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PREVALENCE OF TICK- BORNE PATHOGENS IN SMALL MAMMALS AND WHITE-TAILED DEER IN SOUTHEAST NEBRASKA

By

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A THESIS

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PREVALANCE OF TICK- BORNE PATHOGENS IN SMALL MAMMALS AND WHITE-FOOTED DEER IN SOUTHEAST NEBRASKA

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University of Nebraska, 2015

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The prevalence of tick-borne diseases has been increasing in the United States for the past couple decades. Studies have been conducted throughout the US identifying tick-borne disease pathogens as well as their hosts and prevalence. Research was conducted in Nebraska to determine the presence of some tick-borne disease pathogens, their vectors, and their hosts, with emphasis made on *Borrelia* spp., *Rickettsia rickettsii*, and *Ehrlichia chaffeensis*.

Small rodents in southeast Nebraska were trapped and sampled at eight study sites using live capture traps. Captured rodents were assessed for active parasitism by ticks which were collected and placed in alcohol. Tissue samples were then taken and analyzed using PCR for *Rickettsia rickettsii* and *Borrelia* sp. spirochetes. All 504 ticks collected from the 212 various rodents were identified as either larval or nymphal American dog ticks, *Dermacentor variabilis* (Say). Infestation prevalence was 51% (109/212) total with highest prevalence seen in *Peromyscus maniculatus* (58%), *Peromyscus leucopus* (51%) and *Mus musculus* (35%). No evidence of either pathogen was identified by PCR.

Retropharyngeal lymph node samples from across Nebraska were taken from hunter-killed deer during the open rifle season and analyzed for the presence of *Ehrlichia chaffeensis* and *Borrelia* sp. spirochetes by PCR. Lymph node samples from southeast

Nebraska, south-central Nebraska, and mid-central Nebraska were selected for analysis. *Ehrlichia chaffeensis* was identified in lymph node samples from all three study sets. Southeast Nebraska had 10 positive samples (10/49 – 20%), south-central had 14 positive samples (14/50 – 28%) and mid-central had one positive result (1/50 – 2%). The overall prevalence from all localities tested was 17% (25/149). No positive PCR samples were identified containing *Borrelia* sp. DNA.

This study demonstrates that lone star tick-associated pathogens are present in Nebraska wildlife. Transmission of these pathogens is probable as white-tailed deer are reservoirs for some of the pathogens demonstrated in this study. Further epidemiological and ecological studies are warranted as these organisms are of veterinary and public health importance.

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CHAPTER 1

Literature Review

Tick biology Introduction

Ticks are the most common vectors of zoonotic diseases in temperate regions, including Nebraska (Kalluri, Gilruth, Rogers & Szczur, 2007). The incidence of many tick-borne diseases has increased over the past couple decades (CDC, 2013). The risk of acquiring a tick-borne disease depends on a variety of factors such as habitat, climate, presence and density of a tick species, the presence and density of appropriate tick and disease pathogen hosts, and the occurrence of human-tick interactions within tick-infested areas.

In Nebraska, the recent establishment and range expansion of the lone star tick (*Amblyomma americanum* L.*)* is concerning, since the ticks transmit zoonotic diseases, including *Francisella tularensis* (tularemia), *Ehrlichia chaffeensis* (human monocytic ehrlichiosis), and *Ehrlichia ewingii (*ehrlichiosis ewingii*)* (Cortinas & Spomer, 2013). The overpopulation of the whitetail deer (*Odocoileus virginianus* Zimmerman) is thought to be one of the major contributors for the expansion of these ticks, as well as changes in climate and habitat (Paddock, 2007).

The purpose of this study is to assess the presence of lone star tick-borne pathogens in small mammal and deer hosts and determine prevalence of infection with

the lone star tick-borne pathogens in white tailed deer in the south-eastern region Nebraska, where lone star ticks are established.

The transmission of pathogen-based disease is shown in Figure 1. The diagram shows the needed interplay between host, pathogen, environment and vector. In this study, the hosts were small rodents (not in figure) and white-tailed deer. The pathogens associated with these hosts were *E. chaffeensis* (ehrlichiosis), *B. lonestari* (STARI rash), and *Rickettsia rickettsia* (Rocky Mountain spotted fever). The specific habitat was forests in southeast Nebraska and the vectors were the lone star tick and the American dog tick (*Dermacentor variabilis*). Figure 1 depicts the relationship for white-tailed deer, lone star ticks, and *Ehrlichia* sp.

The Vector-- Ticks

Ticks are large ectoparasitic arthropods, belonging to the order Ixodida. They are ovular in shape, dorsoventrally flattened and range in size from 2 or 3 mm to greater than 20 mm (Sonenshine, 2005). Ticks have three anatomical sections which are the capitulum, containing the mouthparts, the body or idiosoma, and the legs. Unlike most mites, ticks possess a hypostome used for piercing a target host to gain access to a blood meal. They also possess a sensory structure on the distal portion of the first pair of legs, the Haller's organ, which is specific to ticks and is used for host detection (Waladde, 1987).

Ticks are split into three families, Ixodidae (hard ticks), Argasidae (soft ticks) and a single species in Nuttalliellidae (Horak, Camicas & Keirans, 2002). There are 867 known species, of which 683 are Ixodids and 183 are Argasids (Horak et al., 2002). The

hard ticks are so called because they have a hard shield-like structure, known as a scutum, on their dorsal side. Common hard ticks found in Nebraska are the American dog tick, the lone star tick, the Rocky Mountain wood tick (*Dermacentor andersoni*), the brown dog tick (*Rhipicephalus sanguineus*), and the winter tick (*Dermacentor albipictus*). The spinose ear tick (*Otobius megnini*) is a soft tick found in western Nebraska (Cortinas & Spomer, 2014)

Ixodid Life History

Ixodid ticks have three life stages and undergo a hemimetabolous development cycle. They are obligate parasites that only feed on blood, which is required for development and reproduction. The life cycle begins with an egg that hatches into a six-legged, sexually indistinct larva. The larva will find a host and feed for one long 3-7 day timeframe (Oliver, 1989). Depending on the species the larva will drop off the host and bury in the litter layer. The larva undergoes diapause and molts into a nymph. Nymphs will seek out a new host, attach, and feed for 4-8 days and will repeat the diapause phase. After the final molt, sexually dimorphic adults emerge and begin host seeking (Oliver, 1989).

Most ticks encountered by people in North America are ixodid ticks (Merten & Durden, 2000). These ticks usually have a three-host life cycle, meaning that each developmental stage must find a different host for feeding. Some ixodids are one or two host ticks, meaning the tick remains on the host for either one or two life stage shifts respectively (Sonenshine, 2005). The winter tick (*D. albipictus*) is an example of a one

host tick. It is believed this adaptation is to prevent possible starvation while host seeking.

Metastriate ticks have an anal grove posterior to the anus and only mate while on host. The prostriate ticks, consisting of only one genus, *Ixodes*, possess an anal groove anterior to the anus and can mate on or off host (Kiszweski, et al., 2001). On-host mating occurs when feeding females release pheromones attracting males in the immediate vicinity. The males stop feeding to locate the female and copulation takes place, while the female continues to feed. Afterwards the male can return to feeding and the female proceeds to increase her feeding rate, swelling up to 100 times her unfed weight (Oliver, 1989; Sonenshine, 2005). The female will then drop off the host and locate a secure place to oviposit. Oviposition can be delayed if the environmental conditions are unfavorable, as during inappropriate seasons. Oviposition lasts several days while the female lays thousands of eggs on the substrate. Upon completion of oviposition, the female dies.

Host Seeking Behaviors

Sonenshine (2005) described two methods of host seeking behaviors possessed by ticks: ambushing and hunting. The ambush strategy refers to ticks crawling up vegetation, such as grass or bushes, and waiting for a potential host to pass by. This method is also called passive host seeking or questing.

Many factors influence questing behaviors of ticks. The further a tick climbs the vegetation, the more exposure it experiences, increasing the risk of desiccation. Many

factors influence the desiccation rate of questing ticks including, air temperature, humidity, sunlight exposure, and wind.

The height from which some tick species quest is life stage dependent. Larvae tend to quest lower to the ground and litter layer, where they are more likely to encounter a proper host, such as small mammals, reptiles, or ground feeding birds. Lower questing heights also decrease the likelihood of desiccation as these areas are more shaded and have higher moisture content (Randolph and Storey, 1999). Nymphs and adults tend to quest higher on the vegetation, where they encounter medium to large mammals or birds. Nymphs and adults are less likely to desiccate (or are more resistant), so they can ascend further.

Questing ticks can remain inactive for many hours provided they are properly hydrated and the environmental conditions are favorable. Cooler temperatures cause higher relative humidity, which can affect questing heights (Vail & Smith, 2002). When humidity decreases, questing ticks begin to retreat to lower heights to seek cooler temperatures and higher humidities in the shade. There, ticks regain lost water by absorbing atmospheric moisture (Knülle & Rudolph, 1982; Sonenshine, 2005) or by secreting a hyperosmotic solution from their salivary gland which is involved in the uptake of atmospheric moisture during nonparasitic phases (Gaede & Knülle, 1997). After rehydration, they will once again, ascend the vegetation to seek out a host.

Host seeking ticks utilize a unique structure to help find hosts, known as the Haller's organ. This highly complex structure is located at the apex of the front tarsi on the front pair of legs and contains a number of sensory sensilla. This structure is used by

the tick to detect chemical sisngals in the air such as ammonia and carbon dioxide (Waladde & Rice, 1982; Steullet & Guerin, 1992; Sonenshine, 2005). Ticks will often wave their front tarsi in the air while questing to detect environmental shifts due to host activities, such as odor production or breathing. Ticks are also highly responsive to other stimuli, like shadows or movement.

When a tick receives the proper stimuli from the passing host, they will extend their front limbs and cling to the host. Once attached to a host, they will seek out a safe location on the body to begin feeding (Nicholson, Sonenshine, Lane & Uilenberg, 2009). Ticks will seek a place that is unable to be groomed, such as below the hair coat, around the face and ears, or in places where two body parts interact frequently such as joints or points of clothing attachment on humans. This is done by odor and heat stimuli (Sonenshine, 2005).

The hunter strategy is a more active method of finding a host. Some species of *Hyalomma* ticks and the African veld-inhabiting bont tick, *Ambylomma hebraeum*, seek out hosts in a more active or hunter-like strategy (Dennis & Piesman, 2005; Sonenshine, 2005). These hunter ticks hide in substrate, often buried in soil or sand to protect themselves from the environment and dehydration. The ticks lie in wait until they receive proper stimuli from a host, upon which they will emerge and rapidly move towards it. Such stimuli include sensing carbon dioxide shifts, odors produced by hosts, or even pheromones produced by attached females seeking a mate (Norval, Sonenshine, Allan & Burridge, 1996; Sonenshine, 2006).

Vector Competence

Transmission of disease causing pathogens is by no means simplistic, nor is it alike for all pathogens. This is especially true for tick-borne disease organisms. Ticks must possess the ability to acquire a pathogen and be able to transmit it to a host. This idea is known as vector competence. For a vector to be considered competent, both internal and external factors must be met.

Internal factors refer to internal physiological factors that control the ability of a vector to transmit an infection agent (Sonenshine and Mather, 1994). Some internal factors include the ability of the tick to acquire a host and bloodmeal, others are passage of microorganism via transtadial or transovarial transmission. An unfed tick is incapable of acquiring a new microorganism, unless that microorganism is capable of transovarial transmission. Pathogen transmission also requires that the pathogen and vector occur in the same habitat. The vector must acquire the pathogen, and in turn, the pathogen must be able to bypass the immune system, refrain from killing the vector upon acquisition, multiply or undergo a life stage change, then be able to exit the vector to infect a new host. Feeding duration plays a role in determining vector competence. Time requirements for pathogen acquisition from the host need to be met otherwise transmission to the vector will not take place. In one study, 36% of *Ixodes scapularis* that were attached to an infected hamster for 24 hours became infected while 68% of the ticks attached for 48 hours became infected (Piesman 1991, Sonenshine and Mather 1994). External vector competence factors not related to the vector, but those relating to tick host, such as population or host immunity, factors relating to the microorganism such as infectivity, and factors related to environment or climate (Sonenshine and Maher, 1994).

Microbial competition can occur in some tick species. This was noted by Burgdorfer et al. in 1981 wherein they noticed that ticks infected with nonpathogenic *Rickettsia peacockii* were refractive to infections with the virulent *R. rickettsii* (Burgdorfer, Hayes, & Mavros, 1981; Parola, Paddock, & Raoult 2005).

The Host—Small Rodents

Types of Hosts

Though ticks feed on a wide variety of vertebrate hosts, the type of host a tick feeds on has an effect on pathogen transmission. A primary host or principal host is a host for which a parasite seems to have a preference for feeding or development. Principal host could also be a host species most commonly encountered by questing ticks. These hosts often act as a source of pathogens for vector transmission. Definitive hosts are the hosts in which a parasite reaches maturity and sexually reproduces if possible. One example is *A. americanum* where large mammals, especially white-tailed deer are the definitive host. Ticks can also act as definitive hosts for microorganisms such as *Babesia microti*, in which *I. scapularis*, is the primary host (Homer, Aguilar-Delfin, Telford, Krause & Persing, 2000). Bacteria and viruses are the most common disease pathogens of ticks.

A secondary or intermediate host only harbors the parasite for a short time during which a developmental change of state takes place within the parasite. One example of a secondary host for a tick-borne parasite is the brown dog tick, *R. sanguineus*. This tick has recently been found to be capable of ingesting dermal microfilariae (*Cercopithifilaria* sp.) while feeding on infected canines (Brianti, Otarto, Dantas-Torres, Weigl, Latrofa,

Gaglio et al., 2012). These microfilariae develop into an infective stage within the ticks which suggests the ticks are secondary hosts to these filarioids, whose primary host are canids.

Hosts that are competent in harboring a pathogen for long periods of time in which the pathogen is able to be taken up by a feeding tick are termed reservoir hosts. Vertebrate reservoir hosts usually do not develop acute disease as a result of pathogen infections (Nicholson et al., 2009). Tick reservoir hosts are also the primary hosts for the pathogen. Reservoir hosts can feed and potentially inoculate multiple vectors which could lead the spread of a pathogen.

When a pathogen enters a host that is not an intermediate or competent reservoir, it is termed a dead-end host. Dead-end hosts are those that do not meet the necessary conditions for pathogen transmission and maintenance and generally prevent disease transmission to a definitive host. Often, the activated immune system of the host will inhibit the pathogen and the host will exhibit some form of morbidity. Humans are dead-end hosts for various tick-borne disease pathogens. Lyme disease is a well-known affliction caused by tick bites accompanied by transmission of *Borrelia burgdorferi* to a human host. There is no evidence of person to person transmission of Lyme disease (CDC, 2013). If a tick were to feed on an infected person, the tick would not uptake the pathogen due to humans being dead-end hosts for the pathogen.

Before a pathogen can be transmitted, it must complete its extrinsic incubation period, or the time between acquisition and transmission of a pathogen to a susceptible host. The ability of a pathogen to maintain an active infection throughout the life cycle of

the tick is called transstadial or “horizontal” transmission. A pathogen must be able to survive while a tick molts from one life stage to the next. Internal tissue changes of a tick are slower than other holometablous insects during transition from one life stage to another, which contributes to the high potential of many ticks to transstadially transmit a pathogen (Hoogstraal, 1980; Sonenshine & Mather, 1994). Another life stage transmission also occurs when an infected female lays her eggs. If a pathogen is capable of being transmitted into the eggs it is considered to be capable of transovarial or “vertical” transmission.

External factors are the requirements a host must meet to acquire and transmit a pathogen. They also include environmental factors that can inhibit ticks. Geographic distribution of the host must overlap with the vector containing the pathogen. Susceptibility of preferred hosts must also be met, which is known as host competence. An example is *Borrelia burgdorferi*, the causative agent of Lyme disease, which is highly associated with black-legged or deer ticks (*I. scapularis*). However, the principal host for adult deer ticks is the white-tailed deer, which is not a reservoir for the bacterium, thus making it an incompetent host (Telford, Mather, Moore, Wilson & Spielman, 1988). The effect of incompetent reservoirs is thought of as a “natural zooprophylaxis,” which is the divergence of disease-harboring vectors from humans to animals (Nicholson et al., 2009). Climate, a long-term condition; and weather, a seasonal variation, create conditions that can increase or decrease the chances for host acquisition by limiting potential host activity. Variations in the microclimate of an area can also affect ticks seeking a host. Many ticks enter diapause during winter months to undergo developmental changes, but also to wait until feeding conditions are more favorable.

Host defenses also play an important role in vector competence. The tick must be able to remain on a host in order to feed. Grooming habits of many hosts can remove ticks attempting to feed (Mooring, McKenzie & Hart, 1996). Once a tick is feeding, the pathogenic agent must be able to bypass the immune system of the host, and reproduce to become transmissible and re-acquired by a subsequent vector (Soneshine, 2005). Both innate immunity and acquired immunity affect competence. Some hosts have the ability to develop a resistance to tick infestations by impairing the process of engorgement (Wikel & Allen, 1976). Other hosts inherit a natural resistance to ticks or possess physical barriers to tick attachment, such as pelt density (Imbelli, Ribeiro, Giglioti, Rigitano, Alencar, Chagas et al., 2012).

Host Specificity

Several factors, like habitat, life stage, and host availability, determine the host range or the range of species that can harbor the parasitic organism. Generally, larval ticks feed on smaller hosts while nymph and adults feed on medium to large hosts respectively likely relating to size of the hypostome. Most ticks in both the argasid and ixodid families have relatively strict host specificities (Nicholson et al., 2009). Examples of these ticks include *Ixodes marxi* which feed solely on squirrels and *Boophilus microplus* which feed only on large ruminants, namely cattle. However, some studies suggest that current tick-host-association patterns are possible ecological specificity artifacts caused by biogeographic distributions and incomplete sampling (Klomplen, Black, Keirans, & Oliver, 1996; Nicholson et al., 2009). Some species of host-specific ticks are less stringent and will feed on a particular class of host, such as birds, but no other vertebrate (Sonenshine & Mather, 1994). Many species also have restricted host

range during a particular life stage. The rabbit tick, *Haemaphysalis leporispalustris* feeds strictly on lagomorphs as an adult but is often found on many ground dwelling birds as larvae and nymphs.

Ticks that exhibit less host specificity are called non-specific or opportunistic feeders. These ticks are found on a wide variety of hosts, ranging from small to large mammals, birds, reptiles and rarely amphibians. Examples of these ticks are the black-legged tick, the lone star tick, the American dog tick, and the brown dog tick. Opportunistic ticks are often associated with disease pathogens of humans.

Habitat

Lone star ticks are associated with forests and forest ecotones. The increase in whitetail deer and wild turkey (*Meleagris gallopavo* L.) populations is thought to be one of the major contributors for the geographic expansion of these ticks, as well as warmer winters (Cortinas and Spomer, 2013). White-tailed deer are capable of traveling long distances and with them, any parasitizing ticks. Wild turkeys are also suspected to be important hosts for lone star ticks (Mock, Applegate, & Fox, 2001). Warmer weather can allow for molting ticks to emerge earlier, or eliminate their need to overwinter, allowing them to potentially mate and oviposit within the same year (Subak, 2009).

Medical and Veterinary Importance

Until the early 20th Century, vector-borne diseases were responsible for more deaths in humans than all other causes combined (Kalluri, Gilruth, Rogers, & Szczur, 2007). Today, malaria is still one of the most common causes of deaths in the world, affecting over 1.2 million persons per year (WHO, 2013). Ticks are the prime vectors of

pathogenic organisms to both wild and domestic animals and second only to mosquitoes in their importance as vectors of human disease agents (Keirans and Durden, 2005). Tick-borne pathogens are also the leading cause of vector-borne disease in temperate regions (Kalluri et al., 2007; Munderloh & Kurtti, 2011). Ticks are capable of transmitting the widest variety of pathogenic agents, including bacteria, rickettsia, viruses, and protozoans (Munderloh, Jauron, & Kurrti, 2005). However, concern is not limited to their role as vectors for disease pathogens, but also their ability to cause direct damage to hosts.

The idea that ticks could be potential vectors of pathogens began in the late 19th Century when mysterious cattle deaths occurred in herds being moved from Texas to the northern states. Dr. Cooper Curtice hypothesized that the cattle tick *Boophilus* (*Rhipicephalus) microplus* was responsible for transmitting Texas fever to cattle. Later, Theobald Smith and his associate Frederick Kilbourne would conduct numerous experiments to determine the cause. They found that cattle were dying from an infectious protozoan (now *Babesia bigemina*) that was being transmitted by *Boophilus* (*Rhipicephalus)* ticks. This was the first time anyone demonstrated the ability of ticks to transmit disease pathogens (Assadian & Stanek, 2002). Identification of Texas cattle fever and the vector responsible for transmission of the disease laid the foundation for future work on tick-borne disease and vector transmission (Munderloh et al., 2005). Within the decade after the findings of Smith and Kilbourne were released, Howard Ricketts determined that Rocky Mountain spotted fever was transmitted by *D. andersoni*. In Europe 1937, the discovery of tick-borne encephalitis led to the production of the first tick-borne encephalitis vaccine (VENICE II, 2009). There are numerous other tick-borne

diseases discovered throughout the years, including Lyme disease, tularemia, babesiosis, human monocytic ehrlichiosis, (HME) human granulocytic anaplasmosis (HGA), Q fever, other spotted fevers, relapsing fever, and even bite associated tick paralysis (Sonenshine and Mather, 1994; Munderloh et al., 2005).

Many biological factors contribute to the capacity of a tick as a vector. Ticks feed on multiple hosts consisting of varied species and demographics. They have relatively long life spans, about 2-3 years, and high reproductive potential with few natural enemies making control of both the tick and the pathogen difficult.

In order to obtain a blood meal, a tick must penetrate the skin of the host before it can feed. This process leaves an external wound on the host. While feeding, a tick will also release anticoagulants, antihistamines, apyrase (which inhibits platelet accumulation) and other enzymes from their salivary glands that facilitate successful blood feeding (Sonenshine, 2005). This is often when a pathogen is introduced into the host. Most viruses and many bacteria replicate in the salivary glands of ixodid ticks. Some viruses, such as Powassan virus, can be transmitted in as little as 15 minutes after tick attachment (Ebel & Kramer, 2004). Other pathogens can reside within the midgut and will be expelled into the host when the tick regurgitates digestive enzymes (Sonenshine, 2005).

Ticks can be also harmful livestock production facilities by causing economic losses. Heavy infestations can decrease production of livestock by causing anemia, reducing feeding habits or reducing milk production. Livestock can suffer from weight loss or even death. The direct damage caused by ticks feeding can also be detrimental. Bites can cause hide damage, damage to the udders, teats, or scrotum. Secondary

infections or secondary infestations like myasis are also possible in the lesions caused by feeding ticks (Ndhlovu, Makaya, & Penzhorn, 2009; Norval, 1983; Meltzer & Norval, 1993; Dreyer, Fourie, & Koch, 1998).

Important Ticks of Nebraska

American Dog Tick, *Dermacentor variabilis* (Say) (Figure 1-2)

The American dog tick is the most commonly encountered tick in Nebraska and is a major pest for people and domestic animals (Rapp, 1960; Cortinas and Spomer, 2013). It is found in all the Atlantic states westward to eastern Montana and Wyoming and southward all the way to west-central Texas (Keirans & Durden, 2005) (See Figure 1-3). Several populations of this tick species are also found in the west coast of the US, from California to southern Oregon. This tick has also been found in areas of southeastern Canada (Nicholson et al., 2009). The teardrop shaped adults are reddish/brown with ornate gray markings on their scutum. The scutum of the female of this species is short, covering half of her dorsal surface while the male scutum covers the dorsum. The palps of adult males and females are short and about the same length as the hypostome, which sit anterior to the rectangular basis capitulum. The papal segment is about as long as it is wide.

The American dog tick is a three-host tick. Although typically found feeding on canines, adults have been found on a variety of medium to large-sized mammals, including humans, as well as birds. Larvae will feed on small mammals such as mice while the nymphs are found on small or medium-sized mammals. Adults are active in the late spring and early summer, larvae are active during late winter and earlier spring

(Nicholson et al., 2009; Cortinas and Spomer, 2013), and nymphs are active during both peaks but tend to have higher numbers during the earlier peak. Typical tick habitat includes disturbed forests, along roads, in brushy areas around homes, and along forest field ecotones (Brown, Lane, & Dennis, 2005). This species is considered to be one of the prime vectors of *R. rickettsii*, the causative agent of Rocky Mountain spotted fever, likely due to its wide geographic distribution (Macaluso & Azad, 2005). The American dog tick is also a known vector of tularemia. American dog ticks have also been shown to vector anaplasmosis (*Anaplasma phagocytophilum*) and possibly *E. chaffeensis*, one of the pathogens responsible for HME, although likely not the most important vector (Holden, Boothby, Anand, & Massung, 2003; Dennis & Piesman, 2005; Nicholson et al., 2009). Aside from being vectors for disease, *D. variabilis* has also been responsible for cases of tick paralysis in humans and pets, mostly in the eastern United States. Much about tick paralysis is still unknown but removal of a feeding tick reverses the effects of the paralysis (McCue, Stone, & Sutton, 1948).

Lone Star Tick, *Amblyomma americanum (*L.) (Figure 1-4)

The distribution of this species is from the Atlantic Coast, stretching from Maine to Florida, and westward into Iowa and Nebraska, and southwards into Texas and northeastern Mexico (Brown et al., 2005) (See Figure 1-5). Within the past 20 years, the lone star tick has made quite a large foothold in Nebraska, primarily within the southeastern region (Cortinas & Spomer, 2013). In areas where lone star ticks are established in Nebraska, populations are larger and in higher density compared to American dog tick populations.

Lone star ticks are round in shape and reddish/brown in color. The female scutum is ornamented with an iridescent spot, while males have small markings along their festoons (small areas divided by grooves along the posterior margin of the tick) but are otherwise not ornamented. The adults have a relatively long hypostome and palps. These ticks have a three-host lifecycle where they primarily feed on large birds and medium to large animals during immature and adult stages. The principal host is the white-tailed deer, upon which all life stages have been found (Mount, Haile, Barnard, & Daniels, 1993; Childs & Paddock, 2003; Brown et al., 2005). This aggressive tick will also readily attack humans and due to their long mouthparts, all life stages can feed on people (Childs & Paddock, 2003). Adults are most active in the spring while the larvae show peak activity during the late summer and early fall. Nymphal ticks occur during both seasons but typically have higher numbers during the spring. These ticks are generally associated with woodlands, namely hardwood forests with young trees and dense brush, often associated with deer (Brown et al., 2005).

Until the 1990's, *A. americanum* was primarily regarded as a nuisance, not a vector for disease. After decades of study, *A. americanum* has been identified as hosting various pathogens including *E.chaffeensis, E. ewingii, F. tularensis, Coxiella burnetti*, Q fever, and *B. lonestari*. Southern tick-associated rash illness presents with an erythema migrans rash, very similar to the rash seen in patients with Lyme disease (caused by *Borellia burgdorferi*). Reports of Lyme disease in areas without the primary vector (*I. scapularis*) are likely misdiagnosis of STARI. It has been shown that *A. americanum* is not a competent vector of *B. burgerdorferi*, but is capable of hosting and transmitting *B. lonestari* (Piesman & Happ, 1997). Varela-Stokes (2007) showed that white-tailed deer

serve as the reservoir for *B. lonestari*. This study also showed that white-tailed deer are major reservoirs for *E. chaffeensis* and *E. ewingii*. Another pathogen associated with lone star ticks is *Rickettsia amblyomii*. The direct implications and role of this pathogen as a causative agent of disease are also under investigation (Apperson et al., 2008, Goddard & Varela-Stokes, 2009, Jiang et al., 2010).

The list of pathogens transmitted by *A. americanum* continues to grow as well. In 2009, two farmers in Missouri were admitted to a hospital for Ehrlichiosis-like symptoms. These men both lived in geographically distant areas and both reported have a tick feed on them. The discovery of a new *Phlebovirus* named Heartland virus was isolated (McMullan, Folk, Kelly, MacNeil, Goldsmith Metcalfe, et al., 2012). Further studies identified the vector as *A. americanum* (Savage, Godsey, Lambert, Panella, Burkhalter, Harmon, et. al, 2013). In 2014, another virus suspected to be transmitted lone star ticks was identified as Bourbon virus. Bourbon virus is a member of the *Thogotovirus* genus and was found in a patient who had several tick bites and an engorged tick removed from his person before developing clinical illness. The patient failed to respond to treatments and eventually died 11 days after onset of illness (Kosoy, Lamber, Hawkison, Pastula, Goldsmith, Hunt, et. al, 2015).

Brown Dog Tick, *Rhipicephalus sanguineus* (Latreille) (Figure 1-6)

These ticks are the most widely distributed tick in the world and are found circumglobally (Kierans & Durden, 2005) (Figure 1-7). Often called the kennel tick, it can complete its entire life indoors and primarily feeds on dogs, which can become heavily infested (Nicholson et al., 2009). Though generally not a parasite of humans, it

has been known to feed on people as well as other domesticated animals. Adults are reddish/brown with no visible markings or ornamentation. Males appear teardrop in shape while females seem more ovular. The hypostome and palps are fairly wide and relatively short. The hexagonal shape of the basis capitulum makes for a key identifying feature. The brown dog tick is a three-host with all life stages capable of feeding on dogs. Immature stages have been found on rodents and small mammals (Dantas-Torres, 2008). Seasonal activity in the wild peaks by mid-summer, however this species can inhabit homes and will thrive year-round if the homes are temperature controlled (Nicholson et al., 2009). Brown dog ticks are a vector of veterinary importance because it transmits various canine pathogens, including *Ehrlichia canis*, which causes canine ehrlichiosis. These ticks have also been linked to canine babesiosis, caused by *Babesia canis* and a small *B. gibsoni*-like species (Nicholson et al., 2009). When a human becomes the accidental host of a brown dog tick, there is some cause for concern since these ticks have been shown to be competent vectors of *Rickettsia conorii*, and *R. rickettsii*, which cause Mediterranean spotted fever and Rocky Mountain spotted fever respectively (Fournier & Raoult, 2005).

Rocky Mountain Wood Tick, *Dermacentor andersoni* (Stiles) (Figure 1-8)

Rocky mountain wood ticks are distributed in western North America, from the Canadian coastal mountain range in British Columbia, southwards to northern Arizona and New Mexico. The lateral distribution stretches from the eastern slopes of the Cascade Mountains to western Nebraska and South Dakota (Keirans & Durden, 2005) (see Figure 1-9). Rocky Mountain wood ticks are reddish/brown in color and appear very similar to American dog tick (*D. variabilis)*. Adult females have a greyish/white scutum

with some markings that covers half of the dorsal surface while adult males possess a larger scutum, covering most of the dorsum, with many grey/white markings and reddish/brown spots. Adults are shaped like teardrops. The mouthparts are also similar to *D. variabilis* in that they are short and stubby. The basis capitulum is also rectangular. In the wild, these three-host ticks will feed typically on small mammals as larvae, small- to medium-size mammals as nymphs and medium- to large-size mammals as adults. Common hosts include mice, ground squirrels, chipmunks and woodrats as immatures, and deer and carnivores (Brown et al., 2005). The Rocky Mountain wood tick is also an important pest attacking pets, livestock, and humans. They are commonly found in a variety of wooded and brushy habitats in the Rocky Mountains with elevations above 1000 meters to over 3000 m (Brown et al., 2005). Adult activity peaks in the spring and early summer while larvae appear during mid to late summer. These ticks are a vector of Rocky Mountain spotted fever (*R. rickettsii)*, as well as tularemia. They have also been found to transmit Colorado tick fever, a *Coltivirus*, and anaplasmosis (*Anaplasma marginale*) (Nicholson et al., 2009). They can also cause tick paralysis while feeding on humans as well as livestock.

Other Ticks

Blacklegged or Deer Tick, *Ixodes scapularis* (Say) (Figure 1-10)

Blacklegged ticks are found in the northeastern United States, along the eastern coast states down to the southeastern United States. These ticks are also found in the northern Midwest states around the Great Lakes and in the southern Midwest states extending from the Gulf Coast. These ticks are small (about the size of a sesame seed)

with a dark brown dorsum and black legs, mouthparts and scutum. Blacklegged ticks are widely known for transmitting Lyme disease among other pathogens (Aliota, Dupuis, Wilxzek, Peters, Ostfeld & Kramer, 2014). These ticks exhibit a three-host lifecycle similar to other three-host ticks. While not established in Nebraska at this time, it is important to understand the contribution of this species to disease causing pathogen transmission.

Winter or Moose Tick, *Dermacentor albipictus* (Packard) (Figure 1-12)

Winter ticks are distributed nation-wide including Canada and northern Mexico (Bishopp & Trembley, 1945). This species of tick are a one-host tick, meaning all three life stages, including feeding, molting, and mating take place on the same host. These ticks only leave the host to deposit their eggs in the substrate. These ticks are similar in appearance to other Dermacentor ticks with a variation in the markings and coloration pattern found on the scutum. Common hosts include larger domestic and wild animals, including deer, moose, horse and cattle (Bishopp & Trembley, 1945). Winter ticks are so named because they are one of the few species that are active during the fall and winter months. Their role as vectors for disease pathogens is limited and was not assessed in this study.

Co-infections

The ability of a tick to transmit a pathogen might not be limited to one pathogen per feeding. Co-infections can occur when a tick is the host of multiple infectious agents simultaneously. This is a cause for concern because hosts, like humans, could contract multiple infections from a single tick bite (Belongia, 2002). The most common example

of co-infection is that of *B. burgdorferi* (Lyme disease) and *A. phagocytophilum* (anaplasmosis) in black-legged ticks (Swanson, Neitzel, Reed, & Belongia, 2006). However, co-infections of *B. burgdorferi* and *B. microti,* as well as *B. microti* and *A. phagocytophilum* can occur. Co-infections of all three pathogens are less frequent but have been found (Swanson et al., 2006). Co-infections are also found in other tick species. Various combinations of *B. lonestari, E. chaffeensis, E. ewingii,* and *R. amblyommii* have been found in 87 ticks (*A. americanum*) across six states (Mixson, Campbell, Gill, Reichard, Schulze, & Dasch 2006). Co-infecting pathogens might alter the efficiency of transmission, cause cooperative or competitive pathogen interactions, and alter disease severity among hosts (Swanson et al., 2006).

Tick Prevention and Control

Modern American society espouses the idea of living closer to nature and maintaining the natural or original settings while simultaneously indulging in recreational activities that place them within the natural habitats of ticks (Munderloh & Kurtti, 2011). As humans encroach upon tick habitats, increased risk of accidental parasitism occurs. A good example of this is the increased cases of Lyme disease in residential areas in or near naturally wooded areas (Barbour & Fish 1993; Linard, Lamarque, Heyman, Ducoffre, Luyasu, Tersago et al., 2007; Munderloh & Kurrti, 2011). This being the case, prevention of tick bites becomes increasingly more important. There are a number of methods for preventing tick bites, often broken into two categories, physical barriers and chemical barriers. The physical barriers refer to objects worn or actions taken to prevent ticks from attaching to a host. Examples include wearing long pants and sleeves, tucking one’s shirt into his or her pants, tucking pants into socks, and wearing hats.

Avoiding tick habitats is another barrier to prevent tick bites. Physical alteration of the environment, such as removal of brush and trees, is a form of physical barrier. Chemical barriers are solutions applied to an area or oneself to prevent tick feeding. Examples are acaricides (insecticides specific for mites and ticks) applied to brush, repellents such as DEET or permethrin, and prevention products for pets like Frontline® flea and tick control (Merial Company, Duluth, GA) or K9 Advantix® II dog flea and tick drops (Bayer Healthcare LLC, Shawnee Mission, KS). Much research has also been conducted in efforts to lower tick burdens on hosts (Ostfeld, Miller, & Hazler, 1996; Brunner & Ostfeld, 2008). When *B. (Rhipicephalus) microplus* was determined to be the vector of Texas cattle fever, the animals were treated with immense amount of acaricides applied to the body of the cattle. Such treatment methods were considered expensive, wasteful and potentially dangerous. With new technologies, safer and alternative methods have been developed like use of pyrethroids and avermectins, and using tick pheromones as a source of tick control (Sonenshine, 2006).

Another means of control is the use of natural enemies to ticks as a form of control. This is called biological control. This form of control is thought to be more environmentally friendly as it does not require the use of harsh chemical agents. One form of biological control is deemed “classical biocontrol” and is defined by the use of non-native predators or parasites being introduced to an area in attempts to quell a pest species (Ostfeld, Price, Hornbostel, Benjamin and Keesing, 2006). Some examples of biocontrol agents include fungi, parasitoids, birds, bacteria, viruses, and nematodes (Samish, Ginsberg, and Glazer, 2004). This form of control is difficult to use on a large

scale and requires direct interaction between the control agent and ticks and would be difficult to produce or institute in residential areas bordering tick habitats.

One form of control comes from changing the habitat or environment making the area less favorable for tick survival or tick-host interactions. Environmental modification can be accomplished by reducing the litter layer, preventing a favorable hiding and egg-laying environment for ticks. It could also be removal or routine maintenance of grassy fields. Placing a wooded or paved area at the edge of forests also creates an environment that is unfavorable for ticks. Another environmental control method is the removal of any or all potential hosts from an area. Trapping and relocating ticks hosts, or setting up barriers to prevent ticks host from accessing a certain area can help decrease tick-human interaction. This method of control is useful for a personal home or residential areas bordering on forested areas, but has its limitations when considering use for large tick-host areas or woodlands.

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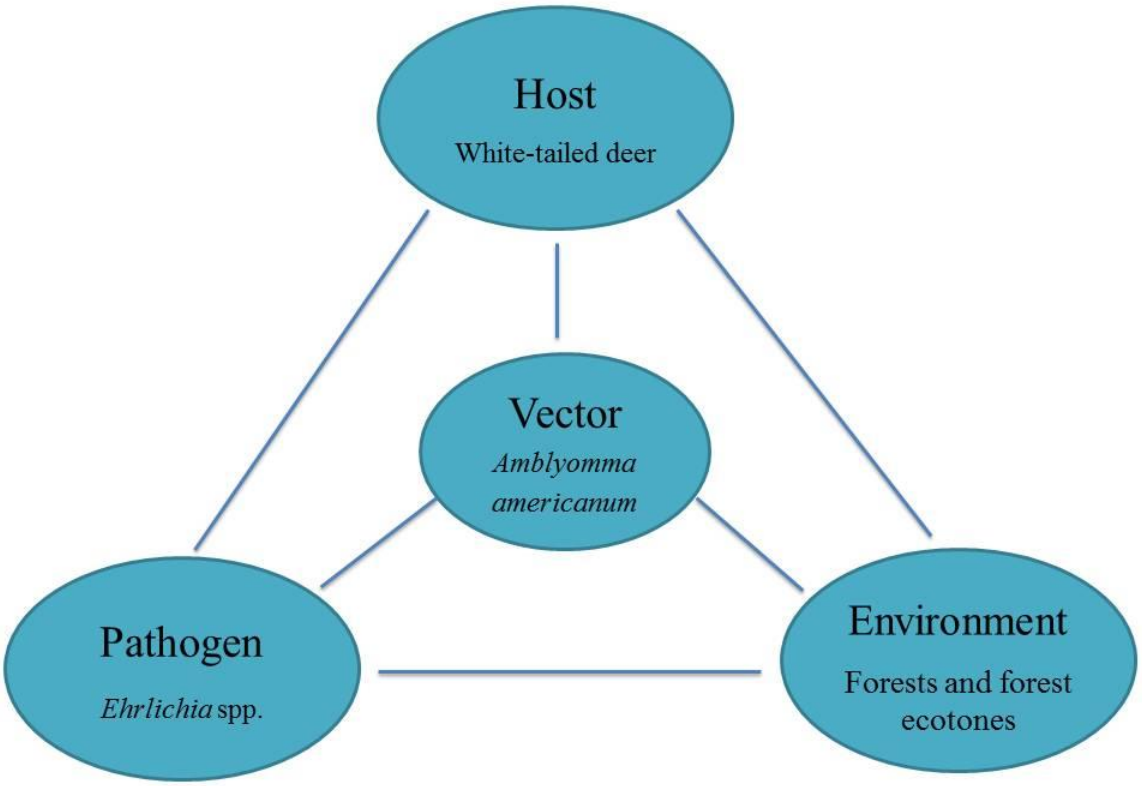

Figure 1-1: Epidemiological Model for *Amblyomma americanum*, White-tailed Deer, and *Ehrlichia* sp.

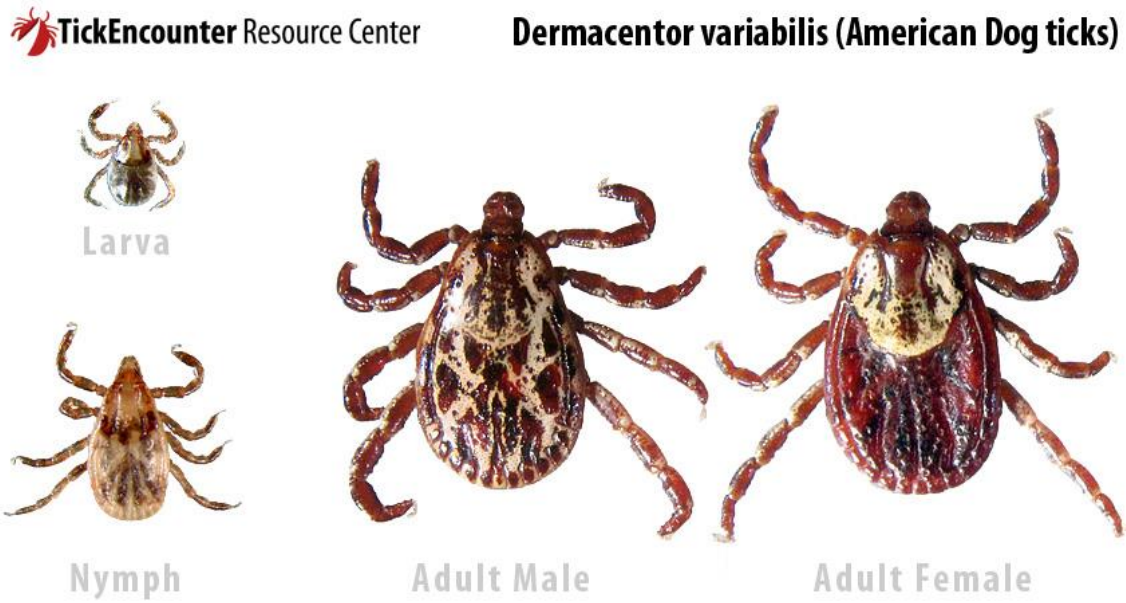

Figure 1-2: Life Stages of the American Dog Tick (*Dermacentor variabilis*)
From: http://www.tickencounter.org/tick_identification/dog_tick#top

Figure 1-3: Geographic Distribution of the American Dog Tick (*Dermacentor variabilis*)
From: http://www.cdc.gov/ticks/maps/american_dog_tick.html

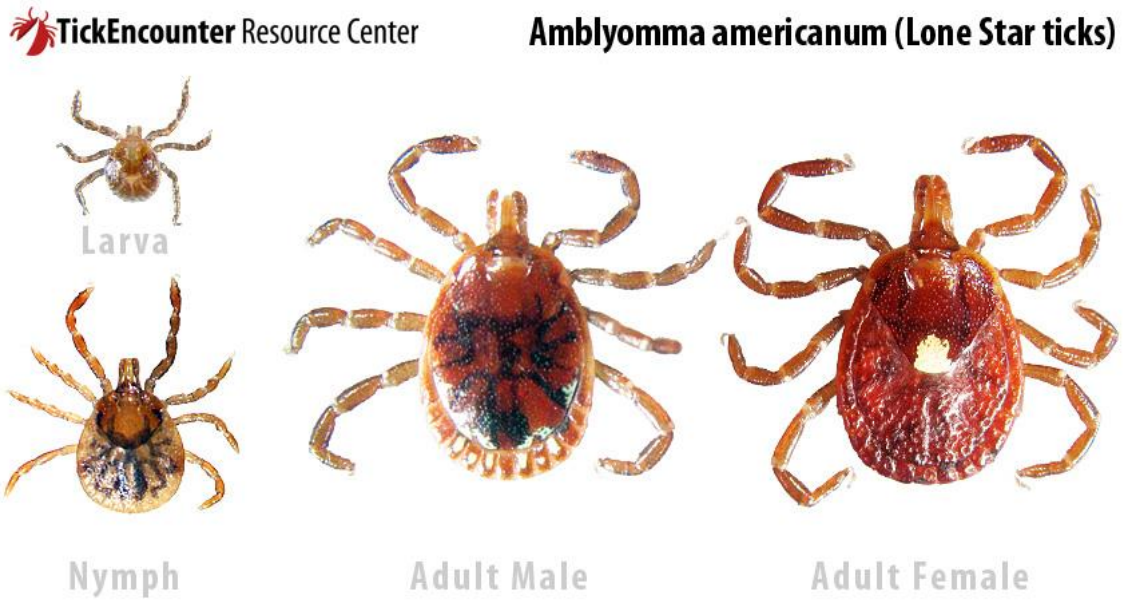

Figure 1-4: Life Stages of the Lone Star Tick (*Amblyomma americanum*)
From: http://www.tickencounter.org/tick_identification/lone_star_tick#top

Figure 1-5: Geographic Distribution of the Lone Star Tick (*Amblyomma americanum*)
From: http://www.cdc.gov/ticks/maps/lone_star_tick.html

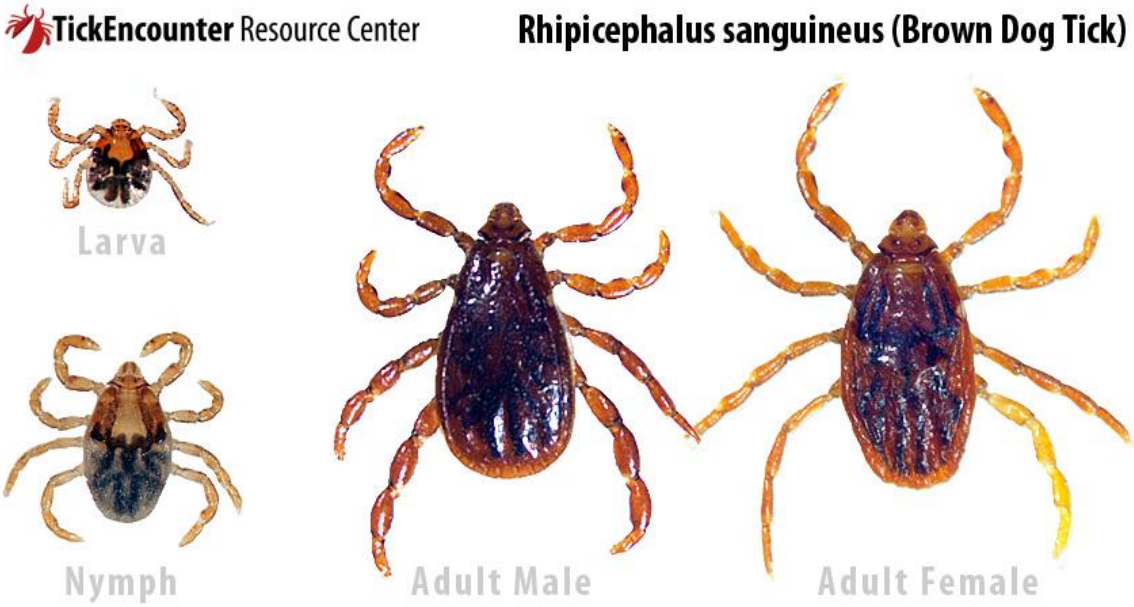

Figure 1-6: Life Stages of the Brown Dog Tick (*Rhipicephalus sanguineus*)
From: http://www.tickencounter.org/tick_identification/brown_dog_tick#top

Figure 1-7: Geographic Distribution of the Brown Dog Tick (*Rhipicephalus sanguineus*)
From: http://www.cdc.gov/ticks/maps/brown_dog_tick.html

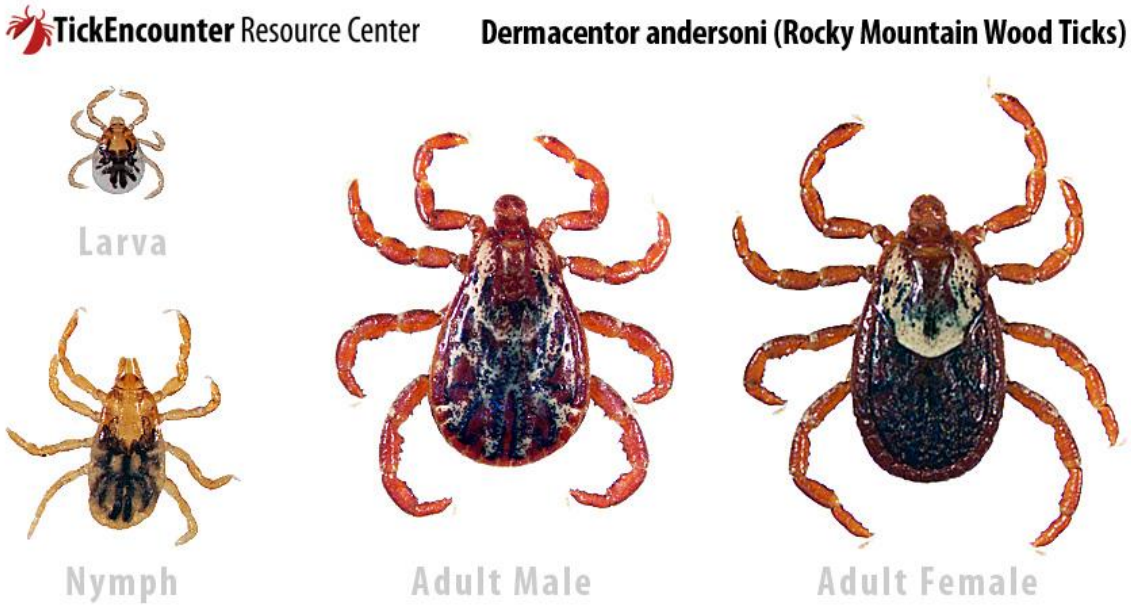

Figure 1-8: Life Stages of the Rocky Mountain Wood Tick (*Dermacentor andersoni)*
From:
http://www.tickencounter.org/tick_identification/rocky_mountain_wood_tick#top

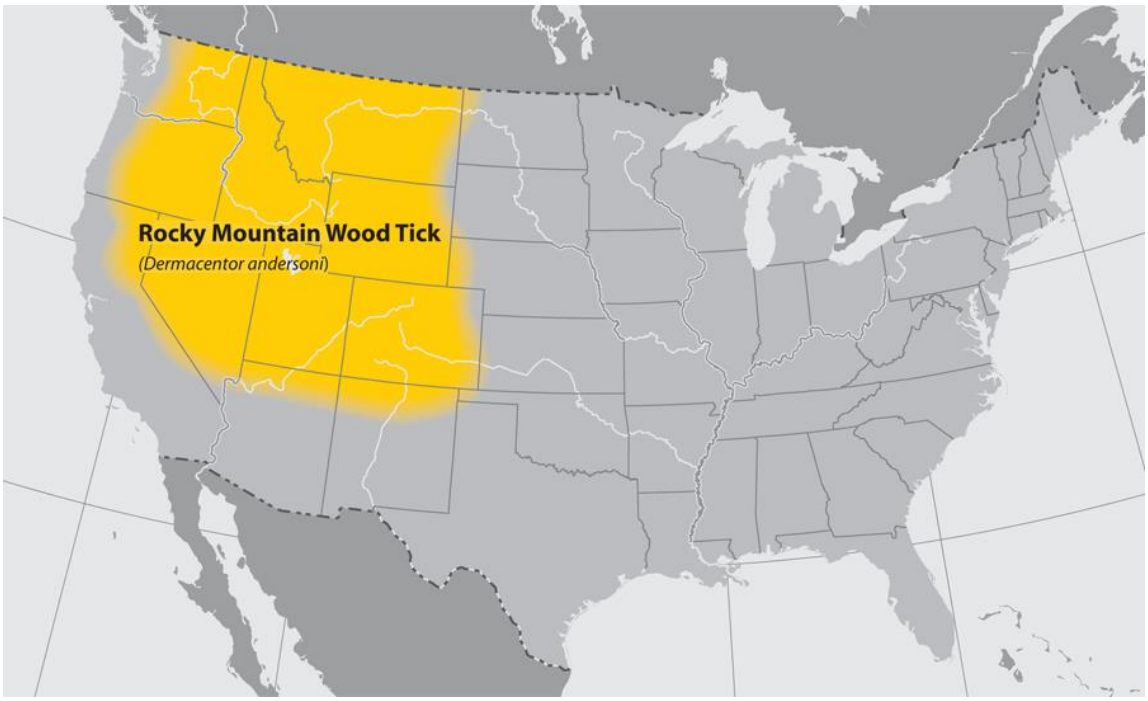

Figure 1-9: Geographic distribution of the Rocky Mountain Wood Tick (*Dermacentor andersoni*)
From: http://www.cdc.gov/ticks/maps/rocky_mountain_wood_tick.html

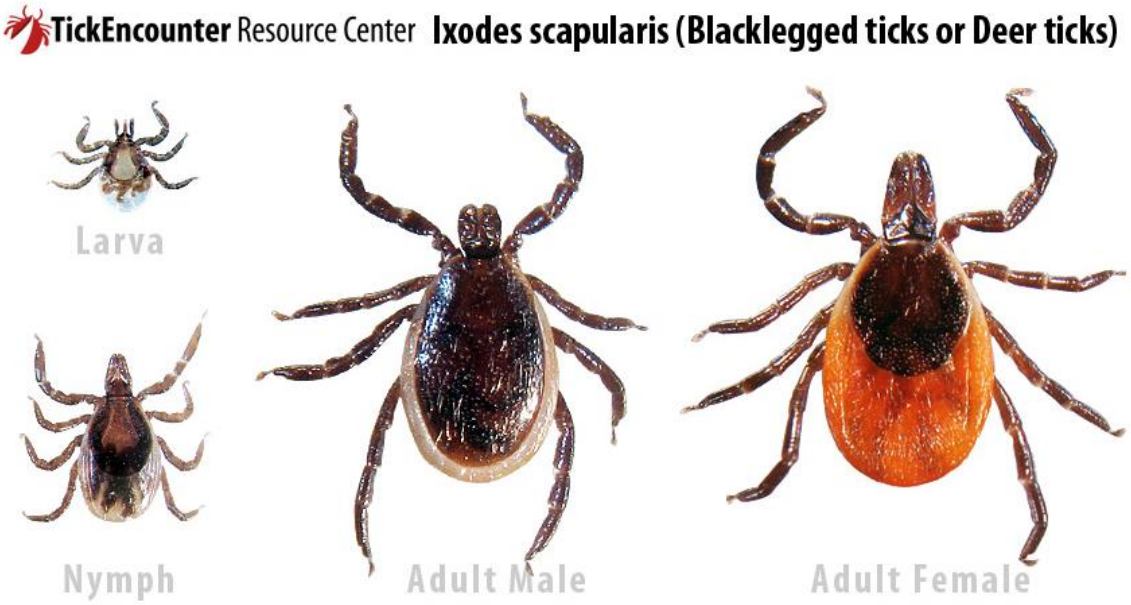

Figure 1-10: Life stages of the Blacklegged or Deer tick (*Ixodes scapularis)*
From: http://www.tickencounter.org/tick_identification/deer_tick

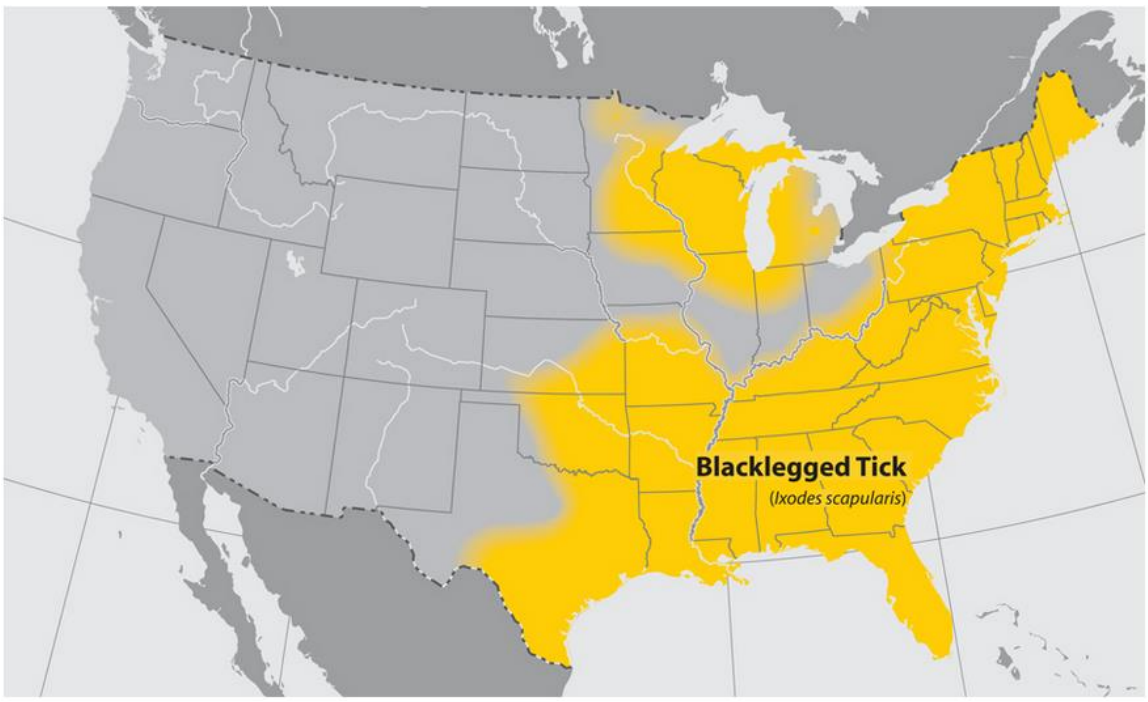

Figure 1-11: Geographic Distribution of the Blacklegged or Deer Tick (*Ixodes scapularis)*
From: http://www.cdc.gov/ticks/images/geo/lgmap-blacklegged_tick.jpg

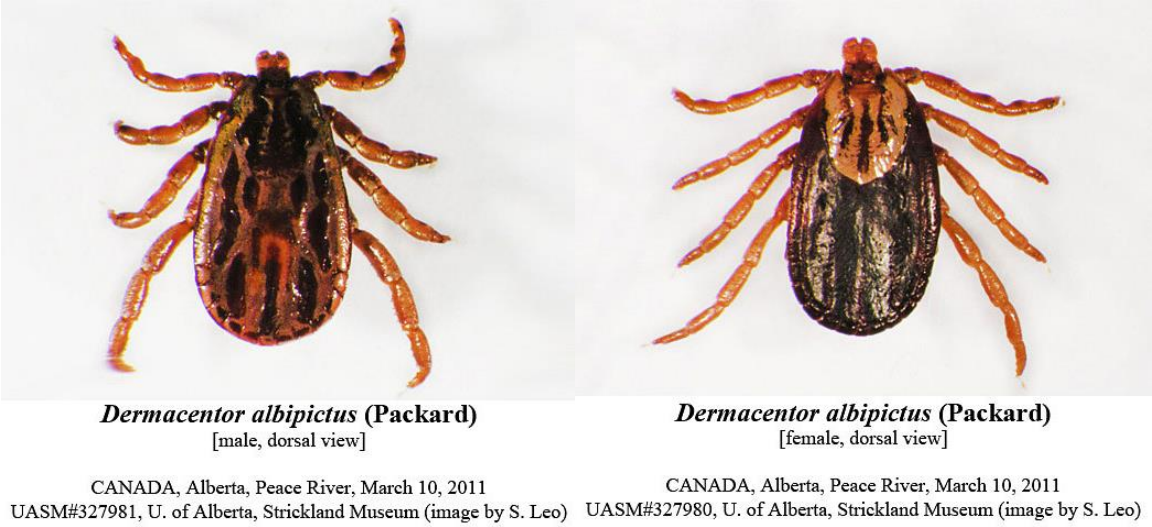

Figure 1-12: Adult Male and Female Winter Ticks (*Dermacentor albipictus)*
From:
http://www.entomology.ualberta.ca/searching_species_details.php?b=Acari&c=7&s=31506

Figure 1-13: Nation-wide Geographic Distribution of the Winter Tick (*Dermacentor albipictus*)
From: http://tickapp.tamu.edu/maps/DA_distribution_map.jpg

Chapter 2

Role of Small Mammals as Tick (Acari: Ixodidae) Hosts and Pathogen Reservoirs in Southeast Nebraska

Introduction

In the US the incidence of human tick-borne disease cases has been increasing (CDC, 2013). In the Midwest, important zoonotic tick-borne infections including, but not limited to, Rocky Mountain spotted fever (*Rickettia rickettsii*) (RMSF), human monocytic ehrlichiosis (HME; *Ehrlichia chaffeensis*) and Lyme disease (*Borrelia burgdoreri*), have been increasing. Rocky Mountain spotted fever cases in the United States rose dramatically from 343 cases in 1993 to 2,553 in 2008 (Centers for Disease Control, 2013). In 2010, the incidence in Nebraska was calculated at 1.5-19 cases per million persons, which is a moderately high rate (Figure 2-1 and 2-2).

There has been a similar increase in the incidence of human monocytic ehrlichiosis. From 2000 to 2008, when cases of ehrlichiosis became reportable, the number of cases rose from 200 to 961 cases (see Figure 2-3 and 2-4). In Nebraska, annual incidence ranged from 1-3.3 cases per million persons in 2010. In nearby states, the highest incidence rates of the disease are found in Missouri, Oklahoma and Arkansas (Centers for Disease Control, 2013).

Lyme disease is a serious tick-borne disease caused by *B. burgdorferi* and transmitted by blacklegged ticks. While the tick and the disease are not known to be established in Nebraska, its presence in nearby states and the potential of permanent

neurological damage makes it an important disease to monitor for public health. A map of the reported Lyme disease cases is seen in Figure 2-5. Figure 2-6 shows the lowest number of Lyme disease cases reported (1995) was 11,700 and the highest number of confirmed cases was in 2009 with 29,959. In 2013 there was an incidence of 0.4 cases per 100,000 persons in Nebraska (Centers for Disease Control, 2013). Even though the data suggest that cases of Lyme disease are rare in Nebraska, the seriousness of the disease merits the identification of potential vectors and hosts within Nebraska. As a result, the identification of tick-borne disease foci and assessment of tick exposure risks have become paramount in the region.

Extensive tick-host surveys in Nebraska had not been conducted since the late 1950's and 1960's (Rapp, 1957; 1960). In these studies, the American dog tick (*Dermacentor variabilis*) (Say) was well established in Nebraska. Today, the American dog tick distribution ranges from the east coast of the United States to portions of the Great Plains, including almost all of Nebraska (see Figure 1-3) (Centers for Disease Control, 2010).

American dog ticks are a vector for RMSF (Azad & Beard, 1998) and HME (Holden, Boothby, Anand & Massung, 2003). Other infectious pathogens transmitted by *D. variabilis* include tularemia (*Francisella tularensis*) (Salbia, Harmston, Diamond, Zymet, Goldenbe & Chin, 1966); tick-borne ehrlichiosis (*Ehrlichia ewingii*) in humans (Buller, Arens, Hmiel, Paddock, Sumner, Rikihisa, et al., 1990) and canine ehrlichiosis (*E. ewingii and Ehrlichia canis)* (Steiert & Gilfroy, 2002; Johnson Ewing, Barker, Fox, Crow & Kocan, 1998).

Since the late 1980's, the lone star tick (*Amblyomma americanum* (L.)) has become the second most-frequently and second most-widely reported tick in Nebraska (Cortinas & Spomer, 2013). The lone star tick is the principal vector of ehrlichiosis (Anderson, Sims, Olson, Childs, Piesman, Happ, et al, 1993; Ewing, Dawson, Kocan, Barker, Warner, Paniciera, et al, 1995; Lockhart, Davidson, Stallknecht, Dawson, Little el al., 1997). The lone star tick can also transmit the agents of human granulocytic anaplasmosis (*Anaplasma phagocytophilum*) (Schulze, Jordan, Schulze, Mixson, & Papero, 2005), tularemia (*Francisella tularensis)* (Calhoun, 1954), tick-borne ehrlichiosis in humans (*Ehrlichia chaffeensis*) (Buller, Arens, Hmiel, Paddock, Sumner, Rikihisa. 1990) and canine ehrlichiosis (*Ehrlichia ewingii*) (Anziani, Ewing, & Barker, 1999). Another bacterium speculated to be transmitted by lone star ticks is *Coxiella burnetti* (Q fever); however, researchers have shown that lone star ticks host a symbiotic species of *Coxiella* that does not cause Q fever (Heise, Elshahed, & Little, 2010). Lone star ticks are also infected with a spirochete, *Borrelia lonestari*. The spirochete was thought to be the agent of southern tick-associated rash illness (STARI) (Masters, Grigery & Masters, 2008) but recent studies suggest that the spirochete is not pathogenic to humans (Stromdahl, Nadolny, Gibbons, Auckland, Vince, Etkins et al., 2015). Recent studies (Yans, 2011; Cortinas & Spomer, 2013, 2014) have shown that the *A. americanum* have become well established in the southeastern region of Nebraska.

The blacklegged tick, or deer tick, is the principal vector of *B. burgdorferi* (Lyme disease) (Daniels, Fish, Levine, Greco, Eaton & Padgett, 1993). *Ixodes scapularis* is also a vector of *Anaplasma phagocytophilum* (human granulocytic anaplasmosis), *Babesia microti* (causative agent of human babesiosis) and Powassan virus or deer tick virus

(Aliota, Dupuis, Wilczek, Peters, Ostfeld & Kramer, 2014). Currently, blacklegged ticks are not established in Nebraska, however, it is speculated that with the migrating habits and of white-tailed deer, birds and other tick hosts, there is a possibility of this tick becoming established within Nebraska.

White-footed mice (*Peromyscus leucopus* (Rafinesque) are competent reservoirs for both *B. burgdorferi* (Donahue, Piesman, Speilman, 1987; Mather, 1993) and *R. rickettsii* (McDade & Newhouse, 1986). *Peromyscus maniculatus* (Wagner) (deer mouse) is a relative of the white-footed mouse and has also been demonstrated as a competent reservoir for *B. burgdorferi* and *R. rickettsii* (Burkot, Clover, Happ, DeBess & Maupin, 1999; Adjemian, Adjemian, Foley, Chomel, Kasten & Foley, 2008).

Due to the paucity of information on the diversity of reservoirs for tick borne disease pathogens in Nebraska, this study was designed to determine (1) the diversity and prevalence of ticks on small mammals and (2) the current reservoirs for tick- borne disease in South Eastern Nebraska. Procedures for this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Nebraska in Lincoln, approval number 614.

Materials and Methods

Study Sites

The study took place from June to August 2011 in eight southeast Nebraska locations (Figure 2-7 A-H). All sites are in the distribution range of *D. variabilis* and *A. americanum* (Cortinas and Spomer, 2013).

Schramm Park State Recreation Area (SPSRA, Lat: 40.02403°, Lon: -96.25092°), located in Sarpy County Nebraska, is a 330-acre public park containing a figure-eight shaped trail, approximately 3 mi in length, through forest covered hills. Tree cover contains a mixture of hardwoods and evergreens. The majority of the park consists of oak trees, nettles, Virginia creeper (*Parthenocissus quinquefolia*), black raspberries (*Rubus occidentalis*), eastern red cedar (*Juniperus virginiana*), fir (*Abies* sp.), poison ivy (*Toxicodendron radicans*), pin oak (*Quercus palustris*) and ash (*Franxinus americana*) (Maegli, 2013). Traps were placed along the trail system (Figure 2-7A).

Eugene T. Mahoney State Park (MSP, Lat: 41.02601°, Lon: -96.31223°), located in Cass County, is a 690-acre, state-managed recreation area. The park consists of two areas, one containing public attractions, buildings, and lakes, and the other is a restricted large evergreen and hard-wooded area adjacent to the park used specifically for ground maintenance, research, and special deer hunts to help control local populations of white-tailed deer (*Odocoileus virginianus* (Zimmerman)). The woodlands consist mostly of oak, hickory and cottonwood trees bordering tall grass prairies. Traps were placed in the restricted area to avoid manipulation or damage (Figure 2-7B).

Platte River State Park (PRSP, Lat: 40.9925°, Lon: -96.210833°), located in Cass County, contains 519-acres of highly forested terrain. The park contains an elaborate trail system covering a large rugged area. The woodlands consist mostly of oak, hickory and cottonwood trees bordering tall grass prairies. The habitat is similar to that found at Mahoney State Park. Traps were placed following the trail systems (Figure 2-7C)

Prairie Pines (Lat: 40.8484°, Lon: -96.5651°) is a 145-acre plot of land located in Lancaster County that is owned by the University of Nebraska Foundation and managed by the University of Nebraska-Lincoln School of Natural Resources. The area contains a mixed tree woodland, prairie fields, and evergreen plantations. Shelterbelts at Prairie Pines are adjacent to native grass fields that have minimal management practices (Yans, 2011). 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). The site is designated for research and education. Traps were placed following wooded area boundary lines, windbreaks, and evergreen plantations lines (Figure 2-7D)

The Agriculture Research and Development Center (ARDC, Lat: 41.155443°, Lon: -96.491005°), is a 9,663-acre research and education facility of the University of Nebraska-Lincoln in Saunders County. Most of the land use is in row crops and cool and warm season pastures. The facility also houses over 6,000 research animals. Traps were placed along large shelterbelts used for impact studies on crop yields. Shelterbelts were composed of three types of plantings; eastern red cedar (*Juniperus virginiana*); eastern red cedar and Austrian pine (*Pinus nigra*); eastern red cedar, Austrian pine, and green ash (*Fraxinus pennsylvanica*) (Yans, 2011). Multiple ticks have been found within these

shelterbelts (Yans, 2011). Traps were placed in several different shelterbelts throughout the site in an effort to limit site bias (Figure 2-7E).

Indian Cave State Park (ICSP, Lat: 40.262106°, Lon: -96.56715°), is a state managed park stretching 3,052-acres. The park is situated in Nemaha and Richardson counties and borders the Missouri River. The park contains a vast mixture of deciduous and coniferous trees, wetlands, and marsh areas covering multiple trail systems habitat types. The dominant plants found at the park include 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*) (Farrar 2013; National Audubon Society 2014; NGPC 2013). The traps were placed on trails (Figure 2-7F).

Table Rock State Wildlife Management Area (TRWMA, Lat: 40.183857°, Lon: -96.061835°), is approximately 420-acres of protected land near Table Rockin Pawnee County. The area is covered by wooded stretches of oak and hickory trees, a small area of warm season prairie fields, crop grounds and annual weeds following the Nemaha River. Traps were placed following the edge of the wooded areas adjacent to prairie fields (Figure 2-7G)

Rock Creek Station State Historical Park (RCS, Lat: 40.11167°, Lon: -97.06528°), is a 350-acre historical location in Jefferson County. The area consists of

open warm season prairie hills and wooded lowlands following creek beds. A forested area consisting of oak, walnut, maple, ash, and eastern red cedar covers a large portion of the park. Traps were placed following a few trails extending across the park grounds (Figure 2-7H).

Trapping Methodology and Data Collection

Approximately 200 Sherman live capture traps (5 x 6.5 x 23 cm) (H. B. Sherman Traps, Jackson, MS) were placed in transects following tree lines or trails at each location. Traps were placed about 20 m apart. Transects were used over grids because they have been shown to result in higher species diversity and they cover a larger sampling area (Read, Malafant, & Myers, 1988; Pearson & Ruggiero, 2003). All transect or travel lines were assigned an alphabet letter at each location. Traps were placed in shaded areas to keep captured animals cool and to help conceal the trap. Any naturally occurring small animal trails were prioritized for trap placement at each interval. Multiple trail sites and trap lines were chosen to cover the widest area and to place as close to 200 traps as possible.

Traps were placed in the late afternoon and early evening. Each trap was baited with small portions of peanut butter sandwiches. The traps were left open overnight and were retrieved the following morning, leaving a maximum time in trap for each animal less than 24 hours. Unsuccessful traps were emptied of any remaining bait and stowed while successful traps were labeled with trap line and number in the line. A rough visual identification of the trap area was taken for release efforts. Animals were released near the captured points.

Up to three locations were visited every week and each location was visited every three weeks. Each site was visited twice during the course of the study except Prairie Pines, which was visited three times. Once traps and data were collected, the research team traveled to the next location in the study where unused traps were baited and placed. Traps were only used once a week in efforts to eliminate possible residual smells / stress pheromones and infectious disease transmission from a previous capture. Traps were cleaned with soapy water and diluted bleach before reuse.

Once all traps were recovered, rodent data were collected. Each rodent was placed into a re-sealable, plastic zipper storage bag connected to a hanging scale. The weight was recorded, correcting for the weight of the storage bag and any debris. After the rodents were weighed, they were placed into a field use inhalation narcosis chamber (Bel-Air Products, Wayne NJ). Approximately 5ml of 20% isoflurane in propylene glycol was added via a funnel system to cotton balls placed at the bottom of the narcosis chamber. The anesthesia mixture allowed for greater manipulation of induction rate as well as decreased recovery times (Itah, Gittleman & Davis, 2004). A built-in platform separated the rodents from direct contact with the anesthesia mixture and a side-mounted stopcock allowed fresh air to enter the chamber.

All animals were monitored carefully during induction through the clear acrylic cover of the narcosis chamber. Induction was denoted by depressed, regular breathing and the ceasing of movement. Successful anesthesia was achieved within 2 minutes. After induction, the animals were removed from the chamber and identification was conducted and measurements were collected. Maintenance of anesthesia was achieved by placing a 50 ml tube with cotton and 2ml of anesthetic at the bottom, in front of the

nose of the animal. The tube was placed approximately 1 centimeter from animal. The tube was only used if the mouse showed signs of recovery during the data collection. Placement of the tube never lasted longer than one second and each animal was carefully monitored the maintenance phase. A screw-on lid was placed on the 50 ml tube to maintain vapor pressure of the anesthetic while not in use.

Ear punch biopsies were taken for use in molecular analysis and use as identification for future captures. The punch biopsies were assigned in numerical order following a numbering scheme shown below (Figure 2-8). Tissue from the ear punch was placed in 75% ethanol in a plastic vial. Ear samples would later be placed into a -70°C freezer for storage. Measurements (aside from weight) taken included, overall length, length of the hind foot, tail length, and ear length. Coloration and pelt patterns were also documented to assist in field identification of each rodent species. Any ticks attached to the animals were removed and placed in 95% ethanol in glass vials and labeled with host information for further identification.

After data collection was completed, the animals were placed in a 23” x 16” x 12” plastic tub which was open to the air for recovery. All animals were observed for signs of recovery, such as increased breathing rate, righting reflex, and restoration of motions. When each animal showed signs of recovery they were replaced back into the trap, and periodically checked on for full recovery. Once all samples were collected from each animal and recovery was completed, the animals were released near their capture location. All animals were allowed to fully recover before release.

Larval ticks were mounted on microscope slides to assist with identification and nymphs were identified under a dissecting microscope. The species of ticks were identified using Clifford et al. (1961), Brinton, Beck and Alfred, (1965) and Keirans and Durden (1998).

DNA Extractions and PCR Amplification

Extraction, polymerase chain reaction (PCR), and gel electrophoresis were conducted at the Veterinary Diagnostic Center (VDC) at the University of Nebraska-Lincoln. Ear punch biopsies were taken from the collection tubes and placed into a sterile 1.5 ml tubes for DNA extraction. Each specimen was processed according to the Thermo Scientific GeneJET Genomic DNA Purification Protocol for mammalian tissue (Thermo Fisher Scientific Inc. Waltham, MA). The procedure consisted of an initial digestion phase followed by a lysis phase, before the DNA isolation phase. DNA presence was determined using a NanoDrop-® Spectrophotometer. Extracted samples were stored at -20°C until further analysis.

Each PCR group included a positive control and a negative control was included when available. Fetal bovine serum was used as a negative control. Polymerase chain reaction procedures for *Rickettsia* citrate synthase gene (gltA) and *Borrelia* flagellin (*fla*B) were modeled after Heise et al. (2010) and Stromdahl, Williamson, Kollars, Evans, Barry, Vince, & Dobbs, (2003) respectively. Positive controls for *Rickettsia prowazekii* and *B. lonestari* were obtained from Dr. Susan Little at Oklahoma State University. PCR reactions were run using Peltier Thermal Cycler-220 DNA Engine DYAD ® Cycler (MJ Research Inc, Roche Molecular Systems Inc., Alameda, CA).

Rickettsia sp. Protocol

Using the protocol described by Heise and colleagues (2010), PCR procedures assessing the presence of *Rickettsia* sp. were conducted on each sample. The primers RpCS.877 (5'-GGGGGCCTGCTCACGGCGG-3') and RpCS.1258 (5'-ATTGCAAAAAGTACAGTGAACA-3') that were used are specific for the *Rickettsia* citrate synthase gene (gltA). All primers were received from IDT (Integrated DNA Technologies, Inc. Coralville, IA) and are listed in Table 2-1. Twenty-five µl reactions were prepared with 12.5 µl of QIAGEN Taq PCR Master Mix (QIAGEN Inc. Valencia, CA), which contained 2.5U of *Taq* DNA polymerase, 1x QIAGEN PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate (dNTP), 2.5 µl of each primer, 1.5 µl of RNA-free water, an addition 1 µl of $MgCl_2$, and 5 µl of sample DNA. The final concentrations of the reaction are 2.5U of *Taq*, 2.5 µM $MgCl_2$, 200 µM of each dNTP, and 1 µM of each primer. Positive, negative, and no template reactions (using water) were prepared with only 1 µl of template and 4 µl of water, instead of 5 µl to save on reagents.

Each reaction was initially denatured for 5 minutes at 95°C followed by 35 cycles of denaturing at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 45 seconds, followed by 5 minutes at 72°C. Reaction products were analyzed via gel electrophoresis in 1.5% agarose gel stained with ethidium bromide. Visualization by ultraviolet exposure was done using a UVP BioDoc-It ™ System 2UV transilluminator (UVP Inc. Upland, CA).

Borrelia sp. Protocol

Each sample was also assessed for the presence of *Borrelia* sp. DNA. A nested PCR reaction was conducted using the *Borrelia* flagellin gene (*fla*B). The primary *fla*B primers FLALL (5′-ACATATTCAGATGCAGACAGAGGT-3′) and FLARL (5′-GCAATCATAGCCATTGCAGATTGT-3′) were used in the initial reaction. Twenty-five µl reactions were prepared using 12.5 µl of QIAGEN Taq PCR Master Mix, containing 2.5U of *Taq* DNA polymerase, 1x QIAGEN PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 2.5 µl of each primer, 2.5 µl of RNA-free water, and 5 µl of sample DNA. Positive, negative, and no template reactions were run using 1 µl of template with 4 µl of water. The nested reaction mixture used 1 µl of the primary PCR product as the template and was prepared with the FLALS (5′-AACAGCTGAAGAGCTTGGAATG-3′) and FlaRS (5′-CTTTGATCACTTATCATTCTAATAGC-3′) in place of the initial primers. Final concentrations of the reaction are 2.5U of *Taq*, 1.5 µM $MgCl_2$, 200 µM of each dNTP, and 1 µM of each primer.

Cycling conditions for both reactions involved an initial denaturing for 3 minutes at 95°C followed by 40 cycles of 1 minute denaturing at 95°C, 1 minute of annealing at 55°C, and 1 minute of extension at 75°C (Barbour, Maupin & Teltow, 1996). Products from the nested reaction were analyzed by gel electrophoresis in 1.5% agarose gel stained with ethidium bromide and visualized by a UV transillumination.

Results

Small-Mammal Captures

Eight mammal species were caught during the study period totaling 212 captures (Table 2-2). The two most common captures were white-footed mice (N = 136), and deer mice (N = 43). Two juvenile *Peromyscus* sp. and two adult *Peromyscus* sp. were also captured but were unable to be identified using field identification practices. Two unknown adult *Peromyscus* sp. escaped prior to data collection.

The highest trapping success was at Prairie Pines (N = 39), Table Rock (N = 37) and ARDC (N = 35). The lowest trapping rate was at Indian Cave State Park (N = 3).

Tick Species Collected

A total of 504 ticks were collected from small-mammal hosts and consisted of 343 larvae and 161 nymphs (Table 2-3). All ticks were identified as *D. variabilis*. Previously caught larval and nymphal lone star ticks were compared during identification. Prairie Pines had the highest number of ticks (N = 246 or 49%) (Table 2-3).

Infestation Prevalence

A total of 212 mice were captured, 109 were infested with tick (51.4% prevalence). Of the 136 *P. leucopus*, 70 were infested (51%) and 25 of the 43 *P. maniculatus* mice were also infested (58%). Prevalence data are presented in Table 2-4.

Molecular Analysis

Ear punch biopsies were tested on 190 specimens for *Rickettsia* sp. and *Borrelia* sp. using the previously mentioned techniques. All samples were negative for *Rickettsia*

sp. and *Borrelia* sp. Representative photographs of transillumination results from *Rickettsia* and *Borrelia* reactions are shown in Figure 2-9 and 2-10.

Discussion

The author found *D. variabilis* on 51.4% of all mice captured in eight sites in southeastern Nebraska. Earlier studies conducted by Rapp (1957, 1960) and Cortinas (2013, 2014) established the presence of the tick in southeastern Nebraska, but provided no data on the degree of infestation on the host. The principal host for immature *Dermacentor variabilis* is *P. leucopus*. Dallas and Foré (2013) also reported that that male gender and larger body size of the *P. leucopus* had a significant effect on the parasite load. Small mammals, like *P. leucopus* were found to play an important role in the development of immature *D. variablis* in Nebraska. *Peromyscus leucopus* is a reservoir for *R. rickettsii* and *B. burgdorferi*; hence, it was important to assess of the infection rate in *P. leucopus*. Findings of this study suggest that the mice in southeastern Nebraska do not currently act as a major reservoir for these pathogens, or that the study was not specific enough to identify infection hosts using the parameters established. Another possibility is that the sample size was not large enough to detect the prevalence of *R. rickettsii* or *B. burgdorferi*. The *B. burgdorferi* findings are also consistent with the lack of the establishment of its primary vector, *I. scapularis*, in Nebraska.

This study did not find *Rickettsia* sp. and *Borrelia* sp. using PCR methods. Another study technique that could have been used to identify the presence of pathogens is antibody assays via serological tests. Serological testing may show that hosts have been exposed to infections. Polymerase chain reaction was chosen over serological assay

to identify active reservoirs over hosts that have exhibited and cleared a previous infection or hosts that have not yet produced antibodies. Another study to identify the presence of these disease pathogens using serological test would identify if the pathogens are in Nebraska. However, a serological study would show exposure levels instead of active infections. A subsequent study analyzing prior exposure to either pathogen could help determine whether or not the pathogens are present in Nebraska. Serological identification of *R. rickettsii* or *B. burgdorferi* would not determine if an animal was hosting the pathogen, simply that they were exposed to the pathogen and elicited and immune response.

This study also identified various immature tick species infesting small mammals in Nebraska. Immature life stages of *A. americanum* were not present on any small mammals captured, which is consistent with findings from Kansas (Brillhart, Fox & Upton, 1994), Missouri (Kollars, Oliver, Durden & Kollars, 2000) and Tennessee (Zimmerman, McWhertet & Bloemer, 1988). These studies suggest that medium to large-sized mammals are more common hosts for immature lone star ticks. A similar pattern would likely be seen in Nebraska but further studies would need to be conducted in order to confirm this.

A subsequent study to identify the role of squirrels in Nebraska as pathogen reservoirs was attempted during the summer and fall of 2012. Tomahawk live capture traps were placed at Mahoney State Park, Schramm Park State Recreation Area, Indian Cave State Park and Table Rock Wildlife Management Area. Traps were placed in late May and were left in place during the course of the study. Each week two locations were visited and traps were left open and baited for three days, and then closed until the next

visiting week. Traps were checked twice a day, once in early morning after being left open overnight, and once in late afternoon. Results of the study however, were inconclusive due to severely low capture rates. A repeat of this study might be warranted after reanalysis and reforming the study parameters in effort to increase capture rates.

Another study, in efforts to identify the role of turkeys (*Meleagris gallopavo*) in pathogen transmission was attempted in the spring of 2011 and 2012. In 2011, 1000 randomly selected registered turkey hunters were asked to examine wild turkeys harvested during the spring hunting season. Hunters were sent a letter explaining the project, an information card to fill out about the bird taken, and a vial to place removed ticks in. Hunters were also sent a pre-paid return mailing envelope to send the vial in to the researchers. Of the 1000 hunters issued a kit, 102 returned the response card. Only 99 of the 102 responses had shot a bird, and only 12 vials returned contained ticks. The data set appeared too small to draw significant conclusions so the test was repeated in 2012 spring season, except hunters were asked beforehand for participation and only those that agreed were issued packets. The 2012 season resulted in five returned forms. No significant results were able to be extrapolated from this study.

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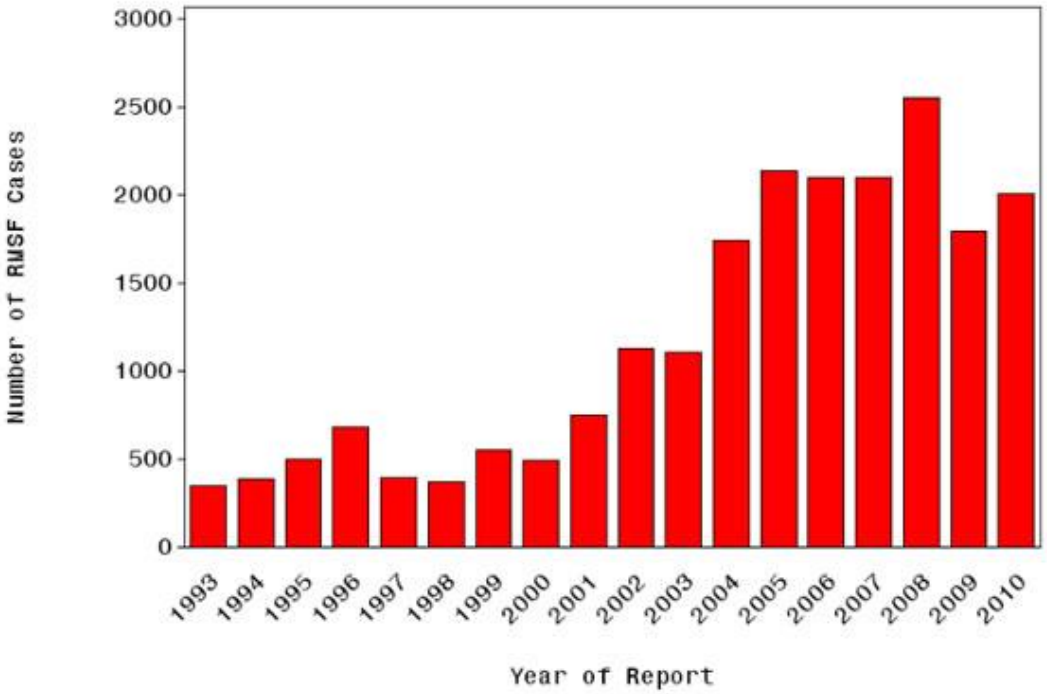

Figure 2-1: Annual number of Rocky Mountain spotted fever cases reported to CDC in the United States, 1993-2010
From: http://www.cdc.gov/rmsf/stats/

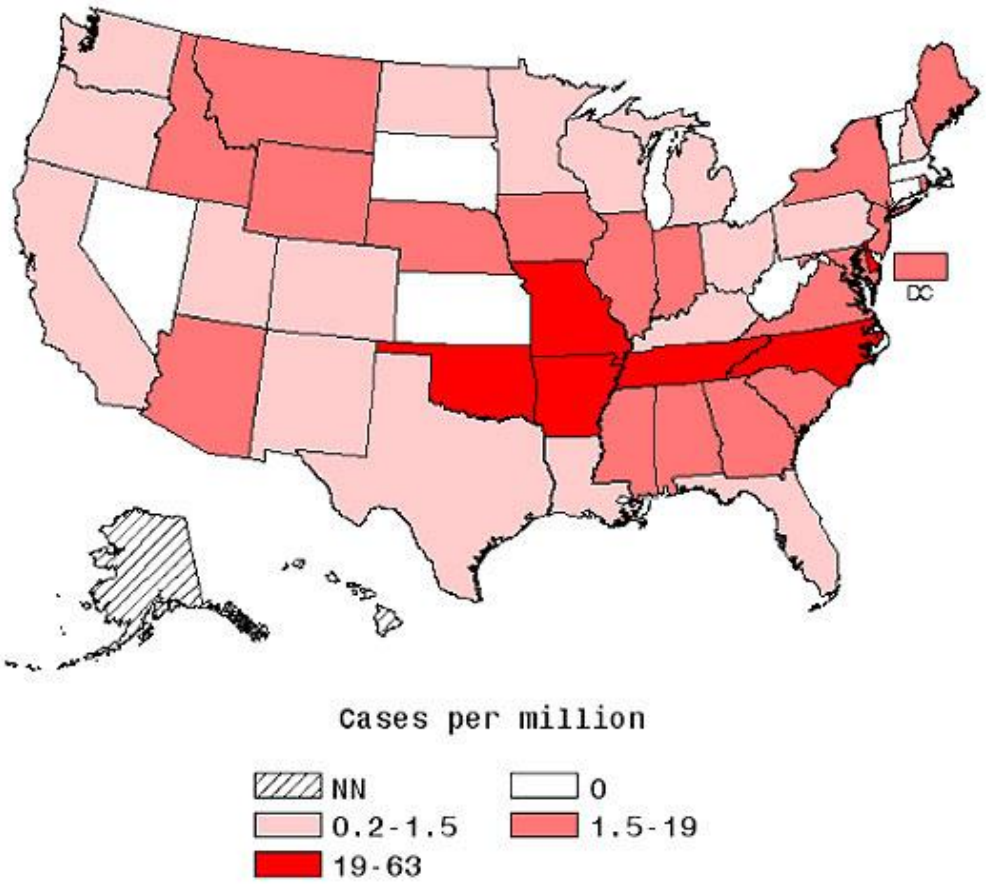

Figure 2-2: Geographic distribution of Rocky Mountain spotted fever incidence in 2010 in the United States
From: http://www.cdc.gov/rmsf/stats/

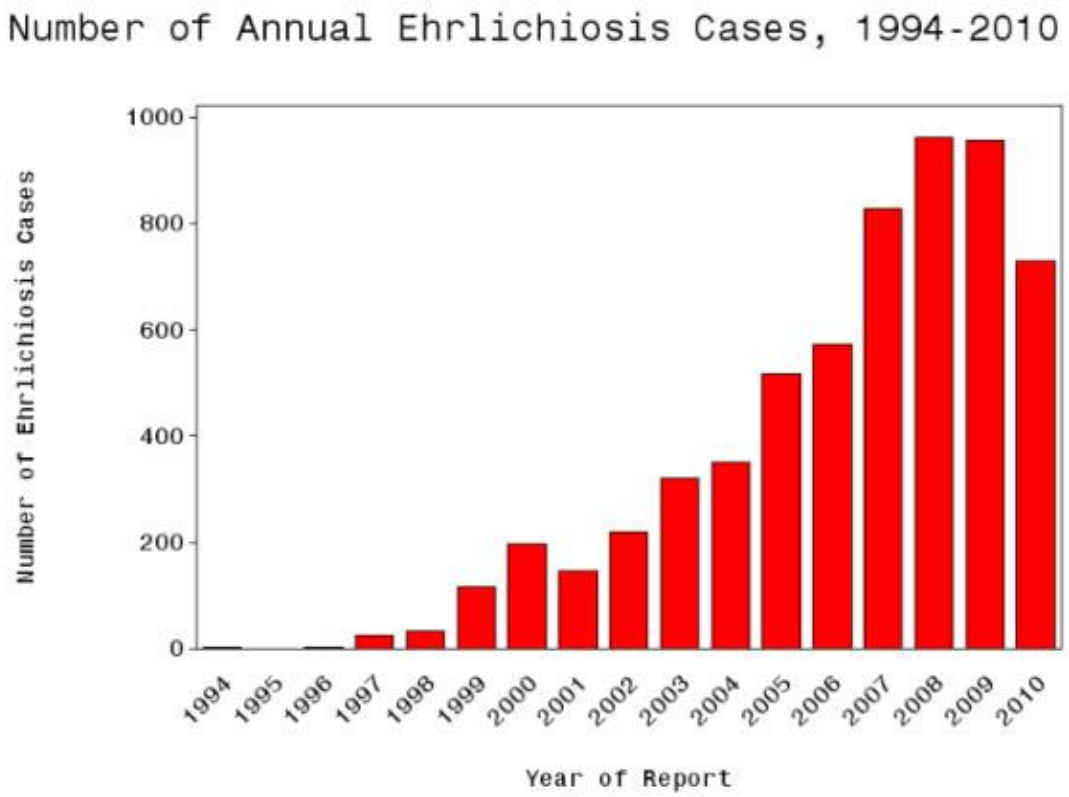

Figure 2-3: Annual number of *Ehrlichia chaffeensis* cases reported to the CDC in the United States, 1994-2010
From: http://www.cdc.gov/ehrlichiosis/stats/

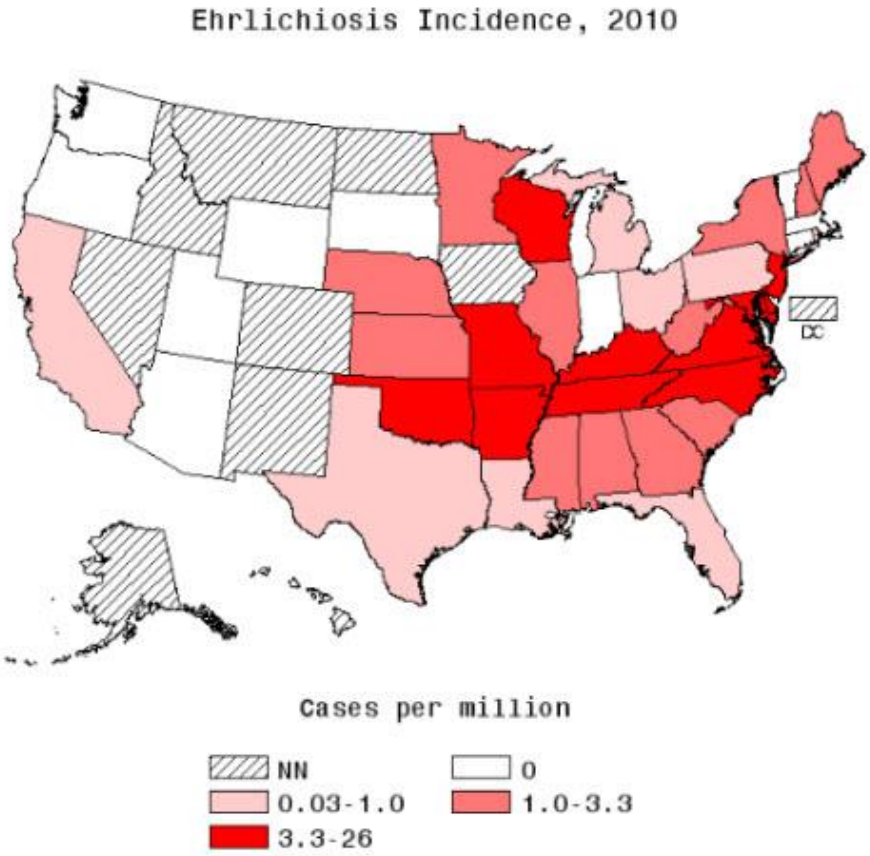

Figure 2-4: Geographic distribution of *Ehrlichia chaffeensis* within the United States incidence for 2010
From: http://www.cdc.gov/ehrlichiosis/stats/

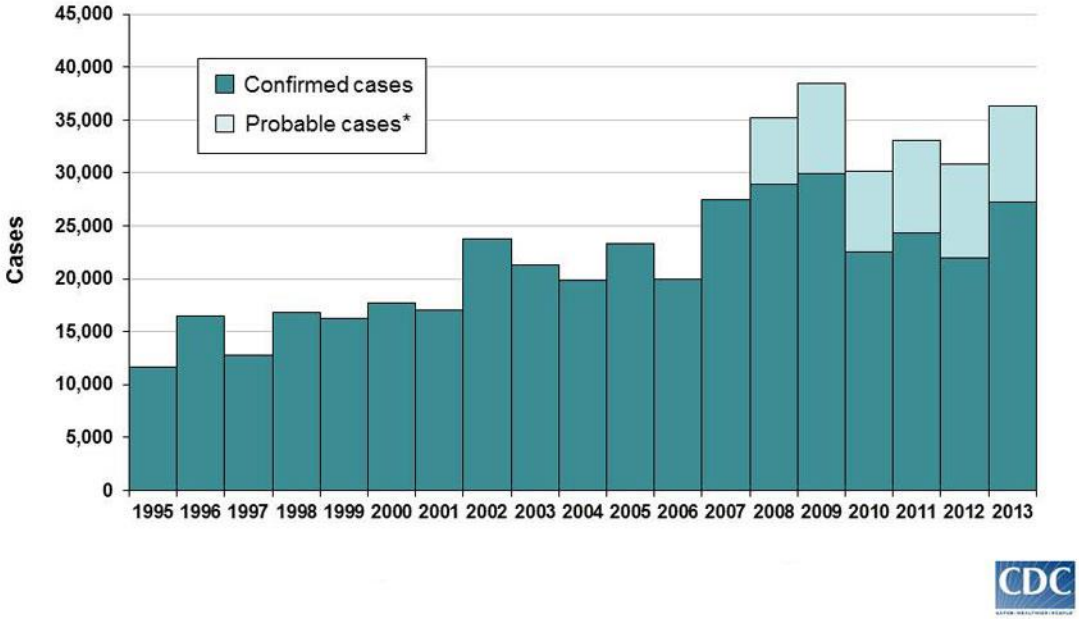

Figure 2-5: Reported Cases of Lyme disease by year in the United States, 1995-2013
From: http://www.cdc.gov/lyme/stats/chartstables/casesbyyear.html

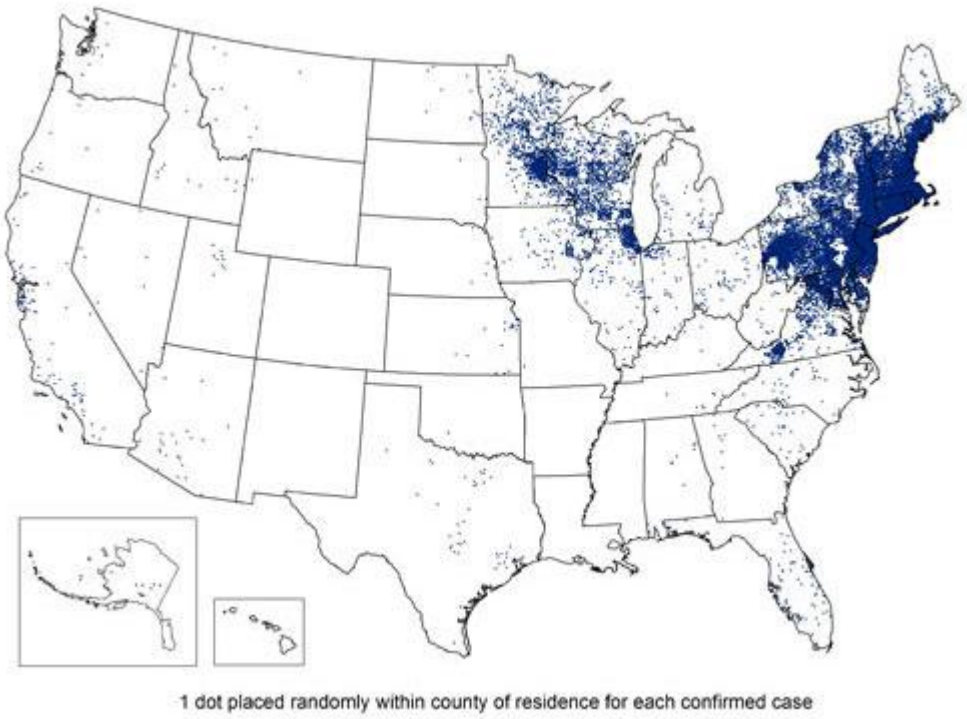

Figure 2-6: Geographic distribution of reported cases of Lyme disease in the United States, 2013
From: http://www.cdc.gov/lyme/stats/maps/map2013.html

Figure 2-7A: Schramm State Recreation Area
Red line indicates location where traps were placed.
Image created using Google Earth © (2158 m)

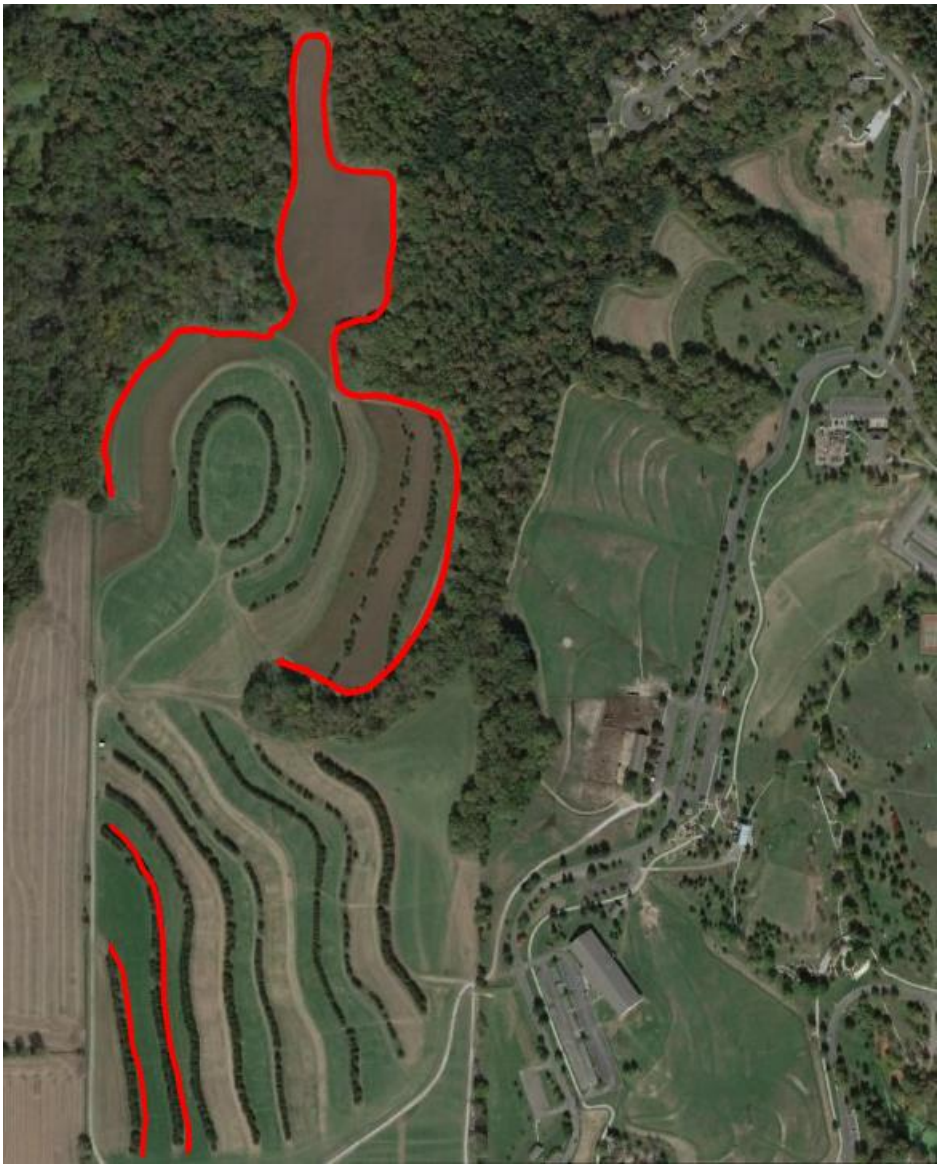

Figure 2-7B: Eugene T. Mahoney State Park
Red line indicates location where traps were placed.
Image created using Google Earth © (2156 m)

Figure 2-7C: Platte River State Park
Red line indicates location where traps were placed.
Image created using Google Earth © (2167 m)

Figure 2-7D: Prairie Pines
Red line indicates location where traps were placed.
Image created using Google Earth © (1913 m)

Figure 2-7E: UNL Agriculture Research and Development Center
Red line indicates location where traps were placed.
Image created using Google Earth © (2432 m)

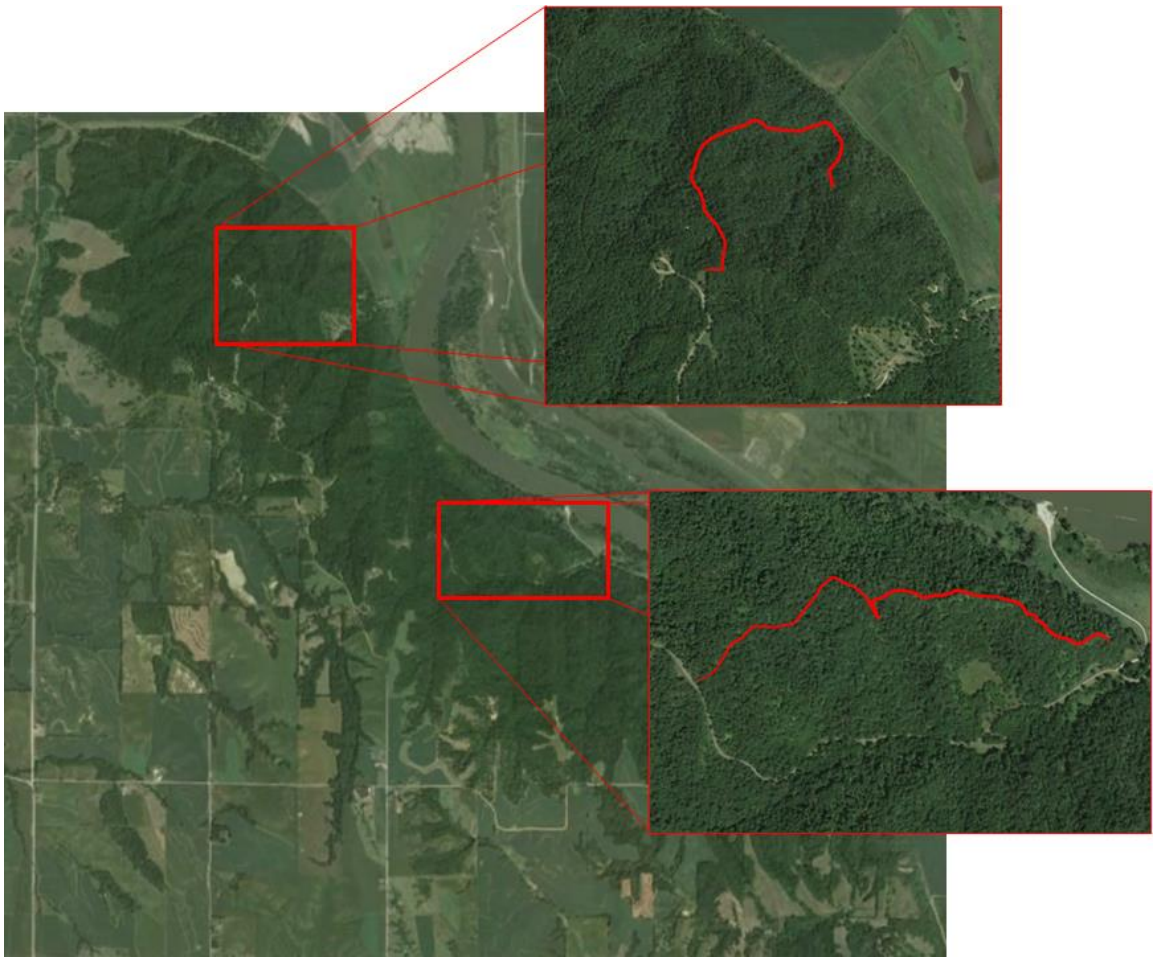

Figure 2-7F: Indian Cave State Park
Red line indicates location where traps were placed.
Image created using Google Earth © (2378 m)

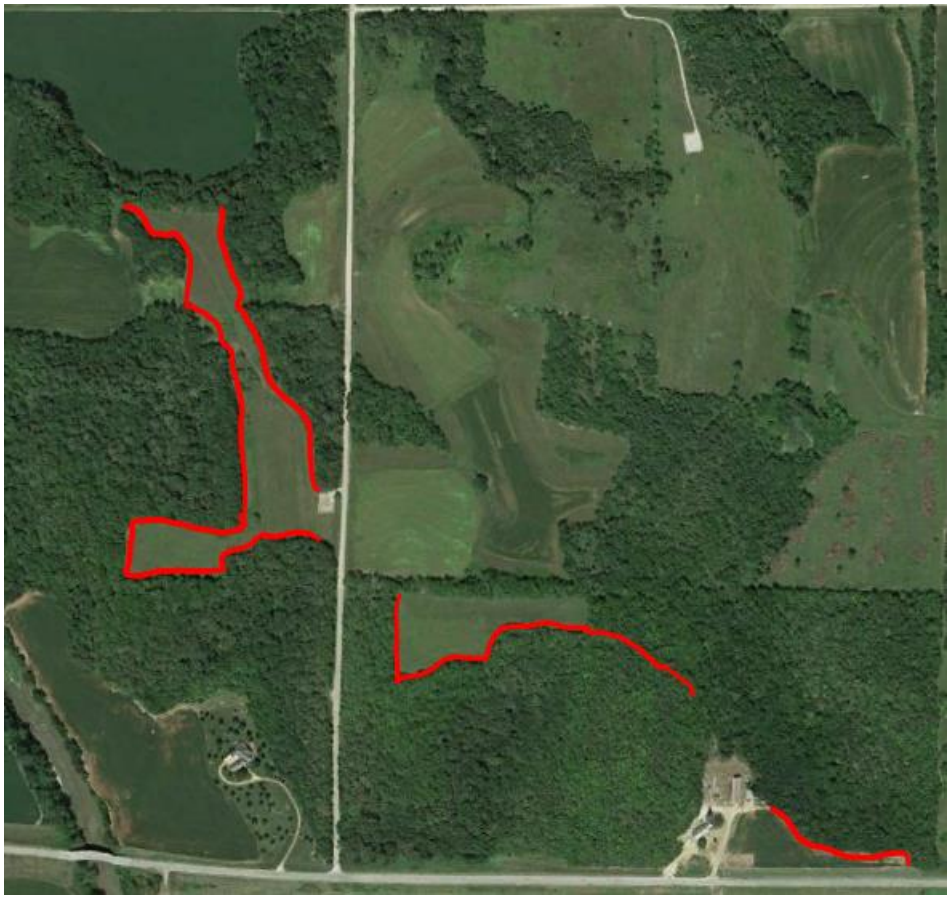

Figure 2-7G: Table Rock Wildlife Management Area
Red line indicates location where traps were placed.
Image created using Google Earth © (2260 m)

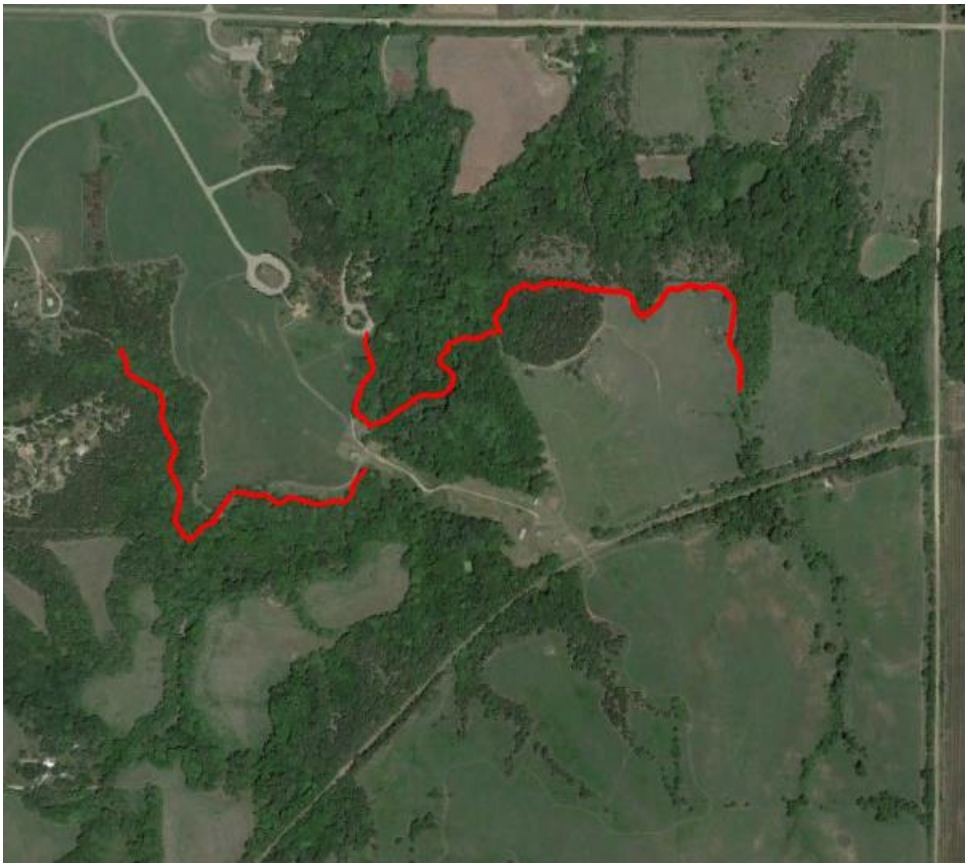

Figure 2-7H: Rock Creek Station State Historical Park
Red line indicates location where traps were placed.
Image created using Google Earth © (2020 m)

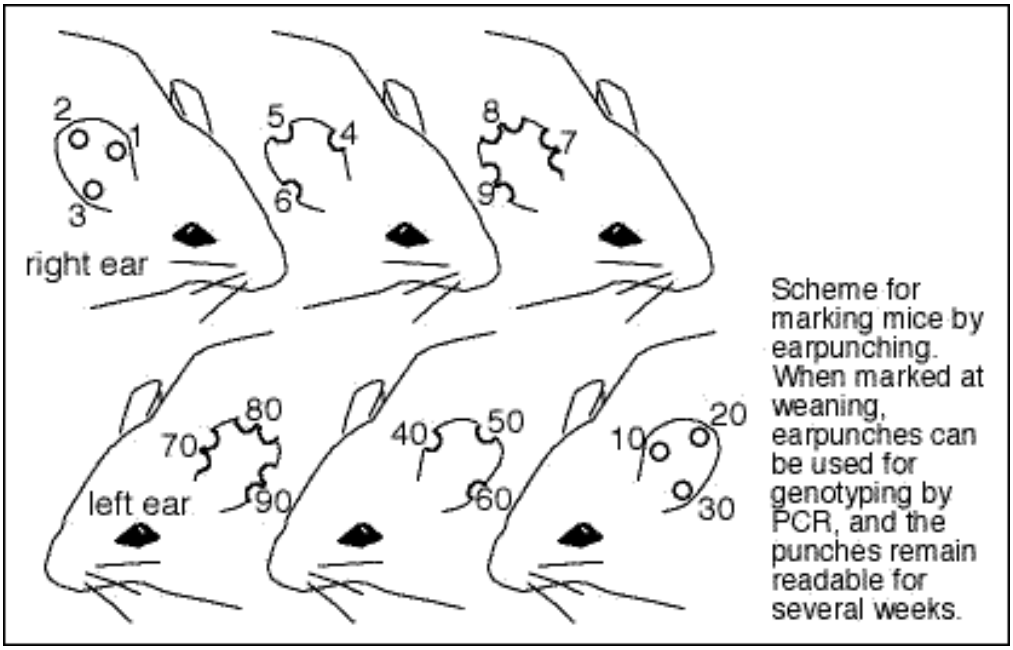

Figure 2-8: Ear punch biopsy scheme
http://ko.cwru.edu/images/punchpattern.gif

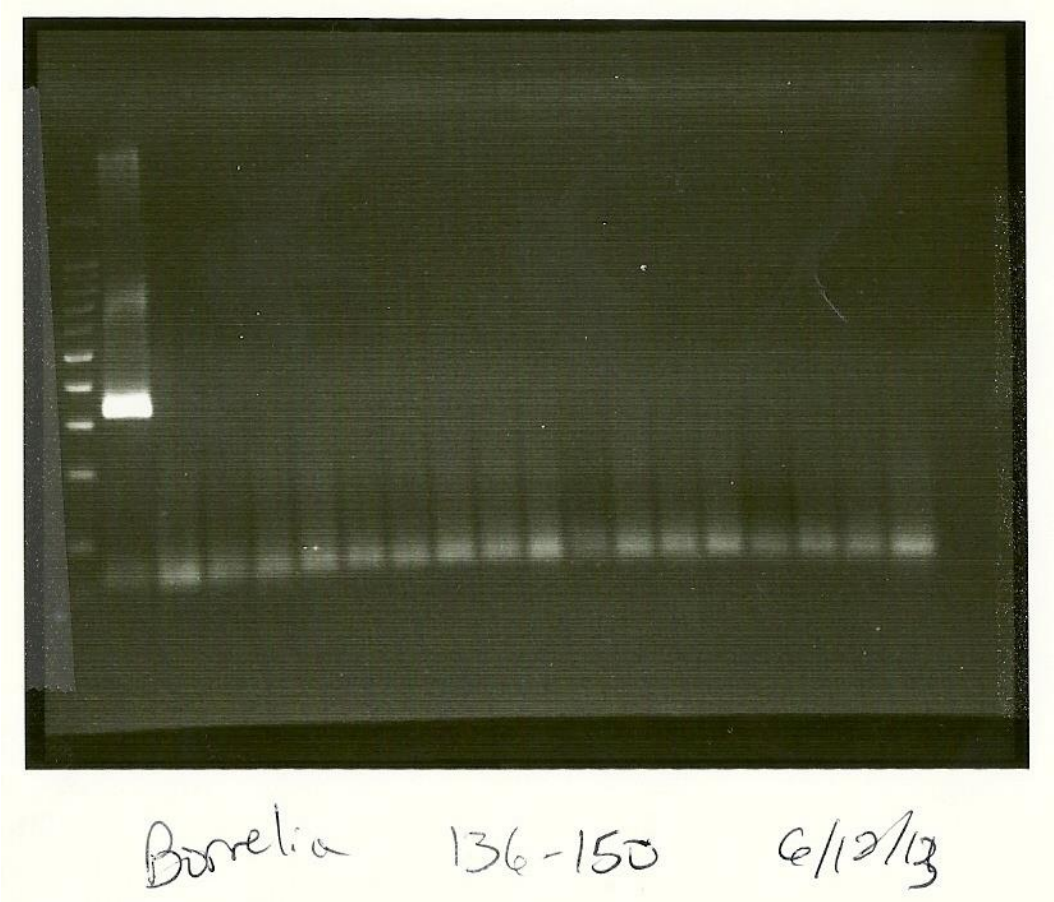

Figure 2-9: Photograph of *Borrelia* sp. PCR results of samples 136-150 after gel electrophoresis

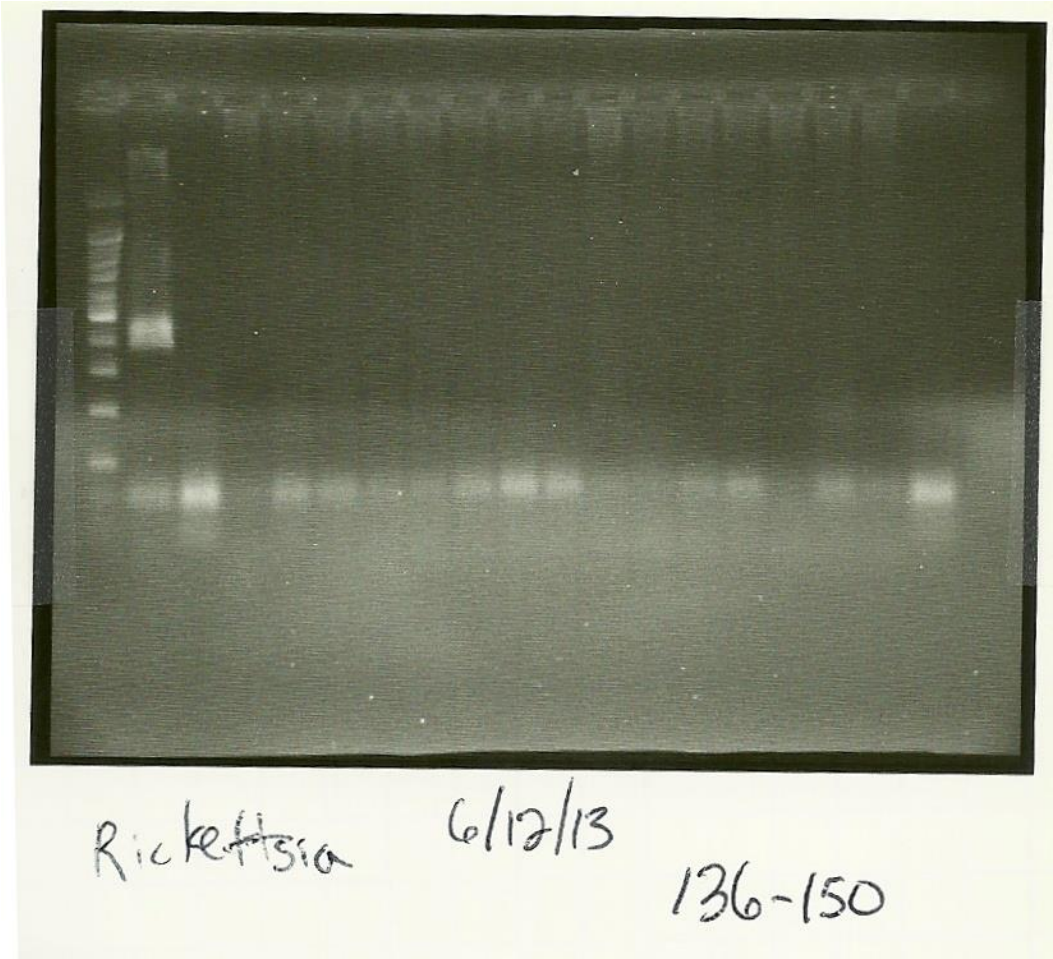

Figure 2-10: Photograph of *Rickettsia* sp. PCR results of samples 136-150 after gel electrophoresis

Primer	**Target gene**		**Sequence**
FLALL	*Borrelia* flagellin (flaB)	1°	5′-ACATATTCAGATGCAGACAGAGGT-3′
FLARL			5′-GCAATCATAGCCATTGCAGATTGT-3′
FLALS		2°	5′-AACAGCTGAAGAGCTTGGAATG-3′
FLARS			5′-CTTTGATCACTTATCATTCTAATAGC-3′
RpCS.877	*Rickettsia* citrate synthase (gltA)		5′-GGGGGCCTGCTCACGGCGG-3′
RpCS.1258			5′-ATTGCAAAAAGTACAGTGAACA-3′

Table 2-1: Polymerase chain reaction primers used in this study

	PP	ARDC	MSP	Schramm	PRSP	ICSP	TR WMA	RCS SP	Total
Peromyscus leucopus	19	22	19	15	13	3	26	19	136
Peromyscus maniculatus	9	6	3	3	6	0	8	8	43
Reithrodontomys megalotis	2	0	1	0	0	0	1	0	4
Mus musculus	3	5	4	3	1	0	0	1	17
Microtus ochrogaster	1	1	0	0	0	0	0	0	2
Microtus pennsylvanicus	2	0	1	0	0	0	0	0	3
Cryptotis parva	2	0	0	0	0	0	0	0	2
Zapus hudsonius	0	0	0	0	0	0	1	0	1
Juveniles	1	1	0	0	0	0	0	0	2
Unknown	0	0	1	0	0	0	1	0	2
								Total	212

Table 2-2: Mice capture data from each location

Location	Larvae/Location	Nymphs/Location	Total
Prairie Pines	137	109	246
ARDC	74	13	87
Mahoney	25	5	30
Schramm	3	4	7
Platte River	2	3	5
ICSP	1	2	3
Table Rock	38	16	54
Rock Creek	63	9	72
Total	343	161	504

Table 2-3: *Dermacentor variabilis* collected by life stage and location

	# Infested	# Captured	Prevalence
Total	109	212	0.51
Peromyscus leucopus	70	136	0.51
Peromyscus maniculatus	25	43	0.58
Mus musculus	6	17	0.35
Reithrodontomys megalotis	2	4	0.5
Microtus ochrogaster	1	2	0.5
Microtus pennsylvanicus	2	3	0.67
Crytpotis parva	0	2	0
Zapus hudsonius	0	1	0

Table 2-4: Prevalence of mice captured hosting ticks

Chapter 3

Prevalence of Tick-borne Disease Pathogens in Deer of Southeastern Region of Nebraska

Introduction

White-tailed deer (*Odocoileus virginianus* (Zimmerman)) are the principal host for several tick species, including black-legged tick or "deer tick" adults (*Ixodes scapularis* Say), and all stages of the lone star tick (*Amblyomma americanum* L.) and the winter tick (*Dermacentor albipictus* Packard) (Apperson et al., 1990; Durden et al., 1991; Kollars et al., 2000; Yabsley, 2010). The lone star tick has become well established in the southeastern region of Nebraska (Cortinas and Spomer, 2013) and this species is capable of transmitting the pathogens that cause tularemia, ehrlichiosis, and some recently identified viruses.

The lone star tick is an aggressive ectoparasite that infests birds and medium to large animals including rabbits and white-tailed deer, and is capable of transmitting many pathogens of veterinary importance (Goddard and Varela-Stokes, 2009). These ticks were originally found in the southeastern United States; however, the geographic range of this tick has expanded within the past few decades to include the northeast and central US (Childs and Paddock, 2003; Mixson et al., 2006; Goddard and Verela-Stokes, 2009). In Nebraska, lone star tick presence was first consistently noticed in 1987 in the extreme southeast (Cortinas and Spomer, 2013). Since then, the geographic range has pushed northwest into the state, and the lone star tick has become the second most frequently encountered tick in Nebraska.

The importance of this tick lies in its ability to transmit veterinary and zoonotic pathogens. Among these pathogens include *Francisella tularensis* (tularemia) (Hopla and Downs, 1953), *Ehrlichia chaffeensis* (human monocytic ehrlichiosis) (Anderson et al., 1992; Anderson et al. 1993, Dawson et al., 1996) *Ehrlichia ewingii* (canine granulocytic ehrlichiosis) (Mixson et al. 2006; Heise et al., 2010), and some spotted fever rickettsials including *Rickettsia parkeri* (Macaluso and Azad, 2005) and *Rickettsia amblyommii* (Apperson et al., 2008; Jiang et al., 2010). Viruses such as Hearltand virus (Savage, Godsey, Lambert, Panella, Burkhalter, Harmon, et. al, 2013) and Bourbon virus (Kosoy, Lamber, Hawkison, Pastula, Goldsmith, Hunt, et. al, 2015) are also transmitted by lone star ticks.

Human monocytic ehrlichiosis (HME) is associated with nonspecific symptoms including a rash-less fever, headache, and other aches and pains (Dawson et al. 2005), coupled with thrombocytopenia, leukopenia, and anemia (Eng, 1990). The primary host and vector for HME is *A. americanum* (Anderson et al, 1993; Dawson et al, 2005), while the primary reservoir is the white-tailed deer (Lockhart et al., 1997a; Dawson et al, 2005). There is some evidence of field collected American dog ticks (*Dermacentor variabilis* Say) naturally hosting *E. chaffeensis* (Anderson, Summer, Dawson, Tzianabos, Greene, Olson, et al., 1992; Roland, et al. 1998). Serological surveys have also found other animals, both wild and domestic, to have antibodies for *E. chaffeensis*. These include raccoons (*Procyon lotor*) (Lockhart et al., 1997b; Comer et al., 2000), opossums (*Didelphis maruspialis*) (Lockhart et al., 1997b), red foxes (*Vulpes vulpes*) (Dawson et al., 2005), gray foxes (*Urocyon cinereoargenteus*) (Dawson et al., 2005), dogs (*Canis*

lupus familiaris) (Dawson et al., 1996; Murphy et al., 1998 Kordick et al., 1999;) and goats (*Capra hircus* Linneaus) (Dugan et al., 2000).

Southern tick-associated rash illness is a bacterial infection that appears similar to Lyme disease by presenting with erythema migrans or "bulls-eye rash". Unlike Lyme disease, which is caused by *Borrelia burgdorferi*, STARI was believed to be caused by *B. lonestari* (Masters, 1998; James et al., 2001; Moore et al., 2003; Varela et al., 2004). *Borrelia lonestari* has been isolated in *A. americanum* but researchers are still debating if it is the causative pathogen (Wormser et al., 2005; Masters et al., 2008). Other research suggests that *B. lonestari* is nonpathogenic (Stromdahl, Nadolny, Gibbons, Auckland, Vince, Elkin, et al., 2015). *Amblyomma americanum* is an incompetent vector for *B. burgdorferi* and studies have shown the saliva of *A. americanum* to be borreliacidal (Ledin et al., 2005; Zeidner et al., 2009), yet it is capable of harboring *B. lonestari* which suggests something within *B. lonestari* allows its survival as opposed to *B. burgdorferi*. There is a correlation between lone star ticks and STARI, but causation has yet to be proven definitively.

Researchers believe the reservoir for *B. lonestari* is the white-tailed deer (Moore et al, 2003; Moyer et al., 2006; Paddock and Yabsley, 2007). White-tailed deer have also been shown to be experimentally capable of developing a reservoir state (Moyer et al., 2006). Other animals such as rodents, domestic canines and calves have also been experimentally tested as potential reservoirs, but failed to develop viable bacteremias (Moyer, 2005).

The goal of this study was to assess the prevalence of *E. chaffeensis* and *Borrelia* sp. in white-tailed deer of Nebraska using retropharyngeal lymph nodes collected during the annual deer hunt.

Materials and Methods

Study Sites

The study took place during the 2012 rifle hunting season. Data were collected on November 10, 11, and 18, 2012. The deer check stations in Auburn, NE (Nemaha County, 40.3883° N, 95.8422° W) and Tecumseh, NE (Johnson County, 40.3700° N, 96.1917° W) were visited. The Auburn station was visited once during the opening weekend and again on the final day of rifle season, and the Tecumseh station was visited only once. The check stations were selected because of their historically high number of deer checks from four Nebraska counties (Johnson, Nemaha, Pawnee and Richardson) where lone star ticks are most dense and frequently encountered (Cortinas and Spomer, 2013). Other deer check stations included in the study were locations fielded by the Nebraska Games and Parks Commission (NGPC) officers across central and western Nebraska. Data collected from these sites were used for a NGPC study on chronic wasting disease (CWD). Deer sex, age, harvest location (and other data related to the hunters) were collected.

Sample Collection

At the sites visited by the author, hunters were asked for their participation in the study. Those participating allowed the collection of retropharyngeal lymph nodes from their white-tailed deer. Lymph node collection was conducted following a NGPC

protocol for collecting lymph nodes for CWD testing. Lymph nodes were collected by incising the ventral aspect of the neck transversely, palpating the tissues for the structures, and removing them. Photos demonstrating lymph node location and removal technique are show in Figures 3-1 through 3-3. Both lymph nodes were removed if possible. Lymph nodes were then placed in a sterile Whirl-Pak® (Nasco, Fort Atkinson WI) which were then placed in a cooler with ice packs until they could be stored at -20°C. Each sample was labeled with an identification number, time of removal, and county from where the deer was taken. Hunters were also asked if they could provide the location where the deer was shot, via coordinates or estimation by pointing on a map. Deer were also inspected for ticks. Ticks were collected and placed into a glass vial with 95% ethanol. The ticks were identified using Brinton et al. (1965).

White-tail and mule deer (*Odocoileus hemionus*) lymph nodes were collected by the NGPC in central and western Nebraska during 2012 CWD testing. Following testing for CWD, remaining lymph node tissue was used in this study. Samples retained CWD accession numbers. Samples were randomly selected from counties on the observed western-most boundaries where lone star ticks were collected (Cortinas and Spomer, 2013). Counties were broken into two testing groups. One group consisted of deer from Dawson and Custer counties (mid-central Nebraska), and the other group contained samples from Gosper, Furnas, Harlan, and Phelps counties (south-central Nebraska). Fifty samples from each group were selected. Tissue samples from the south-central and mid-central Nebraska regions were selected using a random number generator (www.random.org) (Haahr, 1998). These lymph node samples were stored at -20°C until processing could be conducted.

DNA Extraction and PCR Amplification

Extraction of DNA, polymerase chain reactions (PCR) and gel electrophoresis was conducted at the University of Nebraska-Lincoln Veterinary Diagnostic Center. Lymph nodes were thawed, and approximately half of each node was cut smaller using scissors or a flat edge razor. If only one lymph node was available, the entire lymph node was processed. Samples were placed into a sterile bag containing minimal essential medium, a fluid suspension for cells. The bag was placed into a Stomacher 400C Lab Blender (Brinkmann Instruments Westbury, NE) at 230 rpm for 30 seconds. The suspension was poured into a 50ml centrifuge tube and centrifuged for 10 minutes at 3000 rpm. The supernatant was extracted into a 14ml tube and placed into a -20°C freezer if extraction would occur within a couple days; otherwise tubes were kept at -80°C.

To isolate the DNA from the supernatant, the protocol for mammalian blood in the Thermo Scientific GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific Inc. Waltham, MA) was used. The purification process consisted of a lysis phase, followed by column assisted purification. Extracted samples were stored at -20°C until further analysis.

After DNA isolation, PCR was conducted in a clean room. PCR procedures targeting the *Borrelia* flagellin (*fla*B) gene (Stromdahl et al. 2003) and the 16S rDNA gene for *Ehrlichia chaffeensis* (Anderson et al., 1992, Dawson et al., 1996) were performed. A positive control and a negative control using fetal bovine serum were included in each reaction. Positive controls for *E. chaffeensis* and *B. lonestari* were

provided by Oklahoma State University (Dr. Susan Little). Reactions were run in a Peltier Thermal Cycler-220 DNA Engine DYAD ® Cycler (MJ Research Inc. Roche Molecular Systems Inc., Alameda CA).

***Borrelia* sp. Protocol**

Lymph nodes were assessed for *Borrelia* to identify the presence of *Borrelia* spp., including *B. lonestari* or *B. burgdorgeri*. Each sample was tested using a nested PCR reaction. The primary reaction assessing for the *fla*B gene used the FLALL (5′-ACATATTCAGATGCAGACAGAGGT-3′) and FLARL (5′-GCAATCATAGCCATTGCAGATTGT-3′) primers. All primers used are listed in Table 5. Each reaction was assembled in 25 µl reactions using 12.5 µl of QIAGEN *Taq* PCR Master Mix, 2.5 µl of each primer, 2.5 µl of RNA-free water, and 5 µl of sample DNA were prepared. The QIAGEN Master Mix contained 2.5U of *Taq* DNA polymerase, 1x QIAGEN PCR buffer, 1.5 mM $MgCl_2$, and 200 µM of each dNTP. Each reaction series included a positive sample and no template preparation using 1 µl of control and water respectively. Positive control tests used 1 µl of control DNA in effort to minimize waste. Positive results were still seen in each test performed at this concentration. Negative controls remained using 5 µl of template. The secondary reactions used 1 µl of the primary reaction product as the template. Each series contained between 10 and 15 samples plus the positive control, negative control and no template reactions. Secondary reactions were prepared similar to the primary reactions using instead the FLALS (5′-AACAGCTGAAGAGCTTGGAATG-3′) and FLARS (5′-CTTTGATCACTTATCATTCTAATAGC-3′) primers. A list of the primers used is

found in Table 3-1. Final concentrations of each reaction were 2.5U of *Taq*, 1.5 µM $MgCl_2$, 200 µM of each dNTP, and 1 µM of each primer.

Cycling conditions for both the primary and secondary reactions began with a 3 minute denaturing phase at 95°C, followed by 40 cycles of denaturing at 95°C for 1 minute, annealing at 55°C for 1 minute, and extension at 75°C for 1 minute (Barbour, Maupin, & Teltow, 1996). PCR products were separated by gel electrophoresis in 1.5% agarose gel stained with ethidium bromide. Visualization was achieved using a UVP BioDoc-It ™ System 2UV transilluminator (UVP Inc. Upland, CA).

***Ehrlichia chaffeensis* Protocol**

All samples were tested for *E. chaffeensis*. The reaction protocol consisted of a nested procedure, in which the first reaction tested for the prevalence of *Ehrlichia* and *Anaplasma* 16S rDNA using the ECB (5'-CGTATTACCGCGGCTGCTGGCA-3') and the ECC (5'-AGAACGAACGCTGGCGGCAAGCC-3') primers (Table 5). These primers were used in a 25 µl reaction containing 12.5 µl of QIAGEN *Taq* PCR Master Mix, 2 µl of each primer, 3.5 µl of RNA-free water, and 5 µl of sample DNA. The Master Mix retained a concentration 2.5U *Taq* DNA polymerase, 1x QIAGEN buffer, 1.5 mM $MgCl_2$, and 200 µM of each dNTP. Positive and no template reactions were included during each series as well as a negative reaction test. Positive controls only used 1 µl of sample and no template reactions used water. Negative controls used 5 µl of sample. Each series contained between 10 and 15 samples as well as the positive and negative controls and the no template reactions. The secondary reactions were prepared similar to the primary reaction but utilized 1 µl of the primary product with HE1 (5'-

CAATTGCTTATAACCTTTTGGTTATAAAT-3') and HE3 (5'-TATAGGTACCGTCATTATCTTCCCTAT-3') primers. Table 3-1 shows the primers used in the study. Concentrations for both primary and secondary reactions were 2.5U of *Taq*, 1.5 µM $MgCl_2$, 200 µM of each dNTP, and .8 µM of each primer.

The cycling conditions were three initial cycles of denaturing for 1 minute at 94°C, followed by 2 minutes of annealing at 55°C, then 1 minute and 30 seconds of extension at 70°C. These three cycles were then followed by 37 more cycles of 1 minute denaturing at 88°C, then 2 minutes of annealing at 55°C, followed by 1 minute 30 seconds of extension at 70°C. Cycling conditions were the same for both primary and secondary reactions. Products of the secondary reactions were analyzed by gel electrophoresis in a 1.5% agarose gel. The gel was stained with ethedium bromide and results were visualized by transillumination. Any test series that failed to produce a positive control result was completely rerun on the following day.

Results

Retropharyngeal lymph nodes were collected from 49 deer from four counties in southeastern Nebraska and 100 lymph nodes were obtained from the CWD study. The distribution of lymph node samples per county are shown in Table 3-2. Figure 3-4 shows the locality of each deer participating in this study was originally taken. Figure 3-5 demonstrates the locations where deer were harvested during CWD testing. Figure 3-6 shows CWD samples selected for the study.

All lymph node samples from the southeastern counties and from the CWD group tested negative for the *Borrelia flab* gene. Lymph node samples from 10 deer tested

positive for *E. chaffeensis* specific DNA in the southeast Nebraska counties. Samples of lymph nodes from the CWD study also tested positive for *E. chaffeensis.* Maps of the animals with positive tests for *E. chaffeensis* are found in Figures 3-7 and 3-8. Distribution per county is shown in Table 3-3. The prevalence of *E. chaffeensis* was 16.7% in the total sample, with variance per county. The highest prevalence was 33.3 % in Furnas and Phelps counties and 27% seen in Harlan County, all in south central Nebraska. No positive cases identified in Gosper or Custer counties. Representative photographs of PCR results for both *Borrelia* and *E. chaffeensis* are seen in Figures 3-9 and 3-10 respectively.

The distribution of *E. chaffeensis* in the deer sampled in southeast Nebraska was 20.4% compared to 15% in the lymph nodes from the CWD study. This difference is not statistically significant ($\chi^2 = 0.4835$, $p > 0.05$).

Tick Species Collected from Deer

Five of the 49 deer had ticks present at the time of the lymph node extraction. Ticks collected are represented in Table 3-2. All ticks collected were identified as adult and nymphal *Dermacentor albipictus* (Packard).

Discussion

Of the 149 deer analyzed in this study, 25 tested positive for *E. chaffeensis* while no positive samples were observed for *Borrelia* sp. The lack of positive results for *Borrelia* sp. is likely due to white-tailed deer being poor reservoirs for *Borrelia* spirochetes. Researchers have shown that white-tailed deer have a restricted serological response to infection with *Borrelia* (Moore et al., 2003; Moyer et al. 2006; Valera-

Stokes, 2007; Murdock et al. 2009), even though white-tailed deer are the putative reservoir for *B. lonestari*. Analysis was made on the genus level to identify initially if the spirochetes were in the animals in Nebraska. Had positive samples been found, subsequent PCR analysis would have been able to determine the species. Polymerase chain reaction analysis was chosen over serological assessment in order to ascertain current infections. Serological testing could provide a more comprehensive look at exposure and may provide different results. Based on the study parameters, PCR analysis was more favorable since the author was working in association with hunter killed deer.

It is possible that the medial retropharyngeal lymph node is not a good location within the deer to find *Borrelia*. As stated previously, *B. lonestari* DNA was isolated from blood samples taken from white-tailed deer. No studies, to the knowledge of the author have previously demonstrated *Borrelia* isolation from white-tailed deer lymph nodes. However, lymph nodes have been used to identify *Borrelia* infections in mule deer (Nieto, Teglas, Stewart, Wasley, & Wolff, 2012) as well as laboratory mice (Pahl, Kühlbrandt, Brune, Röllinghoff, & Gessner, 1999; Tunev, Hastey, Hodzic, Feng, Barthold, & Baumgarth, 2011).

Another study taking blood samples from deer might provide different results as *B. lonestari* has been isolated from *A. americanum* ticks collected in southeast Nebraska (Maegli, unpublished 2014).

Sampling during this study was limited by participation and collection methodology. Samples were only obtained during the open rifle season of deer hunting. Hunters who brought their deer into the check stations in the southeastern counties were

asked if they would participate in the study. Many declined during the course of sample collection. Check stations were only visited three total days during open season. Initially eight samples were retrieved from the Auburn check station, while 25 were collected from the Tecumseh station. Auburn was revisited the following Saturday in efforts to increase sample size, which resulted in an additional 16 specimens for a total of 24 from Auburn, and 49 total. Collection was limited to the author alone, and ideally collection would have taken place throughout the open season week and at both locations simultaneously. The small sample size from the southeastern collection resulted in every sample being analyzed for the study, while a random sampling of the specimens was analyzed from the lymph nodes provided by the CWD testing. Sampling of the CWD lymph nodes was also restricted due to CWD laboratory protocols. Samples from the collection stations were sent in individual bags that were bundled in larger bags by 50. If a sample tested positive for CWD, the entire bag, rather than the positive specimen was sent to another facility for confirmation and those 50 specimens had to be excluded in the random selection process for PCR analysis. This sample exclusion eliminated the possibility to test some samples from certain counties as all the samples from those counties may have been entirely within a bag 50. This was especially apparent in the central counties of Nebraska as only samples from Custer County and Dawson County were selected from by the random sampling. The inclusion of the samples from other central counties would have provided a better snapshot of the deer infected by allowing a greater and more comprehensive sample size. Some regions of Nebraska were not tested due to the current geographic distribution of the ticks responsible for the specific

pathogens assessed in this study. It is also possible that the sample size was not large enough to detect the presence of *B. lonestari* infected deer samples.

One other study conducted by Little, Stallknecht, Lockhart, Dawson and Davidson (1998) isolated *E. chaffeensis* from white-tailed deer lymph nodes using PCR analysis, in which only one deer was found to PCR positive for *E. chaffeensis*. This study is not only the first to identify *E. chaffeensis* in white-tailed deer in Nebraska, but it is also the first to demonstrate such a high prevalence using PCR analysis.

Results from this study show that deer residing in areas where high populations of lone star ticks, such as those demonstrated in Cortinas and Spomer (2013), are at risk of acquiring *E. chaffeensis.* It also shows that in areas where lone star ticks are not abundant, there is little or no disease. Not only are deer host susceptible to acquiring new infections, but the risk of uninfected deer becoming reservoir hosts is much greater in these areas. As deer migrate, there is a possibility for them to carry pathogens from an area that has not been exposed to the pathogen previously. Providing the new areas have a competent vector, further pathogen transmission becomes highly probable.

Samples in this study were analyzed specifically for *E. chaffeensis*. Another *Ehrlichia* species, *E. ewingii*, is an emerging pathogen that is also responsible for causing ehrlichiosis in humans (Childs and Paddock, 2003; Paddock et al. 2005). *E. ewingii* has been isolated in lone star ticks (*Amblyomma americanum)* as well as American dog ticks (*Dermacentor variabilis*) (Murphy et al. 1998; Steiert and Gillfoy, 2002). Both of these ticks are associated with white-tailed deer (*O. virginianus)* infestations. It is also speculated that white-tailed deer are an active reservoir for *E. ewingii* (Yabsley et al.

2002; Arens et al. 2003). It has been shown that *A. americanum* ticks in Nebraska are hosting both *E. chaffeensis* and *E. ewingii* (Maegli, 2013). Given more time and funding, a thorough analysis of the prevalence of *E. ewingii* in white-tailed deer in Nebraska would further our understanding of the pathogen lifecycle as well as developing potential risk factors for transmission of disease pathogens not only sylvatic, but also zoonotic for both *Ehrlichia* sp. This is the first evidence that *Ehrlichia* is found within wild animal populations in Nebraska.

The primary purpose of this study was to assess the prevalence of tick-borne disease pathogens in white-tailed deer in Nebraska. However, not all samples in this study came from white-tailed deer. Eighteen total samples came from mule deer shot in Nebraska during the open season. Mule deer are not as prevalent in the eastern part of Nebraska presently. A map of the distributions of mule deer and white-tailed deer is presented in Figure 3-11. The majority of the mule deer samples (17) came from those selected in Custer County (mid-central region). The remaining sample was one of the three samples from Gosper County (south central region). These samples were included in the study as there has been some evidence of *E. chaffeensis* found in mule deer (Foley et al. 1998; Yabsley et al. 2005). More accurate results specifically pertaining to white-tailed deer could be assessed to provide a more precise snapshot for strictly white-tailed deer. The presence of *E. chaffeensis* in mule deer in Nebraska is also worth evaluating since the range of these deer overlaps with the expanding range of the lone star tick. Indications from this study would provide more insight into how far the lone star ticks have expanded and a better potential exposure risk analysis could be performed.

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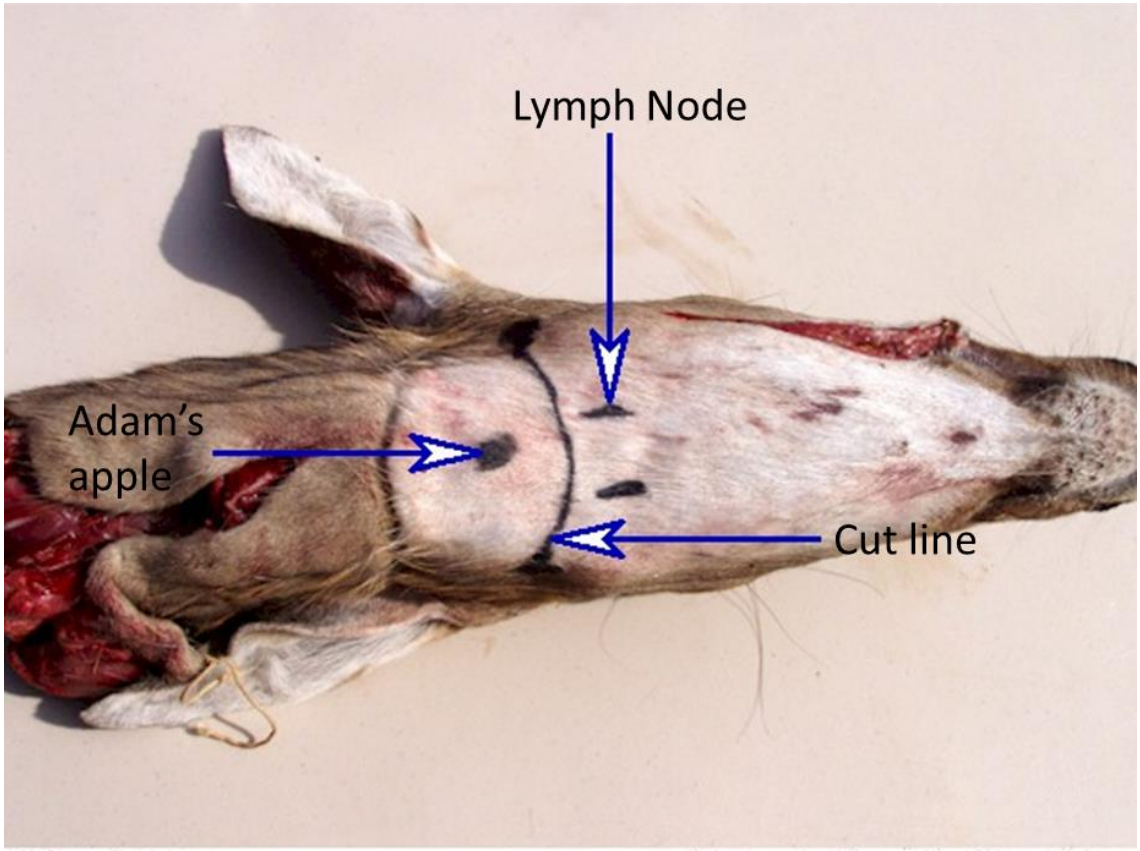
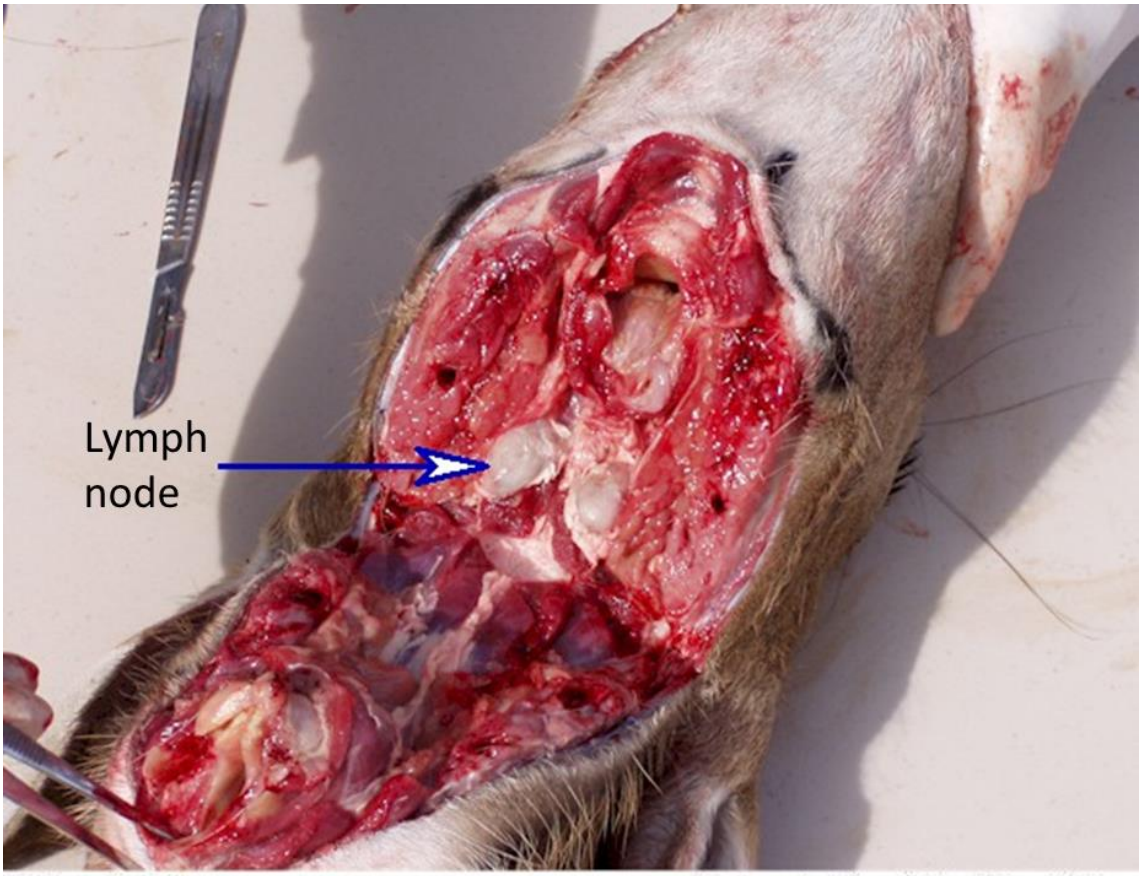


White-tailed deer
Odocoileus virginianus

Photograph / Copyright - Richard Nelson

The above photo identifies the adam's apple (largest laryngeal cartilage), the approximate location of the retropharyngeal lymph nodes, and a cut line from the back of the jaw to 1/2 inch anterior to the adam's apple on this White-tailed doe.

Figure 3-1: Photograph identifying location of medial retropharyngeal lymph nodes and acquisition line in white-tailed deer



White-tailed deer
Odocoileus virginianus

Photograph / Copyright - Richard Nelson

This photo reveals the retropharyngeal lymph nodes that were 1/2" anterior to the above described cut and were exposed with forceps.

Figure 3-2: Photograph isolating the medial retropharyngeal lymph nodes

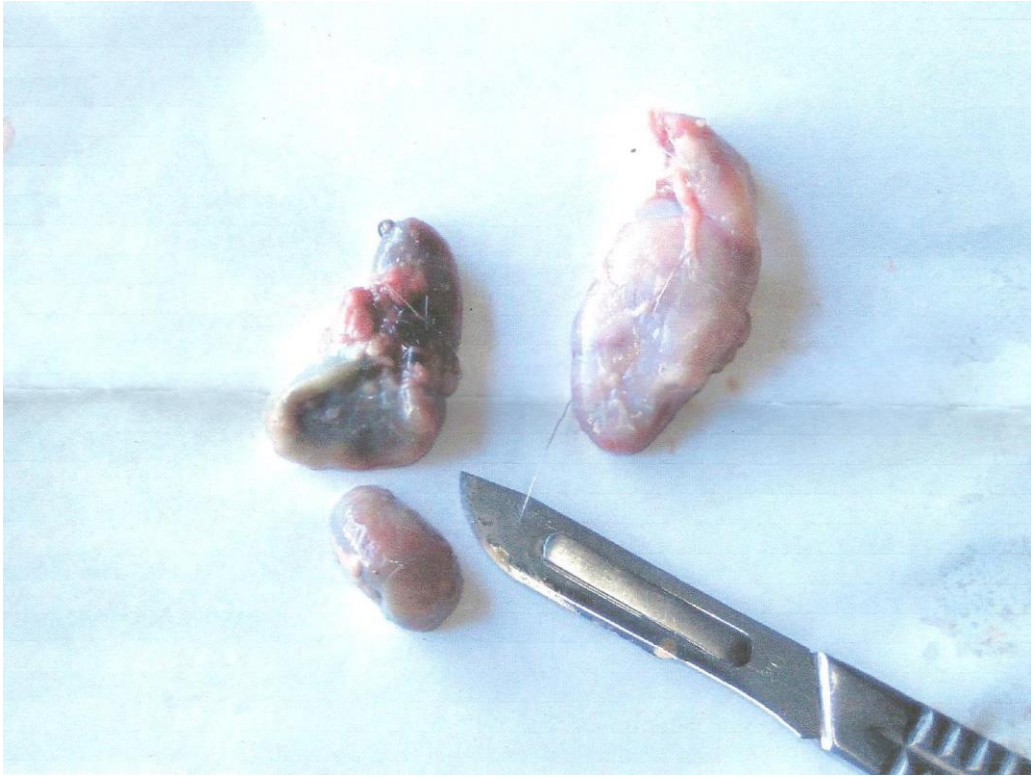


Figure 3-3: Isolated retropharyngeal lymph nodes
Lymph node on left has a portion cut off.

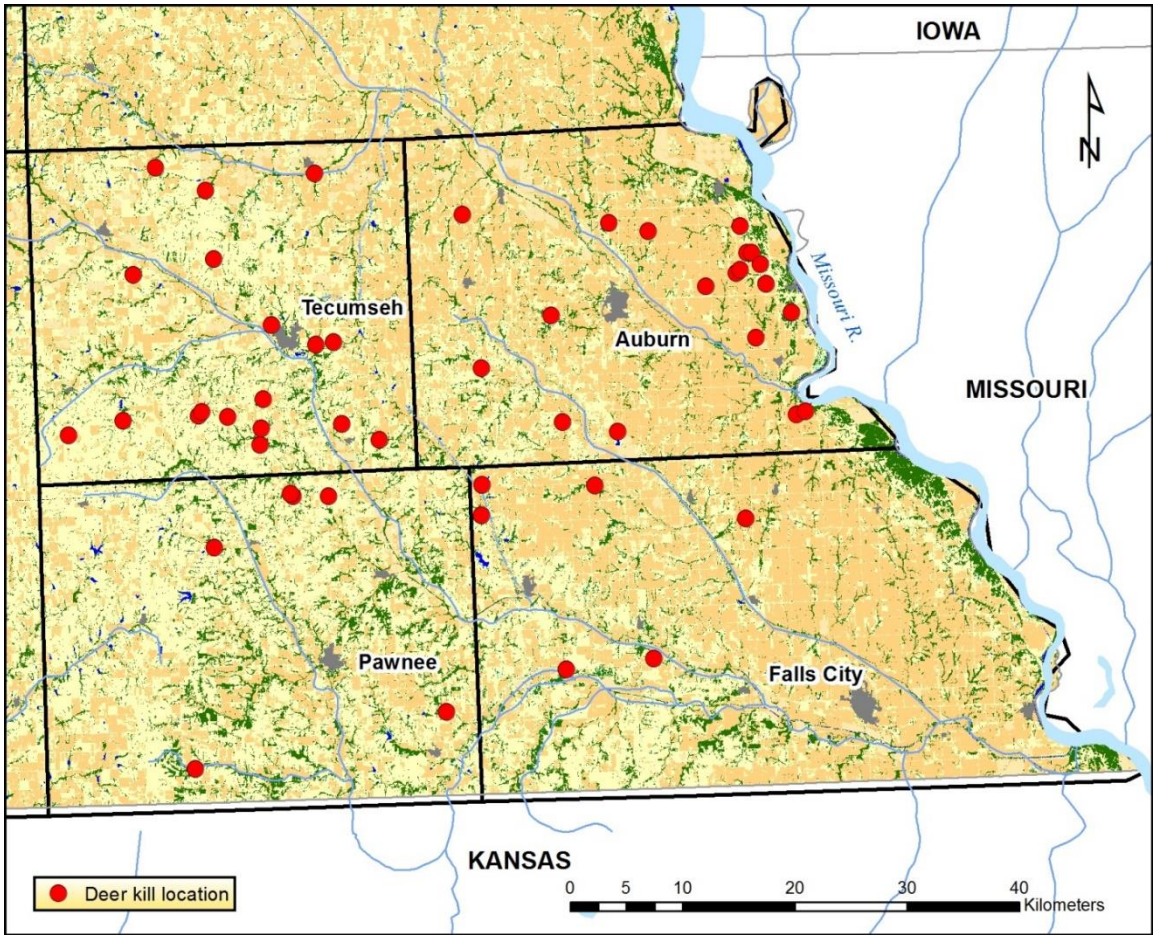


Figure 3-4: Map showing localities of deer killed during lymph node collection in southeast Nebraska

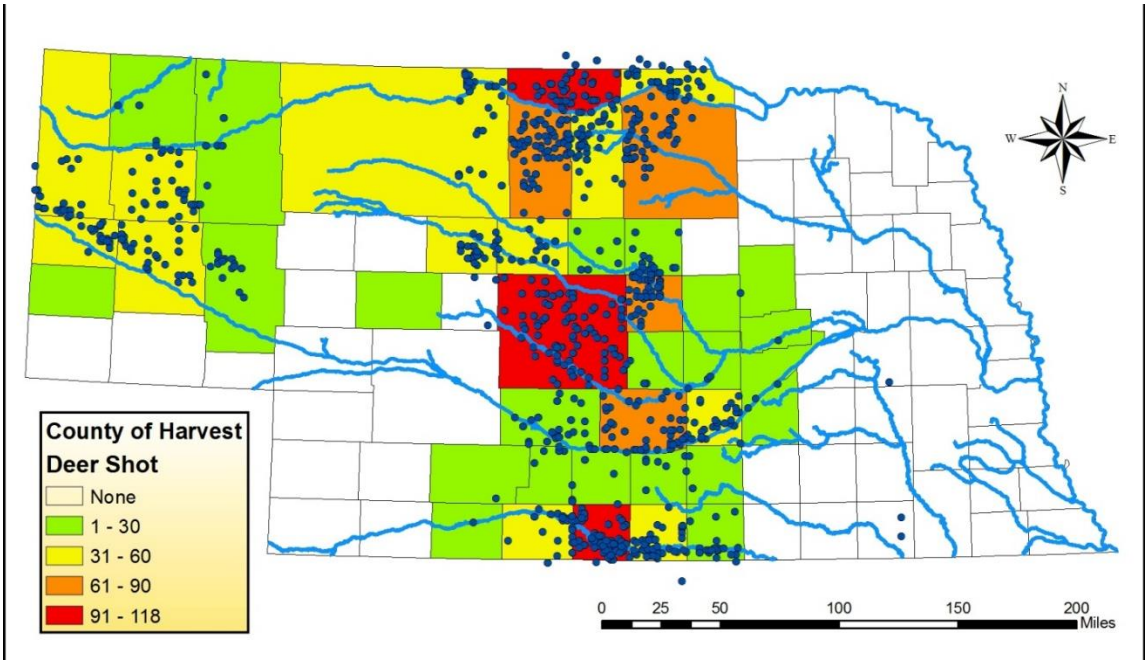


Figure 3-5: Map showing localities of deer taken during chronic wasting disease sampling

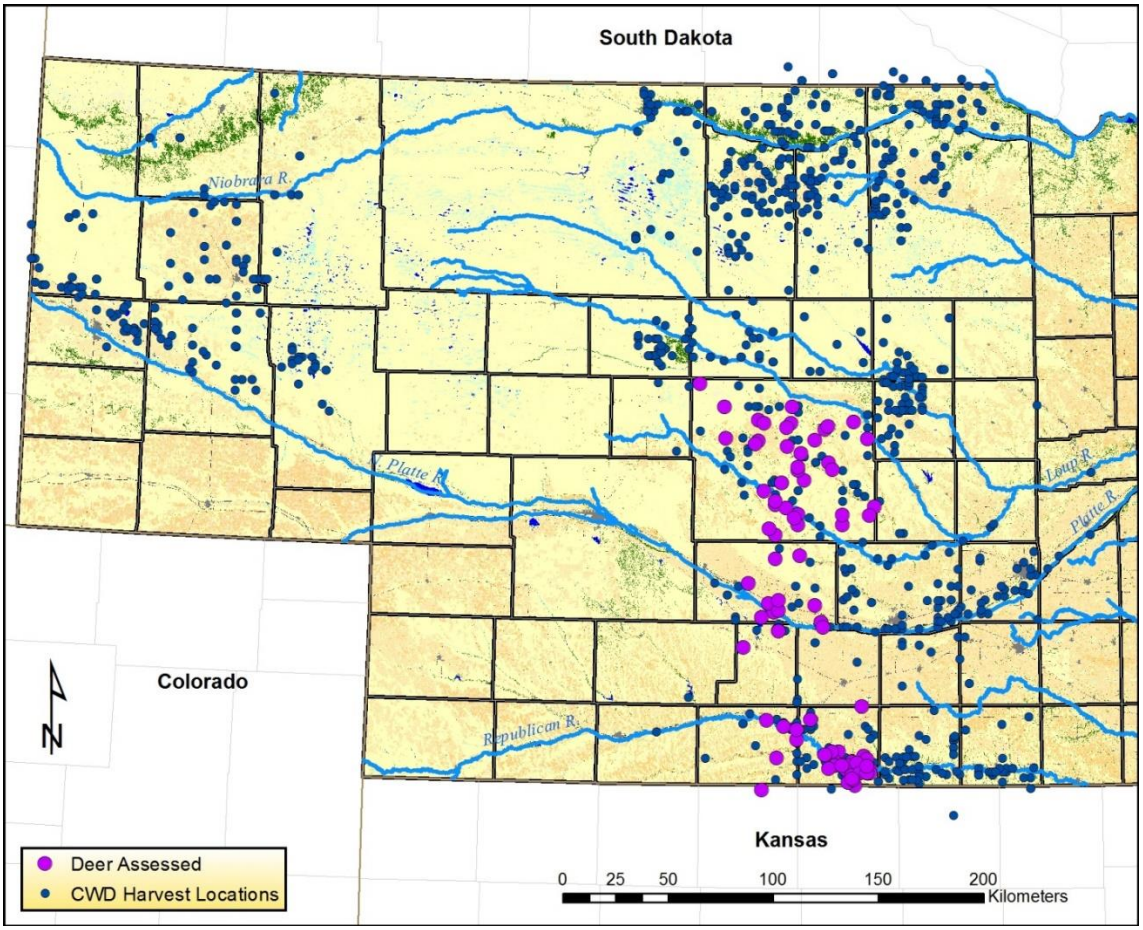


Figure 3-6: Map of chronic wasting disease deer samples assessed for *Borrelia* sp. and *Ehrlichia chaffeensis*

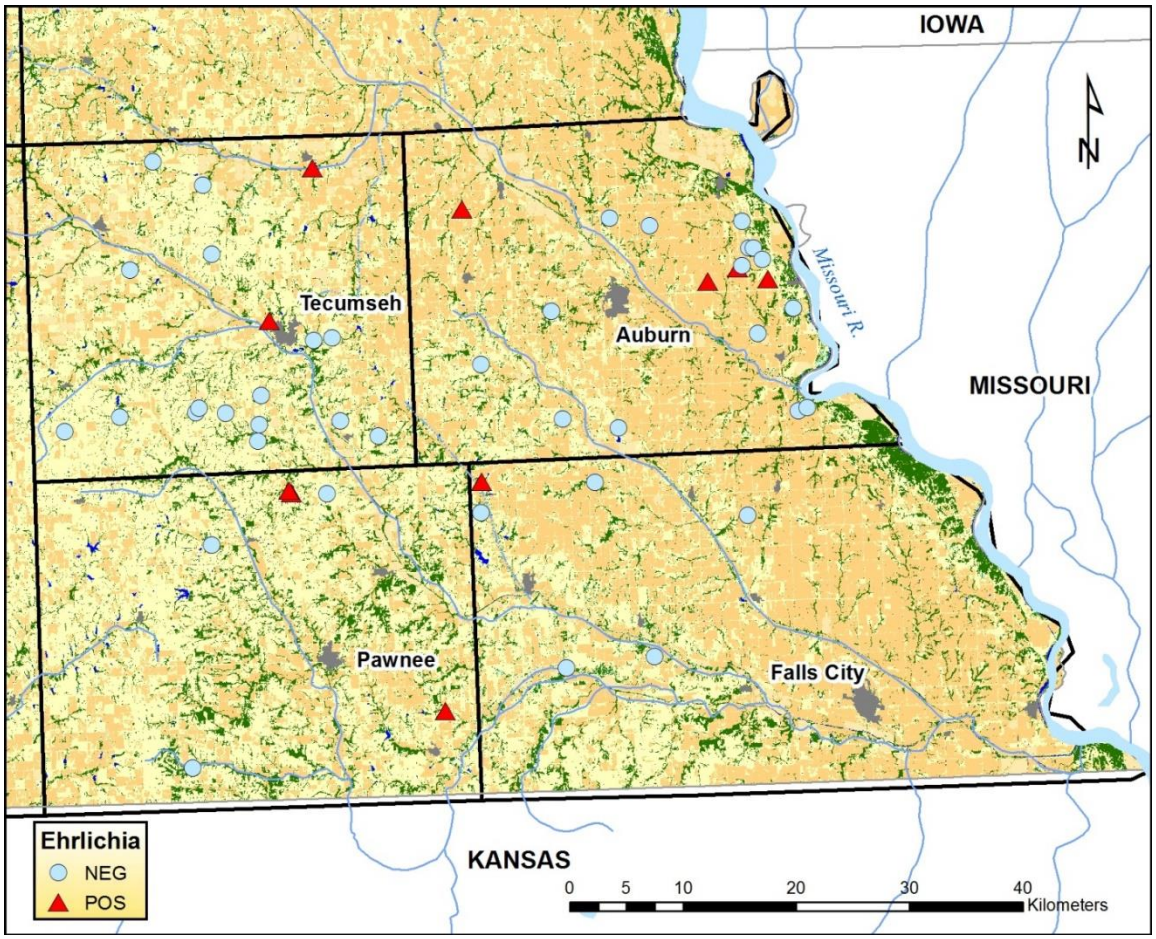


Figure 3-7: Map of deer samples from southeast Nebraska with positive tests for *Ehrlichia chaffeensis*

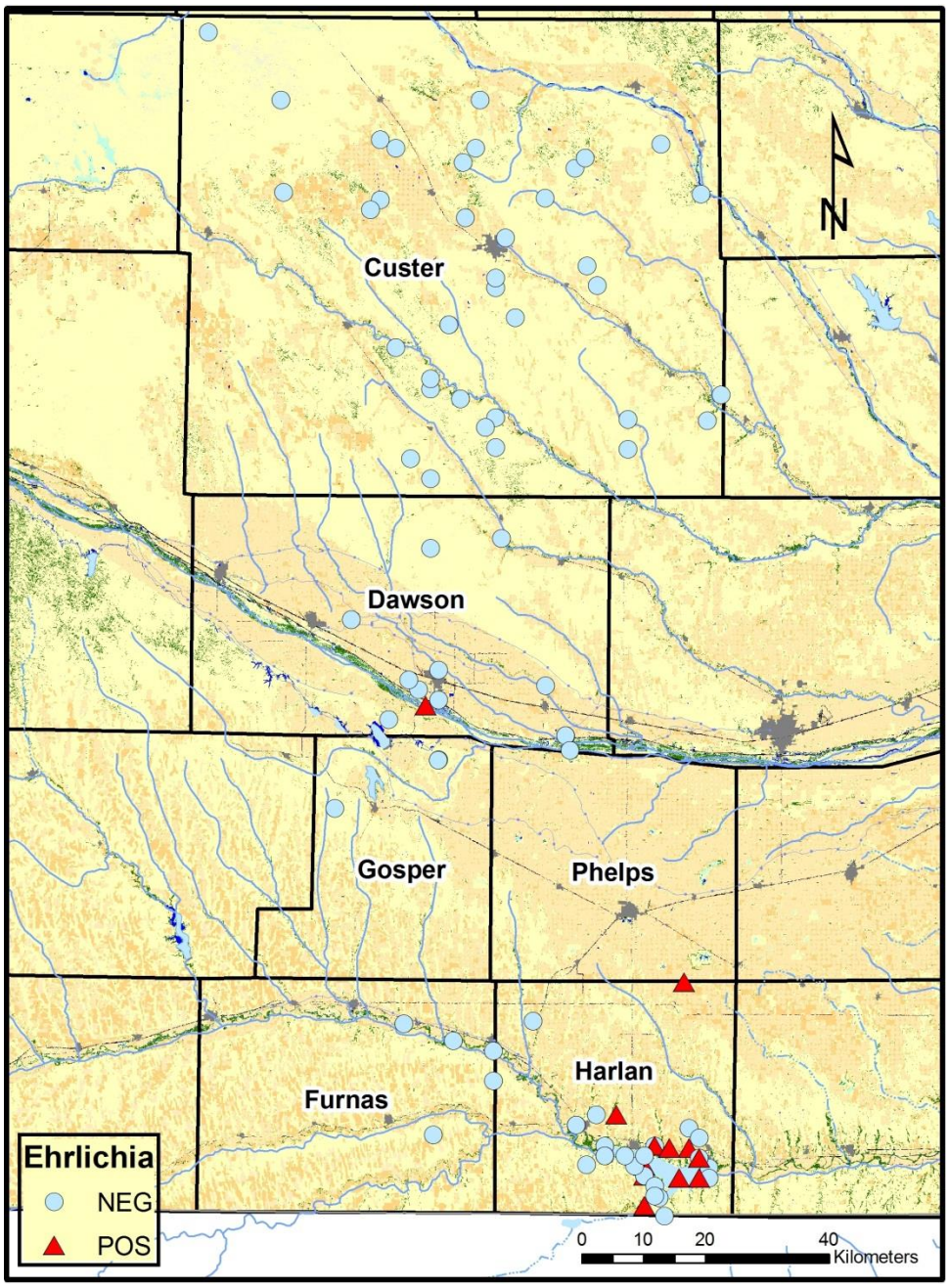


Figure 3-8: Map of positive *Ehrlichia chaffeensis* tests from chronic wasting disease samples

Two positive whitetail deer shot in Furnas County were not mapped due to lack of GPS coordinate information. Two negative mule deer and one negative whitetail from Custer, and one negative whitetail from Furnas are also not shown due to lack of GPS data.

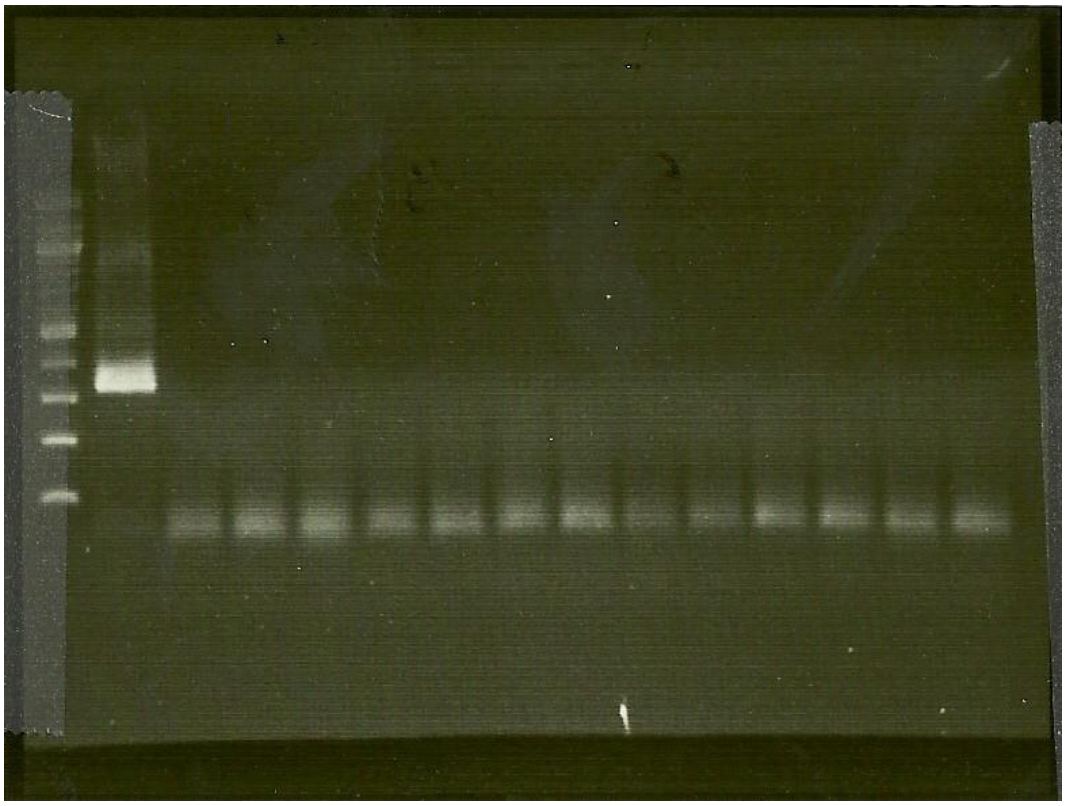


Figure 3-9: Photograph of representative PCR results for *Borrelia* from deer lymph node analysis

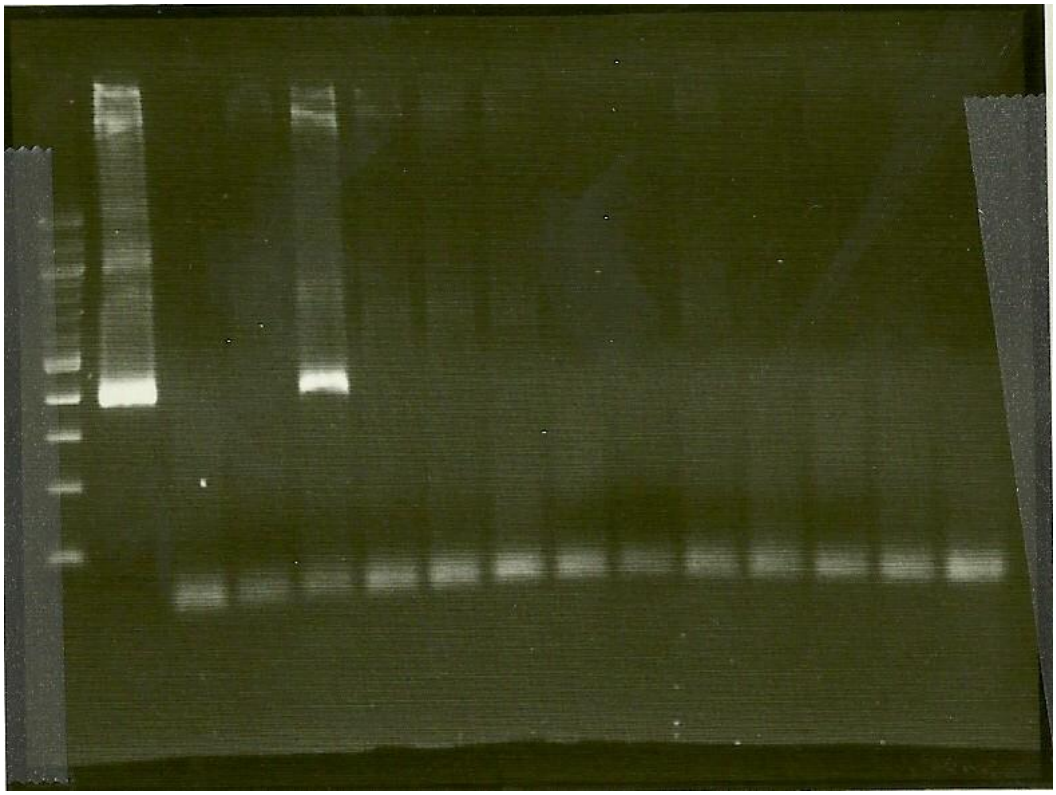


Figure 3-10: Photograph of representative PCR results for *Ehrlichia chaffeensis* from deer lymph nodes

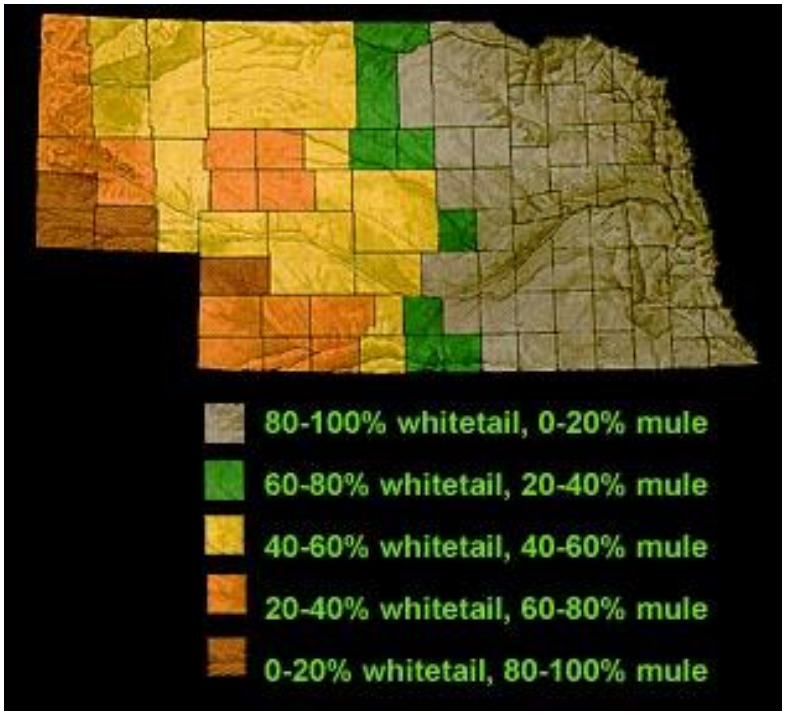


Figure 3-11: Distribution map of whitetail versus mule deer
http://outdoornebraska.ne.gov/hunting/guides/biggame/deer/bgdeermaps.asp

Primer	Target gene		Sequence
FLALL	*Borrelia* flagellin (*fla*B)	1°	5′-ACATATTCAGATGCAGACAGAGGT-3′
FLARL			5′-GCAATCATAGCCATTGCAGATTGT-3′
FLALS		2°	5′-AACAGCTGAAGAGCTTGGAATG-3′
FLARS			5′-CTTTGATCACTTATCATTCTAATAGC-3′
ECB	*Ehrlichia* and *Anaplasma* 16S rDNA	1°	5'-CGTATTACCGCGGCTGCTGGCA-3'
ECC			5'-AGAACGAACGCTGGCGGCAAGCC-3'
HE1		2°	5'-CAATTGCTTATAACCTTTTGGTTATAAAT-3'
HE3			5'-TATAGGTACCGTCATTATCTTCCCTAT-3'

Table 3-1: List of polymerase chain reaction primers used during the study

County	# Taken	# *E. chaffeensis*	Prevalence	# *Borrelia* sp.
Richardson	8	2	0.25	0
Nemaha	17	3	0.18	0
Johnson	18	2	0.11	0
Pawnee	6	3	0.5	0
Custer	40	1	0.03	0
Dawson	11	1	0.09	0
Harlan	37	10	0.27	0
Gosper	3	0	0	0
Furnas	6	2	0.33	0
Phelps	3	1	0.33	0
Southeast	49	10	0.20	0
South central	50	14	0.28	0
Mid-central	50	1	0.02	0
Overall	149	25	0.17	0

Table 3-2: Prevalence of *Ehrlichia chaffeensis* and *Borrelia* sp. per county

Deer ID #	Ticks collected	Adult	Nymph
19	1	1	0
30	1	1	0
35	3	2	1
47	13	12	1
48	3	2	1
Total	21	18	3

Table 3-3: Ticks collected from deer taken in southeast Nebraska during lymph node collection