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Population occurrence and pathogen prevalence of lone star (Acari: Ixodidae) ticks collected from southeast Nebraska

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POPULATION OCCURRENCE AND PATHOGEN PREVALENCE OF LONE STAR (ACARI: IXODIDAE) TICKS COLLECTED FROM SOUTHEAST NEBRASKA

By

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POPULATION OCCURRENCE AND PATHOGEN PREVALENCE OF LONE STAR (ACARI: IXODIDAE) TICKS COLLECTED FROM SOUTHEAST NEBRASKA

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Lone star tick, *Amblyomma americanum* (L.), has recently become established in Nebraska; therefore, local biology, ecology, and tick-borne disease risk are not known. Research was conducted to determine monthly questing activity, establishment, and pathogenic microorganisms associated with the lone star tick in Nebraska.

Lone star tick populations were collected from May through August, 2012 in six sites in southeast Nebraska using carbon dioxide (CO_2) traps. A total of 747 adults, 3076 nymphs, and 1289 larvae were collected. Total ticks collected and monthly activity were significantly different for each site.

A semi-randomized sample of 251 adult ticks were selected for polymerase chain reaction (PCR) analysis for *Rickettsia* spp., *Ehrlichia chaffeensis*, and *E. ewingii*. Adult ticks were 51.8% (130/251) positive for *Rickettsia* spp., and prevalence was almost equal for both sexes 52.1% (73/140) females and 51.4% (57/111) males. Approximately 2% (4/251) of adult ticks tested positive for *E. chaffeensis*, all from Table Rock Wildlife Management Area where 12% (3/25) of female and 4% (1/25) of males tested positive.

Ticks collected in Indian Cave State Park, Schramm SRA, and Table Rock WMA were almost 2% (4/251) positive for *E. ewingii*, including 2.1% (3/140) female and 0.9% (1/111) male ticks. Co-infection with *Rickettsia* spp. and *E. chaffeensis* was detected from 8.0% (2/25) of female and 4.0% (1/25) of male adult ticks from Table Rock Wildlife Management Area. Lone star ticks are established at the six collection sites in southeast Nebraska, and lone star tick-associated disease microorganisms are present in Nebraska.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: LITERATURE REVIEW

INTRODUCTION

The lone star tick, *Amblyomma americanum* (L.), is an aggressive ectoparasite with a three-host life cycle that is a vector of both medical and veterinary pathogens (Goddard and Varela-Stokes 2009). Lone star ticks were originally found in the southeastern and south central United States, but in the past 20 years, its geographic range has expanded northeast to Maine, New York, and in the Midwest region (Childs and Paddock 2003; Mixson et al. 2006b; Goddard and Verela-Stokes 2009; Heise et al. 2010). The lone star tick has recently become established in Nebraska (Cortinas and Spomer 2013). Retrospective analysis of human case reports demonstrate that the tick appeared in southeastern Nebraska during the late 1980s and has continued to spread in a northerly and westerly direction (Cortinas and Spomer 2013).

There have been studies conducted on the lone star tick pertaining to biology, prevalence, hosts, reservoirs, and vector ecology in other Midwestern states (Kollars et al. 2000; Mixson et al. 2006a; Allan et al. 2010; Heise et al. 2010); however, little is known about the lone star tick in Nebraska. The state of Nebraska has a small human population compared to the entire United States population, comprising only 0.59% of the US population (U.S. Census Bureau 2011). The majority (56%) of Nebraskans live in the Omaha and Lincoln metro areas (US Census Bureau 2011). Throughout the rest of the state, the population is dispersed with only 23.8 persons per square mile (U.S. Census Bureau 2011). Lone star ticks in Nebraska could be competent vectors of diseases, but people are not often going into tick-infested areas and are not coming into contact with lone star ticks. There is a lack of knowledge of lone star ticks in Nebraska pertaining to

which pathogens lone star ticks are capable of harboring and transmitting, the prevalence of these vector-borne pathogens, and if Nebraska's habitats provide the proper hosts and reservoirs required for the life cycle of vector-borne diseases and parasites.

The objective of this chapter is to review lone star tick biology and ecology, the importance of the lone star tick, microorganism diversity within lone star ticks, and provide relevant details on pathogens associated with the lone star tick.

BIOLOGY

Classification

The lone star tick is an acarid in the family Ixodidae. It is a metastriate tick within the genus *Amblyomma* that also includes *Amblyomma maculatum* (Gulf Coast tick) (Parola and Raoult 2001). It is an ectoparasite that is hematophagous or obligate blood-feeder

Life cycle

The lone star tick is hemimetabolous and has three active life stages: larva, nymph, and adult. Larvae emerge in June and begin questing for hosts, feed, detach, and molt to the nymph stage in leaf litter on the forest floor (Goodman et al. 2005). Larvae reach peak populations from July through September and increase in activity in August (Lancaster 1955; Kollars et al. 2000). The larvae molt into nymphal stage and overwinter as unfed nymphs. If the larvae do not successfully molt to the nymph stage, they will not survive over the winter (Lancaster 1955). Nymphs emerge in March after diapause and are active through October (Lancaster 1955; Cortinas and Spomer 2013). Nymphs have

two population peaks from April through May and August (Lancaster 1955; Kollars et al. 2000). Nymphs feed to repletion, molt, and overwinter as adults. Adults emerge from winter diapause in April and are active until July or August (Fleetwood et al. 1984; Kollars et al. 2000). Adults quest for suitable hosts, preferably large mammals, to feed, find a mate, and copulate (Lancaster 1957). After the adult female has fed to repletion and mated, the female tick detaches from the host, oviposits a cluster of approximately 3,500 eggs in the leaf litter of the forest floor, and dies (Sacktor et al. 1948; Goodman et al. 2005). Lone star ticks can take up to three years to complete their life cycle, but in regions with milder winters, such as the south-central and southern United States, lone star ticks can complete its life cycle in less than three years (Drees and Jackman 1998).

Habitat

Lone star ticks are generally found in young, second growth deciduous habitats with dense under story vegetation and mature forests (Sonenshine 1993, Paddock and Yabsley 2007). In the south-central United States, lone star ticks are associated with oak-hickory, post-oak, blackjack oak, and persimmon-sassafras-winged elm forests (Sonenshine 1993). These woodland habitats provide habitats necessary for tick survival and for white-tailed deer and other suitable lone star tick hosts.

Hosts

Lone star ticks are generalists and will quest, attach, and take a blood meal from mammals, birds, and reptiles (Sonenshine 1993). Hosts are essential as a source of the blood meal required to molt to the next life stage as well as ovarian development, as a location for finding mates, and as a means of dispersal (Lancaster 1955, 1957; Gladney

and Drummond 1969; Kollars et al. 2000). The principal host of the adult lone star tick is the white-tailed deer (*Odocoileus virginianus*) (Bloemer et al. 1988), but adults also feed on medium to large-sized mammals including raccoons (*Procyon lotor*), opossums (*Didelphis maruspialis*), red foxes (*Vulpes vulpes*), coyotes (*Canis latrans*), and wild turkeys (*Meleagris gallopavo*) (Kollars et al. 2000, Paddock and Yabsley 2007). Nymphs and larvae are found on birds and medium and large-sized mammals including wild turkeys, white-tailed deer, raccoons, gray squirrels (*Sciurus carolinensis*), eastern cottontail rabbits (*Sylvilagus floridanus*), opossums, red foxes, and Carolina wrens (*Thryothorus ludovicianus*) (Kollars et al. 2000).

The white-tailed deer is a keystone host for all three life stages of the tick and has a major impact on the zoonotic transmission cycle of tick-borne infectious agents including *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, and *Borrelia lonestari* (Bloemer et al. 1988; Kollars et al. 2000; Paddock and Yabsley 2007; Goddard and Varela-Stokes 2009; Allan et al. 2010; Eisen et al. 2012). As white-tailed deer populations have increased, lone star tick populations have also increased (Paddock and Yabsley 2007). White-tailed deer populations have increased due to hunting restrictions, reforestation of agricultural lands, and lack of natural predators, such as wolves (Childs and Paddock 2003). In a study where white-tailed deer were excluded from two 1-ha enclosures in woodland tracts on Fire Island, New York, the reduction of white-tailed deer was associated with a reduction in nymphal lone star tick densities by approximately 48% during four years post-treatment (Paddock and Yabsley 2007). Populations of lone star ticks are increasing and spreading along with their primary host, the white-tailed deer (Childs and Paddock 2003; Paddock and Yabsley 2007). Additionally, ticks are being spread by white-tailed

deer to new habitats, expanding the tick's geographic distribution and possibly increasing the chance of humans coming into contact with the lone star tick (Childs and Paddock 2003; Paddock and Yabsley 2007).

Behavior

Lone star ticks quest most actively in late morning and early evening during periods of higher temperatures above 4.4^{o}C and lower humidity (Sonenshine 1993; Schulze et al. 2001a). Adults quest between 1300 and 1700 h and nymphs at approximately 1600 h (Schulze et al. 2001a; Schulze and Jordan 2003). Interestingly, lone star tick questing periods are the opposite of the tick's preferred host, white-tailed deer, which are most active during dawn and dusk (Bloemer et al. 1988). Researchers believe that lone star ticks are a "hunting tick", capable of tolerating desiccation and are attracted to carbon dioxide created by resting deer and other hosts (Schulze and Jordan 2003).

Importance

Lone star ticks can be a nuisance for humans and animals (companion animals, livestock, and wildlife). However, the lone star tick is not only a nuisance to humans and animals, but has important economic impacts and is a competent vector of pathogens and symbiotic microorganisms. A study by Brennan (1945) found one man with 294 attached lone star ticks. Another study, 1,150 lone star ticks were removed from a man who sat in a tick-infested thicket for 2 h (Webb 1952). White-tailed deer in eastern Oklahoma have extensive numbers of lone star ticks as well, resulting in anemia, tissue damage, and death in fawns (Bolte et al. 1970). Approximately 57% of the annual fawn crop was lost

in 1969 due to high tick infestations in eastern Oklahoma (Bolte et al. 1970). Heavy infestations can also cause decreased weight gains in cattle (Barnard 1985; Ervin et al. 1987). A study by Ervin et al. (1987) found that an average of 40 female lone star ticks reduced weight gains by $40 per head of cattle. Additionaly, tick bites may cause hide damage, anemia, and secondary infections (Johnson et al. 2013).

People come into contact with lone star ticks in reforested agricultural lands, in rural wooded areas, and as a result of going into tick-infested habitats for recreation (Standaert et al. 1995; Gubler 1998; Harrus and Baneth 2005). Also, immunocompromised individuals have access to treatments that allows them to go into areas where lone star ticks and reservoirs are found naturally (Paddock et al. 2001). This increased contact with lone star ticks may be associated with increased diagnosis of tick-borne diseases like human monocytic ehrlichiosis (HME) and canine granulocytic ehrlichiosis (CGE).

Microorganisms known to be symbionts of lone star ticks, such as *Rickettsia amblyommii*, are now considered to be pathogenic. *Rickettsia amblyommii* was once considered non-pathogenic (Burgdorfer et al. 1981b, Goddard and Noment 1986) but has now been associated with a mild spotted fever disease (Parola et al. 2005; Billeter et al. 2007), and it is believed that reported cases of Rocky Mountain spotted fever (RMSF) may have been misdiagnosed and caused by *R. amblyommii* (Apperson et al. 2008; Stromdahl et al. 2008; Jiang et al. 2010). Increasing health and public awareness of ticks and potential microorganisms that they are capable of harboring is important for public health.

Habitat impact on lone star ticks

Habitats not only have an impact on lone star ticks ability to survive, quest, and reproduce, but also determine host abundance and diversity. Lone star ticks are located specifically forest floor leaf litter, which provides shelter and protection from desiccation between questing and molting (Paddock and Yabsley 2007). Forest habitats consist of abiotic and biotic factors that create a habitat where the lone star tick can successfully establish a population.

Two primary abiotic factors that impact lone star tick populations are humidity and temperature. Lone star ticks require a cool, moist environment to rest between questing to avoid desiccation due to their small size and high surface area (Paddock and Yabsley 2007). Ticks avoid water loss by obtaining water through a blood meal and vapor absorption from the air (Needham 1991; Paddock and Yabsley 2007). Ticks are located at the ground level and shrub layer of vegetation which is cooler and more humid than the surrounding climate due to the forest canopy (Schulze et al. 2001b). The canopy provides a barrier to the wind and shade that reduces evaporation rates (Sonenshine 1993). The second abiotic factor is temperature. Adult ticks are most abundant in May through July in Nebraska (Cortinas and Spomer 2013). However, in the southern United States, the lone star tick may be active throughout the year, but is less active in midwinter (Kollars et al. 2000). Differences in seasonal activity are in response to humidity and temperature (Sonenshine and Mather 1994; Kollars et al. 2000).

Lone star ticks and their hosts are also affected by the biotic factors of the forest habitat. Hosts prefer an environment with a suitable food source, space, temperature, and

humidity for survival. However, many forests are becoming fragmented due to deforestation (Eisen et al. 2012). According to the island biogeography theory, as island size decreases and isolation increases, host diversity will decrease (LoGiudice et al. 2003; Eisen et al. 2012). Since forests are becoming smaller and more isolated host diversity will decrease leaving the forest with either competent or non-competent hosts. As forest size decreases, the chance of lone star ticks coming into contact and feeding on remaining hosts will increase. If the hosts left in the forest habitat are competent hosts, it would lead to an increased chance of ticks becoming infected at early stages with disease causing pathogens that can then be transstadially transmitted (Schmidt and Ostfeld 2001; LoGiudice et al. 2003). If the tick acquired the pathogen as a larva, it would have two chances of transmitting the pathogen to two different hosts as a nymph and adult since lone star ticks are three host ticks. In a model created by Schmidt and Ostfeld (2001) on blacklegged ticks (*Ixodes scapularis*), it was observed that increasing biodiversity would increase chance of blacklegged ticks feeding on non-competent hosts that do not have vector-borne microorganisms, thus decreasing the chance of ticks feeding on white-footed mice (*Peromyscus leucopus*) and acquiring and transmitting Lyme disease (*Borrelia burgdorferi*) in later life stages. As forest biodiversity increases, the number of host species will increase leading to decrease in vector-borne disease but also a growth in tick population (Schmidt and Ostfeld 2001; LoGiudice et al. 2003).

Microorganism diversity within the lone star tick

The association of vector-borne microorganisms and ticks has been recognized for more than a century (Kilborne and Smith 1893). Endosymbionts of lone star ticks include *Ehrlichia chaffeensis* (human monocytic ehrlichiosis) (Anderson et al. 1993; Ewing et al.

1995; Lockhart et al. 1997a, b), *Rickettsia amblyommii* (Burgdorfer et al. 1981b), *Rickettsia parkeri* (eschar maculatum agent) (Macaluso and Azad 2005; Cohen et al. 2009), *Borrelia lonestari* (Southern tick-associated rash illness) (Masters 1998), and *Francisella tularensis* (tularemia) (Hopla and Downs 1953). Each of these vector-borne diseases has the capacity to cause illness, potentially long-term health problems, and possibly mortality in humans (Paddock and Childs 2003). Non-pathogenic microorganisms considered symbionts associated with lone star tick include non-Q fever *Coxiella* spp., *Bacillaceaea*, *Micrococcaceae*, *Methylobacterium*, *Enterobacteriaceae*, *Strenotrophommonas*, and *Pseudomonas* (Heise et al. 2010). Researchers have also found evidence of a novel spotted fever group *Rickettsia* species within the tick that has not yet been identified (Heise et al. 2010).

Microorganism diversity within the lone star tick is determined by several factors including interspecific interactions within the tick, blood feeding (Heise et al 2010), habitat (Perlman et al. 2006), and mammalian reservoir competency (Niebylski et al. 1997). Co-occurring microorganisms within the tick can cause competitive exclusion, mutual interference, or mutual facilitation (Clay et al. 2006). Symbionts within the tick have the capacity to compete with the pathogenic microorganisms such that they interfere with maintenance of the pathogenic microorganism in nature (Niebylski et al. 1997; Perlaman et al. 2006). For example the symbiont, *Rickettsia peacockii*, interferes with maintenance of *Rickettsia rickettsii* (Rocky Mountain spotted fever) in Rocky Mountain wood ticks (*Dermacentor andersoni*) (Burgdorfer et al. 1981a). Blood feeding also has an impact on microbial diversity. The primary hosts of lone star ticks are white-tailed deer which are competent reservoirs for vector-borne

infectious agents (Bloemer et al. 1988; Allan et al. 2010). Lone star ticks have a seasonal host-seeking pattern where each life stage emerges from diapause or molting at different times of the year (Kollars et al. 2000). White-tailed deer are competent reservoir hosts but must become infected first (Bowman and Nuttall 2008). The adults and nymphs harboring transstadially transmitted tick-borne infectious agents from previous life stages must attach, feed, and successfully transmit the pathogen to the deer so the infectious agent has enough time to reach the critical transmission threshold (CTT) value before larval ticks emerge to feed (Eisen et al. 2012). If a host is at the proper CTT value, emerging larvae feeding on the host will become infected and be able to harbor and transmit the tick-borne infectious agent as nymphs and adults due to transstadial transmission (Bowman and Nuttall 2008). Infected ticks will feed on two different hosts as a nymph and adult, and transmit pathogens to hosts that could include humans.

The vector, reservoir, and infectious agent need to be present for ticks to become infected and transmit tick-borne infectious agent to hosts. To demonstrate effective tick-borne microorganism transmission of *E. chaffeensis*, the lone star tick (vector), white-tailed deer (reservoir), and *E. chaffeensis* (infectious agent) must be present at the same time in the forest habitat. Ticks quest during late morning and afternoon and will come in contact with mid- to large-sized mammals including white-tailed deer, a known reservoir of *E. chaffeensis* (Schulze et al. 2001a; Bowman and Nuttall 2008). For effective transmission to occur, the lone star adult or nymph must transmit *E. chaffeensis* to white-tailed deer while blood feeding to ensure the infectious agent will reach proper CTT values before naïve larvae emerge and quest (Eisen et al. 2012). *Ehrlichia chaffeensis* is

transstadially transmitted between each molt (Anderson et al. 1993; Ewing et al. 1995). Once lone star ticks acquire *E. chaffeensis*, they are able to harbor and transmit the infectious agent to subsequent hosts during the rest of their life cycle (Bowman and Nuttall 2008). Most human *E. chaffeensis* cases occur during spring and summer, May through July, when adult and nymph lone star tick populations peak (Goodman et al. 2005).

Pathogenic microorganisms associated with lone star ticks

Rickettsia spp.

Several *Rickettsia* species within the spotted fever group (SFG) are found within and transmitted by the lone star tick (Burgdorfer et al. 1981b; Goddard and Norment 1986; Walker 1998; Mixson et al. 2006a). Pathogenic *Rickettsia* species include *Rickettsia rickettsii* (Rocky Mountain spotted fever), (Maver 1911; Macaluso and Azad 2005), *Rickettsia parkeri* (Paddock et al. 2004; Macaluso and Azad 2005) and *Rickettsia amblyommii* (Burgdorfer et al. 1981b; Stromdahl et al. 2008; Jiang et al. 2010).

Lone star ticks have been implicated as vectors of *Rickettsia rickettsii* (Maver 1911, Parker et al. 1943). The ticks are naturally infected (Berrada et al. 2011) and capable of transmitting a spotted fever virus to guinea-pigs (Maver 1911). Parker et al. (1943) recovered *R. rickettsii* from 114 unfed lone star tick nymphs collected in Oklahoma. However, natural infection does not mean that the ticks are competent vectors of *R. rickettsii*. Many RMSF cases may be misdiagnosed. Burgdorfer et al. (1981b) studied 1,700 lone star ticks from Arkansas, South Carolina, and Tennessee and did not find any ticks positive for *R. rickettsii*. A study of 2,333 lone star ticks collected from

Mississippi and Kentucky along with 734 ticks from Oklahoma and Texas yielded no positive results for *R. rickettsii* also (Goddard and Norment 1986). It is believed that RMSF cases may have been caused by former endosymbionts of lone star ticks that include *R. parkeri* and *R. amblyommii* (Paddock et al. 2004; Apperson et al. 2008; Stromdahl et al. 2008; Jiang et al. 2010).

Rickettsia parkeri was first recovered in *Amblyomma maculatum* (Gulf Coast tick) in 1939 (Parker et al. 1939) and later in lone star ticks (Macaluso and Azad 2005; Cohen et al. 2009). *Rickettsia parkeri* was initially believed to be an endosymbiont of the ticks, but is now considered a human pathogen and has been associated with a case of "rickettsial pox" in a southeastern Virginia patient (Paddock et al. 2004). Symptoms of *R. parkeri* include rapid onset of fever, headache, malaise, diffuse myalgias and arthralgias, and multiple eschars that start as small erythematous papules that develop into pustules and ulcerate (Paddock et al. 2004). The rash then spreads from the flanks and trunk to face and extremities (Paddock et al. 2004).

Rickettsia amblyommii was discovered in 1974 by Burgdorfer et al. (1981b) and tentatively named WB-8-2 agent. Lone star ticks are naturally infected and *R. amblyommii* is transovarially and transstadially transmitted (Burgdorfer et al. 1981b; Macaluso and Azad 2005; Stromdahl et al. 2008). Lone star tick larvae from Arkansas, South Carolina, and Tennessee were 100% (1,320 larvae) positive for *R. amblyommii* (Burgdorfer et al. 1981b). Adult infection rate varied from 37 to 75% positive for *R. amblyommii* (Apperson et al. 2008, Jiang et al. 2010), and prevalence between male and female ticks was not significantly different (Mixson et al. 2006a).

Pathogenicity of *R. amblyommii* is unknown (Stromdahl et al. 2008). It was considered non-pathogenic (Burgdorfer et al. 1981b; Goddard and Noment 1986) but has now been associated with a mild spotted fever disease (Parola et al. 2005, Billeter et al. 2007). It is hypothesized that reported cases of RMSF may have be misdiagnosed and were caused by *R. amblyommii* (Apperson et al. 2008; Stromdahl et al. 2008; Jiang et al. 2010). Patients with *R. amblyommii* have ranged from 2 to 83 years old and symptoms included mild sudden onset, fever, thrombocytopenia, headache, and an erythematous maculopapular rash on trunk, arms, and legs (Dasch et al. 1993; Apperson et al. 2008). No patients required hospitalization.

Ehrlichia ewingii

Ehrlichia ewingii is the etiologic agent of canine granulocytic ehrlichiosis (CGE) (Stockham et al. 1985) an emerging disease in the United States (Childs and Paddock 2003). *Ehrlichia ewingii* was discovered in 1971 as a new strain of *Ehrlichia canis* effecting granulocytes that caused a mild form of canine ehrlichiosis (Ewing et al. 1971). It was finally recognized as a novel species in 1992 (Anderson et al. 1992). However, there is not much known about *E. ewingii* because it is a newly recognized pathogen, difficult to cultivate in the laboratory, and is present in only a small number of cases (Anderson et al. 1992; Paddock et al. 2001; Telford and Goethert 2008).

Canine granulocytic ehrlichiosis was originally a disease of veterinary importance in domesticated dogs (*Canis lupus familiaris*) until 1999 when the first reported human case of CGE occurred in a patient from Missouri (Stockham et al. 1985; Paddock et al. 2005; Telford and Goethert 2008). The disease is commonly diagnosed in

immunosuppressed individuals and all cases have been reported from the south-central and southeastern United States (Fishbein et al. 1994; McQuiston et al. 1999; Paddock et al. 2001). By 2002 only eight infections in humans had been reported and included patients from Missouri, Oklahoma, and Tennessee (Buller et al. 1999; Paddock et al. 2001).

The primary vector of *E. ewingii* is the lone star tick (Anziani et al. 1990; Mixson et al. 2006a; Heise et al. 2010). The ticks acquire *E. ewingii* as larvae or nymphs and transstadially transmit it between life stages (Anderson et al. 1992; Sumner et al. 2000; Wolf et al. 2000; Paddock et al. 2005). Lone star ticks collected from nine states by Mixson et al. (2006a) found the highest tick infection prevalences in New Jersey (8.2%) and North Carolina (4.9%); however, no ticks collected from midwestern sites were infected. Lone star ticks infected with *E. ewingii* have been detected from Missouri, North Carolina, New Jeresy, and Oklahoma (Murphy et al. 1998; Wolf et al. 2000; Steiert and Gilfoy 2002; Schulze et al. 2004; Mixson et al. 2006b). Sumner et al. (2000) found that 5.4% of 579 adult ticks and 0.6% of 115 nymphs from Missouri were positive for *E. ewingii*.

The primary reservoir for *E. ewingii* is the domestic dog (Ewing et al. 1971; Goldman et al. 1998; Buller et al. 1999; Goodman et al. 2003; Liddell et al. 2003). However, other wildlife species are involved in maintenance of *E. ewingii* (Childs and Paddock 2003) such as white-tailed deer (Yabsley et al. 2002; Arens et al. 2003) and possibly wild canids that include foxes and coyotes (Telford and Goethert 2008). In a study done in Boone County in central Missouri, 217 hunter-killed white-tailed deer had an infection prevalence of approximately 20% (Arens et al. 2003).

Canine granulocytic ehrlichiosis can infect both animals and humans, and causes different symptoms in each. Symptoms in domestic dogs caused by *E. ewingii* are less severe than *E. canis* (Ewing et al. 1971; Anderson et al. 1992). Symptoms include polyarthritis, stiffness, stilted gait, mild to moderately severe thrombocytopenia, anemia, neutropenia, head tilt, tremors, fever (Stockham et al. 1985; Bellah et al. 1986; Anziani et al. 1990; Stockham et al. 1990; Goodman et al. 2003; Paddock et al. 2005), petechial hemorrhages, vomiting, diarrhea, and meningitis (Carrilo and Green 1978; Anziani et al. 1990; Murphy et al. 1998; Goodman et al. 2003). Human cases have symptoms that are non-specific and indistinguishable from *E. chaffeensis* in humans (Buller et al. 1999). CGE is a milder illness than HME (Paddock and Childs 2003) in people with no underlying immune conditions and few seek medical attention (Paddock et al. 2005). Symptoms include febrile syndrome, headache, discomfort, and muscle pain (Paddock et al. 2005). No patients to date have exhibited multi-system organ failure or deaths (Buller et al. 1999; Paddock et al. 2001).

Ehrlichia chaffeensis

Ehrlichia chaffeensis is the causative agent of Human Monocytic Ehrlichiosis (HME) which is an emerging disease in humans. As of 2013, there are currently 1,025 presumptive cases of HME reported in the United States (CDC 2013). Historically, most cases were reported from the south-central and southeastern United States, but now HME has been reported from 30 states in the south and central Midwest, southeast, and mid-Atlantic regions (Fishbien et al. 1994; Standaert et al. 1995; McQuiston et al. 1999; Rikihisa 1999). The states with the highest reported average incidence rates are Arkansas, North Carolina, Missouri, and Oklahoma (Rikihisa 1999). Even with a high number of

cases being reported, the fatality rate was between 2-5% and mortality was more probable among patients that were 60 years or older, delayed treatment eight or more days after onset of symptoms, or were immunocompromised (Fishbien et al. 1994; Dumler and Bakken1995; McQuiston 1999; Rikihisa 1999; Paddock and Childs 2003). Only ten deaths have been attributed to HME in the United States (Paddock et al. 1997).

The main vector of *E. chaffeensis* is the lone star tick and can only be transstadially transmitted (Anderson et al. 1993; Ewing et al. 1995; Lockhart et al. 1997b). Sixty-eight percent of human cases develop in May, June, or July which coincides with the highest seasonal activity of the lone star tick (Fishbien et al. 1994; Rikihisa 1999), and 70% of patients reported having a tick bite or attached tick (Fishbien et al. 1994; Rikihisa 1999).

Symptoms of HME are nonspecific and range from being asymptomatic to a mild or severe disease (Dumler and Bakken 1995; McQuiston et al. 1999). Most human patients with *E. chaffeensis* are asymptomatic (Fishbien et al. 1994; Dumler and Bakken 1995). Early symptoms of HME develop nine days after a tick bite (Fishbien et al. 1994; Rikihisa 1999; Paddock and Childs 2003). Early symptoms include fever, headache, joint and muscle pain, nausea, and decreasing leukocyte and platelet counts (Fishbien et al. 1994; Rikihisa 1999; Paddock and Childs 2003). Rashes develop in 36% of patients; however, they are infrequent and are variable in character, occurrence, and location on the body (Fishbien et al. 1994; Dumler and Bakken 1995; Rikihisa 1999). If left untreated, HME symptoms can develop into upper respiratory infections, sepsis, influenza, pharyngitis, gastroenteritis or prostatitis (Fishbien et al. 1994, Rikihisa 1999).

Severe reactions to HME include multi-system failure, cardiomegaly, seizures, coma, and death (Fishbien et al 1994; Dumler and Bakken 1995; Paddock and Childs 2003).

Patients seeking medical assistance with early symptoms receive proper diagnosis are treated with doxycycline or tetracycline, and are able to leave the hospital (Fishbien et al. 1994; Rikihisa 1999). However, approximately 61% of patients are hospitalized (Fishbien et al. 1994; Rikihisa 1999). Patients could also be misdiagnosed with *Rickettsial* infections including Rocky Mountain spotted fever, ehrlichiosis, or another *Rickettsial* illness of unspecified cause (Fishbien et al. 1994; Rikihisa 1999). Misdiagnosis and delay of treatment could lead to clinical complications, severe reactions, and secondary bacterial or fungal infections in patients (Fishbien et al 1994; Dumler & Bakken 1995; Rikihisa 1999; Paddock and Childs 2003).

Host reservoirs for *E. chaffeensis* include the white-tailed deer (*Odocoileus virginianus*) (Ewing et al. 1995; Lockhart et al. 1997a; Irving et al. 2000; Arens et al. 2003), coyotes (*Canis latrans*) (Kocan et al. 2000), red foxes (*Vulpes vulpes*) (Davidson et al. 1999), gray foxes (*Urocyon cinereoargenteus*) (Davidson et al. 1999), domestic goats (*Capra aegagrus hircus*) (Dugan et al. 2000), raccoons (*Procyon lotor*) (Comer et al. 2000), opossums (*Didelphis maruspialis*) (Lockhart et al. 1997b), and domestic dogs (*Canis lupus familiaris*) (Dawson et al. 1996; Murphy et al. 1998; Kordick et al. 1999). However, the main reservoir is the white-tailed deer and has been shown to be experimentally and naturally infected with *E. chaffeensis* (Lockhart et al. 1997a; Irving et al. 2000; Arens et al. 2003).

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CHAPTER 2: LONE STAR TICK COLLECTIONS

ABSTRACT

From May through August 2012 field collections were conducted using carbon dioxide traps in six sites in southeast Nebraska to determine *Amblyomma americanum* (L.) (lone star tick) establishment, compare tick populations among sites, and to gather large samples of lone star ticks to determine pathogen prevalence. Two species of ticks were collected: lone star ticks and American dog ticks (*Dermacentor variabilis* (Say)). A total of 747 adult (395 females, 352 males), 3076 nymphal, and 1289 larval lone star ticks were collected. Adults (403), nymphs (1469), and larvae (945) were most abundant at Table Rock Wildlife Management Area. Total number of *D. variabilis* collected at all six sites was 164 adults (83 females, 81 males). Data indicated that lone star ticks are established in southeast Nebraska, and that there are significant differences between adult and nymph lone star ticks between sites, and differences between visits for adult lone star ticks. Table Rock Wildlife Management Area had the highest number of lone star ticks for all three life stages and the highest rate of lone star ticks per trap.

INTRODUCTION

The lone star tick, *Amblyomma americanum* (L.) is an aggressive ectoparasite with a three-host life cycle capable of being a competent vector of both human and animal pathogens. Lone star ticks were originally found in the southeastern and south-central United States, but in the past 20 years, its geographic range has expanded northeast to Maine and New York and into the Midwest (Childs and Paddock 2003; Mixson et al. 2006a; Goddard and Verela-Stokes 2009; Heise et al. 2010). The tick has

recently become established in Nebraska (Cortinas and Spomer 2013). Retrospective analysis of human case reports demonstrate that the tick appeared in southeastern Nebraska during the late 1980s and has continued to spread in a northerly direction within Nebraska (Cortinas and Spomer 2013).

Lone star ticks are now becoming established in Nebraska in a corridor between Omaha and Lincoln where 56% of the Nebraska human population lives (US Census Bureau 2011), thus the chance of human and tick contact increases (Cortinas and Spomer 2013). Lone star ticks have been shown to be competent vector of vector-borne pathogens including *Ehrlichia chaffeensis* (human monocytic ehrlichiosis) (Anderson et al. 1993; Ewing et al. 1995; Lockhart et al. 1997a,b), *E. ewingii* (canine granulocytic ehrlichiosis) (Anziani et al. 1990; Mixson et al. 2006b; Heise et al. 2010), *Rickettsia amblyommii* (Burgdorfer et al. 1981), *R. parkeri* (eschar maculatum agent) (Macaluso and Azad 2005; Cohen et al. 2009), *Borrelia lonestari* (Southern tick-associated rash illness) (Masters 1998), and *Francisella tularensis* (tularemia) (Hopla and Downs 1953). In 2010 the incidence of ehrlichiosis cases in Nebraska ranged from 1 to 3.3 cases per million persons (CDC 2013) indicating that either Nebraska citizens are acquiring *Ehrlichia* pathogens in Nebraska or have been diagnosed in Nebraska after traveling to areas where lone star ticks were prevalent and capable of transmitting *Ehrlichia* pathogens. There is a lack of research on lone star ticks in Nebraska pertaining to the prevalence of lone star tick pathogens and if Nebraska's habitats encompass the proper hosts and reservoirs required for effective vector-borne disease transmission. The objectives of this study are to 1) determine if lone star ticks are established in various sites in southeast Nebraska, 2) to

compare tick population density among the sites, and 3) to gather large samples of lone star ticks to determine pathogen prevalence.

MATERIALS AND METHODS

Study Sites

Lone star ticks were collected from May through August 2012, when adult and nymphal stages are actively questing (Lancaster 1955; Fleetwood et al. 1984; Kollars et al. 2000; Goodman et al. 2005). Six sites were selected in southeastern Nebraska: Indian Cave State Park (SP), Kinter's Ford Wildlife Management Area (WMA), Prairie Pines, Schramm Park State Recreation Area (SRA), Table Rock WMA, and Wilderness Park (Figure 1). Each site was selected based on lone star tick presence and high densities ascertained from a previous study of lone star ticks in Nebraska (Cortinas and Spomer 2013). Collection sites were located within and near forested areas that are favorable habitats for lone star ticks and their hosts (Sonenshine 1993; Kollars et al. 2000; Paddock and Yabsley 2007).

Indian Cave State Park (40.26, -95.57) is a popular recreation area used by people for camping, hiking, backpacking, horseback riding, picnicking, and nature and wilderness activities. The park is located in Richardson Co. and Nemaha Co., Nebraska along the Missouri River. The park consists of 3,052 acres of forest, wetland and marsh areas. Eastern black oak (*Quercus velutina*), white oak (*Quercus alba*), blackjack oak (*Quercus marilandica*), Chinquapin oak (*Quercus muehlenbergii*), bur oak (*Quercus macrocarpa*), shellbark hickory (*Carya laciniosa*), bitternut hickory (*Carya cordiformis*), sycamore (*Platanus occidentalis*), black cherry (*Prunus serotina*), redbud (*Cercis canadensis*), pawpaw (*Asimina triloba*), bladdernut (*Staphylea trifolia*), cottonwood

(*Populus deltoids*), common hackberry (*Celtis occidentalis*) and highbush blackberry (*Rubus argutus*) are the dominant vegetation (Farrar 2013; National Audubon Society 2013; NGPC 2013a). Wildlife and domestic animals inhabiting and surrounding the park include horses (*Equus ferus caballus*), cattle (*Bos primigenius*), white-tailed deer (*Odocoileus virginianus*), wild turkeys (*Meleagris gallopavo*), fox squirrels (*Sciurus niger*), song birds (Farrar 2013; NGPC 2013a), and white-footed mice (*Peromyscus leucopus*) (Hotaling unpublished).

Kinter's Ford WMA (40.05, -95.99) is located in Richardson Co., Nebraska approximately seven miles south of Humboldt, Nebraska. The area includes a natural bedrock ford and oxbow lake along the south fork of the Nemaha River and is comprised of approximately 196 acres of open grass, cropland, and mixed deciduous forests used for hiking, hunting, and fishing. Vegetation includes eastern black walnut (*Juglans nigra*), common hackberry, American elm (*Ulmus americana*), burr oak (*Quercus macrocarpa*), elderberry (*Sambucus canadensis*), highbush blackberry, honey locust (*Gleditsia triacanthos*), silver maple (*Acer saccharinum*), shellbark hickory, bitternut hickory, sumac, Curly dock (*Rumex crispus*), milkweed (*Asclepias syriaca*), ragweed, cottonwood, American pokeweed (*Phytolacca americana*), common nettles (*Urtica dioica*), and poison ivy (*Toxicodendron radicans*) (NGPC 1991). Wildlife inhabiting the WMA included fox squirrel (*Sciurus niger*), white-tailed deer, bobwhite quail (*Colinus virginianus*), and the common pheasant (*Phasianus colchicus*).

Prairie Pines (40.85, -96.57) is located in Lancaster Co. near Lincoln, Nebraska. The site is affiliated with University of Nebraska-Lincoln School of Natural Resources and Nebraska Statewide Arboretum as an environmental refuge, arboretum, horticultural

study area (UNL 2013), and research area used by the University of Nebraska-Lincoln. Prairie Pines consists of 145 acres that has been transformed from farmland and Christmas tree plantation to woodland and grassland through succession (UNL 2013). Vegetation include fir, spruce, juniper, and pine trees, eastern red cedar (*Juniperus virginiana*), pawpaw, American sycamore (*Platanus occidentalis*), hackberry, elm, mulberry, bitternut hickory, chestnut, oak, sweet birch (*Betula lenta*), American basswood (*Tilia americana*), cottonwood, quaking aspen (*Populus tremuloides*), chokecherry (*Prunus virginiana*), black cherry, honey locust, black locust (*Robinia pseudoacacia*), dogwood, maple, boxelder (*Acer negundo*), sumac, poison ivy, white ash (*Fraxinus americana*), and stinging nettles (*Urtica dioica*) (Bagley 2010). Animals found at the site include wild turkey, blue jay (*Cyanocitta cristata*), American robin (*Turdus migratorius*), white-tailed deer, Eastern cottontail (*Sylvilagus floridanus*), coyote (*Canis latrans*), fox squirrel (*Sciurus niger*), Virginia opossum (*Didelphis virginiana*), raccoon (*Procyon lotor*), skunk, eastern mole (*Scalopus aquaticus*) (Loomis 2010), white-footed mouse (Peromyscus leucopus), house mouse (*Mus musculus*), deer mouse (*Peromyscus maniculatus*), western harvest mouse (*Reithrodontomys megalotis*), meadow vole (*Microtus pennsylvanicus*), North American least shrew (*Cryptotis parva*) (Hotaling unpublished), and domestic cat (*Felis catus*).

Schramm SRA (41.02, -96.25) is used for hiking, picnicking, and camping. It is located in Sarpy Co., Nebraska. The park encompasses 331 acres of deciduous forests that consist of oak trees, nettles, Virginia creeper (*Parthenocissus quinquefolia*), black raspberries (*Rubus occidentalis*), eastern red cedar, fir, poison ivy, pin oak (*Quercus palustris*), and ash. Wildlife of Schramm includes fox squirrel (*Sciurus niger*), white-

footed mouse (*Peromyscus leucopus*), deer mouse (*Peromyscus maniculatus*), and house mouse (*Mus musculus*) (Hotaling unpublished).

Table Rock WMA (40.18; -96.06) is used for hiking, hunting, and fishing. The area is located in Pawnee Co., Nebraska and consists of 400 acres of woodland, grassland, and cropland. Vegetation includes eastern redbud (*Cercis canadensis*), eastern red cedar, bur oak, red oak (*Quercus rubra*), poison ivy, Virginia creeper, black walnut, hickory, shagwood, dogwood, prickly ash (*Zanthoxylum* spp.), sumac, elm, parsnip, wild currant (*Mahonia trifoliolata*), hackberry, buckbrush, black raspberries, honey locust, black locust, golden rod, tick clover (*Desmodium illinoense*). Wildlife includes quail, doves, rabbits, squirrels, pheasants, and white-tailed deer (NGPC 1991).

Wilderness Park (40.75, -96.72) is an urban park within the city of Lincoln located in Lancaster Co., Nebraska. The park is located in the Salt Creek flood plain and is used for hiking, off-road cycling, and horseback riding (Lincoln Parks and Recreation 2013). The park consists of 1,472 acres with woodland and grasslands. Vegetation includes hackberry, honey locust, black walnut, buckbrush (*Symphoricarpos* spp.), poison ivy, mulberry, dandelions, brome grass, green ash (*Fraxinus pennsylvanica*), bur oak, red oak, locust, eastern red cedar, silver maple, wild current, snake root (*Ageratina altissima*), honey suckle, and black raspberry. Domestic and wildlife include white-tailed deer, wild turkey, raccoons, robins, song birds, frogs, and horses (NGPC 2013b).

Tick Collection and Preservation

Collection sites were visited three to six times every other week from May through August 2012 to gather a large, representative sample of lone star ticks to determine lone star tick establishment and seasonal activity. Questing ticks were

collected by carbon dioxide traps constructed of a cardboard piece (2 ft x 1.5 ft) with masking tape along the edges so most of the tape was sticking out (Figure 2). The traps were placed with the adhesive side of the tape facing up, and dry ice (~0.5 kg) was placed in the middle of the trap and allowed to sublimate. Approximatly 20 traps were placed on the ground, approximately 20 m apart, in forested areas of the study site along hiking and deer trails. Trap placement was determined based on lone star tick questing behavior and included young, second growth deciduous habitats with dense under story vegetation and mature forests (Sonenshine 1993; Paddock and Yabsley 2007). Traps were left for two hours during which time questing ticks were attracted to the carbon dioxide and became trapped on the adhesive side of the masking tape. Traps were then collected by folding the masking tape over onto the cardboard and transported back to lab.

In the laboratory, ticks were removed from the masking tape with fine forceps and 100% ethyl alcohol; then identified to life stage, sex, and species (Cooley and Kohls 1944; Keirans and Litwak 1989; Keirans and Durden 1998). Ticks were then surface sterilized by placing individuals into a 1.5 mL microcentrifuge tube containing 100% ethyl alcohol and vortexed for 5 min with a Vortex Genie 2 (DAIGGER, Vernon Hills, IL). Ticks were then transferred to a 1.5 mL microcentrifuge tube containing pure water from Nanopure Barnstead (Thermo Scientific, Waltham, MA) using fine forceps that were surface sterilized with BACTI-CINERATOR*IV (McCormick Scientific) and vortexed for 30 s. Individual ticks were placed into individually labeled vials containing 95% ethyl alcohol and stored in a freezer at -20^{o}C.

Lone star tick establishment at a site was determined if requirements by Dennis et al. (1998) were met wherein at least 6 ticks or two of the three tick life stages (larvae,

nymphs, adults) had been identified in a single collection period. Six ticks represent a critical mass and more than one stage represents a reproductive population.

Voucher Specimens

Voucher specimens were deposited at the Harold W. Manter Laboratory of Parasitology of the University of Nebraska State Museum, University of Nebraska-Lincoln.

Statistical Analysis

Data were analyzed using GLIMMIX with a negative binomial distribution using SAS software (SAS Institute 2001) to evaluate the differences in populations between sites based on life stage (adult and nymph). The significance level was $\alpha=0.05$ used to test the null hypothesis that tick populations were the same for all locations, and tick populations were the same for all visits. Analysis was adjusted for unequal visits between sites. Kinter's Ford WMA and Table Rock WMA were visited three times. The three visits for Kinter's Ford WMA and Table Rock WMA were aligned with visits 1, 3, and 6 while visits 2, 4, and 5 were missing data. Rates were determined for the number of ticks per trap, while relative rate was calculated by taking the exponential of the estimate between two different locations to determine how many more ticks were collected at one location compared to another.

RESULTS

Two species of ticks were collected: *Amblyomma americanum* (L.) and *Dermacentor variabilis* (Say) (American dog tick). The total number of lone star ticks collected at all six sites was 747 adults (395 females, 352 males), 3076 nymphs, and 1289 larvae after all sites were visited a total of 30 times. Table Rock WMA had the greatest

number of adults (403), nymphs (1469), and larvae (945). Total number of *D. variabilis* collected at all six sites was 164 adults (83 females, 81 males). However, visits and number of traps placed at each site were not equal so total tick populations were represented as the average number of ticks per trap.

Only adult *D. variabilis* ticks were collected during this study. The collection sites with the highest average of *D. variabilis* were Indian Cave SP (2.3 ticks/trap) and Table Rock WMA (2.2 ticks/trap), followed by Kinter's Ford WMA (1.6 ticks/trap), Prairie Pines (1.2 ticks/trap), Wilderness Park (1.1 ticks/trap), and Schramm SRA (0.1 ticks/ trap) (Figure 3). American dog ticks were collected from May through July. Adult *D. variabilis* reached peak activity in late June and early July and were collected from all six collection sites.

Sites with the highest average number of lone star ticks per trap were Table Rock WMA (178.2 ticks/trap), Kinter's Ford WMA (53.51 ticks/trap), and Prairie Pines (25.0 ticks/trap) (Figure 5). Table Rock WMA and Kinter's Ford WMA consistently had the highest number of ticks with all three life stages: adult (36.3, 12.5 ticks/trap), nymph (104.1 and 26.9 ticks/trap), and larva (37.8, 14.1 ticks/trap) respectively (Figure 6, 8, 9).

The greatest number of adult lone star ticks were collected at Table Rock WMA (36.3 ticks/trap) and Kinter's Ford WMA (12.5 ticks/trap), followed by Indian Cave State Park (4.4 ticks/trap), Schramm SP (2.2 ticks/trap), Prairie Pines (1.1 ticks/trap). Wilderness Park had the lowest average number of adult ticks (0.78) (Figure 6). The number of males and females were almost equal for each site; however, slightly more females were collected than males at five of six sites (Figure 7). The only site that had more males than females was at Table Rock WMA (Figure 7).

Adult tick collections were significantly different between locations ($P < 0.0001$) and between visits ($P < 0.0001$). Data for rates are presented in Table 1 and 2, and data for relative rate comparison are presented in Table 3 and 4. Table Rock WMA had the highest rate of ticks collected with 3.26 ticks collected. Table Rock WMA had a relative rate of 3.84 times the number of ticks collected than from Kinter's Ford WMA and a relative rate of 64.97 times the number of ticks collected than from Wilderness Park (Table 3). After Table Rock WMA, the rates for the other sites were as follows: Kinter's Ford WMA (0.85), Indian Cave SP (0.30), Schramm SRA (0.20), Prairie Pines (0.07), and Wilderness Park (0.05) (Table 1; Figure 16). Table Rock WMA and Kinter's Ford WMA rate of ticks collected are significantly different than other collection sites, while Indian Cave SP and Schramm SP, and Prairie Pines and Wilderness Park had a similar rate of ticks collected (Table 3). The rate for adult tick collections between visits decreased from visit 1 (2.17) to visit 5 (0.023) and then there was an increase in adult ticks collected at visit 6 (0.034) (Table 2). Seasonality was accounted for by comparing visits, and the rate of adult ticks collected over visits was almost linear and decreased from visit 1 to 5 then increased slightly at visit 6 (Figure 17).

Lone star nymphal stage was highest at Table Rock WMA with 104.1 ticks per trap. The next two sites that had high numbers of nymphs were Kinter's Ford WMA (26.9 ticks/trap) and Prairie Pines (23.2 ticks/trap), followed by Wilderness Park (19.7 ticks/trap), Indian Cave SP (9.1 ticks/trap), and Schramm SP (6.9 ticks/trap) (Figure 8). The greatest number of larval lone star ticks were collected at Table Rock WMA (37.8 ticks/trap), followed by Kinter's Ford (14.1 ticks/trap), Wilderness Park (2.2 ticks/trap),

and Prairie Pines (0.7 ticks/trap) (Figure 9). The sites with the lowest number of larval ticks were Indian Cave SP and Schramm SP (0.05 ticks/trap) (Figure 9).

Populations of nymphs showed a significant difference between locations (P: 0.0173), however there was not a significant difference between visits over all sites (P: 0.3031). Data for rates are presented in Table 5, and data for relative rate comparison are presented in Table 6. Table Rock WMA had the highest rate of nymphs collected with 45.16 ticks and had a relative rate of 3.87 times the number of nymphs collected from Kinter's Ford WMA and a relative rate of 32.49 times the number of nymphs collected at Wilderness Park (Table 5, 6). After Table Rock WMA, the rates for the other sites were as follows: Kinter's Ford WMA (11.67), Indian Cave SP (2.53), Schramm SP (1.71), Prairie Pines (1.41), and Wilderness Park (1.39) (Table 5; Figure 18). Table Rock WMA and Kinter's Ford WMA had similar rates for adult ticks collected compared to other sites.

The presence of all life stages of lone star ticks at each site indicate that these ticks are established in southeast Nebraska using Dennis et al. (1998) criteria. Larvae were collected beginning late July and August. Nymphs were collected from May through August with population peaks in May or June and then in August. Adult ticks were collected beginning in May or June then decreased throughout summer into August with population peaks in May through July depending on site. Seasonal activity for each life stage was different for each collection site. Indian Cave SP (Figure 10) had an adult peak in population of 1.4 ticks per trap on 11 June, and then steadily declined into July until no adults were collected in August. Nymphs had a bell-shaped curve with a peak of

2.6 ticks per trap on 25 June then declined into August. As both adult and nymph life stages decreased, larvae appeared and were collected on 16 August.

Kinter's Ford WMA (Figure 11) had an adult peak in population on 1 June (11.8 ticks/trap) then steadily declined into July and few adult ticks were collected in August (0.05 ticks/trap). Nymphs had a peak in population on 1 June (15.9 ticks/trap) and steadily decreased throughout the summer. Larvae were collected beginning 14 July and peaked on 17 August (13.7 ticks/trap).

Prairie Pines (Figure 12) had two adult peaks in population, 7 June (0.6 ticks/trap) and 9 July (0.3 ticks/trap), then steadily declined into July until no adults were collected in August. Nymphs had two peaks in population, 13 June (0.7 ticks/trap) and 30 July (16.62 ticks/trap). Larvae were collected beginning 30 July and increased into August.

Schramm SRA (Figure 13) also had two adult peaks in population occurring in early summer on 6 June (0.8 ticks/trap) and in late summer on 23 August (0.05 ticks/trap). There were also two peaks in population for nymphs, 8 May (2.2 ticks/trap) and 23 August (0.4 ticks/trap). Larvae were collected beginning 23 August (0.05 ticks/trap).

Table Rock WMA (Figure 14) had an adult peak in population on 10 May (27.3 ticks/trap) then steadily declined into July. Nymphs had a peak in population on 23 June (50.93 ticks/trap). Larvae were collected beginning 25 July (37.8 ticks/trap).

Wilderness Park (Figure 15) had an adult peak in population on 9 July (0.2 ticks/trap) then steadily declined until 28 August (0.05 ticks/trap). Nymphs had two peaks in population, 19 June (0.5 ticks/trap) and 28 August (11.0 ticks/trap). Larvae were collected beginning 15 August (1.4 ticks/trap) but decreased into late August.

DISCUSSION

Although two species of ticks were encountered in this study, *Dermacentor variabilis* is the most common and widespread tick in Nebraska (Cortinas and Spomer 2013). However, *A. americanum* was collected more frequently than *D. variabilis* in all three life stages and at all collection sites. Tick collections for both species may have been different due to the trapping technique and habitats chosen for trap placement. The study used carbon dioxide traps which are a more efficient trapping technique for lone star ticks because of their active questing behavior (Sonenshine 1993). American dog ticks have a different questing technique characterized by "sitting and waiting" and do not respond to carbon dioxide as well as lone star ticks (Sonenshine 1993). The two tick species also differ in habitats. Lone star ticks are generally found in young, second growth deciduous habitats with dense under story vegetation and mature forests (Sonenshine 1993, Paddock and Yabsley 2007). In southeast Nebraska lone star ticks have been associated with oak/hickory deciduous forests (Cortinas and Spomer 2013). While American dog tick is associated with grassy meadows, young second growth forests especially in mesic deciduous forest along edges of roads, trails, and clearings around homes and barns (Sonenshine 1993). In this study, lone star ticks were collected more frequently than American dog ticks except at Prairie Pines and Wilderness Park (Figure 4).

Although seasonal activity of the lone star ticks was addressed, research was only performed over one season. To have proper seasonal activity data, research should be conducted over multiple seasons. Larvae were collected beginning in late July and August. This coincides with known seasonal activity (Kollars et al. 2000) and data

collected by Cortinas and Spomer (2013) who collected larvae in late summer and had peak numbers in August. Nymphs were collected from May through August with population peaks in May or June and then again in August. Nymphs have been collected in Nebraska and elsewhere from March until September reaching peak populations in May and August (Kollars et al. 2000; Cortinas and Spomer 2013). Adult ticks were collected beginning in May or June then decreased throughout the summer into August with population peaks from May through July depending on site. This coincides with known seasonal activity (Kollars et al. 2000) and data collected by Cortinas and Spomer (2013) for adult ticks that are present from April until August and are most abundant in May and early summer, July.

Seasonal activity for each collection site may have varied from other studies due to latitude, temperature, and humidity. The three northern sites (Prairie Pines, Schramm SRA, and Wilderness Park) and three southern sites (Indian Cave SP, Kinter's Ford WMA, and Table Rock WMA) differed in latitude. For the southern sites, seasonal temperatures may have increased earlier in the season, thus lone star ticks would emerge earlier and begin questing earlier than at northern sites. Temperature and humidity has an impact on tick questing behavior. Lone star tick questing was not only affected by moisture availability but also temperature because lone star ticks are small and have a greater surface area to volume ratio and are therefore more susceptible to desiccation. Lone star ticks quest most actively in late morning and early evening during periods of higher temperatures above 4.4^{o}C and lower humidity (Sonenshine 1993; Schulze et al. 2001). Temperatures were very high during the summer of 2012 and got hot quickly. At Wilderness Park and Indian Cave SP when collections started in May, temperatures were

25.6°C and 27.2°C (respectively); as summer continued temperature highs in July were in the upper 32.2°C and lower 37.8°C; and temperatures in August ranged from 34.4°C and 36.1°C for Wilderness Park and 26.7°C for Indian Cave State Park (Figure 19 and 20). At Wilderness Park, there was a decrease in adult and nymphs collected during July when temperatures were highest, but high numbers of nymphs and larvae were collected in August when temperatures started to decrease. Indian Cave SP had nymphs and larvae collected in August when temperatures started to decline. Possibly, lone star ticks might have been present during high temperatures, but waited until lower temperatures and higher humidity to quest for hosts.

The summer of 2012 was unusual in that high temperatures appeared much earlier in the year than normal. Peaks for adults and nymphs may have been missed due to a late start in May, and because of drought conditions experienced that year. Southeast Nebraska went from "abnormally dry" in late May when dryness began to slow plant growth and soil moisture was at 21-30% to "extreme drought" conditions in late August that included major crop/pasture losses, widespread water shortage, and soil moisture at 3-5% (U.S. Drought Monitor Data Archive 2013). The drought might not have impacted the lone star tick populations, but would have impacted tick questing behavior and number of ticks collected (Jones and Kitron 2000). Drought conditions were evident by drying of creek beds, drying of ground and extreme cracking, wilting plants, premature tree defoliation, and minimal precipitation. Moisture at ground level is the most important factor in long-tern survivorship rather than questing levels because the lone star ticks need to acquire water from the unsaturated air to survive (Yoder and Speilman 1992). Lone star tick nymphs greatly increased in late July and August at Prairie Pines and

Wilderness Park. At each site low numbers of nymphs were collected throughout the summer until after late July and mid-August, respectively. At Indian Cave SP, high numbers of lone star ticks were found in a small area where a creek continued to provide moisture and vegetation during the drought, potentially serving as a focal point for host-parasite interactions. While at Trail 7 along the bluff the ground was dry, vegetation was wilting, and not as many ticks were collected.

The objectives of this study were to 1) determine if lone star ticks are established in various sites in southeast Nebraska, 2) to compare tick population density among the sites, and 3) to gather large samples of lone star ticks for polymerase chain reaction (PCR) analysis for prevalence of pathogenic microorganisms. Objective 1 was achieved, and it was determined that lone star ticks are established at each site in southeast Nebraska using criteria in Dennis et al. (1998). The second objective of this research was to gather a large, representative sample of lone star ticks from six different sites in southeastern Nebraska and to compare tick populations of southeastern Nebraska was achieved. If this project was to be expanded on in the future, I would recommend going to all of the collection sites an equal number of times, start collecting from early March into September, and placing more CO_2 traps at each location. The high density of lone star ticks collected during this study, especially in sites in the corridor between Lincoln and Omaha where 56% of the human population is located, could mean that Nebraska citizens will have higher chance of coming into contact with infected ticks. The infected ticks could be infected with vector-borne pathogens including *Ehrlichia chaffeensis* (Anderson et al. 1993; Ewing et al. 1995; Lockhart et al. 1997a, b), *Ehrlichia ewingii* (Anziani et al. 1990; Mixson et al. 2006b; Heise et al. 2010), *Rickettsia amblyommii*

(Burgdorfer et al. 1981; Apperson et al. 2008), *Rickettsia parkeri* (Macaluso and Azad 2005; Cohen et al. 2009), *Borrelia lonestari* (Masters and Girardeau 1998), and *Francisella tularensis* (Hopla and Downs 1953). Lone star ticks from this study were analyzed for prevalence of *Ehrlichia* and *Rickettsia* pathogens in a later study.

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TABLES

Table 2.1: Rate of adult lone star ticks collected by location, 2012.

Location	Mean	Std Err	DF	Alpha	Lower Mean	Upper Mean
Table Rock WMA	3.262	1.090	19	0.05	1.6206	6.567
Kinter's Ford WMA	0.849	0.310	19	0.05	0.395	1.824
Indian Cave SP	0.293	0.0826	19	0.05	0.162	0.528
Schramm SRA	0.196	0.0621	19	0.05	0.101	0.381
Prairie Pines	0.0706	0.0249	19	0.05	0.0339	0.148
Wilderness Park	0.0502	0.0196	19	0.05	0.0222	0.114

Table 2.2: Rate of adult lone star ticks collected per visit, 2012.

Visit	Mean	Std Error	DF	Alpha	Lower Mean	Upper Mean
1	2.173	0.447	19	0.05	1.412	3.342
2	1.192	0.336	19	0.05	0.661	2.153
3	0.876	0.190	19	0.05	0.556	1.379
4	0.322	0.120	19	0.05	0.148	0.702
6	0.0338	0.0143	19	0.05	0.0139	0.0818
5	0.0229	0.0237	19	0.05	0.00261	0.200

Table 2.3: Relative rates of adult lone star ticks collected by location, 2012. For the labels "I" is Indian Cave SP, "K" is Kinter's Ford WMA, "P" is Prairie Pines, "S" is Schramm SRA, "T" is Table Rock WMA, and "W" is Wilderness Park.

Label	Est	Std Err	DF	T value	Prob	Alpha	Lower	Upper	1 vs 2	2 vs. 1
I vs K	-1.065	0.405	19	-2.630	0.017	0.05	-1.913	-0.218	0.345	2.901
I vs P	1.420	0.383	19	3.711	0.002	0.05	0.619	2.222	4.139	0.242
I vs S	0.398	0.354	19	1.123	0.275	0.05	-0.344	1.140	1.489	0.672
I vs T	-2.412	0.382	19	-6.306	4.7E-06	0.05	-3.212	-1.611	0.088	11.152
I vs W	1.762	0.417	19	4.230	0.001	0.05	0.890	2.634	5.827	0.172
K vs P	2.486	0.434	19	5.728	1.6E-05	0.05	1.577	3.394	12.007	0.0832
K vs S	1.463	0.437	19	3.349	0.003	0.05	0.549	2.377	4.319	0.231
K vs T	-1.346	0.391	19	-3.441	0.003	0.05	-2.165	-0.527	0.260	3.844
K vs W	2.827	0.468	19	6.039	8.3E-06	0.05	1.848	3.807	16.903	0.059
P vs S	-1.022	0.410	19	-2.494	0.022	0.05	-1.881	-0.164	0.360	2.781
P vs T	-3.832	0.421	19	-9.099	2.4E-08	0.05	-4.714	-2.951	0.022	46.16
P vs W	0.342	0.460	19	0.744	0.466	0.05	-0.620	1.304	1.408	0.710
S vs T	-2.810	0.414	19	-6.787	1.8E-06	0.05	-3.676	-1.943	0.060	16.603
S vs W	1.364	0.443	19	3.082	0.006	0.05	0.4377	2.291	3.914	0.256
T vs W	4.174	0.455	19	9.169	2.1E-08	0.05	3.221	5.127	64.98	0.015

Table 2.4: Relative rates of adult lone star ticks collected per visit, 2012.

Label	Est	Std Err	DF	t-value	Probt	Alpha	Lower	Upper	1 vs 2	2 vs. 1
1 vs 2	0.599	0.368	19	1.632	0.119	0.05	-0.169	1.369	1.822	0.549
1 vs 3	0.908	0.305	19	2.977	0.008	0.05	0.270	1.546	2.479	0.403
1 vs 4	1.909	0.442	19	4.316	0.0004	0.05	0.983	2.834	6.743	0.148
1 vs 5	4.554	1.063	19	4.284	0.0004	0.05	2.329	6.778	94.970	0.011
1 vs 6	4.164	0.455	19	9.152	2.2E-08	0.05	3.212	5.117	64.360	0.016
2 vs 3	0.308	0.349	19	0.883	0.388	0.05	-0.422	1.038	1.361	0.735
2 vs 4	1.309	0.443	19	2.957	0.0081	0.05	0.382	2.235	3.701	0.270
2 vs 5	3.954	1.062	19	3.722	0.0014	0.05	1.730	6.177	52.125	0.019
2 vs 6	3.565	0.529	19	6.736	1.9E-06	0.05	2.457	4.672	35.325	0.028
3 vs 4	1.001	0.424	19	2.361	0.0291	0.05	0.113	1.888	2.720	0.368
3 vs 5	3.646	1.055	19	3.455	0.0027	0.05	1.437	5.854	38.303	0.026
3 vs 6	3.256	0.471	19	6.908	1.3E-06	0.05	2.270	4.243	25.958	0.039
4 vs 5	2.645	1.089	19	2.429	0.0252	0.05	0.366	4.924	14.083	0.071
4 vs 6	2.256	0.582	19	3.878	0.0010	0.05	1.038	3.474	9.544	0.105
5 vs 6	-0.389	1.128	19	-0.3449	0.734	0.05	-2.749	1.972	0.678	1.476

Table 2.5: Rate of nymph lone star ticks collected by location, 2012.

Location	Mean	Std Err	DF	Alpha	Lower Mean	Upper Mean
Table Rock SWMA	45.16	34.002	19	0.05	9.340	218.354
Kinters Ford WMA	11.67	8.901	19	0.05	2.365	57.597
Indian Cave SP	2.53	1.396	19	0.05	0.797	8.027
Schramm SRA	1.71	0.909	19	0.05	0.564	5.203
Prairie Pines	1.41	0.793	19	0.05	0.435	4.573
Wilderness Park	1.39	0.853	19	0.05	0.385	5.018

Table 2.6: Relative rates of lone star tick nymphs collected by location, 2012. For the labels “I” is Indian Cave SP, “K” is Kinter’s Ford WMA, “P” is Prairie Pines, “S” is Schramm SRA, “T” is Table Rock WMA, and “W” is Wilderness Park.

Label	Est	Std Err	DF	T value	Probt	Alpha	Lower	Upper	1 vs 2	2 vs. 1
I vs K	-1.529	0.911	19	-1.678	0.110	0.05	-3.436	0.378	0.217	4.614
I vs P	0.585	0.884	19	0.661	0.516	0.05	-1.265	2.434	1.794	0.557
I vs S	0.390	0.711	19	0.550	0.590	0.05	-1.099	1.879	1.477	0.677
I vs T	-2.882	0.895	19	-3.221	0.004	0.05	-4.755	-1.010	0.056	17.855
I vs W	0.599	0.923	19	0.649	0.524	0.05	-1.334	2.531	1.820	0.549
K vs P	2.114	1.016	19	2.080	0.051	0.05	-0.013	4.240	8.279	0.121
K vs S	1.919	0.869	19	2.207	0.040	0.05	0.099	3.739	6.814	0.147
K vs T	-1.353	0.941	19	-1.439	0.167	0.05	-3.322	0.615	0.258	3.869
K vs W	2.128	1.066	19	1.996	0.061	0.05	-0.104	4.360	8.398	0.119
P vs S	-0.195	0.849	19	-0.229	0.821	0.05	-1.973	1.584	0.823	1.215
P vs T	-3.467	1.010	19	-3.431	0.003	0.05	-5.582	-1.352	0.031	32.035
P vs W	0.014	0.700	19	0.0203	0.984	0.05	-1.451	1.480	1.014	0.986
S vs T	-3.272	0.864	19	-3.787	0.001	0.05	-5.080	-1.463	0.038	26.366
S vs W	0.209	0.901	19	0.232	0.819	0.05	-1.677	2.095	1.232	0.811
T vs W	3.481	1.057	19	3.295	0.004	0.05	1.270	5.693	32.495	0.031

FIGURES

Figure 2.1: Map of collection sites southeast Nebraska. Sites include Indian Cave State Park, Kinter's Ford Wildlife Management Area, Prairie Pines, Schramm State Recreational Area, Table Rock Wildlife Management Area, and Wilderness Park in respect of two major cities, Lincoln and Omaha, in Nebraska.

Figure 2.2: Carbon dioxide (CO_2) trap at Table Rock Wildlife Management Area.

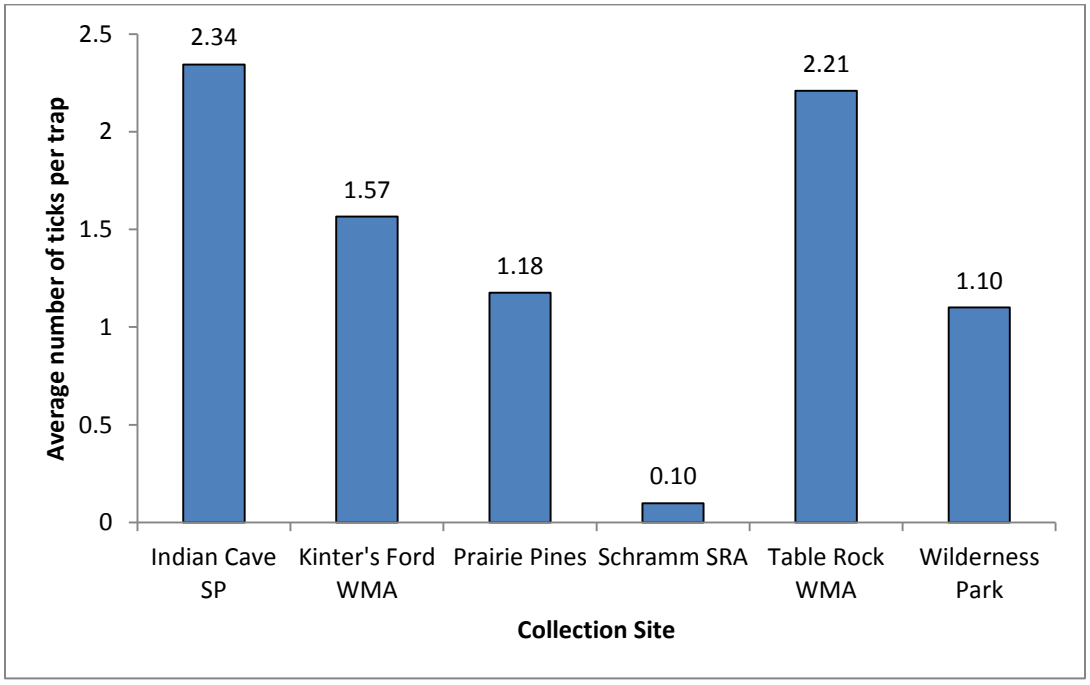

Figure 2.3: Average number of adult *Dermacentor variabilis* per trap, 2012.

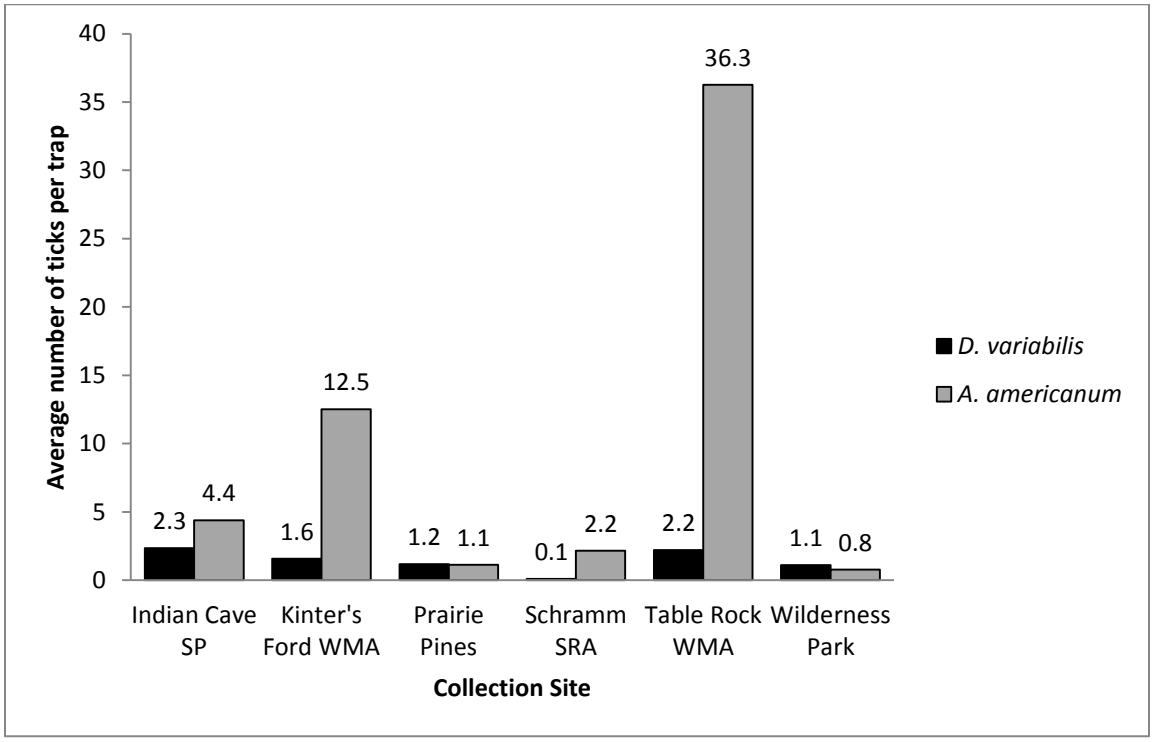

Figure 2.4: Average number of adult *Amblyomma americanum* vs. *Dermacentor variabilis* per trap, 2012.

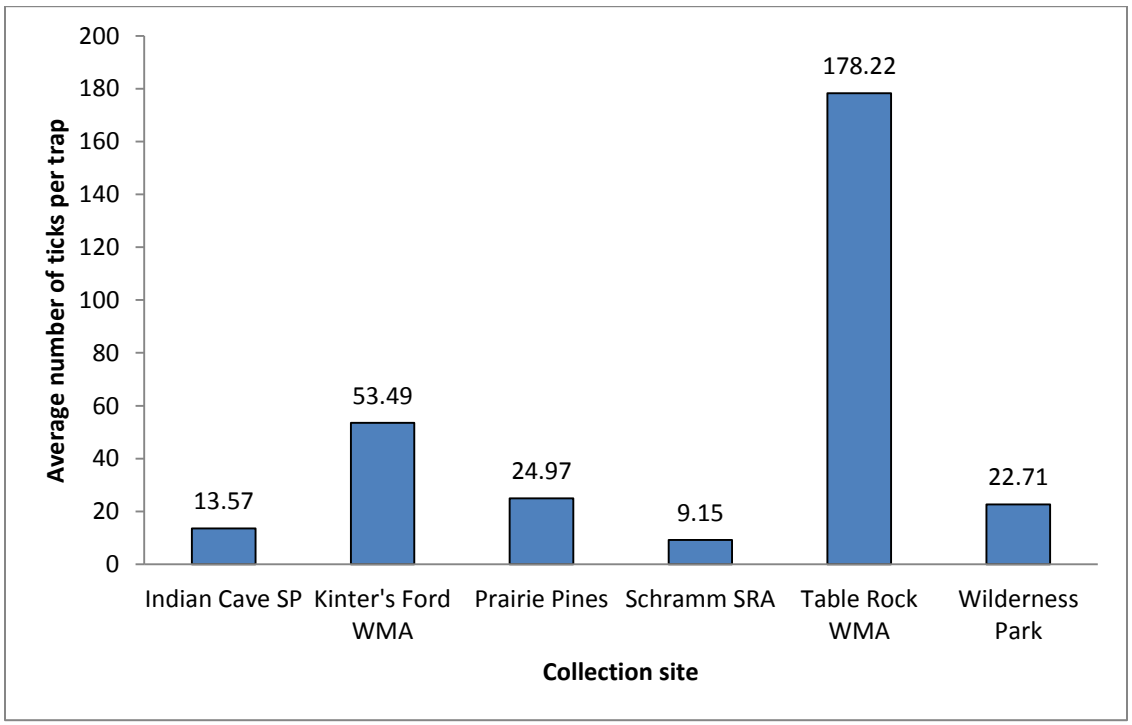

Figure 2.5: Average number of *Amblyomma americanum* (all life stages) per trap, 2012.

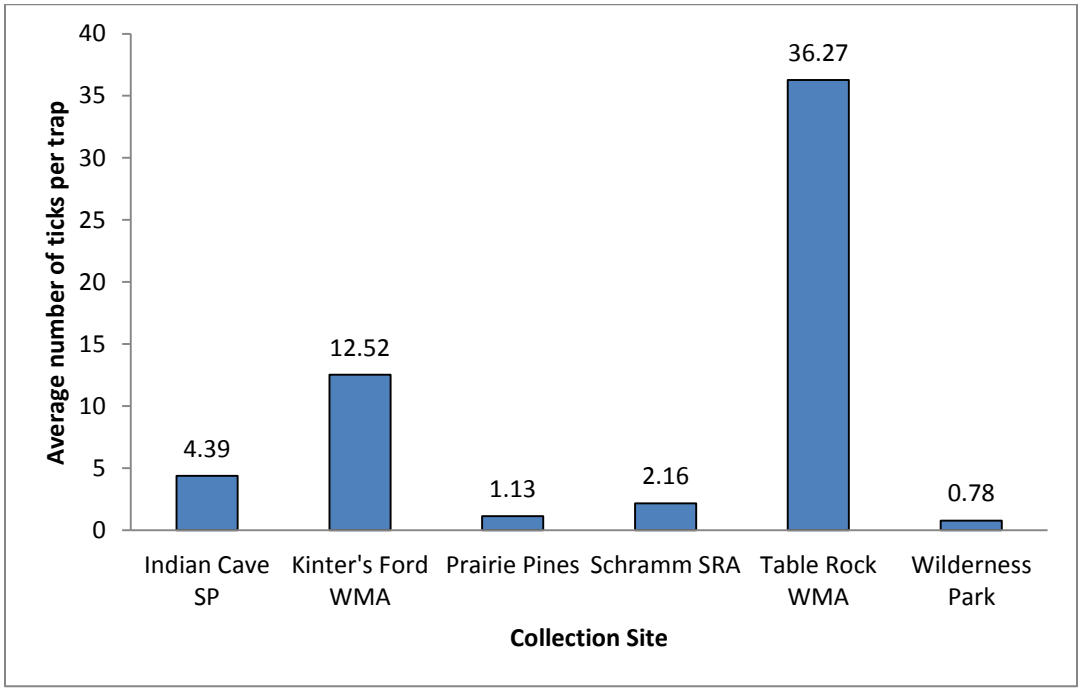

Figure 2.6: Average number of adult *Amblyomma americanum* per trap, 2012.

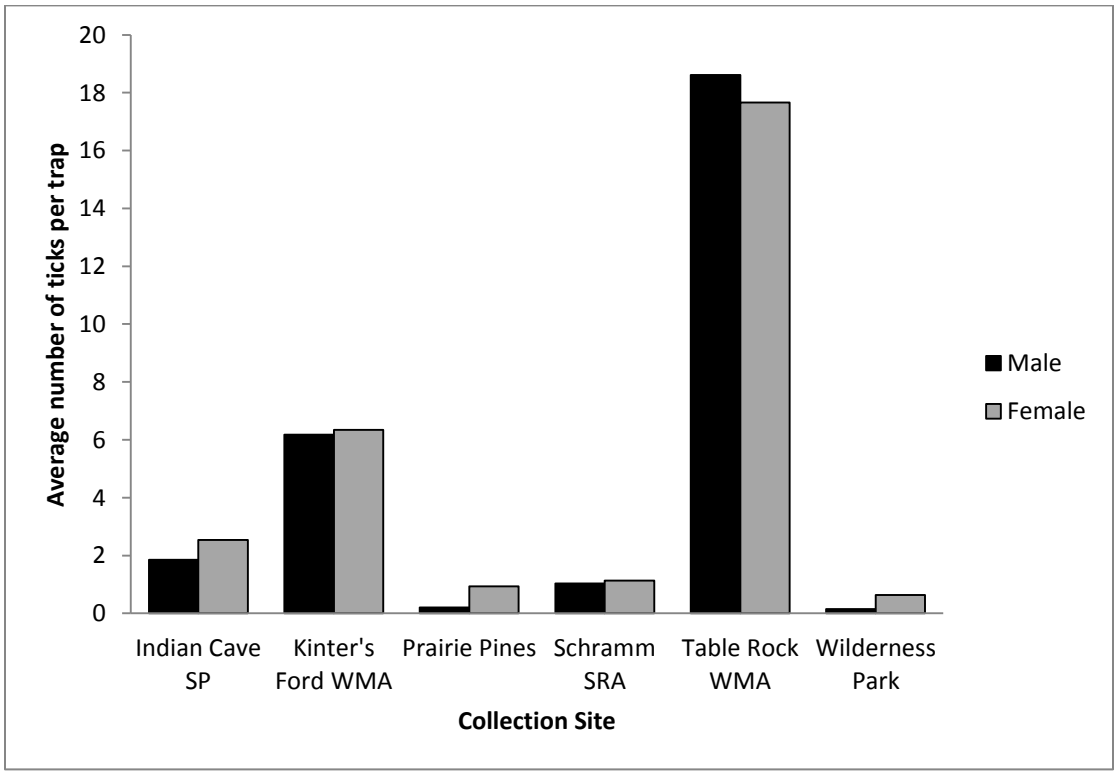

Figure 2.7: Average number of male vs. female *Amblyomma americanum* per trap, 2012.

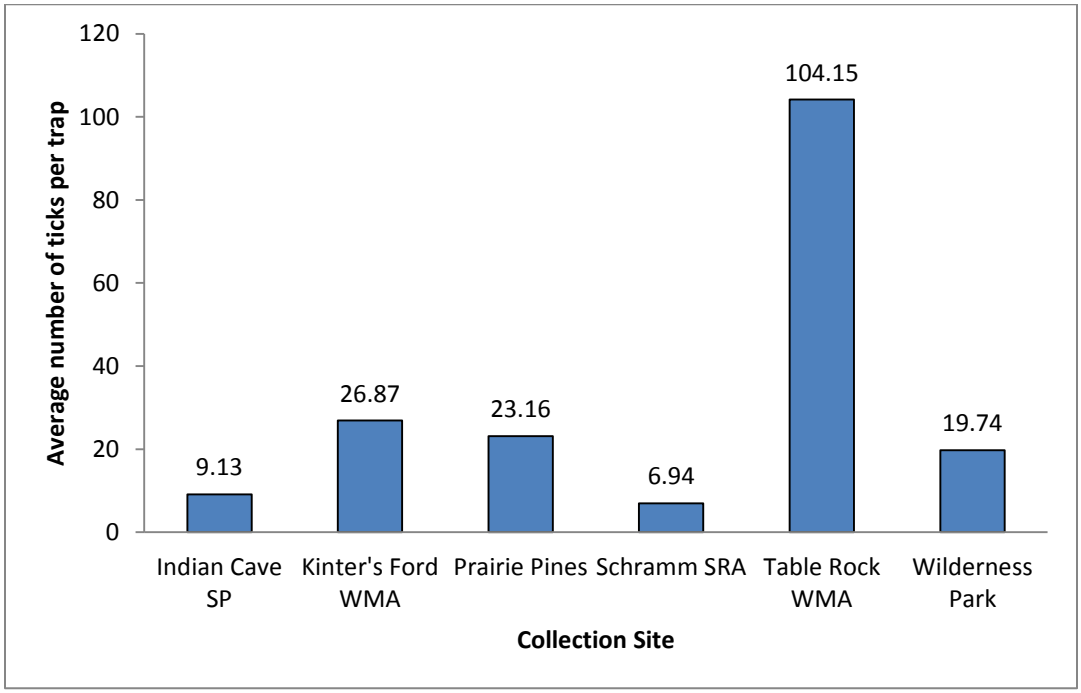

Figure 2.8: Average number of nymphal *Amblyomma americanum* per trap, 2012.

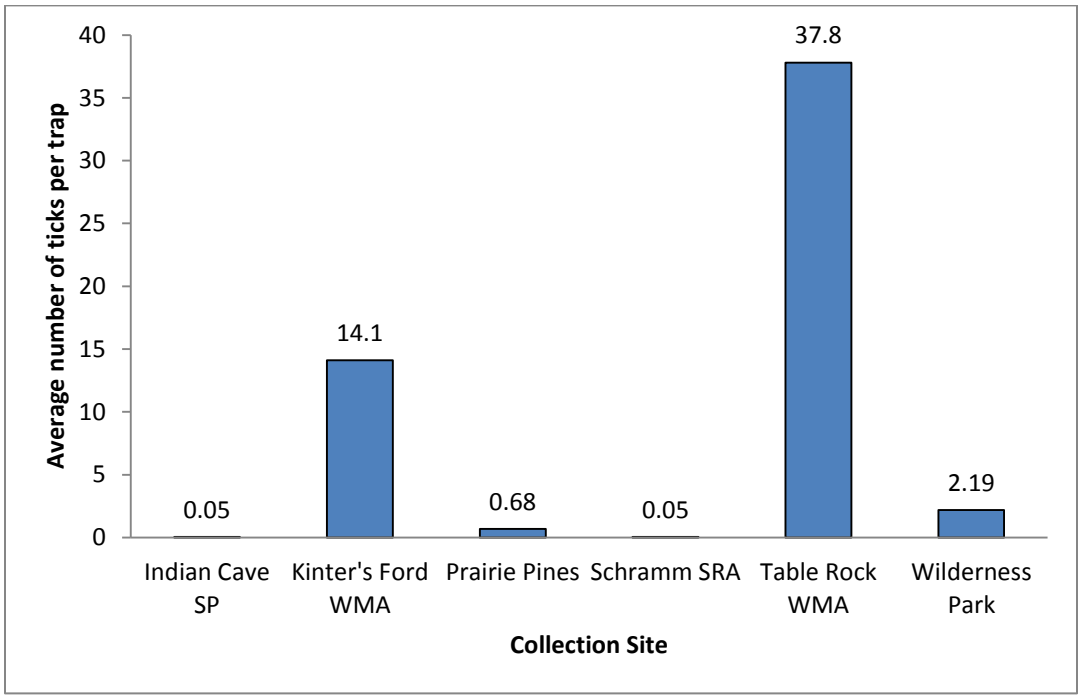

Figure 2.9: Average number of larval *Amblyomma americanum* per trap, 2012.

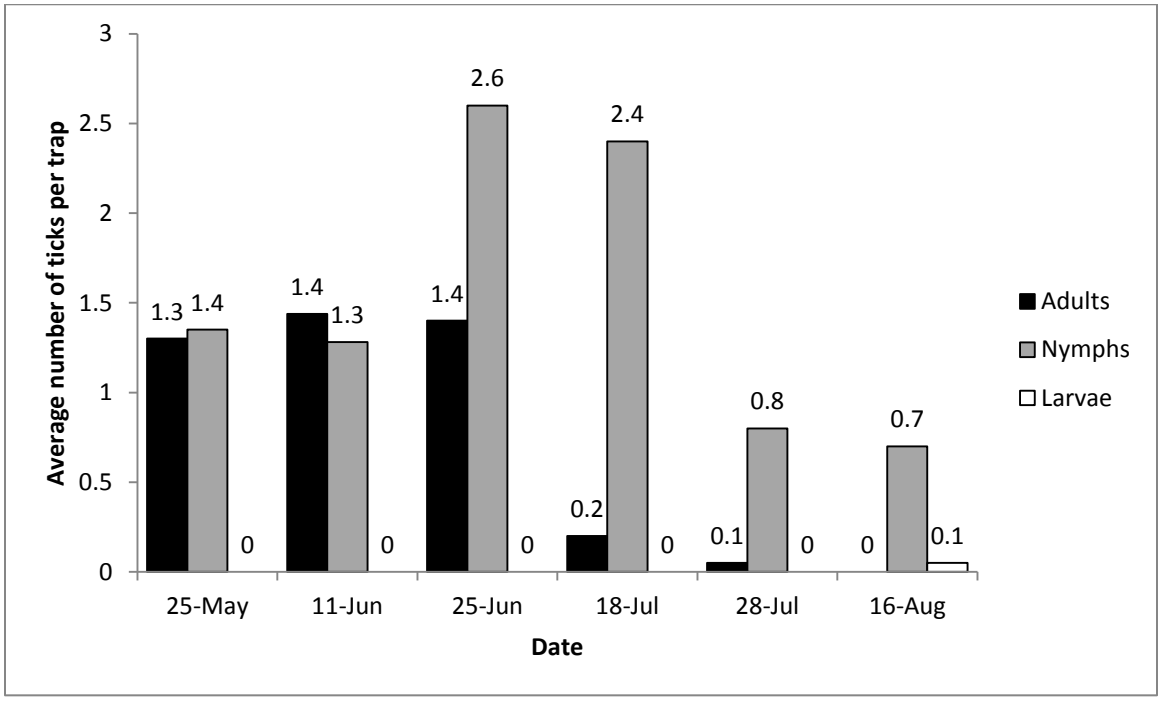

Figure 2.10: *Ambyomma americanum* populations at Indian Cave State Park. Adult ticks had a peak in population on 11 June (1.4 ticks/trap) and then steadily declined into July until no adults collected in August. Nymphs had a bell-shaped curve that had a peak on 25 June (2.6 ticks/trap) then declined into August. As both adult and nymph life stages decreased larvae were collected on 16 August.

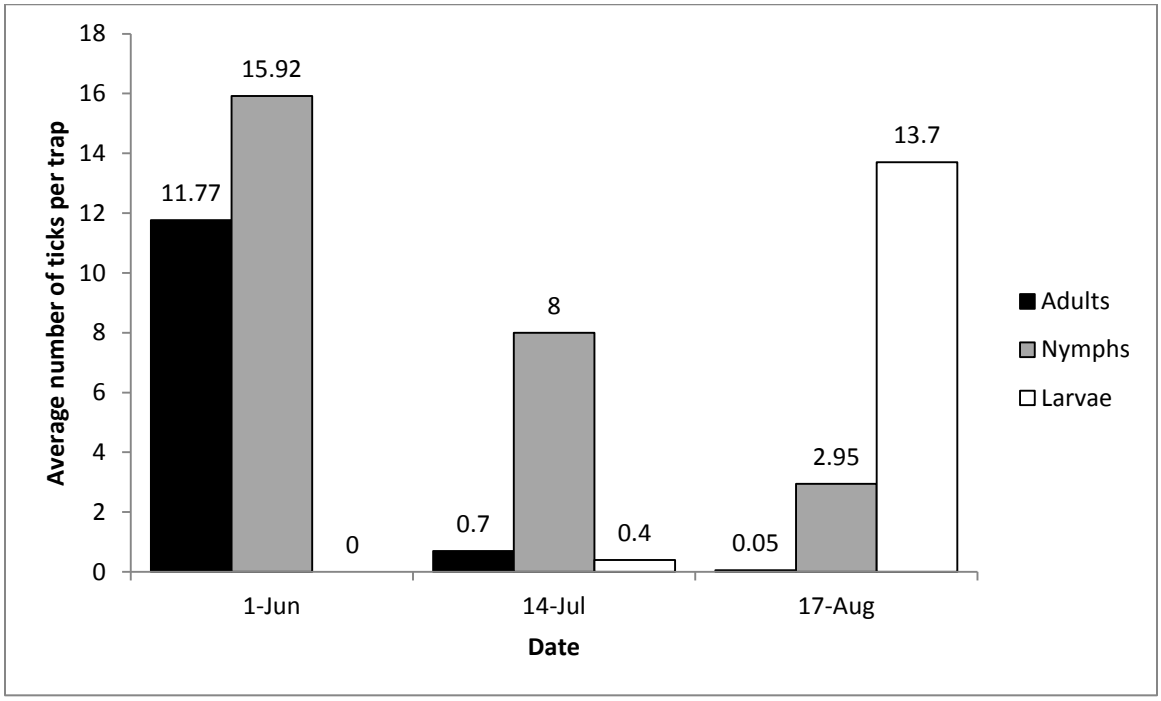

Figure 2.11: *Ambyomma americanum* populations at Kinter's Ford Wildlife Management Area. Adult ticks had a peak in population on 01 June (11.77 ticks/trap) then steadily declined into July, and adult ticks were collected in August (0.05 ticks/trap). Nymphs had a peak in population on 01 June (15.92 ticks/trap) and steadily decreased through the summer. Larvae were collected beginning 14 July and had a peak in population on 17 August (13.7 ticks/trap).

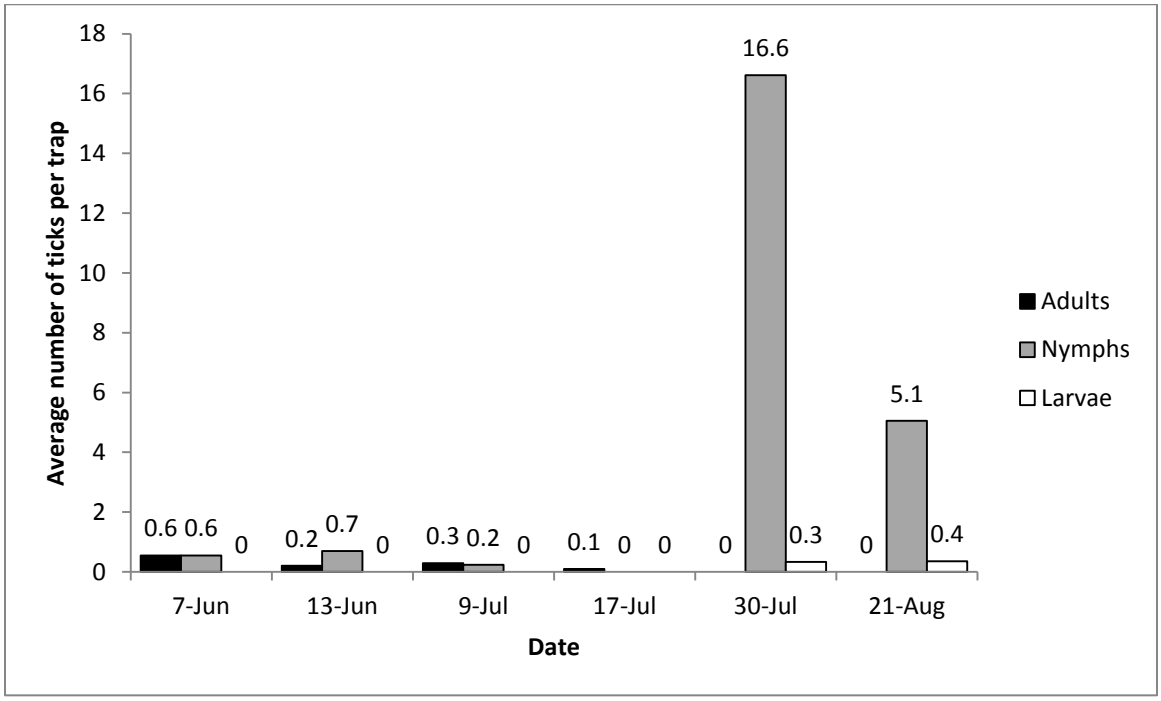

Figure 2.12: *Ambyomma americanum* populations at Prairie Pines. Adult ticks had two peaks in population on 07 June (0.55 ticks/trap) and 09 July (0.29 ticks/trap) then steadily declined into July until no adults collected in August. Nymphs had two peaks in population on 13 June (0.70 ticks/trap) and 30 July (16.62 ticks/trap). Larvae were collected beginning 30 July and started to increase in August.

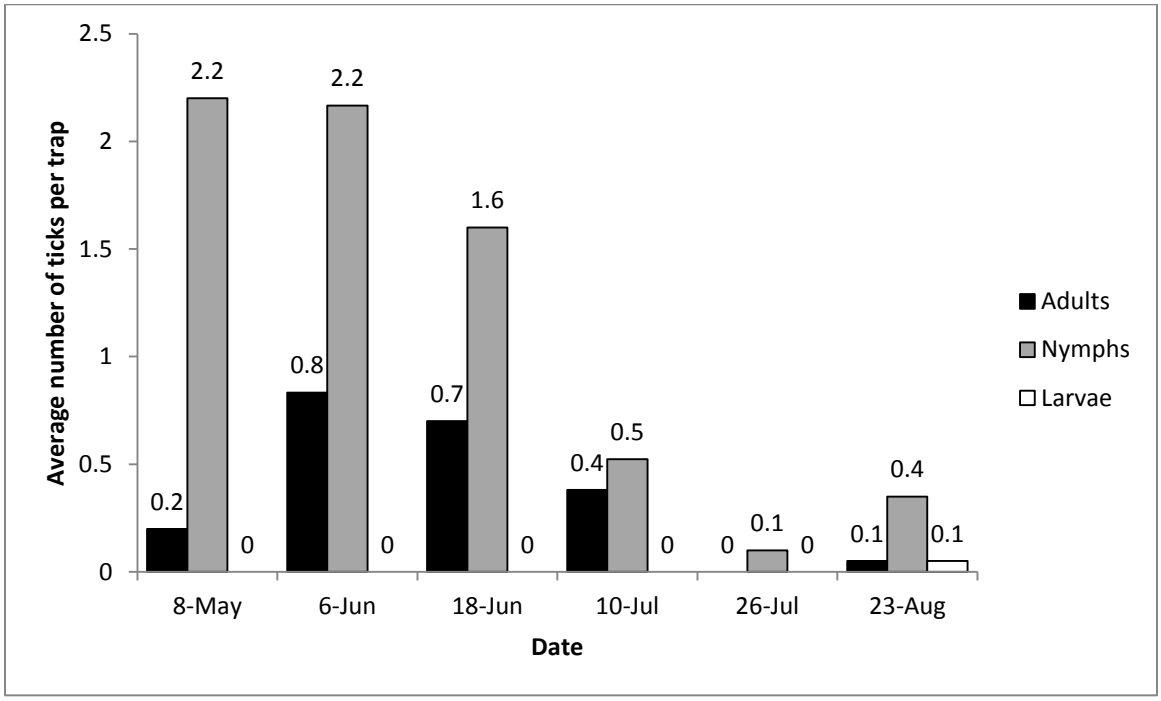

Figure 2.13: *Ambyomma americanum* populations at Schramm State Park Recreational Area. Adult ticks had two peaks in population during early summer on 6 June (0.83 ticks/trap) and in late summer on 23 August (0.05 ticks/trap). There were also two peaks in population for nymphs on 08 May (2.2 ticks/ trap) and 23 August (0.35 ticks per trap). Larvae were collected beginning 23 August (0.05 ticks/trap).

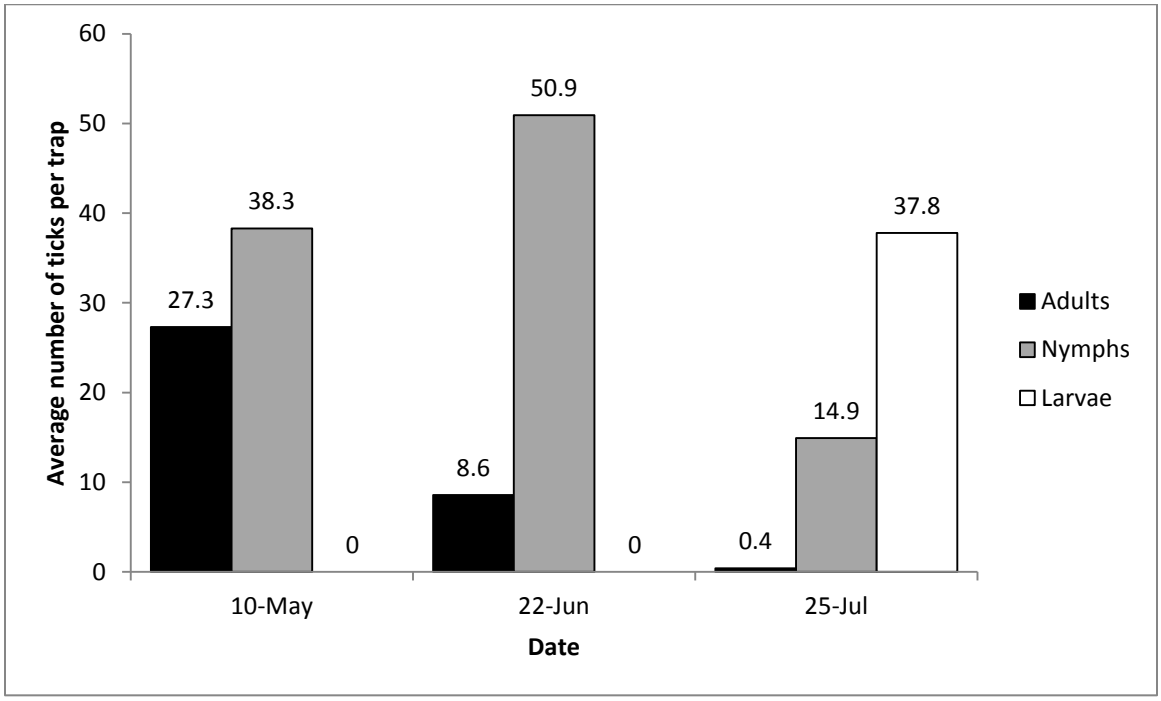

Figure 2.14: *Ambyomma americanum* populations at Table Rock Wildlife Management Area. Adult ticks had a peak in population on 10 May (27.3 ticks/trap) then steadily declined into July. Nymphs had a peak in population on 23 June (50.93 ticks/trap). Larvae were collected beginning 25 July (37.8 ticks/trap).

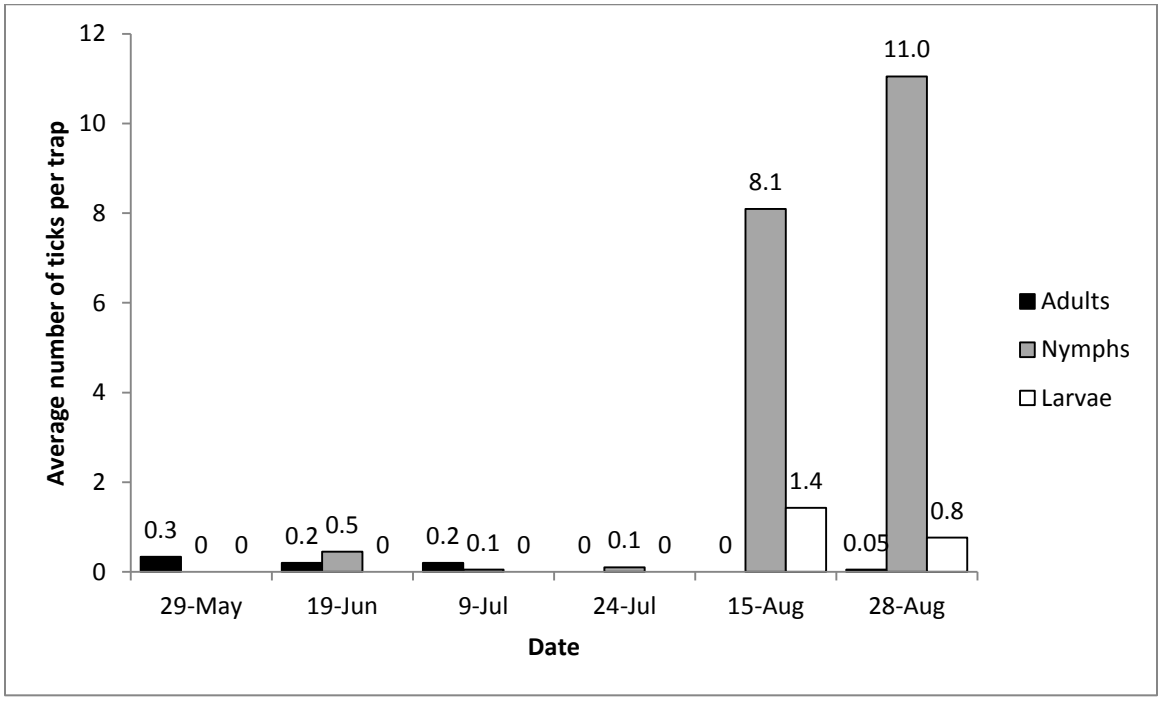

Figure 2.15: *Ambyomma americanum* populations at Wilderness Park. Adult ticks had a peak in population on 09 July (0.2 ticks/trap) then steadily declined until collected on 28 August (0.05 ticks/trap). Nymphs had two peaks in population on 19 June (0.45 ticks/trap) and 28 August (11.05 ticks/trap). Larvae were collected beginning 15 August (1.43 ticks/trap) and started to decrease into late August.

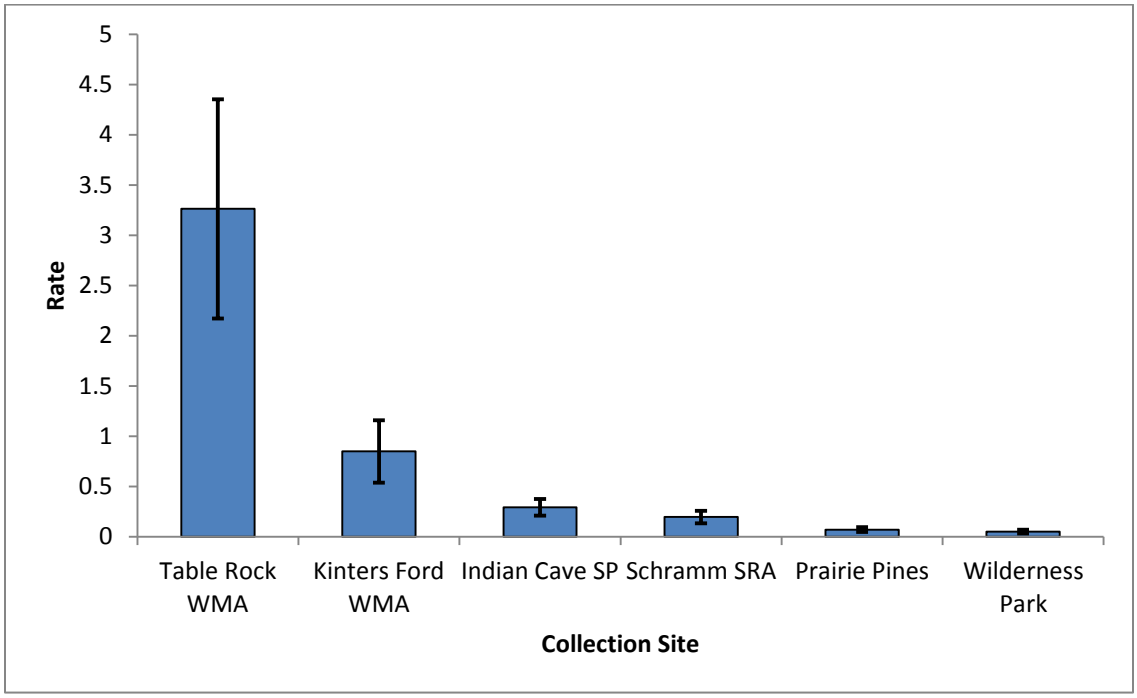

Figure 2.16: Rate of adult lone star ticks (*Amblyomma americanum*) at each location.

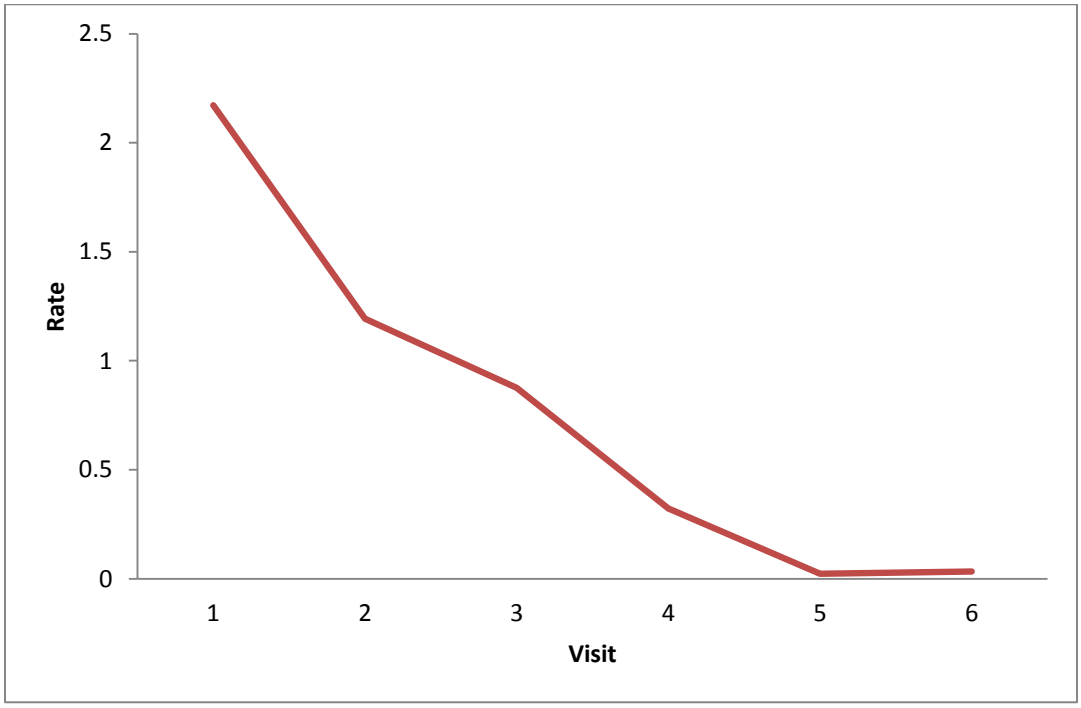

Figure 2.17: Rate of adult lone star ticks (*Amblyomma americanum*) at each visit.

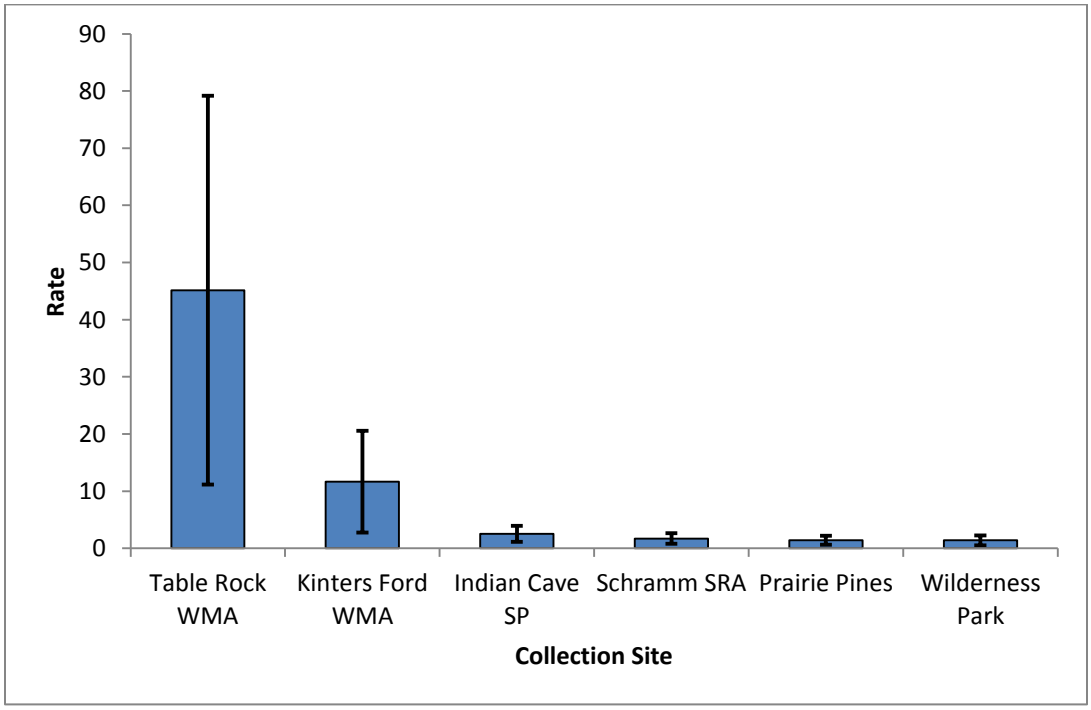

Figure 2.18: Rate of nymphal lone star ticks (*Amblyomma americanum*) at each location.

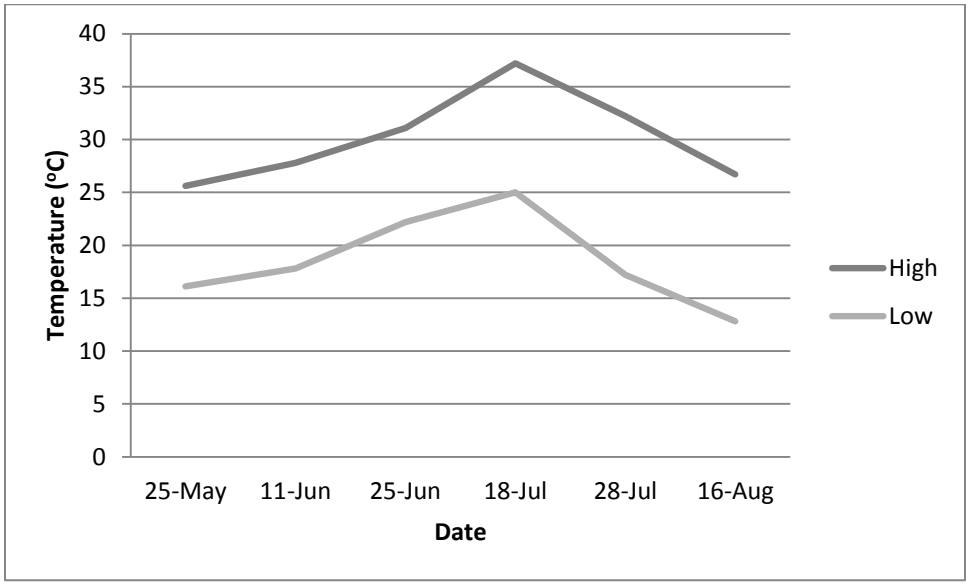

Figure 2.19: Temperature (Celsius) at Indian Cave State Park, 2012.

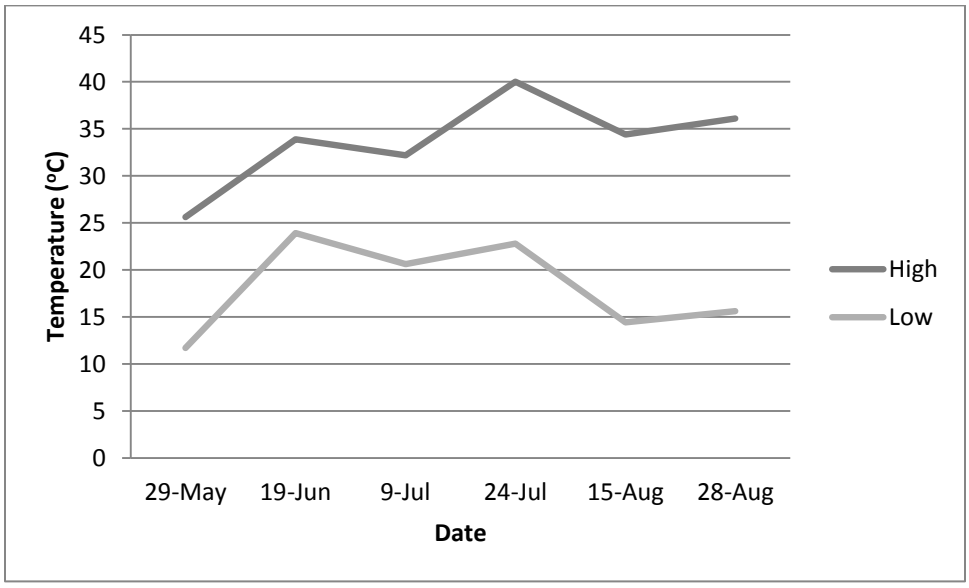

Figure 2.20: Temperature (Celsius) at Wilderness Park, 2012.

Chapter 3: PREVALENCE OF *RICKETTSIA* AND *EHRLICHIA* SPP. HARBORED BY *AMBLYOMMA AMERICANUM* (ACARI: IXODIDAE) TICKS FROM SOUTHEAST NEBRASKA

ABSTRACT

A sample of 251 questing adult lone star ticks, *Amblyomma americanum* (L.) (Acari: Ixodidae), collected at six sites in southeast Nebraska were analyzed for detection of three pathogens: *Rickettsia* spp., *Ehrlichia chaffeensis*, and *Ehrlichia ewingii*. *Rickettsia* spp. were detected in adult ticks collected from all six sites in southeast Nebraska. Adult ticks testing positive for *Rickettsia* were almost equal for both sexes: 73/140 (52.1%) females and 57/111 (51.4%) males. Ticks testing positive for *E. chaffeensis* 1.6% (4/251) were found only at Table Rock Wildlife Management Area (WMA) and consisted of 12% (3/25) female and 4% (1/25) male ticks. Indian Cave State Park, Schramm State Recreational Area (SRA), and Table Rock WMA had ticks testing positive for *E. ewingii*. Overall 2.1% (3/140) female and 0.9% (1/111) male ticks were positive. Total lone star tick adults testing positive for *E. ewingii* were 1.6% (4/251). Lone star ticks co-infected with *Rickettsia* spp. and *E. chaffeensis* were found only at Table Rock WMA, where 8% (2/25) of female ticks and 4% (1/25) of males for total of 6% (3/50) of lone star ticks were coinfected.

INTRODUCTION

The lone star tick, *Amblyomma americanum* (L.), is an aggressive ectoparasite capable of being a vector of both human and animal pathogens including *Ehrlichia chaffeensis* (human monocytic ehrlichiosis) (Anderson et al. 1993; Ewing et al. 1995; Lockhart et al. 1997a, b), *Ehrlichia ewingii* (canine granulocytic ehrlichiosis) (Anziani et

al. 1990; Mixson et al. 2006; Heise et al. 2010), *Rickettsia amblyommii* (Burgdorfer et al. 1981b; Apperson et al. 2008), *Rickettsia parkeri* (eschar maculatum agent) (Macaluso and Azad 2005; Cohen et al. 2009), *Borrelia lonestari* (Southern tick-associated rash illness) (Masters 1998), and *Francisella tularensis* (tularemia) (Hopla and Downs 1953). Lone star ticks have a three-host life cycle; thus, if the lone star tick acquired a pathogen as a larva, the tick would transstadially transmit the pathogen to the nymph. The lone star tick would be able to transmit the pathogen to two different hosts, including humans, as a nymph and adult.

Lone star ticks are now established in southeastern Nebraska (Cortinas and Spomer 2013). Prevalence of pathogens harbored in lone star ticks has been studied across the Midwest, northeast, and south-central United States (Anderson et al. 1993; Burket et al. 1998; Ijdo et al. 2000; Irving et al. 2000; Kollars et al. 2000; Wolf et al. 2000; Steiert and Gilfoy 2002; Mixson et al. 2006; Allan et al. 2010; Heise et al. 2010); however little research has been conducted in Nebraska. Ehrlichioses were diagnosed in Nebraska between 1986 and 1997, and there were 17 probable cases and one confirmed case of human monocytic ehrlichiosis (HME) (McQuiston et al. 1999). Diagnosed cases do not necessarily indicate that Nebraska citizens are acquiring *Ehrlichia* spp. from ticks in Nebraska. Nebraska citizens are either acquiring infections from lone star ticks in Nebraska or have travelled to areas where lone star ticks and pathogens are prevalent and return home to get diagnosed.

The two main genera of pathogens transmitted by lone star ticks are *Rickettsia* spp. and *Ehrlichia* spp. The *Rickettsia* spp. shown to be transmitted by lone star ticks include *Rickettsia rickettsii* [Rocky Mountain spotted fever (RMSF)] (Maver 1911;

Macaluso and Azad 2005), *Rickettsia parkeri* (Paddock et al. 2004; Macaluso and Azad 2005) and *Rickettsia amblyommii* (Burgdorfer et al. 1981b; Stromdahl et al. 2008; Jiang et al. 2010). Two *Ehrlichia* spp. that are emerging pathogens in the United States include *Ehrlichia chaffeensis* [human monocytic ehrlichiosis (HME)] (Anderson et al. 1993; Ewing et al. 1995; Lockhart et al. 1997a, b) and *E. ewingii* [canine granulocytic ehrlichiosis (CGE)] (Anziani et al. 1990; Mixson et al. 2006; Heise et al. 2010).

Lone star ticks have been implicated as vectors of *Rickettsia rickettsii* (Maver 1911, Parker et al. 1943). However, other studies have shown that lone star ticks do not transmit *R. rickettsii* (Burgdorfer et al. 1981a; Goddard and Norment 1986). Lone star ticks were analyzed from Mississippi, Kentucky, Oklahoma, and Texas (Goddard and Norment 1986), Arkansas, South Carolina, and Tennessee (Burgdorfer et al. 1981a) and none were positive for *R. rickettsii*. It is believed that RMSF cases were misdiagnosed and may have been caused by former endosymbionts of lone star ticks that include *R. parkeri* and *R. amblyommii* (Paddock et al. 2004; Apperson et al. 2008; Stromdahl et al. 2008; Jiang et al. 2010).

Rickettsia parkeri was first recovered in *Amblyomma maculatum* (Gulf Coast tick) in 1939 (Parker et al. 1939) and later in *Amblyomma americanum* (lone star ticks) (Macaluso and Azad 2005; Cohen et al. 2009). *Rickettsia parkeri* was initially believed to be an endosymbiont of the ticks but is now considered a human pathogen and has been associated with a case of "rickettsial pox" in a Virginia patient (Paddock et al. 2004). Symptoms of *R. parkeri* include rapid onset of fever, headache, malaise, diffuse myalgias and arthralgias, and multiple eschars that start as small erythematous papules that develop

into pustules and ulcerate, and the rash then spreads from the flanks and trunk to face and extremities (Paddock et al. 2004).

Rickettsia amblyommii was discovered in 1974 and was tentatively named WB-8-2 agent (Burgdorfer et al. 1981b). Lone star ticks have been shown to be naturally infected with *R. amblyommii*, and *R. amblyommii* is transovarially and transstadially transmitted (Burgdorfer et al. 1981b; Macaluso and Azad 2005; Stromdahl et al. 2008). Adult tick infection rate varies from 37 to 75% positive for *R. amblyommii* (Apperson et al. 2008, Jiang et al. 2010), and prevalence between male and female ticks not significantly different (Mixson et al. 2006). Pathogenicity of *R. amblyommii* is unknown (Stromdahl et al. 2008). It was considered non-pathogenic (Burgdorfer et al. 1981b, Goddard and Noment 1986) but has now been associated with mild spotted fever disease (Parola et al. 2005, Billeter et al. 2007). Patients with *R. amblyommii* have symptoms such as mild sudden onset, fever, thrombocytopenia, headache, and an erythematous maculopapular rash on trunk, arms, and legs (Dasch et al. 1993; Apperson et al. 2008).

The emerging pathogen, *Ehrlichia ewingii*, is the etiologic agent of canine granulocytic ehrlichiosis (CGE) (Stockham et al. 1985). It is an emerging disease in the United States (Childs and Paddock 2003) and not much is known about *E. ewingii* because it is a newly recognized disease, it has shown a failure to cultivate in the laboratory, and has been reported from small number of cases (Anderson et al. 1992; Paddock et al. 2001, Telford and Goethert 2008). CGE was originally a disease of veterinary importance in domesticated dogs (*Canis lupus familiaris*) until the first reported case in humans in 1999 (Stockham et al. 1985; Paddock et al. 2005). Cases have been reported from south-central and southeastern United States (Fishbein et al. 1994;

McQuiston et al. 1999; Paddock et al. 2001), and by 2011 there were 13 confirmed cases in the United States (CDC 2013). Canine granulocytic ehrlichiosis can infect both animals and humans. Symptoms in domestic dogs include polyarthritis, stiffness, stilted gait, anemia, head tilt, tremors, fever (Stockham et al. 1985; Bellah et al. 1986; Anziani et al. 1990; Stockham et al. 1990; Goodman et al. 2003; Paddock et al. 2005), vomiting, diarrhea, and meningitis (Carrilo and Green 1978; Murphy et al. 1998). Human symptoms are non-specific and indistinguishable from *E. chaffeensis* in humans (Buller et al. 1999). Canine granulocytic ehrlichiosis is a milder illness than HME (Paddock and Childs 2003) in people with no underlying immune conditions (Paddock et al. 2005). Human symptoms include febrile syndrome, headache, discomfort, and muscle pain (Paddock et al. 2005). No patients to date have died due to *E. ewingii* (Buller et al. 1999; Paddock et al. 2001).

Ehrlichia chaffeensis is the causative agent of Human Monocytic Ehrlichiosis (HME). Over 850 cases of HME are known, and it is also an emerging pathogen. A total of 16 cases have been reported in Nebraska since the first case of HME in 2007 (CDC 2013). Historically most cases were reported from the south central and southeastern United States, but now HME has been reported from 30 states in the south and central Midwest, southeast, and mid-Atlantic regions (Fishbien et al. 1994; Standaert et al. 1995; McQuiston et al. 1999; Rikihisa 1999). The states with highest reported average incidence rate are Arkansas, North Carolina, Missouri, and Oklahoma (Rikihisa 1999). Even with a high number of cases, the mortality is low, between 2-5% (Dumler and Bakken 1995; Fishbien et al. 1994; McQuiston 1999; Rikihisa 1999; Paddock and Childs 2003). Only ten deaths have been attributed to HME in the United States (Paddock et al.

1997). Symptoms of HME are nonspecific and range from being asymptomatic or mild disease to being a severe, fatal disease (Dumler and Bakken 1995; McQuiston et al. 1999). Early symptoms include fever, headache, joint and muscle pain, and nausea (Fishbien et al. 1994; Rikihisa 1999; Paddock and Childs 2003). Rashes developed in 36% of patients but are variable in character, occurrence, and location on body (Fishbien et al. 1994; Dumler and Bakken 1995; Rikihisa 1999). Severe reactions to HME include multi-system failure, cardiomegaly, seizures, coma, and death (Fishbien et al 1994; Dumler and Bakken 1995; Paddock and Childs 2003).

Research on prevalence of vector-borne microorganisms transmitted by the lone star tick in Nebraska is important because of high densities of lone star ticks, the ticks aggressive, generalist questing behavior, and their ability to feed on humans in all three life stages. Lone star ticks are established and spreading throughout Nebraska along a corridor between Omaha and Lincoln where 56% of Nebraska's population is located (Cortinas and Spomer 2013). As lone star ticks become established in new foci, the probability of people coming into contact with the ticks increases, thus increasing the chance of humans acquiring pathogens transmitted by the ticks. The objective of this study is to determine prevalence of *Rickettsia* and *Ehrlichia* spp. in lone star tick adults collected from six sites of southeastern Nebraska.

MATERIALS AND METHODS

Tick Collection

Lone star ticks were collected from May to August 2012, during the time when lone star ticks are actively questing (Fleetwood et al. 1984; Lancaster 1955; Kollars et al. 2000; Goodman et al. 2005). Six sites were selected in southeastern Nebraska: Indian

Cave State Park (SP), Kinter's Ford Wildlife Management Area (WMA), Prairie Pines, Schramm SRA, Table Rock Wildlife Management Area, and Wilderness Park (Figure 1) based on the presence of lone star tick and high frequency of encounter rates in Nebraska (Cortinas and Spomer 2013) (See Chapter 2 for site descriptions).

Collection sites were visited three to six times every other week over the summer. Questing ticks were collected by carbon dioxide traps constructed of a cardboard piece (2 ft x 1.5 ft) with masking tape along the edges so that most of tape was sticking out (Figure 2). Traps were inverted and placed on the ground, approximately 20 m apart, in forested areas of each study site along hiking and deer trails where lone star ticks known to quest, including young, second growth deciduous habitats with dense under story vegetation and mature forests (Sonenshine 1993, Paddock and Yabsley 2007). Traps were left for two hours during which time questing ticks were attracted to the carbon dioxide and became trapped on the sticky side of the masking tape. Traps were then collected by folding masking tape over on to the cardboard and transported back to lab.

In the laboratory, ticks were removed from the masking tape with fine forceps, stored in 100% ethyl alcohol, and identified to life stage, sex, and species (Cooley and Kohls 1944; Keirans and Litwak 1989; Keirans and Durden 1998). Ticks were then surface sterilized by placing individuals into 1.5 mL microcentrifuge tube containing 100% ethyl alcohol and vortexed for 5 min with a Vortex Genie 2 (DAIGGER, Vernon Hills, IL). Ticks were then transferred to 1.5 mL microcentrifuge tube containing pure water from Nanopure Barnstead (Thermo Scientific, Waltham, MA) using fine forceps that were surface sterilized with BACTI-CINERATOR*IV (McCormick Scientific) and

vortexed for 30 s. Individual ticks were placed into individually labeled vials containing 95% ethyl alcohol and stored in a freezer.

A total of 251 (140 females, 111 males) out of 747 adult ticks were randomly selected for PCR from pools of sexed ticks collected at each site and each date (Table 1). Because not all sites were visited the same number of times, ticks were collected at different times over the summer, and high numbers of adults were not collected at each site. Due to low adult tick numbers collected at Prairie Pines, Schramm SRA, and Wilderness Park, all ticks from these sites were processed.

Analysis of Tick Specimens

DNA Preparation and Extraction

DNA was extracted using the Animal Tissues Spin-Column protocol from DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Ticks were removed from 95% ethanol and placed on a paper towel to dry for 30 min, and then each tick was turned over using fine tweezers after 15 min to ensure alcohol had evaporated from both surfaces. Individual ticks were transferred to autoclaved 1.7 mL microcentrifuge tubes (VWR International, Randor, PA). Each tube containing a single tick was placed into liquid nitrogen in a Styrofoam® container. After the liquid nitrogen evaporated, the microcentrifuge tube was placed back into liquid nitrogen but only so that the bottom of the tube was immersed. Ticks were then ground using two inch pestles (Fisher Scientific). Buffer ATL (Qiagen) was pipetted into a microcentrifuge tube to wash off the pestle and to ensure no tick sample was lost. The pestle was then disposed into a dirty container to prevent cross contamination between samples. After the pestle was removed, 20 µL of proteinase K and 4 µL polyacryl carrier (Molecular Research Center, Inc.,

Cincinnati, OH) were pipetted into microcentrifuge tube and the tube was vortexed to mix the solution. Animal Tissues Spin-Column protocol provided with DNeasy Blood and Tissue Kit (Qiagen) to extract DNA from adult ticks was followed. Tick DNA was placed into a -20^{o}C freezer until needed.

Extraction verification

Verification was performed to ensure DNA was extracted and present within samples, and to exclude false negatives from study. DNA extractions were verified using mitochondrial polymerase chain reaction (PCR) and Nanodrop analysis.

Mitochondrial PCR used primers specific for the 16+2 and 16-1 mitochondrial primers (Integrated DNA Technologies) specific for ~300 bp amplicon of the bacteria-wide 16S rRNA gene as described by Trout et al. (2010). Mitochondrial primers were used to detect lone star tick deoxyribonucleic acid (DNA). PCR primers 16+2 and 16-1 (Table 2) were used in a 50 µL reaction that contained 25 µL of Taq PCR Master Mix Kit (Qiagen) that contained 2.5 U *Taq* DNA Polymerase, 1x QIAGEN PCR Buffer, 1.5 mM $MgCl_2$, and 200 µM of each deoxyribonucleotide triphosphates (dNTP), 19 µL PCR Grade Water (Roche), 1 µL of each primer (10 µM) (Integrated DNA Technologies), and 4 µL (0.04 to 55.26 ng/µL) of sample DNA. PCR was carried out at an initial temperature of 94^{o}C for 2 minutes followed by 35 cycles of 95^{o}C for 30 s, 55^{o}C for 30 s, and 72^{o}C for 60 s. Final extension was carried out at 72^{o}C for 5 min. The reactions were analyzed using QIAxcel automated system used for nucleic acid separation (Qiagen).

Nanodrop analysis performed using the Nano Drop Spectrophotometer ND-1000 (Program: ND-1000 V3.5.2) to detect the amount of nucleic acid within each extraction sample and to determine if DNA was present.

PCR Amplification

Multiple established PCR protocols were used to identify organisms in these samples, including Rickettsial citrate synthase gene (gltA), *Ehrlichia chaffeensis*, and *Ehrlichia ewingii*. All primers, sequences, protocols, and references are listed in Table 2.

General *Rickettsia* species analysis

Rickettsia species analysis performed by amplification of the citrate synthase (gltA) fragment (380 bp) that is present in all *Rickettsia* species. PCR was based on a journal article by Heise et al. (2010). PCR primers RpCS.877 and RpCS1258 (Table 2) were used in a 50 µL reaction that contained 35 µL PCR Grade Water (Roche), 5 µL 25µM $MgCl_2$ (Invitrogen), 5 µL 10X Buffer (Invitrogen), 1 µL of each primer (10 µM) (Integrated DNA Technologies), 0.5 µL 10 µM dNTPs (Vet lab), 0.5 µL Taq DNA Polymerase (Invitrogen), and 2 µL (0.04 to 55.26 ng/µL) of sample DNA. Cycling conditions were 95^{o}C for 5 min followed by 35 cycles of 94^{o}C for 30 s, 60^{o}C for 30 s, 72^{o}C for 45 s, followed by 72^{o}C for 5 min.

***Ehrlichia chaffeensis* analysis**

Amplification of *Ehrlichia chaffeensis* 16S rDNA fragment (~389-bp) for *Ehrlichia chaffeensis* (Anderson et al. 1992) was performed using a nested PCR protocol using general primers ECB and ECC for *Ehrlichia* spp. (Table 2) in a 50 µL reaction for individual samples that contained 25 µL Taq PCR Master Mix Kit (Qiagen), 15 µL PCR Grade Water (Roche), 4 µL of each primer (10 µM) (Integrated DNA Technologies), and 2 µL of sample DNA. Pools (consisted of three tick sample DNA from individual extractions) used external primers ECB and ECC (Table 2) in a 50 µL reaction that contained 25 µL Taq PCR Master Mix Kit (Qiagen) which contained 2.5 U *Taq* DNA

Polymerase, 1x QIAGEN PCR Buffer, 1.5 mM $MgCl_2$, and 200 µM of each deoxynucleoside triphosphate (dNTP), 11 µL PCR Grade Water (Roche), 4 µL of each primer (10 µM) (Integrated DNA Technologies), and 6 µL of sample DNA (0.04 to 55.26 ng/µL) (2 µL of sample DNA from 3 different samples). Cycling conditions in the primary reaction were 3 cycles of 94°C for 1 min, 56°C for 2 min, 70°C for 1.5 min, followed by 37 cycles of 88°C for 1 min, 56°C for 2 min, 70° C for 1.5 min (Anderson et al. 1992).

For the secondary reaction, 2 µL (individual samples) or 6 µL (pools) of primary product was used as template in a 50 µL reaction containing the same PCR components with the exception of the primers HE3 and HE1 (Table 2) and cycling conditions in the secondary reaction were 3 cycles of 94°C for 1 min, 56°C for 2 min, 70°C for 1.5 min, followed by 37 cycles of 88°C for 1 min, 56°C for 2 min, 70°C for 1.5 min (Anderson et al. 1992).

***Ehrlichia ewingii* analysis**

Amplification of *Ehrlichia ewingii* 16S rDNA fragment (~407-bp) for *Ehrlichia ewingii* (Steiert and Gilfoy 2002) was achieved using a nested PCR protocol using external primers ECB and ECC (Table 2) in a 50 µL reaction for individual samples that contained 25 µL Taq PCR Master Mix Kit (Qiagen), 15 µL PCR Grade Water (Roche), 4 µL of each primer (10 µM) (Integrated DNA Technologies), and 2 µL of sample DNA. Pools (consisted of three tick sample DNA from individual extractions) used external primers ECB and ECC (Table 2) in a 50 µL reaction that contained 25 µL Taq PCR Master Mix Kit (Qiagen) which contained 2.5 U *Taq* DNA Polymerase, 1x QIAGEN PCR Buffer, 1.5 mM $MgCl_2$, and 200 µM of each deoxyribonucleotide triphosphates

(dNTP), 11 µL PCR Grade Water (Roche), 4 µL of each primer (10 µM) (Integrated DNA Technologies), and 6 µL (0.04 to 55.26 ng/µL) of sample DNA (2 µL of sample DNA from 3 different samples) (Yabsley et al. 2002). Cycling conditions in the primary reaction were 94^{o}C for 3 min, followed by 30 cycles of 94^{o}C for 35 s, 60^{o}C for 2 min, 72^{o}C for 2 min, followed by 72^{o}C for 5 min (Steiert and Gilfoy 2002).

For the secondary reaction, 2 µL (individual samples) or 6 µL (pools) of primary product was used as template in a 50 µL reaction containing the same PCR components with the exception of the primers HE3 and EE72 (Table 2) and cycling conditions in the secondary reaction were 94^{o}C for 3 min, followed by 35 cycles of 94^{o}C for 35 s, 55^{o}C for 2 min, 72^{o}C for 1.5 min, followed by 72^{o}C for 5 min (Steiert and Gilfoy 2002).

Sequencing

Sequencing was performed on randomly selected positive controls and samples for *Ehlichia chaffeensis* and *E. ewingii* from PCR analysis. The samples were sent to Eurofins MWG Operon (Huntsville, Alabama) for DNA sequencing. DNA sequences were aligned using Align Sequences Nucleotide Basic Local Alignment Search Tool (BLASTn) (NCBI) and then inserted into Standard Nucleotide BLAST (NCBI) program to compare the samples nucleotide sequences to the databases and calculate the statistical significance.

RESULTS

***Rickettsia* spp.**

Rickettsia was detected in adult lone star tick specimens collected from all six sites in southeast Nebraska. Adult ticks positive for *Rickettsia* were almost equal for both sexes (Figure 3): 73/140 (52.1%) females and 57/111 (51.4%) males (Table 3 and 4). Of

the lone star ticks selected for analysis 51.8% (130/251) of all adult specimens were positive for *Rickettsia* species. For each site approximately 50% of males and female lone star ticks were positive for *Rickettsia* spp. (Table 4). However, Kinter's Ford WMA, Prairie Pines, and Wilderness Park did not have 50% of ticks testing positive for *Rickettsia* spp. Kinter's Ford WMA had 66.7% (18/27) of female ticks positive; 100% (4/4) of male ticks were positive from Prairie Pines; and 33.3% (1/3) of male ticks from Wilderness Park were positive for *Rickettsia* spp. (Table 4). The reason for 100% and 33.3% of male tick specimens from Prairie Pines and Wilderness Park, respectively, could be because of the small sample size from these sites. The site with the highest proportion of tick specimens positive for *Rickettsia* spp. was Kinter's Ford WMA (56.6%) (Figure 4). Prairie Pines also had a high proportion of ticks positive but had a low number of adult male tick specimens analyzed. Indian Cave SP had the lowest proportion (47.4%) of adult tick specimens positive for *Rickettsia* spp. (Figure 4).

Lone star tick adults positive for *Rickettsia* spp. were diagnosed from May through August. The number of ticks positive for *Rickettsia* spp. was different over the collection dates for each site. Prairie Pines had positive female and male tick specimens from June to July, Indian Cave SP from May to July, Kinter's Ford WMA from June to August (Figure 1), Schramm SRA from June to August, Table Rock WMA from May to July, and Wilderness Park from May to July.

Ehrlichia chaffeensis

Of the 251 lone star tick specimens analyzed from all six sites, 1.6% (4/251) were positive for *E. chaffeensis* (Table 5 & 6). Female ticks had a higher percentage of

positives (2.1%) compared to male ticks (0.9%) (Table 5 and 6). The only site that had positive tick specimens was Table Rock WMA. Table Rock WMA had 12% (3/25) of female and 4% (1/25) of male tick specimens positive for *E. chaffeensis*. The total number of tick specimens positive for *E. chaffeensis* from Table Rock WMA was 8% (4/50) (Table 5 and 6). Positive ticks were collected from 10 May and 22 June with a higher number of positive ticks in June (Figure5).

Ehrlichia ewingii

Indian Cave State Park, Schramm SP, and Table Rock WMA had ticks positive for *E. ewingii*. Overall 2.1% (3/140) female and 0.9% (1/111) male ticks were positive (Table 7 and 8). Total lone star tick adults positive for *E. ewingii* was 1.6% (4/251) (Table 7 and 8). Indian Cave SP had the highest number of positive ticks with 2.6% (1/38) for both female and male ticks; Schramm SP had 5% (1/20) of female and zero (0/15) male ticks positive for total of 2.9% (1/35) ticks from Schramm SP; and Table Rock WMA had 4% (1/25) of female and zero (0/25) male ticks positive for total of 2% (1/50) testing positive for *E. ewingii* (Table 7 and 8). All ticks positive for *E. ewingii* were collected in July. Individual dates for positive ticks collected at each site are Indian Cave SP (18 July), Schramm SRA (10 July), and Table Rock WMA (25 July).

Co-infection

Lone star ticks were co-infected with *Rickettsia* spp. and *E. chaffeensis* only at Table Rock WMA. Ticks testing positive for *Rickettsia* spp. and *E. chaffeensis* were 8% (2/25) of females and 4% (1/25) of males. Total coinfected female ticks were 1.4% (2/140) and for males 0.9% (1/111) for male lone star ticks. Lone star ticks at Table Rock

WMA were coinfected with *Rickettsia* spp. and *E. chaffeensis* on 10 May (one female) and 22 June (one male and female).

Sequencing

Sequences for *E. chaffeensis* control were 100% identical to *E. chaffeensis* 16S ribosomal RNA gene (GenBank accession number KF034786.1) and *E. chaffeensis* str. Arkansas strain Arkansas 16S ribosomal RNA (GenBank accession number NR_074500.1), and sequence for *E. ewingii* control was 100% identical to *E. ewingii* genotype Panola Mountain 16S ribosomal RNA gene (GenBank accession number DQ365880.1). Sequences for samples randomly selected that were positive for *E. chaffeensis* were 100% identical to the *E. chaffeensis* control sequence (GenBank accession number KF034786.1 and NR_074500.1), and sequences for positive samples for *E. ewingii* were 100% identical to the *E. ewingii* control sequence (GenBank accession number DQ365880.1).

DISCUSSION

Lone star ticks are not only established in southeast Nebraska, but are also infected with *Rickettsia* spp., *Ehrlichia chaffeensis*, and *E. ewingii*. Approximately 50% of male and female lone star tick specimens from each site were positive for *Rickettsia* species, similar to other studies where adult lone star infection with *R. amblyommii* was between 37 to 75% (Mixson et al. 2006; Apperson et al. 2008, Jiang et al. 2010). However, since lone star ticks were only analyzed to the *Rickettsia* genus level, it is uncertain if the lone star ticks are transmitting *Rickettsia rickettsii, R. parkeri,* or *R. amblyommii*. Lone star ticks have been shown to be naturally infected with *R.*

amblyommii and it is considered to be an obligate endosymbiont of lone star ticks since it has been found in ovarian tissue of adult females and is transovarially and transstadially transmitted (Burgdorfer et al. 1981b; Macaluso and Azad 2005; Stromdahl et al. 2008). Burgdorfer et al. (1981b) determined that the infection rate of 1,320 larvae collected from Arkansas, South Carolina, and Tennessee were 100% positive for *R. amblyommii*. Therefore through previously conducted studies, it is possible that the *Rickettsia* spp. within lone star ticks collected in southeastern Nebraska is *R. amblyommii*.

Seasonal activity and pathogen transmission is important for lone star ticks to be competent vectors. Ticks positive for *E. chaffeensis* were collected in Nebraska from May through June with a higher number of positive ticks in June from Table Rock WMA. *Ehrlichia* cases peak in May through July (Eng et al. 1990; Fishbein et al. 1994; Standaert et al. 1995; Rikihisa 1999; Paddock and Childs 2003). At Table Rock WMA, abundance of adult ticks collected was highest in May and for nymphs in June (Figure 6). Adult ticks that emerge from winter diapause in April (Fleetwood et al. 1984; Kollars et al. 2000) have either been infected as larvae or nymphs and transstadially transmitted *E. chaffeensis* to the next life stage, or the adult ticks emerge as a naïve, uninfected tick. To have a successful disease transmission, the adult ticks positive for *E. chaffeensis* must emerge before the nymphal peak and larvae emerge, quest for competent reservoirs, such as white-tailed deer (*Odocoileus virginianus*) (Bowman and Nuttall 2008), and transmit *E. chaffeensis* to an uninfected reservoir to allow enough time for *E. chaffeensis* to reach the proper critical transmission threshold (CTT) value before nymphal and larval ticks emerge to feed (Eisen et al. 2012). If a host is at the proper CTT, emerging larvae feeding

on the host will become infected and be able to harbor and transmit the tick-borne infectious agent as nymphs and adults (Bowman and Nuttall 2008).

Approximately 1.6% of lone star ticks were positive for *E. chaffeensis*. This is significant because this is the first time lone star ticks in Nebraska have been shown to be infected with pathogens. Only one other study analyzed lone star tick adults of Nebraska and found that none of the ticks were positive for *E. chaffeensis* (Anderson et al. 1993). *Ehrlichia chaffeensis* has been reported in 0.4 to 9.8% of lone star ticks tested across the United States (Burket et al. 1998; Wolf et al. 2000; Steiert and Gilfoy 2002). The state with the highest number of adult ticks positive for *E. chaffeensis* was Missouri (Steiert & Gilfoy 2002) and the highest number of human cases was from Oklahoma, Missouri, and Arkansas (Fishbien et al. 1994). Missouri is adjacent to the southeast border of Nebraska where lone star ticks first became apparent in the 1980s (Cortinas and Spomer 2013). It is hypothesized that ticks in Nebraska were either infected ticks when they became established in Nebraska, or uninfected ticks were established in Nebraska and came into contact with infected reservoirs that migrated from Missouri. Nebraska has both the vector and reservoir. White-tailed deer in Nebraska are infected with *E. chaffeensis* (Hotaling unpublished). White-tailed deer lymph node samples were analyzed for *E. chaffeensis*, and 25% (2/8) of deer from Richardson Co. and 50% (3/6) of deer from Pawnee Co. were positive for *E. chaffeensis* (Hotaling unpublished). Counties with *E. chaffeensis*-positive white-tailed deer are also counties where lone star ticks were collected. Richardson Co. contains Indian Cave SP and Kinter's Ford WMA, and Pawnee Co. includes Table Rock WMA. White-tailed deer populations have risen in Nebraska from 5,600 in 1960 to 304,000 in 2010 based on annual harvest totals (Cortinas and

Spomer 2013). Nebraska has a competent vector and reservoir for successful transmission of *E. chaffeensis*. As lone star tick populations are increasing and spreading along with white-tailed deer populations in Nebraska, increased potential of human-tick interations could lead to an increase in *E. chaffeensis* cases in Nebraska.

Table Rock WMA was the only site to have ticks positive for *Rickettsia* spp., *E. chaffeensis*, and *E. ewingii*. Total lone star tick specimens positive at Table Rock WMA for *Rickettsia* spp. was 52% (26/50), *E. chaffeensis* was 8% (4/50), and *E. ewingii* was 2% (1/50). Lone star ticks at Table Rock WMA were coinfected with *Rickettsia* spp. and *E. chaffeensis*, which could have serious consequences if lone star ticks are capable of simultaneous transmission. Coinfection in *Ixodes scapularis* (blacklegged tick) of *Borrelia burgdorferi* (Lyme disease) and *Babesia microti* (human babesiosis) or *Anaplasma phagocytophilum* (human granulocytic anaplasmosis) (Varde et al. 1998; Levin and Fish 2000; Krause et al. 2002; Steiner et al. 2008) has been documented. Coinfected ticks can readily transmit both of the microorganisms efficiently while feeding (Levin and Fish 2000), and the microorganisms can cause more severe cases of illness in humans (Varde et al. 1998) or lead to complications due to misdiagnosis of only one of the pathogens (Krause et al. 2002). Coinfection has been demonstrated in lone star ticks (Mixson et al. 2006; Clay et al. 2006). Mixson et al. (2006) found that the greatest rate of coinfection (6.1%) was with *E. chaffeensis* and *R. amblyommii* in New York. This coincides with coinfection of lone star ticks with *Rickettsia* spp. and *E. chaffeensis* at Table Rock WMA. If lone star ticks are capable of transmitting both pathogens while blood feeding, humans can be at a significant risk of complications due to multiple pathogens (Mixson et al. 2006).

Lone star ticks are established in southeast Nebraska and are infected by*Rickettsia* and *Ehrlichia chaffeensis* and *E. ewingii*. Infected ticks may have been missed because of the ticks being on hosts, trap placement was not correct, or the temperature and humidity was not conducive to questing. Future studies should analyze more adult ticks from the study collection sites, especially from Table Rock, where ticks were coinfected, run specific PCR analysis on ticks positive for *Rickettsia* spp. to determine the exact species, test other life stages, including larval and nymphal life stage, and determine what pathogens are transovarially and transstadially transmitted in Nebraska. Future studies should be conducted in multiple sites in Nebraska especially in the corridor between Omaha and Lincoln where there is a higher population of people to determine pathogens prevalent in populations of lone star ticks. As the lone star tick population spreads this leads to higher chance of encountering lone star ticks and pathogens transmitted.

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TABLES

Table 3.1: Adult ticks selected for analysis by location.

Site	Females	Males	Total
Indian Cave SP	38	38	76
Kinter's Ford WMA	27	26	53
Prairie Pines	19	4	23
Schramm Park SRA	20	15	35
Table Rock WMA	25	25	50
Wilderness Park	11	3	14
Total	140	111	251

Table 3.2: Gene targets, primers, sequences, and references for PCR protocols.

Targeted gene	Primer	Sequence	Reference
Bacteria-wide 16S rDNA	16S+2	5'-TTGGGCAAGAAGACCCTATGAA-3'	Trout et al. 2010
	16S-1	5'-CCGGTCTGAACTCAGATCAAGT-3'	
Rickettsia citrate synthase (gltA) fragment	RpCS.877	5'-GGGGGCCTGCTCACGGCGG-3'	Heise et al. 2010
	RpCS.1258	5'-ATTGCAAAAAGTACAGTGAACA-3'	
Ehrlichia and Anaplasma 16S rDNA	ECB	5'-CGTATTACCGCGGCTGCTGGCA-3'	Heise et al. 2010
	ECC	5'-AGAACGAACGCTGGCGGCAAGCC-3'	
E. chaffeensis 16S rDNA	HE3	5'-TATAGGTACCGTCATTATCTTCCCTAT-3'	Anderson et al. 1992 Heise et al. 2010
	HE1	5'-CAATTGCTTATAACCTTTTGGTTATAAAT-3'	
E. ewingii 16S rDNA	HE3	5'-TATAGGTACCGTCATTATCTTCCCTAT-3'	Heise et al. 2010 Steiert et al. 2002 Yabsley et al. 2002
	EE72	5'-CAATTCCTAAATAGTCTCTGACTATT-3'	

Table 3.3: Adult ticks positive for *Rickettsia* citrate synthase (gltA) fragment.

Site	Females	Males	Total
Indian Cave SP	18 (n: 38)	18 (n: 38)	36 (n: 76)
Kinter's Ford WMA	18 (n: 27)	12 (n: 26)	30 (n: 53)
Prairie Pines	9 (n: 19)	4 (n: 4)	13 (n: 23)
Schramm Park SRA	10 (n: 20)	8 (n: 15)	18 (n: 35)
Table Rock WMA	12 (n: 25)	14 (n: 25)	26 (n: 50)

Wilderness Park	6 (n: 11)	1 (n: 3)	7 (n: 14)
Total	73 (n: 140)	57 (n: 111)	130 (n: 251)

Table 3.4: Proportion of adult ticks positive for *Rickettsia* citrate synthase (gltA) fragment.

Site	Females	Males	Total
Indian Cave SP	0.474	0.474	0.474
Kinter's Ford WMA	0.667	0.462	0.566
Prairie Pines	0.474	1.00	0.565
Schramm Park SRA	0.500	0.533	0.514
Table Rock WMA	0.480	0.560	0.520
Wilderness Park	0.545	0.333	0.500
Total	0.521	0.514	0.518

Table 3.5: Adult ticks positive for *Ehrlichia chaffeensis* 16SrDNA.

Site	Females	Males	Total
Indian Cave SP	0 (n: 38)	0 (n: 38)	0 (n: 76)
Kinter's Ford WMA	0 (n: 27)	0 (n: 26)	0 (n: 53)
Prairie Pines	0 (n: 19)	0 (n: 4)	0 (n: 23)
Schramm Park SRA	0 (n: 20)	0 (n: 15)	0 (n: 35)
Table Rock WMA	3 (n: 25)	1 (n: 25)	4 (n: 50)
Wilderness Park	0 (n: 11)	0 (n: 3)	0 (n: 14)
Total	3 (n: 140)	1 (n: 111)	4 (n: 251)

Table 3.6: Proportion of adult ticks positive for *Ehrlichia chaffeensis* 16SrDNA.

Site	Females	Males	Total
Indian Cave SP	0	0	0
Kinter's Ford WMA	0	0	0
Prairie Pines	0	0	0
Schramm Park SRA	0	0	0
Table Rock WMA	0.12	0.04	0.08
Wilderness Park	0	0	0
Total	0.021	0.009	0.0159

Table 3.7: Adult ticks positive for *Ehrlichia ewingii* 16SrDNA.

Site	Females	Males	Total
Indian Cave SP	1 (n: 38)	1 (n: 38)	2 (n: 76)
Kinter's Ford WMA	0 (n: 27)	0 (n: 26)	0 (n: 53)
Prairie Pines	0 (n: 19)	0 (n: 4)	0 (n: 23)
Schramm Park SRA	1 (n: 20)	0 (n: 15)	1 (n: 35)
Table Rock WMA	1 (n: 25)	0 (n: 25)	1 (n: 50)
Wilderness Park	0 (n: 11)	0 (n: 3)	0 (n: 14)
Total	3 (n: 140)	1 (n: 111)	4 (n: 251)

Table 3.8: Proportion of adult ticks positive for *Ehrlichia ewingii* 16SrDNA.

Site	Females	Males	Total
Indian Cave SP	0.026	0.026	0.0263
Kinter's Ford WMA	0	0	0
Prairie Pines	0	0	0
Schramm Park SRA	0.05	0	0.0286
Table Rock WMA	0.04	0	0.02
Wilderness Park	0	0	0
Total	0.0214	0.009	0.0159

FIGURES

Figure 3.1: Map of collection sites in southeast Nebraska. Sites include Indian Cave State Park, Kinter's Ford Wildlife Management Area, Prairie Pines, Schramm State Recreational Area, Table Rock Wildlife Management Area, and Wilderness Park in respect of two major cities, Lincoln and Omaha, in Nebraska.

Figure 3.2: Carbon dioxide trap at Table Rock Wildlife Management Area.

Figure 3.3: *Rickettsia* spp. in male and female adult lone star ticks.

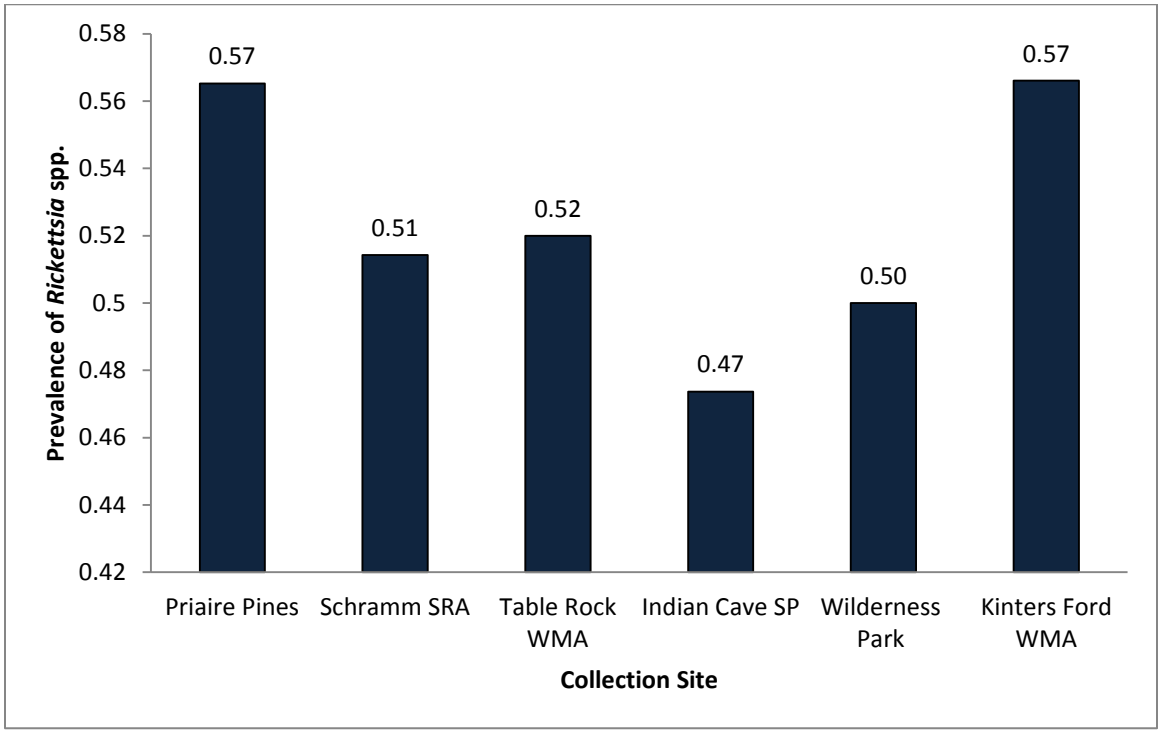

Figure 3.4: Prevalence of *Rickettsia* spp. in lone star ticks by site.

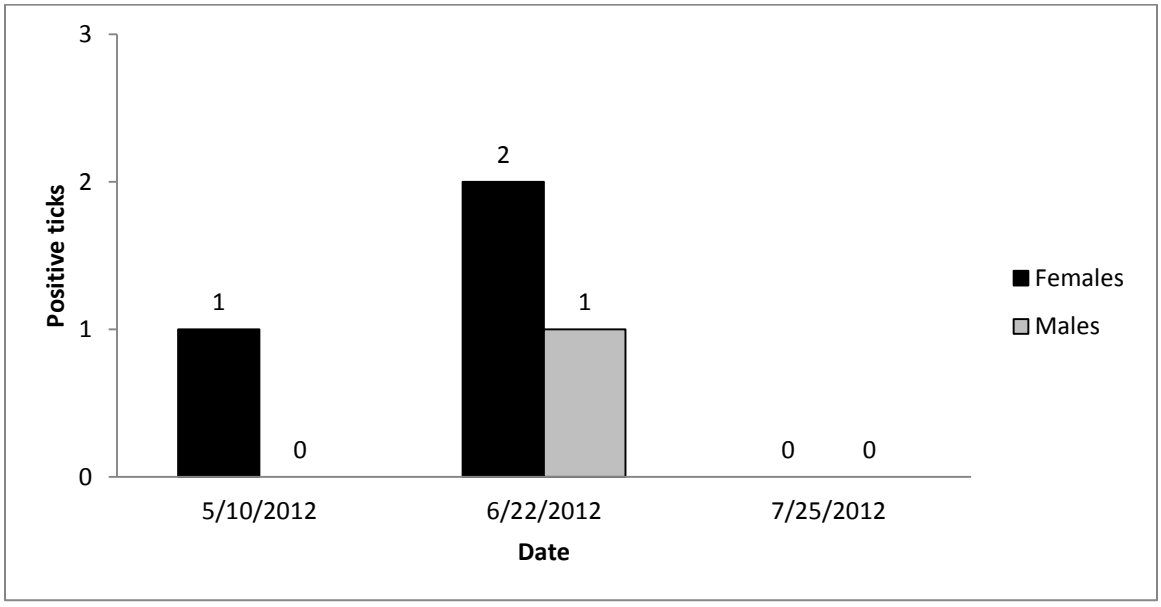

Figure 3.5: Prevalence of *Ehrlichia chaffeensis* in lone star ticks at Table Rock Wildlife Management Area, 2012.

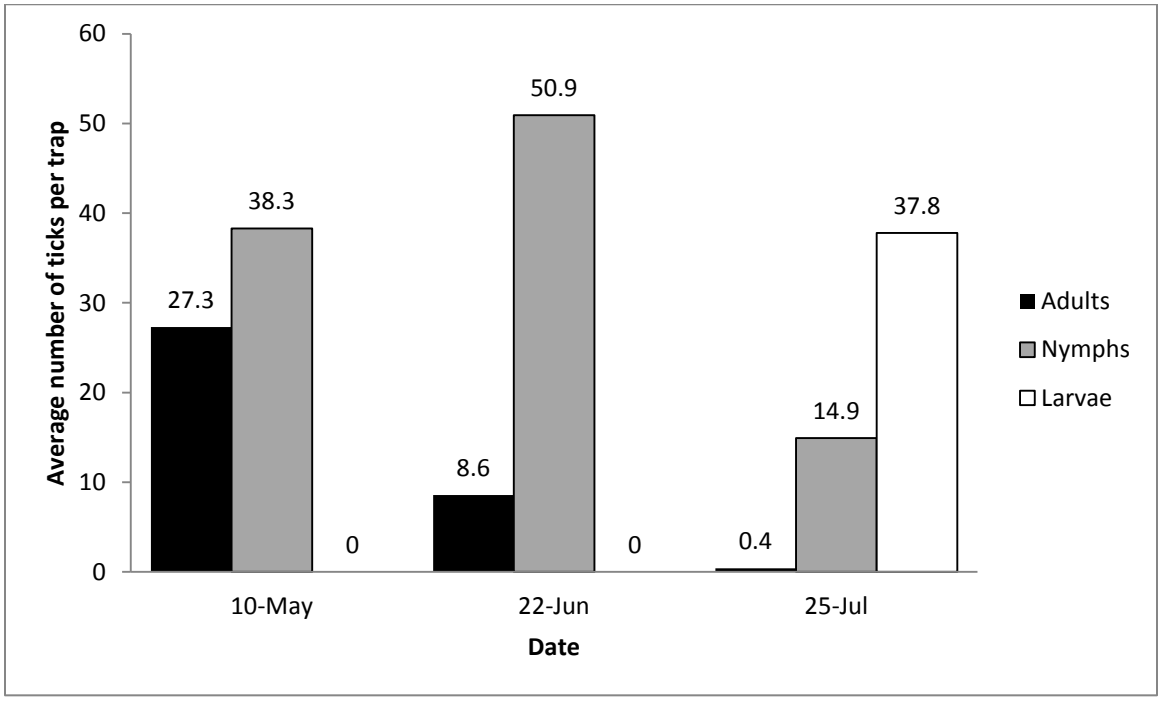

Figure 3.6: *Amblyomma americanum* populations at Table Rock Wildlife Management Area. Adult ticks had a peak in population on 10 May (27.3 ticks/trap) then steadily declined into July. Nymphs had a peak in population on 23 June (50.93 ticks/trap). Larvae were collected beginning 25 July (37.8 ticks/trap).