another site located in middle Tennessee.

3.3 Methods

3.3.1 Sampling method

Ticks were collected from vegetation by drag sampling once a month from March 2009-October 2009 and in May and June of 2010. Researchers pulled a 1x1 meter corduroy cloth along 400m transects at fourteen sites within the retirement community. Additional tested ticks were collected from Henry Horton State Park (HHSP) in Chapel Hill, Tennessee, using similar techniques. Ticks from HHSP were collected by dragging 500m transects at six different sites within the park. A random sample of 100 ticks collected from March 2008-July 2008 at HHSP was used for comparison with the ticks tested from the Cumberland Plateau site to assess any differences of *Ehrlichia* infection rates and host bloodmeal sources of ticks between the two areas.

Ticks were brought to the University of Tennessee's Medical and Veterinary Entomology laboratory, where they were identified and separated by species, life stage, and sex. Total DNA was extracted from adult and nymphal ticks as described by Beati and Keirans (2001) using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA).

3.3.2 *Ehrlichia* Assays

Half of the DNA from each extracted sample was assayed for *Ehrlichia* species using nested PCR for the GroEL operon fragment. Visualization was performed using gel electrophoresis. DNA from all positive bands was isolated using a Zymoclean Gel DNA Recovery Kit (Zymo Research Corporation, Orange, CA) and sequenced using ABI Big-Dye cycle sequencing mix on a 3130 analyzer (Applied Biosystems, Carlsbad, CA). Sequences were analyzed using BioEdit software and BLASTed for species identification. Positive samples that did not match a GenBank sequence for GroEL were subsequently amplified for the gltA (citrate synthase) gene. For GroEL amplification, we used the primary forward and reverse oligonucleotide primers 5' GAAGATGC(A/T)GT(A/T)GG(A/T)TGTAC(T/G)GC-3' and 5' AG(A/C)GCTTC(A/T)CCTTC(A/T)AC(A/G)TC(C/T)TC-3' and the nested forward and reverse primers 5' ATTACTCAGAGTGCTTCTCA(A/G)TG 3' and 5' TGCATACC(A/G)TCAGT(C/T)TTTTCAAC-3' (Takano et al., 2009). Primary forward and reverse oligonucleotide primers used for gltA amplification were 5' GCCACCGCAGATAGTTAGGGA 3' and 5' TTCGTGCTCGTGGATCATAGTTTT 3' and the nested forward and reverse primers 5' TGTCATTTCCACAGCATTCTCATC 3' and 5' TGAGCTGGTCCCCACAAAGTT 3' (Loftis et al., 2006). Negative and positive controls were included in each *Ehrlichia* spp. PCR. A subset of 16 ticks were tested for *E. chaffensis* and *E. ewingii* via 16S nested PCR as described by Yabsley (2005), however observed cross-reactivity between species and high presence of detected *Rickettsia amblyommii* led to sole utilization of the GroEL and gltA PCR techniques.

3.3.3 Blood Meal Analysis:

The remaining half of the DNA from each extracted sample underwent touchdown PCR amplification for the 12S rDNA mitochondrial gene followed by blood meal analysis using the reverse line blot (RLB) hybridization method (Cadenas et al., 2007; Humair et al., 2007). Oligonucleotide probes were developed exclusively for New World species that allowed for determination of host blood meal source from ticks in the southeastern United States (Table 1). Probe verification was done using tissue and blood samples collected from mammalian, avian, and reptilian species that are known to be or have potential to be blood meal hosts for *A. americanum*. Samples were received from throughout the southeastern US, were fresh, frozen, or preserved in alcohol, and DNA was promptly extracted from collected samples using QiagenDNeasy Blood and Tissue Kit. Forty-three oligonucleotide probes were coupled to a Biodyne C nylon membrane that may be stripped of PCR products and reused up to 40 times. Forty-three PCR products were hybridized to the membrane and then treated with a streptavidin-peroxidase conjugate. The membrane was incubated in electrochemiluminescence (ECL) detection liquid and exposed to X-ray film for visualization.

For the purpose of reducing potential contamination, DNA was extracted from ticks in one hood and *Ehrlichia* outer PCR amplification and RLB PCR amplification was performed in another hood in the Medical Entomology laboratory. In the Center for Wildlife Health laboratory, we performed *Ehrlichia* nested PCR amplification in a designated hood, gel electrophoresis and analysis, and RLB hybridization and visualization. All laboratory fume hoods were equipped with built-in UV lamps and were thoroughly sanitized between reactions.

3.3.4 Statistical analysis

Fisher Exact tests were used to compare the prevalence of *E. chaffeensis*, *E. ewingii,* and Panola Mountain *Ehrlichia* between the sites and to compare bloodmeal host determination between sites, life stages, and wildlife host species. Binomial confidence intervals for prevalence comparisons were calculated using http://statpages.org/confint.html. The observed-to-expected ratio was calculated for *Ehrlichia* spp. infection by wildlife host for the top four host species.

3.4 Results and Discussion

Ticks from our retirement community field site were infected with *Ehrlichia* spp. at similar values found in other *Ehrlichia* endemic areas. Of 232 adult and nymphal *A. americanum* tested from our study site, we found two positive for *E. chaffeensis* (0.86%), fourteen positive for *E. ewingii* (6.03%), and four positive for Panola Mountain *Ehrlichia* (1.72%). Of the positives, there were one adult male and one adult female positive for *E. chaffeensis*, nine adult males, four adult females, and one nymph positive for *E. ewingii*, and two adult males and two nymphs positive for Panola Mountain *Ehrlichia*. The *glt*A sequences for Panola Mountain were identical to the reported PME sequence reported by Loftis et al (2008) (GenBank: DQ363995). Of 82 ticks tested from HHSP, two were positive for *E. chaffeensis* (2.4%), one was positive for *E. ewingii* (1.2%), and two were positive for Panola Mountain *Ehrlichia* (2.4%). These results were not significantly different, although a higher prevalence of *E. ewingii* was found in our primary site than at HHSP (Fig. 1).

Bloodmeal source was successfully determined for 47.7% of tested ticks from our primary study site (n=281) and for 63.4% of ticks from HHSP (n=82). This difference in detected blood meal by site was statistically significant ($p<0.001$). The proportion of successful determination for tested adults (48.3%; n=268) was significantly higher than for tested nymphal ticks (30.4%; n=56; $p=0.018$). A range of wildlife species contributed to *A. americanum* bloodmeals (Table 2). Wild turkeys were the most common bloodmeal source for both larval and nymphal ticks at both sites. Considering only successful bloodmeal determinations, turkeys were fed on by 15.1% of tested ticks at our primary study site and 40.4% at HHSP. Deer were an important host bloodmeal source at both sites (11.2% at the study site and 21.2% at HHSP),

however at the primary site 93% of the deer bloodmeals were detected in adult ticks and at HHSP 82% of the deer bloodmeals were detected in nymphal ticks; the difference was highly statistically significant ($p<0.001$). Based on our results, turkeys and squirrels are the two primary bloodmeal sources in the retirement community and are also implicated as reservoirs for all three of the causative agents of ehrlichiosis. This is the first report of Panola Mountain *Ehrlichia* in the state of Tennessee and it was found at both the primary study site in the Cumberland Plateau and in Henry Horton State Park in middle Tennessee. Of the successfully detected bloodmeals from our primary study site, 10% were from squirrels whereas we detected no squirrel bloodmeal in ticks from the comparison site. The retirement community is highly fragmented and the state park comparison site is not, so there may be increased density of squirrels in forested areas of the community due to home range compaction (Sterzik et al., 1988). Squirrels were also routinely seen feeding at and around the '4-poster' acaricide applicators in the retirement community but rarely came into contact with permethrin treated paint rollers. Panola Mountain *Ehrlichia* was detected in nymphal ticks that fed as larvae on squirrels and turkeys, implicating both as reservoirs for PME in this community.

Table 3.1 List of oligonucleotide sequences of primers and probes used to analyze blood meal source for Amblyomma americanum in the southeastern United States.

Oligo primer/probe Name	Nucleotide sequence	Target organism
Primers		
N.A. 12S-6F	CTR GGA TTA GAT ACC CYA CTA TG	Vertebrates
N.A. B-12S-9R	5' biotin-ATT AYA GRA CAG GCT CCT CTA	Vertebrates
Probes		
N. Green Anole	5'-CAG AAA AGC CTA AAA CTC AA	*Anolis carolinensis*
N. Fence Lizard	5'-CAC TAA AAT ATC CGC CAG AAA AC	*Sceloporus undulatus*
E. Six-lined Racerunner	5'-CAC CCC TAC CTA GAG GAG	*Cnemidophorus sexlineatus*
Broadhead, Five-lined skink	5'-GCC AGA GAA CTA CAA GTG A	*Eumeces*
Little brown skink	5'-CAG AGA ACT ACA AGC GAA A	*Scincella lateralis*
All Turtle	5'-ACA AAA ATA TCC GCC AGA GA	*Testudines*
All Birds except Owl	5'-ACG CTT AAA ACT CTA AGG AC	*Aves*
Owls	5'-TTA AAA CCC TAA GGA CTT G	*Strigiformes*
Turkey	5'-CTT GAT ACT AAT ATA CTC ACG TAT CC	*Maleagris gallopavo*
Vulture	5'-CTC ACC AAA GCA TCC G	*Cathartidae*
Quail and Bobwhite	5'-CTA AAT CCA GAT ACC CCC AAT	*Colinus virginianus*
Passerine bird	5-TGC TTA CAC CTA CTA AAG CAT CC	*Passeriformes*
Cat, Thrasher, Mocking..	5'-TTG ATA CTC GAT ATT ACC TGA GT	*Mimidae*
Robin, Thrushes	5'-TGA TGC TCG ATA TTA CCT GA	*Turdidae*
Mourning Dove, Pigeon	5'-TCT AGA TGC TTA TGT TAC TAA AGC AT	*Columbidae*
N. American small rodent	5'-GAC TTG GCG GTA CTT TAT ATC	*Rodentia*
Virginia Opossum	5'-TAGTAATAAACTAAAATAATTTAACAAAC	*Didelphis virginiana*
American short-tailed shrew	5'-GGT AAT TTA ATT AAC AAA ACT ACT CG	*Blarina*
Long-tailed shrew	5'-CTA ACA AAA ATA CCC GCC AGA	*Sorex*
Moles	5'-AAC TAA GAC AAT CCA ACT AAC AAG	*Talpidae*
Deer mouse	5'-GGC CAT CGC TTA AAA CTC	*Peromyscus maniculatus*
White-footed mouse	5'-TAC TGG CTA CCG CTT AAA ACT	*Peromyscus leucopus*
Cotton mouse	5'-CCC TAA ACC TCA AAG ATT AAA TA	*Peromyscus gossypinus*
House mouse	5'-TGC TTA GCC ATA AAC CTA AAT	*Mus musculus*
Cotton rat	5'-TAG CCC TAA ACC ACA ATA ACT TA	*Sigmodon hispidus*
Wood rat	5'-CCTAAACCCTAATAATTCAATAACAAAAT	*Neotoma floridana*
Rat	5'-CTT AGC CCT AAA CCT TAA TAA TTA	*Rattus*
Vole	5'-CAA AAA TAT TTG CCT GAG AAC	*Microtus*
Chipmunk	5'-GCT TAG CCT TAA ACA CAA ATA C	*Tamias striatus*
Squirrel	5'-AGA GAA CTA CTA GCC ACT GCT	*Sciurus carolinensis*
Woodchuck	5'-CAT AAA CAT TCA ACA AAC AAG AA	*Marmota monax*
Cottontail	5'-GCC CTA AAC TTA AAT AAT TCC ATA AC	*Sylvilagus floridanus*
Striped Skunk	5'-GCC ATA AAC ACA GAC AAT TAA TAT	*Mephitis mephitis*
Raccoon	5'-ACG TAA CAA AAT TAT TTG CCA	*Procyon lotor*
Grey Fox	5'-AGT TCT ATA AAA CAA AAT AGT TCG C	*Urocyon cinereoargenteus*
Red Fox	5'-CTA TAA CAA AAC AAT TCG CC	*Vulpes vulpes*
Dog, Coyote	5'-CCC TAA ACA TAG ATA ATT TTA CAA CAA	*Canis*
Cats	5'-CAA AAC TAT CCG CCA GAG AA	*Felidae*
Deer	5'-AGT ACT ACC GGC AAT AGC TTA	*Odocoileus virginianus*
Pig	5'-TAC TAC TCG CAA CTG CCT	*Sus scrofa*
Cattle	5'-CCT AAA CAC AGA TAA TTA CAT AAA C	*Bos primigenius*
Horse	5'-CTA AAA TAG CTT ACC ACA ACA AA	*Equus ferus caballus*
Human	5'-AAA TCA ACA AAA CTG CTC GC	*Homo sapien*

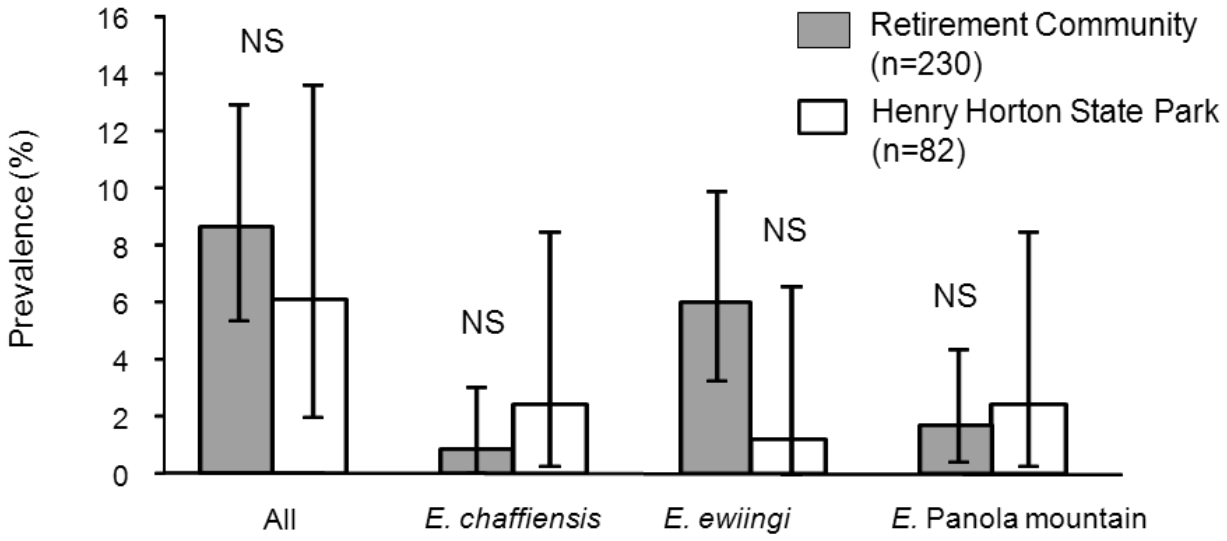

Figure 3.1 Comparison of *Ehrlichia spp.* infection prevalence in ticks from our retirement community study area and from Henry Horton State Park. Comparisons are for all *Ehrlichia* species combined, E. chaffeensis alone, E. ewingii alone, and Panola Mountain *Ehrlichia* alone. NS = not statistically significant; no significant differences were seen between the study area and the comparison site for any of the tested *Ehrlichia* species.

One reason for the lower observed detection of deer and turkeys as bloodmeal hosts at the primary study site is that community managers have been utilizing '4-poster' acaricide applicators (Chapter 2) to kill ticks feeding on deer in an attempt reduce the tick and tick-borne disease risk to residents in the area. Trail cameras have documented turkeys feeding from '4-poster' devices as well, although they do not appear to be treated by the devices (Chapter 2). However, despite treatment efforts in this community, ticks feeding on deer still have the highest observed-to-expected ratio of *Ehrlichia spp.* infection of the top detected wildlife bloodmeal sources (Fig. 2). The proportion of ticks feeding on feral pigs is also much higher in the retirement community than in the comparison site, likely due to the growing population of hogs

found within the community. However, of ticks that fed on feral pigs, none tested positive for *Ehrlichia* species.

Table 3.2 *Ehrlichia* results by each detected bloodmeal host for site one, a retirement community in the Cumberland Plateau of Tennessee. X(neg,A,B,C) where x= number of BMA hits; neg=number negative for *Ehrlichia spp.* A=number *E. chaffeensis* +ve; B=number *E. ewingii* +ve; C=number Panola Mountain *Ehrlichia* +ve. Forty-seven adult female *A. americanum* ticks were tested for bloodmeal host but not *Ehrlichia spp.* infection.

Site One:	**Species**	**All ticks**	**Nymphs**	**Adult F**	**AM**
	turkey	**27**(20,0,1,1)	**8**(7,0,0,1)	**12**(6,0,1,0)	**7**(7,0,0,0)
	squirrel	**16**(13,1,1,1)	**6**(4,0,1,1)	**4**(3,1,0,0)	**6**(6,0,0,0)
	deer	**15**(3,0,2,0)	**1**(1,0,0,0)	**12**(2,0,0,0)	**2**(0,0,2,0)
	pig	**12**(9,0,0,0)	**6**(6,0,0,0)	**5**(2,0,0,0)	**1**(1,0,0,0)
	Passeriformes	**10**(7,0,2,1)	**3**(3,0,0,0)	**2**(2,0,0,0)	**5**(2,0,2,1)
	opossum	**10**(10,0,0,0)	**3**(3,0,0,0)	**1**(1,0,0,0)	**6**(6,0,0,0)
	passerine	**7**(5,0,1,0)	**2**(2,0,0,0)	**3**(1,0,1,0)	**2**(2,0,0,0)
	raccoon	**6**(5,0,0,0)	**2**(2,0,0,0)	**1**(0,0,0,0)	**3**(3,0,0,0)
	shrew	**5**(5,0,0,0)	**0**(0,0,0,0)	**0**(0,0,0,0)	**5**(5,0,0,0)
	white footed mouse	**5**(5,0,0,0)	**2**(2,0,0,0)	**2**(2,0,0,0)	**1**(1,0,0,0)
	thrush/robin	**5**(5,0,0,0)	**3**(3,0,0,0)	**0**(0,0,0,0)	**2**(2,0,0,0)
	bird	**4**(3,0,0,0)	**0**(0,0,0,0)	**3**(2,0,0,0)	**1**(1,0,0,0)
	cow	**4**(3,0,0,0)	**1**(1,0,0,0)	**3**(2,0,0,0)	**0**(0,0,0,0)
	small rodent	**3**(3,0,0,0)	**1**(1,0,0,0)	**1**(1,0,0,0)	**1**(1,0,0,0)
	mole	**2**(2,0,0,0)	**1**(1,0,0,0)	**0**(0,0,0,0)	**1**(1,0,0,0)
	red fox	**2**(1,0,0,0)	**0**(0,0,0,0)	**1**(0,0,0,0)	**1**(1,0,0,0)
	rabbit	**2**(1,0,0,0)	**0**(0,0,0,0)	**1**(1,0,0,0)	**1**(1,0,0,0)
	chipmunk	**1**(0,1,0,0)	**0**(0,0,0,0)	**0**(0,0,0,0)	**1**(0,1,0,0)
	Felids	**1**(1,0,0,0)	**0**(0,0,0,0)	**0**(0,0,0,0)	**1**(1,0,0,0)
	wood rat	**1**(1,0,0,0)	**0**(0,0,0,0)	**0**(0,0,0,0)	**1**(1,0,0,0)
	woodchuck	**1**(1,0,0,0)	**0**(0,0,0,0)	**0**(0,0,0,0)	**1**(1,0,0,0)
	turtle	**1**(0,0,0,0)	**0**(0,0,0,0)	**1**(0,0,0,0)	**0**(0,0,0,0)
	skinks	**1**(0,0,0,0)	**0**(0,0,0,0)	**1**(0,0,0,0)	**0**(0,0,0,0)
	unknown	**147**(117,0,7,1)	**34**(34,0,0,0)	**53**(29,0,2,0)	**60**(54,0,5,1)
Total:		**288**(212,2,14,4)	**73**(70,0,1,2)	**106**(54,1,4,0)	**109**(97,1,9,2)

Table 3.3 *Ehrlichia* results by each detected bloodmeal host for site two, Henry Horton State Park in middle Tennessee. X(neg,A,B,C) where x= number of BMA hits; neg=number negative for *Ehrlichia spp.* A=number *E. chaffeensis* +ve; B=number *E. ewingii* +ve; C=number Panola Mountain *Ehrlichia* +ve.

Site Two:	**Species**	**All ticks**	**N**	**AF**	**AM**
	turkey	**21**(20,1,0,0)	**7**(7,0,0,0)	**7**(6,1,0,0)	**7**(7,0,0,0)
	deer	**10**(10,0,0,0)	**9**(8,0,0,0)	**0**(0,0,0,0)	**2**(2,0,0,0)
	passerine	**6**(6,0,0,0)	**0**(0,0,0,0)	**1**(1,0,0,0)	**5**(5,0,0,0)
	Passeriformes	**2**(2,0,0,0)	**2**(2,0,0,0)	**0**(0,0,0,0)	**0**(0,0,0,0)
	pig	**2**(0,1,1,0)	**1**(0,1,0,0)	**0**(0,0,0,0)	**1**(0,0,1,0)
	Felids	**2**(2,0,0,0)	**1**(1,0,0,0)	**0**(0,0,0,0)	**1**(1,0,0,0)
	small rodent	**2**(2,0,0,0)	**1**(1,0,0,0)	**0**(0,0,0,0)	**1**(1,0,0,0)
	opossum	**1**(1,0,0,0)	**1**(1,0,0,0)	**0**(0,0,0,0)	**0**(0,0,0,0)
	white footed mouse	**1**(1,0,0,0)	**1**(1,0,0,0)	**0**(0,0,0,0)	**0**(0,0,0,0)
	raccoon	**1**(1,0,0,0)	**1**(1,0,0,0)	**0**(0,0,0,0)	**0**(0,0,0,0)
	Other bird	**1**(1,0,0,0)	**0**(0,0,0,0)	**0**(0,0,0,0)	**1**(1,0,0,0)
	gray fox	**1**(1,0,0,0)	**0**(0,0,0,0)	**1**(1,0,0,0)	**0**(0,0,0,0)
	rabbit	**1**(1,0,0,0)	**0**(0,0,0,0)	**0**(0,0,0,0)	**1**(1,0,0,0)
	skunk	**1**(1,0,0,0)	**0**(0,0,0,0)	**0**(0,0,0,0)	**1**(1,0,0,0)
	unknown	**31**(29,0,0,2)	**5**(5,0,0,1)	**14**(14,0,0,0)	**11**(10,0,0,1)
Total:		**83**(78,2,1,2)	**29**(27,1,0,1)	**23**(22,1,0,0)	**31**(29,0,1,1)

The ability to detect bloodmeals from adult ticks much more efficiently than from nymphal ticks is likely a direct result of the amount of bloodmeal that is taken by larval ticks compared to nymphal ticks. However, comparison of bloodmeal source to *Ehrlichia* infection in adult ticks leads to difficulty assessing species that are acting as pathogen reservoirs as there is no certainty that the tick acquired the infection from the wildlife host fed on during the nymphal life stage. By refining an extraction protocol specifically for nymphal ticks, it may be possible to increase the number of successful bloodmeal results for that life stage.

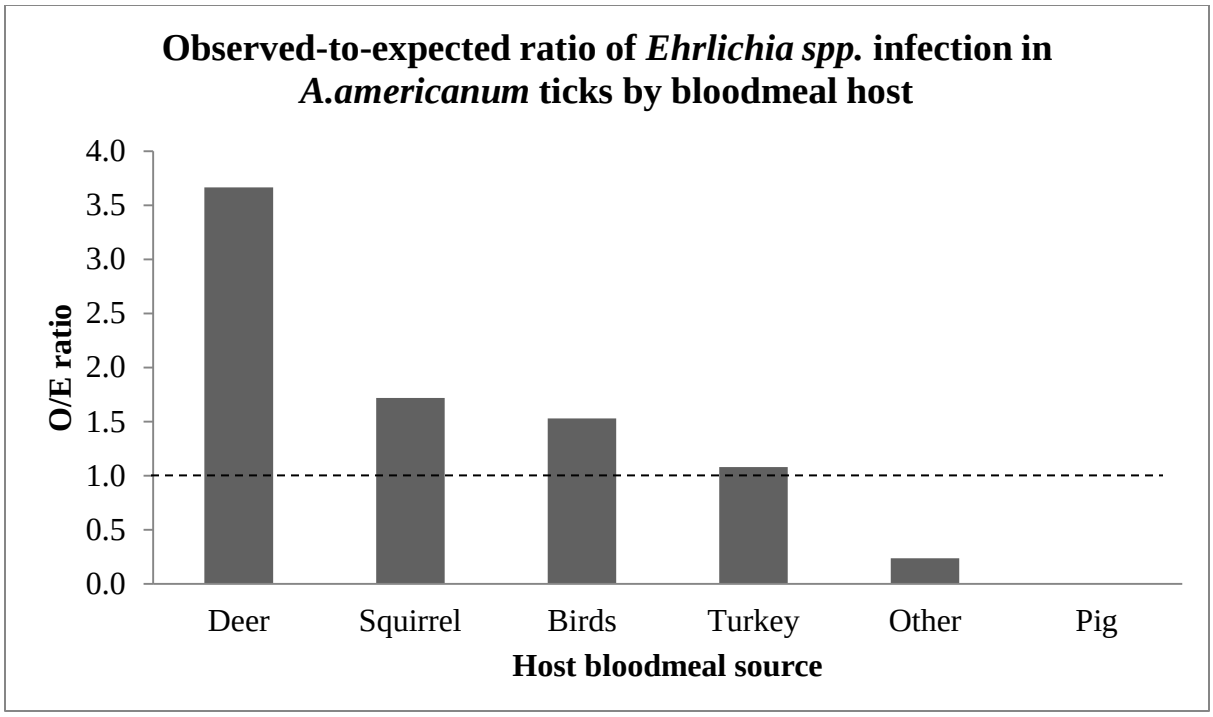

Fig. 3.2 Observed-to-expected ratio of *Ehrlichia spp.* infection in *A. americanum* ticks that fed on the most common bloodmeal hosts. Ratios greater than 1 represent higher *Ehrlichia* infection rates than are expected for that host group, indicating potential for that wildlife species to act as a reservoir for *Ehrlichia*.

The prevalence of *Ehrlichia* spp. infections in *A. americanum* is not significantly different between the two study sites, which implies that the retirement community is not currently a uniquely "hot" spot for *Ehrlichia* infection in Tennessee. However, the primary bloodmeal sources differ between the two sites, as squirrels and feral pigs play a more significant role as bloodmeal hosts in the retirement community and deer and turkeys play a more significant role at the comparison site. Based on the observed-to-expected ratio of *Ehrlichia* spp. per bloodmeal host, deer, squirrels, Passeriform birds, and turkeys are the most prominent species acting as reservoir hosts for *Ehrlichia* infections (Fig. 3.2). This clarification of the

species that are acting as reservoirs for pathogens in Tennessee is the first step in developting targeted management strategies to mitigate the disease risk for residents.

4 CONCLUSION

4.1 Utilization of ‘4-poster’ acaricide applicators as a sole method of tick mitigation in a large-scale community

There are several benefits of the use of ‘4-poster’ acaricide applicators to manage ticks and tick-borne disease. In just one year, it is possible to have an approximately 90% reduction of larval ticks at treatment sites. With additional treatment years, significant reductions of nymphal and adult life stages are also possible. Self-treatment of wildlife species is a simple way to manage ticks without having to handle and treat wildlife species or perform extensive acaricide applications that can result in non-target effects. However, this type of treatment is best used in a small area or at a site that does not have a high density of non-target wildlife species. In our large retirement community study site utilization of ‘4-poster’ acaricide applicators as a sole method for managing ticks cannot efficiently reduce the numbers of ticks or the risk of tick-borne disease to community residents. Regulations on placement of ‘4-poster’ devices in proximity to houses and areas where children may be present greatly limit the area in a community where acaricide applicators can be used. Resident concerns about deer-vehicle collisions due to deer being baited to particular areas of the community lead to additional limits for device placement. Even if community managers were able to afford the cost of buying and maintaining enough ‘4-poster’ acaricide applicators for an area of this size, it is likely that they would be unable to evenly distribute them throughout the community while also maintaining adequate distance from residences and main roads.

Rapid development in the community has led to areas of urbanized land that border heavily wooded land. This fragmented habitat allows several wildlife species to thrive which provides abundant bloodmeal sources for ticks. Many of these wildlife species are also attracted

to the corn supplemented by '4-poster' acaricide applicators but few come into contact with treated paint rollers as readily as deer. The presence of non-target species at '4-poster' sites decreases the efficacy of the devices, as nymphal and adult ticks are likely to be re-deposited by untreated wildlife. Increased maintenance costs can also be expected with the presence of non-target wildlife species due to greater corn consumption and an increase in necessary repairs, for example, repairing paint rollers broken by feral pigs and holes that squirrels chew in the main storage compartment. The attraction of many different wildlife species to these centralized devices also increases the risk that individual '4-poster' devices will become fomites for wildlife disease. In the event of a disease outbreak in this area, the affected wildlife population could be drastically affected by indirect transmission of infection via a '4-poster' acaricide applicator.

In relatively small areas, '4-poster' acaricide applicators appear to effectively reduce the number of questing ticks within the treatment area. However, for a large residential community such as our study site, integrated techniques will likely be necessary to successfully reduce the tick population as management using '4-poster' devices alone is limited by available space and costs of maintenance.

4.2 Implications of *Ehrlichia* and Blood meal analysis

We have reported the first identification of Panola Mountain *Ehrlichia* (PME) in *A. americanum* ticks in the state of Tennessee. This report is not surprising given the widespread distribution of PME in the eastern United States as determined by Loftis et al. (2008), but important to note as this species could be contributing to tick-borne illness within the state. While the prevalence of each species of *Ehrlichia* is not significantly different from Henry Horton State Park (HHSP), the prevalence of *Ehrlichia ewingii* was higher in the retirement

community than in HHSP (Chapter 3). Continued surveillance of the prevalence of these *Ehrlichia* species in *A. americanum* in the community is important for rapid response to any increases in infection rates. It is also imperative that managers educate the residents about their risk of tick-borne disease in the community and how to properly protect themselves to avoid ehrlichiosis outbreaks in the future. Based on a questionnaire survey done by The Human Dimensions Research Lab at the University of Tennessee, 33% of women and 20% of men in Tennessee do not participate in outdoor recreation more often due to concern about tick related diseases (Mark Fly, Human Dimensions Research Lab Director, pers. comm.). Through investments into resident education about prevention of tick-borne illnesses, the community would potentially see an increase in participation in outdoor activities and therefore increased economic gains for course memberships, gear rentals, etc. Resident education needs to be a primary focus of tick-borne disease mitigation in the community, as the greatest gain would likely be seen from this investment.

The wildlife host species that are potentially acting as reservoirs for *Ehrlichia* (turkeys, deer, and squirrels) are species that thrive in fragmented areas like our study site (Chapter 3). These are also species of particular importance to hunters from the nearby wildlife management area. The high populations of wildlife observed within and in proximity to this community results in greater difficulty managing ticks on hosts in an area of this size. Exclusion fencing along the border of the retirement community and the wildlife management area may serve to reduce the density of wildlife that are currently moving freely into and out of the community. Community managers should assess the cost-benefit ratio of using integrated techniques to

manage the tick-borne disease risk in the community as a whole in order to develop a system that best suits their needs.

4.3 Future Research Direction

To determine if Panola Mountain *Ehrlichia* is widespread across Tennessee and to assess the prevalences of the *Ehrlichia* species across the state, *A. americanum* should be collected and tested from various regions of Tennessee. Understanding the distribution of these agents across the state allows for assessment of high-risk areas or potential 'hot' spots for disease. This widespread testing could also lead to comparisons of the effects of different habitat types in Tennessee on both the number of *A.americanum* and prevalence of *Ehrlichia* infection in ticks. Knowledge of what habitat types and conditions are ideal for ticks and the pathogens they carry can provide insight into the best types of landscape management to pursue as means to reduce the number of ticks within the community.

Additional development and optimization of the Reverse Line Blot method of blood meal analysis should be performed, starting with testing different methods of DNA extraction that could reduce contamination issues. The most ideal method of DNA extraction would involve very little opening and closing of sample tubes, as is the case with bead beater methods, however *A. americanum* that have been stored in ethanol need long bead beating times for the tick to be efficiently broken up. Extensive bead beating can potentially lead to shearing of DNA and therefore compromise detection of bloodmeal hosts, so determining a better method is needed to efficiently break up ticks without damaging available DNA or allowing contamination into tubes. Optimization of the DNA extraction technique would likely solve the issues that we were unable to address through modification of PCR and hybridization protocols.

Community managers would likely benefit from focusing mitigation attempts in certain parts of the community, either in areas where tick densities are highest or where humans are

most likely to come into contact with ticks. Consideration should be made into more integrated approaches to manage ticks in areas where 4-posters aren't a feasible option, such as near residences, golf courses, or busy roads. Investments into field mitigation techniques should supplement an education effort to teach residents how best to protect themselves from ticks and tick-borne disease. Future research projects are needed to assess how several different mitigation techniques work together to reduce tick density as well as tick-borne disease risk to residents. A follow-up questionnaire survey should be done to determine resident concern about ticks at a larger community scale than what was achieved by our questionnaire survey (Appendix 1). An increase in response rate can be achieved through direct distribution of questionnaires to residents and sending out reminders about completion. Pre-addressed envelopes could also increase the response rate, as residents would then not be required to personally take completed questionnaires to the community center. A more comprehensive questionnaire would provide more insight into concern of the community as a whole rather than a small subset of the community members, and could allow community managers to determine topics that should be the focus of resident education efforts.

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APPENDICES

4.4 Appendix 1: Resident awareness and concern about tick-borne disease at the site of a previous ehrlichiosis outbreak

4.4.1 Introduction

Our study site is a golf-oriented retirement community located in the Cumberland Plateau of Tennessee. In 1993, four men at the study site came down with symptoms consistent with Human Monocytic Ehrlichiosis, a tick-borne disease transmitted by the bite of the *Amblyomma americanum* tick. These cases prompted a CDC outbreak investigation and led community managers to attempt mitigation of ticks feeding on deer. In recent years, management officials and health care professionals have voiced concern over increased reports of tick-borne disease in the community. Previous studies have found that a high level of concern about being bitten by ticks is strongly associated with the use of preventive measures (Herrington, 2004). Additionally, precautionary behavior has been correlated to the perception that the benefits of prevention outweigh inconvenience as well as certainty about one's ability to find attached ticks (Shadick et al., 1997). This questionnaire survey sought to determine residents' perceived level of concern about tick and tick-borne disease risk in the community through a voluntary questionnaire survey.

4.4.2 Methods

A short survey was conducted at the retirement community during the study period to determine resident knowledge of, concern about, and level of exposure to, tick borne diseases. Paper questionnaires were made available in the community center for pick-up on a voluntary basis (Fig. 5.2 and 5.3). Residents were instructed to complete only one survey per person per household. Completed questionnaires were returned to a locked box at the community center

where they were retrieved every second week by the principal investigator. Consent forms were obtained for all questionnaire participants (Fig. 5.1) allowing respondents the option to participate in a follow-up phone interview. Responses were analyzed using Epi Info software (Centers for Disease Control, Atlanta, GA).

4.4.3 Results and Discussion

We received fifty-three responses to the questionnaire, all but one respondent are permanent residents and twenty-eight reported having a rash or other illness associated with a tick bite obtained in the retirement community. Seventy-eight percent of respondents who reported a tick-bite associated illness also consider themselves avid golfers, and 89% walk or hike regularly compared to 42% that boat or swim and 10% that play tennis (activities that are associated with lower risk of exposure to ticks). Eighty-five percent of those reporting illnesses subsequently sought medical advice. Nearly all participants expressed medium to high concern about ticks and tick-borne disease and all reported seeing deer within 100m of their residences. Sixty-three percent of respondents always checked for ticks after being outdoors and forty-two percent reported usually wearing some form of repellant when going outside. The most commonly utilized repellant was Deep Woods Off® for which DEET is the active ingredient.

Responses to the resident questionnaire indicate a high level of concern about ticks and tick-borne disease in the retirement community. It is likely that the residents who chose to respond to this survey are also those who have a particular interest in the subject, a fact that may be represented by the high percentage of respondents who reported having tick bites associated with rash or illness. Residents who have little to no concern about ticks and tick-borne disease may be more likely to be indifferent about responding to this opportunistic questionnaire.

Response rates for residents of various levels of interest in the topic may have been higher had individual questionnaires and follow ups been mailed to or directly delivered to residents. Nonetheless, residents who did participate still represent high levels of concern among at least a portion of people in the community.

All residents reported seeing deer within 100m of their households and several commented that they have neighbors who routinely feed deer in their back yards. While many residents report practicing methods to protect themselves from ticks and tick-borne disease, i.e. using repellants and performing tick checks, some also attracted wildlife species into human inhabited areas through feeding. It is important that community managers are vigilant in educating the residents about the risk they incur when baiting wildlife species, particularly those that are known to have high tick infestation rates. This resident behavior could also result in fewer deer visiting '4-poster' acaricide applicators, effectively reducing the ability of those devices to manage the ticks and tick-borne disease in this area.

Most respondents who reported using repellants when participating in outdoor activities describe using a products containing DEET. DEET is arguably the most accessible insect repellant, as it can be found in nearly any convenience store. However, DEET has been shown to have very little efficacy against *A. americanum* nymphs, with only 25% of *A. americanum* repelled (Carroll et al., 2004). Efforts are needed in publicizing the availability of alternative repellant options, such as Permethrin, that are more effective against hard ticks.

While it is clear that at least a subset of the residents are knowledgeable and concerned about the risk of ticks and tick-borne disease in this community, the low response rate to a tick

related questionnaire may represent apathy about the subject in other members of the community. A relatively easy way to continue education of residents about ticks would be to distribute flyers one to two times per year with tick and tick-borne disease information of relevance along with the monthly community newsletter. This education method does not require individuals to seek out the information in their community, as it is consistently delivered to their door. Continued follow-up questionnaires after education campaigns could provide confirmation of increased awareness about risk factors in this community.

OFFICE USE ONLY

ID #_____

TICKS IN TENNESSEE – Page 1

A SHORT SURVEY OF YOUR ATTITUDES AND AWARENESS

INTRODUCTION

We invite you to participate in a research study involving attitudes and awareness about ticks and tick-borne disease in Tennessee. This survey is being carried out as part of a Master of Science thesis project by Jessica Harmon in the Entomology and Plant Pathology Department at the University of Tennessee, Knoxville. Information from this study will be used to educate the residents of Tennessee about ticks and tick-borne disease prevention.

The purpose of this study is:

1. To estimate Tennessee residents' exposure to ticks and tick-borne diseases.
2. To determine residents' attitudes and awareness of ticks and tick-borne disease in the state.

Your participation in this study will consist of answering a questionnaire. Completing the questionnaire will take approximately 10 minutes.

CONFIDENTIALITY

Your name will not appear on the questionnaire, so your anonymity will be maintained. Your signed informed consent document and completed questionnaire will be kept for the duration of the project and for at least three years thereafter. These will be stored securely in a locked file cabinet at the University of Tennessee Center for Wildlife Health, and will be used only by the principal investigator and the faculty advisor. When the consent forms and questionnaires are disposed, they will be destroyed by shredding.

CONTACT INFORMATION

If you have questions at any time about the study or the procedures, you may contact the researchers c/o Jessica Harmon, University of TN Department of Entomology and Plant Pathology, Knoxville TN 37996, (865) 974-8602, jharmon4@utk.edu. If you have questions about your rights as a participant, contact the Compliance Section of the Office of Research, University of Tennessee, Knoxville TN at (865) 974 3466.

PARTICIPATION

Your participation is voluntary and you may discontinue participation and choose not to complete the questionnaire at any time. However, we hope you will assist us in this study.

CONSENT

I have read the above information. I have received a copy of this form. I agree to participate in this study.

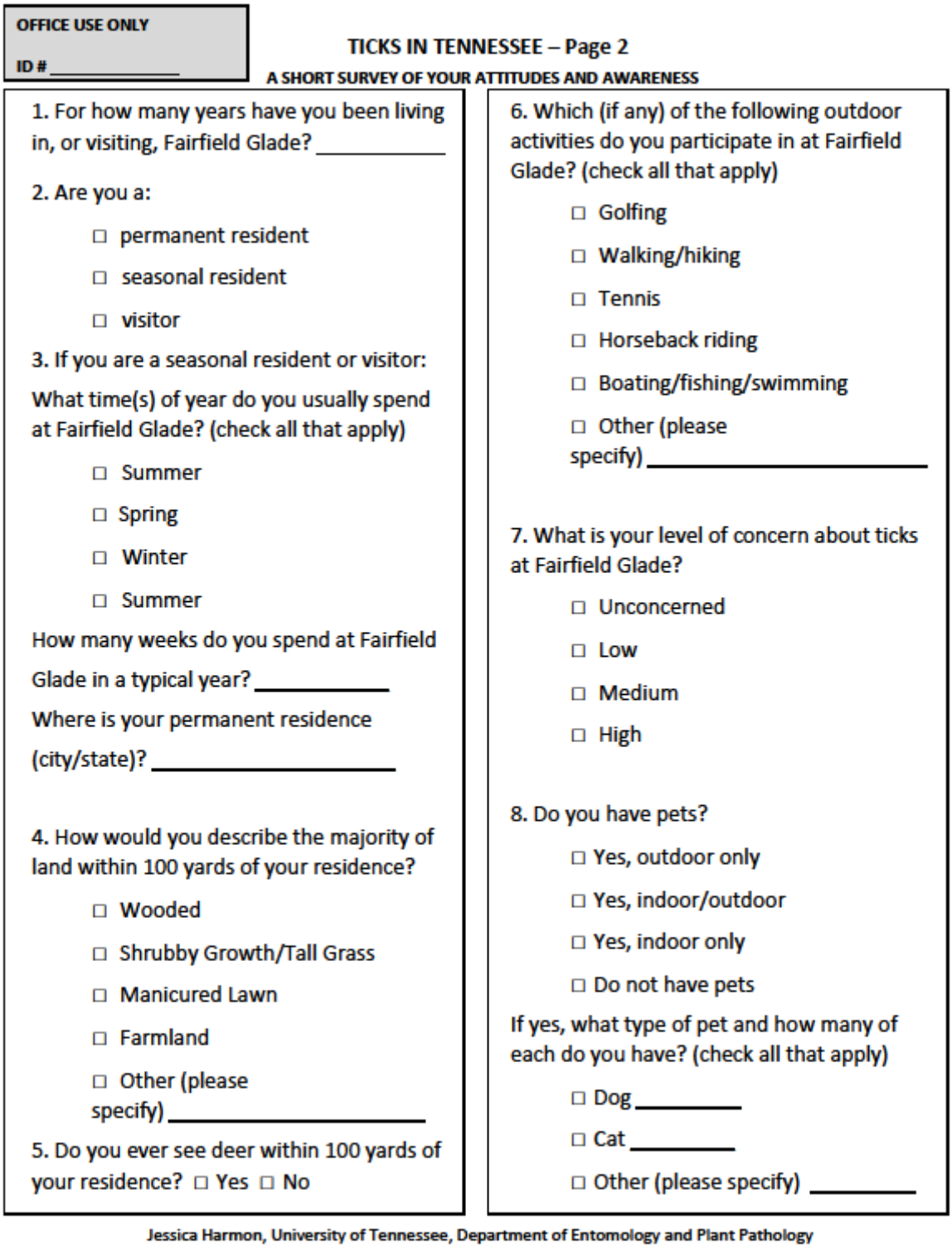
Participant's name (print)_____ Phone (optional) _____

Participant's signature _____Date_____

An electronic version of this questionnaire can be filled out at http://wildlifehealth.tennessee.edu/ ...

Jessica Harmon, University of Tennessee, Department of Entomology and Plant Pathology

Figure 5.1.1 Resident questionnaire consent form



OFFICE USE ONLY

ID # _____

TICKS IN TENNESSEE – Page 2

A SHORT SURVEY OF YOUR ATTITUDES AND AWARENESS

1. For how many years have you been living in, or visiting, Fairfield Glade? _____

2. Are you a:

- ☐ permanent resident
- ☐ seasonal resident
- ☐ visitor

3. If you are a seasonal resident or visitor:

What time(s) of year do you usually spend at Fairfield Glade? (check all that apply)

- ☐ Summer
- ☐ Spring
- ☐ Winter
- ☐ Summer

How many weeks do you spend at Fairfield Glade in a typical year? _____

Where is your permanent residence (city/state)? _____

4. How would you describe the majority of land within 100 yards of your residence?

- ☐ Wooded
- ☐ Shrubby Growth/Tall Grass
- ☐ Manicured Lawn
- ☐ Farmland
- ☐ Other (please specify) _____

5. Do you ever see deer within 100 yards of your residence? ☐ Yes ☐ No

6. Which (if any) of the following outdoor activities do you participate in at Fairfield Glade? (check all that apply)

- ☐ Golfing
- ☐ Walking/hiking
- ☐ Tennis
- ☐ Horseback riding
- ☐ Boating/fishing/swimming
- ☐ Other (please specify) _____

7. What is your level of concern about ticks at Fairfield Glade?

- ☐ Unconcerned
- ☐ Low
- ☐ Medium
- ☐ High

8. Do you have pets?

- ☐ Yes, outdoor only
- ☐ Yes, indoor/outdoor
- ☐ Yes, indoor only
- ☐ Do not have pets

If yes, what type of pet and how many of each do you have? (check all that apply)

- ☐ Dog _____
- ☐ Cat _____
- ☐ Other (please specify) _____

Jessica Harmon, University of Tennessee, Department of Entomology and Plant Pathology

Figure 5.1.2 Page one of questionnaire distributed to residents of the retirement community

OFFICE USE ONLY

ID #_____

TICKS IN TENNESSEE – Page 3

A SHORT SURVEY OF YOUR ATTITUDES AND AWARENESS

	Never	Usually not Less than 50%	Usually More than 50%	Always	N/A
7. Do you wear insect repellant when you go outside? What kind? _____	☐	☐	☐	☐	☐
8. How often do you go into the woods (off of trails or golf courses) at Fairfield Glade?	☐	☐	☐	☐	☐
9. How often do you see ticks at Fairfield Glade?	☐	☐	☐	☐	☐
10. Do you check for ticks after being outside at Fairfield Glade?	☐	☐	☐	☐	☐
11. Have you been bitten by ticks at Fairfield Glade?	☐	☐	☐	☐	☐

12. Have your pets been bitten by ticks while at Fairfield Glade?

☐ Yes ☐ No ☐ N/A

13. Do you treat your pets with tick repellant?

☐ Yes, use repellant

what kind? _____

☐ Do not use repellant

☐ Do not have outdoor pets

14. Have you ever had a rash, fever or other symptoms that you felt might have been associated with a bite of a tick from Fairfield Glade?

☐ Yes ☐ No

15. If yes, did you seek medical advice?

☐ Yes ☐ No

If yes, may we contact you briefly to gather some additional information about the tick bite(s) your received? ☐ Yes ☐ No

Jessica Harmon, University of Tennessee, Department of Entomology and Plant Pathology

Figure 5.1.3 Page two of questionnaire distributed to residents of the retirement community

Appendix 3.2: Tick DNA Extraction Protocol

Modified from: Qiagen DNeasy Blood and Tissue Handbook: Protocol: Purification of Total DNA from Animal Tissues (Spin-Column Protocol) Catalog No. 69506

Tick Prep:

1. Remove ticks from identification/measurement vials and blot dry on a Kimwipe.
2. Zero a clean 1.5ml centrifuge tube, labeled with extraction identification number.
3. Place 1 tick per vial and recorded tick extraction ID and weight.
4. Include 1 positive and 1 negative control tick per batch.

Phase 1- Lysis:

1. Turn on incubator and set to 56˚C. Put beaker for ATL/Pro-K solution and ATL solution in incubator to warm.
2. Place each individual tick/vial into liquid Nitrogen without submerging the vial.
3. Use a pestle to pop and grind the tick in the vial. Leave the pestle in the vial until after the lysis buffer has been added.*
4. Create a master mix of lysis solution. (180µL of Buffer ATL and 20µL Pro-K per sample). Prepare enough for 5% extra ticks due to loss from transfer.
5. When samples have warmed (room temp), add 200µL of Buffer ATL and Pro-K solution to each 1.5ml microcentrifuge tube.** Be careful not to shoot tick particles out of vial. This can be prevented by directing the pipette tip to the side of the vial instead of straight down.
6. Mix thoroughly by vortexing for 5-15 seconds and incubate at 56˚C overnight, rocking. Make certain no tick pieces are stuck to the vial where the lysis buffer can‘t reach.

*Use a clean scalpel to position the tick for cutting within its vial if the tick does not pop with liquid N2. Cut tick into several (at least 4) pieces, with attention to cutting open the midgut.

** 20µl of additional ATL/pro-K can be added to large engorged ticks until the tick is completely submerged.

Phase 2- Extraction

1. Place enough Buffer AE (provided) for final elution to 70˚C. (100ul per sample)
2. Pre-label 1 set of spin-columns and 2 sets of 1.5ml centrifuge tubes with final extraction ID.
3. Create a master mix of 200µl Buffer AL (provided) and 200µl of EtOH (95%-100%) per sample.
4. Remove samples from incubator and vortex for 15 seconds
5. Add 400µl of Buffer AL/EtOH master mix to each sample and mix again thoroughly by vortexing.
6. Pipette the sample mixture from step 5 (including any precipitate) in the corresponding spin-column.
7. Centrifuge each spin-column at ≥6,000 x g (8,000rpm) for 1 min. Discard flow through collection tube and place in a clean collection tube.
8. Add 500µl of Buffer AW1 and centrifuge at ≥6,000 x g (8,000rpm) for 1 min. Discard flow through collection tube and place in a clean collection tube.
9. Add 500µl of Buffer AW2 and centrifuge at 20,000 x g (14,000rpm) for 3 min. Discard flow through collection tube and place spin-column in the corresponding (final) 1.5 microcentrifuge tube. Incubate at 45˚C for 10 minutes.
10. Pipette 50µl of Buffer AE directly onto the spin-column membrane. Incubate at room temperature for 10 min.
11. Centrifuge each sample at ≥6,000 x g (8,000rpm) for 1 min to elute.
12. Place spin-column in the second labeled microcentrifuge tube and repeat step 10.
13. Ensure all microcentrifuge tubes are properly labeled (elution 1 or 2, place a cardboard box and stored in the freezer.

4.5 **Amplification of Ehrlichia sp. GroEL operon fragment** (Takano et al., 2009)

Nested PCR Primer sequences:

GRO607F - Primary forward GAA GAT GCW GTW GGW TGT ACK GC

GRO1294R - Primary reverse AGM GCT TCW CCT TCW ACR TCY TC

GRO677F - Nested forward ATT ACT CAG AGT GCT TCT CAR TG

GRO1121R - Nested reverse TGC ATA CCR TCA GTY TTT TCA AC

Amplification conditions:

Denaturation 5 min @ 95C

Denaturation 30 sec @ 95C

Annealing 30 sec @ 57C 40 cycles for primary PCR; 30-35 cycles for nested PCR

Elongation 30 sec @ 72C

Final elongation 3 min @ 72C

Starting DNA quantity 4 ul for primary PCR, 1-2 ul for nested PCR

1. For each reaction, add the following to a tube containing a PCR bead: Note: Do not mix the tube contents until all the components (below) have been added to the tube containing the bead.

Forward 5pmol/µl	5µl
Reverse 5pmol/µl	5µl
Template DNA*	4 µl for primary 2 µl for nested
Water final volume of 25 µl	11 µl for primary 13 µl for nested

*Start with 50 pg for a simple template such as plasmid DNA, or 50 ng for a complex template such as genomic DNA. Avoid template amounts > 1 µg. Sterile high-quality water

2. Snap the caps (provided) onto the tubes, pushing down firmly to ensure a tight fit. Mix the tube contents by gently flicking the tube with a finger. Vortex gently and then centrifuge the tube for a few seconds to bring the components to the bottom of the tube. The reaction is fully dissolved and mixed when it appears clear.

3. Place the reaction mixtures on ice or in a cold block until ready for cycling. Minimize the time on ice prior to cycling to prevent formation of background reaction products.

4.6 Appendix 3.3: *Ehrlichia* spp. PCR Protocol

Outer Ehrlichia PCR Primers Tm=about 53°c

16s forward (2) Ehrlichia outer(Cathy)-GCAAGCYTAACACATGCAAG
16s reverse Erhrlichia outer(Cathy)-GGGCAGTGTGTACAAGAC

Dilute primers to 10uM or 10pMoles/ µl - Add 10mM Tris to the concentration in nMoles on the IDT sheet for stock concentration of 1nMol/ µl or 1mM. Dilute to 10pMoles/µl in Water.

Assemble an amount of FailSafe Master Mix corresponding to the total number of reactions. Extra Master Mix may be required to offset losses caused by pipeting.

1. Prepare the FailSafe Master Mix. Thaw and thoroughly mix all of the reagents listed below before dispensing; place on ice. Combine on ice, all of the following:

25 µl of E FailSafe PCR 2X PreMix
17.5 µl sterile water
1.0 µl 10 µM primer 1 (0.2 µM final concentration)
1.0 µl 10 µM primer 2 (0.2 µM final concentration)
0.5 µl FailSafe PCR Enzyme Mix (1.25 Units)
45 µl Total volume

2. Add 5 µl DNA Template to each tube (extracted using Qiagen DNeasy tissue kit) into individual wells, using extraction controls as well as PCR controls (5 µl previously-positive extract no-template control (NTC) for negative). Total Reaction volume 50 µl.

PCR Ehrlichia 1

a. 1 cycle as follows:
    i. Denature: 2min at 95°C
b. 10 touch down cycles annealing temper lowered by 1°C each cycle
    i. Denature: 20 sec at 94°C
    ii. Anneal: 30 sec at 60°C
    iii. Extend: 90 sec at 72°C
c. 20 cycles
    i. Denature: 20 sec at 94°C
    ii. Anneal: 30 sec at 52°C
    iii. Extend: 90 sec at 72°C
d. Final extension: 7 min at 72°C
e. 4°C∞

Inner Species specific PCR primers (Stromdahl et al., 2000; Yabsley et al., 2005)- Dilute to 5 µM

HE1 E.chaffeensis(2nd)16SrRNA forward HE1 Anderson et al. (1992) (Anderson et al., 1992b)
CAATTGCTTATAACCTTTTGGTTATAAAT

HE3 E.chaffeensiiandE.ewingii(2nd)16SrRNAreverse HE3 Anderson et al. (1992)
TATAGGTACCGTCATTATCTTCCCTAT

EE5 E ewingii forward Oklahoma coyoteEE5 (Kocan et al., 2000)
CAATTCCTAAATAGTCTCTGACTATTTAG

Two separate reactions one containing HE1+ HE3 and another with HE3 +EE5.

1. Prepare the FailSafe Master Mix. Thaw and thoroughly mix all of the reagents listed below before dispensing; place on ice. Combine on ice, all of the following:

12.5 µl of E FailSafe PCR 2X PreMix
8.25 µl sterile water
1.0 µl 5 µM primer 1 (0.2 µM final concentration)
1.0 µl 5 µM primer 2 (0.2 µM final concentration)
0.25 µl FailSafe PCR Enzyme Mix (0.75 Units)
23 µl Total volume

2. Add 2 µl of reaction from PCR I.

PCR Ehrlichia II

a. 1 cycle as follows:
   Denature: 2min at 95°C
b. 10 touch down cycles annealing temper lowered by 1°C each cycle
   i. Denature: 20 sec at 94°C
   ii. Anneal: 30 sec at 60°C
   iii. Extend: **30 sec** at 72°C
c. 20 cycles for Tick (20 for vertebrate DNA) as follows:
   iv. Denature: 20 sec at 94°C
   v. Anneal: 30 sec at **50°C**
   vi. Extend: **30 sec** at 72°C
d. Final extension: 7 min at 72°C
e. 4°C∞

3. Store samples at 4°C until prepared for QIAxcel or gel electrophoresis

Outer 16s all bacteria primers from (Clay et al., 2008; Marchesi et al., 1998) modified to include Ehrlichia.
16sall 63f CAGGCCTAACACATGCAAGTC
16sallrv (reverse compliment) 1387r GCCTTGTACACWCCGCCC

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4.7 Appendix 3.4: Panola Mountain *Ehrlichia* PCR Protocol (Loftis et al., 2006)

Amplification of Panola Mountain *Ehrlichia* sp. *glt*A (citrate synthase) gene

Nested PCR

Primer sequences:

Ehr3CS-185F-Primary forward	GCC ACC GCA GAT AGT TAG GGA
Ehr3CS-777R-Primary reverse	TTC GTG CTC GTG GAT CAT AGT TTT

Amplification conditions:

Denaturation	3 min @ 95C	
Denaturation	30 sec @ 95C	40 cycles
Annealing	30 sec @ 55C	
Elongation	60 sec @ 72C	
Final elongation	5 min @ 72C	

Primer sequences

Ehr3CS-214F-Nested forward	TGT CAT TTC CAC AGC ATT CTC ATC
Ehr3CS-619R-Nested reverse	TGA GCT GGT CCC CAC AAA GTT

Amplification conditions:

Denaturation	3 min @ 95C	
Denaturation	30 sec @ 95C	40 cycles
Annealing	30 sec @ 60C	
Elongation	60 sec @ 72C	
Final elongation	5 min @ 72C	

Starting DNA quantity 4 ul for primary PCR, 1-2 ul for nested PCR

1. For each reaction, add the following to a tube containing a PCR bead: **Note: Do not mix the tube contents until all the components (below) have been added to the tube containing the bead.**

Forward 5pmol/µl	2.5µl
Reverse 5pmol/µl	2.5µl
Template DNA*	4 µl for primary 2 µl for nested
Water final volume of 25 µl	13.5 µl for primary 15.5 µl for nested

*Start with 50 pg for a simple template such as plasmid DNA, or 50 ng for a complex template such as genomic DNA. Avoid template amounts > 1 µg. Sterile high-quality water

2. Snap the caps (provided) onto the tubes, pushing down firmly to ensure a tight fit. Mix the tube contents by gently flicking the tube with a finger. Vortex gently and then centrifuge the tube for a few seconds to bring the components to the bottom of the tube. The reaction is fully dissolved and mixed when it appears clear.

3. Place the reaction mixtures on ice or in a cold block until ready for cycling. Minimize the time on ice prior to cycling to prevent formation of background reaction products.

4.8 Appendix 3.5: DNA Purification Protocol

Modified from: Zymoclean Gel DNA Recovery Kit (Catalog No. D4001)

Materials Needed:
1.5µl Microcentrifuge tubes—weighed and final set for purified sample
Autoclaved (sterile) pure water (heated to 55C)
Razor blades

Sample Prep:
1. Weigh clean, dry, 1.5µl microcentrifuge tubes (number of samples to be excised)
2. Turn on hot plate to 55C

Purification:
1. Excise DNA fragments from the gel using a new razor blade or scalpel for each sample and transfer samples to corresponding weighed microcentrifuge tube.

Make sure to make the excision as precise as possible and to cut out as little gel as possible.

2. Weigh microcentrifuge tubes and subtract original tube weight to obtain sample weight.
3. Add 3 volumes of ADB to each volume of agarose excised from the gel (i.e. for every 100µl of gel, add 300µl of ADB)
4. Incubate at 55C for 5 to 10 minutes or until the gel is completely dissolved
5. Transfer the melted agarose solution to a Zymo-Spin I column in a collection tube
6. Centrifuge at ≥10,000 x g for 60 seconds. Discard flow-through and place in a new collection tube
7. Add 200µl of Wash Buffer to the column and centrifuge at ≥10,000 x g for 30 seconds. Discard flow through and place in a new collection tube.
8. Repeat step 7.
9. Spin samples for 30 seconds at ≥10,000 x g to remove any additional liquid. Discard collection tube and place into final 1.5µl microcentrifuge labeled with sample name.
10. Add 20µl of sterile pure water to the column and incubate at room temperature for 1 minute.
11. Spin at ≥10,000 x g for 60 seconds to elute DNA.
12. Discard minicolumn and store sample at -20C or prepare for sequencing.

4.9 Appendix 3.6: RLB bloodmeal analysis Protocol

Physically separate areas for DNA extraction, preparationof reagents (including PCR master mix) and storage, PC,post-PCR and RLB, to minimize contamination.

REAGENTS
Streptavidin-peroxidase conjugate
ECL chemiluminescence blotting substrate
Saline Sodium Phosphate–EDTA buffer (SSPE) NaCl–NaHPO4–EDTA
***(BOWN) SSPE 20x* for RLB store at rt for up to 1 year** –more NaCl for specificity

Clear	Conc.	Stock	1L	
NaCl	3.6M	58.44g/M	210.24g	
Na_2HPO_4 x2H_2O X7H_2O	200mM	178.0g/M 268.2	35.6g 53.64g	Only need one
EDTA (not sodium salt)	20mM	292.23g/M	5.84g	
H_2O		mQ	800ml	

- adjust pH 7.4 by 10N NaOH. QS to 1L

SDS CRITICAL- SDS is a critical reagent. Some brands of 'purity analysis' (p.a.) grade SDS may destroy the signal on the blot or yield a completely black image of the membrane on the X-ray. All reagents should be used before their expiry dates to ensure optimal performance.
10% (w/v) SDS! CAUTION Take great care when preparing SDS stock solution from powder, which is corrosive—use a mask and an exhaust fan to prevent breathing in powder and do not allow the solution to come into contact with the skin or eyes. In case of accidental splashing, wash immediately with water.
1% SDS m CRITICAL Must be freshly prepared and used within a few hours.
16% (w/v) EDAC m CRITICAL Must be freshly prepared and used within a few hours.
0.1 M NaOH
0.5 M NaHCO3 (pH 8.4)
0.5 mM EDTA (pH 8.0)
20 mM EDTA
2x SSPE
2x SSPE/0.1% SDS

2x SSPE/0.5% SDS m CRITICAL For buffers 8–10, SSPE buffers with or without SDS should be freshly prepared.

EQUIPMENT
Transparency film (Corporate Express, cat. no. 39547000)
Miniblotter (Immunetics, cat. no. MN100-45)
Foam cushions (Immunetics, cat. no. PC200)
Hybridization oven, rolling bottle
Rocking platform (Model: Belly Dancer, Pegasus Scientific, Inc.)
Imager or X-ray film exposure cassette (Sigma-Aldrich, cat. no. Z36,009-0) Film cartridge,X-ray film developer

PCR

II. Take out all PCR components to thaw including template DNA (keep Taq DNA polymerase on ice).

III. Prepare a mastermix. Using Invitrogen Taq DNA polymerase and primers. Standardize primers such that 1uL contains 40pmol/µl.
New 2010 American 12s forward- 5' CTR GGA TTA GAT ACC CYA CTA TG-3'
New 2010 American 12s reverse biotin- 5'Biotin-ATT AYA GRA CAG GCT CCT CTA-3'

IV. Combine the mastermix reagents **in order** in a multi-channel pipettor boat. Mix thoroughly. Using a multi-channel pipettor set to 30uL for tick or 45uL for vertebrate, fill the appropriate number of well strips on ice. Account for number of samples and controls plus a ~5% error buffer.

Example- 43 samples, then calculate for 45:
Taq DNA polymerase : 0.25 x 45 = 11.25µL
RV primer: 1 x 45 = 45µL

45reactions	µl/Each Rxn	
225	5.0	10x PCR buffer Qiagen
1293.75	28.75	Autoclaved milli-Q water
45	1.0	dNTP mixture 10mM
45	1.0	primer FW 40pmol/ul
45	1.0	primer RV 40pmol/ul
135	3.0	25mM $MgCl_2$
11.25	0.25	*Taq* DNA polymerase Qiagen
add DNA template to each tube		
10.0		DNA
	50 total Rxn	

V. Mix in 10uL (Carbone et al.) or 5ul (vertebrate) of each sample DNA (extracted using Qiagen DNeasy tissue kit) into individual wells, using extraction controls as well as PCR controls (no-template control (NTC) for negative).

VI. Run PCR program on thermocycler as follows:

Touch down PCR

a. Initial denature: 3 min at 94°C,
b. 1 cycle as follows:
    i. Denature: 20 sec at 94°C
    ii. Anneal: 30 sec at 65°C
    iii. Extend: 30 sec at 72°C
c. 8 touch down cycles annealing temper lowered by 1°C each cycle
    i. Denature: 20 sec at 94°C
    ii. Anneal: 30 sec at 65°C
    iii. Extend: 30 sec at 72°C
d. 30 cycles for Tick (20 for vertebrate DNA) as follows:
    i. Denature: 20 sec at 94°C
    ii. Anneal: 30 sec at 53°C
    iii. Extend: 30 sec at 72°C
e. Final extension: 7 min at 72°C
f. 4°C∞

VII. Store samples at 4°C until prepared for hybridization on blot. 20°C long term storage

Check mPCR result using gel electrophoresis (optional) or Qia-Axcel.

Covalent coupling of oligonucleotide probes to the membrane TIMING 3–4 h

Set Belly Dancer to 60 °C – warm 2x SSPE/0.1% SDS

16% EDAC = 3.2g EDAC in 20 mL water
2x SSPE/0.1% SDS = 445 mL water + 50 mL SSPE + 5 mL SDS (ADD WATER FIRST!!)
2x SSPE = 50 mL SSPE in 450 mL water

1. Dilute the oligonucleotides in water to their optimal concentrations, ranging from approximately 100-1000pmol (Cadenas et al., 2007; Humair et al., 2007b) add 10µl to 180 µl 0.5 M NaHCO3 (pH 8.4).

    -Stock concentration 100pmol/µl- IDT sheet has original concentration in nMoles
    -Working concentration 100-1000pmol (look at probe layout sheet). We want to add 10µl so the working concentration will be /10. For example --horses 200pmol you want to make a 20pmol/µl working concentration, so you would dilute the stock 1 to 5 in water.

CRITICAL STEP Make sure all required buffers for steps are warmed and incubators prepared before starting the following procedures.

2. Cut the Biodyne C membrane to 15 cm^2 size.
3. Fold one corner to help hold membrane during manipulation, write membrane identifier along side in pencil for orientation.

4. Activate the Biodyne C membrane in a sealed plastic bag with 15 min incubation in 20 ml of freshly prepared 16% (w/v) EDAC, on a rocking platform, at room temperature 25°C.

CRITICAL STEP EDAC allows amine-labeled probes to bind covalently to the nylon membrane. It is important that the 16% (w/v) EDAC is prepared just before use. (*3.2g EDAC in 20 ml water)*

5. Rinse the membrane with 250 ml milliQ water and place it on a support cushion in the clean Miniblotter. Tighten the screws manually.

6. Remove residual fluid from the slots by aspiration.

7. Fill the slots of the Miniblotter with 170 ml of each of the diluted oligonucleotide solutions.

8. The first and the last slots are filled with red and blue dye to allow for blot orientation.

CRITICAL STEP It is essential to avoid the formation of air bubbles in the slots because they can cause loss of hybridization signal.

9. Incubate for 5 min at room temperature. Do not rock or shake the Miniblotter in this step.

10. Remove excess oligonucleotide probe solutions by aspiration.

11. Remove the membrane from the Miniblotter and incubate it in 250 ml 0.1 M NaOH for 9 min on rocking platform to inactivate the membrane

CRITICAL STEP It is critical that incubation in 0.1 M NaOH be no longer than 10 min.

12. Briefly wash the membrane in a plastic container on the rocking platform in 250 ml 2x SSPE, then incubate in 250 ml prewarmed 2x SSPE/0.1% SDS for 5 min at 60°C in hybridization oven with rocking.

PAUSE POINT If the membrane is to be stored at this point, wash it in a plastic container on the rocking platform in 250 ml 20 mM EDTA for 20 min at room temperature.

Seal it in a plastic sleeve containing about 10 ml 20 mM EDTA to avoid dehydration and store at 4^0C until use. The properly labeled and sealed membranes can be kept for several years at 4^0C until use (proceed to Step 24).

CRITICAL STEP Make sure the membranes are properly sealed and stored; dehydration will render them useless for further hybridization assays.

Hybridization and detection of PCR products TIMING About 5h

Heat tube block above 100 ºC; get ice for denaturation
Heat Belly Dancer to 60 ºC – warm 2x SSPE/ 0.1% SDS
Heat oven to 42 ºC and 55 ºC. Warm miniblotter in 55 ºC

2x SSPE/ 0.1% SDS = 222.5 mL water + 25 mL SSPE + 2.5 mL SDS (ADD WATER FIRST!!)
2x SSPE/0.5% SDS = 850 mL water + 100 mL SSPE + 50 mL SDS (ADD WATER FIRST!!)
2x SSPE= 50 mL SSPE in 450 mL water

1. Prepare test samples by adding 10µl PCR products to 2x SSPE/0.1% SDS to obtain a total volume of 190 ml. (*The optimal concentration of SDS in the hybridization buffer varies between 0.1 and 0.5% and should be determined experimentally. In our experience, 0.1% SDS has been suitable for all assays. (0.1 SDS (Humair et al., 2007b))*

2. Heat-denature the test samples in boiling water for 10 min--VORTEX and cool on ice immediately for at least 5 min VORTEX. Vortexing periodically will insure strand separation!

3. Block membrane for 7 minutes in Casein. Incubate the membrane in 250 ml prewarmed 2x SSPE/0.1% SDS in a plastic container for 5 min in a 62°C hybridization oven with rocking.

4. Place the membrane in the Miniblotter on a support cushion, such that the slots are perpendicular to the previously applied oligonucleotides.

5. Close the Miniblotter and remove residual fluid from the slots by aspiration. Fill the slots with 170 ml of diluted PCR product whilst avoiding air bubbles and overloading. Any empty slots should be filled with 150 ml 2x SSPE/0.1% SDS to prevent the membrane from drying out and to prevent cross-flow between channels due to capillary action, ink slots should be used for orientation.

CRITICAL STEP It is essential to avoid the formation of air bubbles in the slots because they can cause loss of hybridization signal.

6. Hybridize for 30 minutes at 75°C then 60 min at 55°C on horizontal surface. Avoid shaking the Miniblotter in this step to prevent cross-flow to the neighboring slots.

7. Remove excess PCR products by aspiration- wash 2-3x with 2x SSPE/0.5% SDS while still in the miniblotter! Remove the membrane from the Miniblotter.

8. Wash the membrane twice in 250 ml prewarmed 2x SSPE/0.5% SDS for 10 min in a 62°C (Humair et al., 2007b) hybridization oven with rocking. **Turn oven down to 42^0.**

9. During wash mix 15µl neutravidin in 50 ml prewarmed 2x SSPE/0.5% SDS. (1:4000)

10. Place the membrane onto streptavidin- peroxidase conjugate Tupperware container incubate with constant mixing for 45–60 min at 42°C

11. Wash the membrane 2x in 250 ml prewarmed 2x SSPE/ 0.5% SDS for 10 min in a 42°C hybridization oven with rocking. Use dedicated plastic containers. **If stripping membrane – turn belly dancer up to 75^0C.**

12. Wash the membrane 2x in 250 ml 2x SSPE for 5 min at room temperature on the rocking platform.

13. For chemiluminescent detection, incubate the membrane in 20 ml electrochemiluminescence (ECL) detection liquid (10 ml of each of detection reagents 1 and 2), to cover the membrane, for about 2 min, while gently rocking the solution by hand.

14. Place membrane in a film cartridge between two overhead transparency sheets and expose an X-ray film to the membrane for 5–30 min. If the signal is too weak or too strong, the membrane can be used again directly to expose another film for a longer or shorter period. However, it should be noted that the peak of light emission occurs 1 min after incubation with the ECL substrate. After that the signal rapidly diminishes and prolonged exposure may be required to obtain a significant signal. Therefore in the initial stage of a new project, we recommend exposing two X-ray films for different periods to determine the optimal exposure time

CRITICAL STEP Step 14 should be performed in a dark chamber or dark room.

Stripping of the membrane for reuse TIMING 1.25 h

Heat Belly Dancer to 75 ^{0}C – microwave 1% SDS until bubbly

1% SDS = 50mL 10% SDS in 450 mL water
20mM EDTA = 100mL 100mM EDTA in 400 mL water

1. Wash the membrane twice in prewarmed 1% SDS at 80°C for 30 min with rocking.

2. Wash the membrane in 20 mM EDTA, for 15 min at room temperature on the rocking platform.

3. Seal the membrane in a plastic bag with approximately 10 ml of 20 mM EDTA to avoid dehydration.

4. Store at 4°C until reuse.(Cadenas et al., 2007; Kong and Gilbert, 2006; Rijpkema et al., 1995)

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VITA

Jessica Harmon is originally from Germantown, TN. She earned her Bachelor of Science degree in Animal Science with a specialization in Science and Technology at the University of Tennessee, Knoxville. Her interest in wildlife research was peaked when she participated in the Minority Health International Research Training (MHIRT) program which sent her to Brazil to partake in a study on the effects of landscape fragmentation on the carnivore species of Emas National Park. Prior to beginning graduate school, she worked as a field and lab technician in the Center for Wildlife Health lab at the University of Tennessee investigating the maintenance of Lyme Disease and *Ixodes scapularis* in Tennessee. Jessica completed her Master of Science in Entomology and Plant Pathology at the University of Tennessee, Knoxville in December 2010.