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# Ixodes Scapularis In North Dakota: Phenology, Population Genetics, And Local Host Reservoir Competency In An Emerging Vector Population

Michael Dougherty

# IXODES SCAPULARIS IN NORTH DAKOTA: PHENOLOGY, POPULATION GENETICS, AND LOCAL HOST RESERVOIR COMPETENCY IN AN EMERGING VECTOR POPULATION

By

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This dissertation, submitted by Michael William Dougherty in partial fulfillment of the requirements for the Master of Science from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done, and is hereby approved.

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ABSTRACT

Tick surveillance in eastern North Dakota has revealed the presence of established *I. scapularis* populations in fragmented forest habitats previously regarded as unsuitable for this species. Understanding *I. scapularis* phenology, the reservoir competency of local vertebrate hosts, and population structure of blacklegged ticks in this region is essential to determining potential human risk of Lyme disease. Tick surveys were conducted throughout North Dakota by flagging and small mammal trapping. 1,701 ticks were collected, including 297 *I. scapularis* exhibiting a relatively low *Borrelia burgdorferi* infection rate (ca. 3-5%). Seasonal abundance varied greatly among life stages, with adult *I. scapularis* being most common in the early summer months (May-June) and juvenile *I. scapularis* abundance increasing later (July-August). Nearly half of all small mammals captured were red-backed voles (*Myodes gapperi*), and were parasitized by immature blacklegged ticks. To determine whether voles served as reservoirs for Lyme disease F-1 voles were injected with *B. burgdorferi* s.s. and at 2 and 4 weeks, the voles were infested with larval deer ticks. Engorged ticks were allowed to molt to nymphs and the nymphs were re-fed on naïve laboratory mice to determine if the larval ticks got infected and, as nymphs, were able to transmit the borrelia. Analyses of the mitochondrial DNA of deer ticks collected from North Dakota revealed a high haplotype diversity (Hd=0.80), and genetic structure suggests colonization from multiple sources. Populations fit theoretical models of population expansion using multiple test statistics, indicating potential increase in human tick exposure and Lyme disease incidence.

# CHAPTER I

## INTRODUCTION

Ticks are obligate blood sucking arachnids and are members of the subclass Acari along with mites. Ticks are ubiquitously distributed among a wide variety of habitats and are significant pests of humans, livestock, and wildlife (Sonenshine 1991). While tick bites themselves may cause a range of symptoms to both humans and animals (blood loss, tick paralysis, allergies, etc.), ticks also serve as vectors for many pathogenic organisms including viruses, bacteria, and protozoa. In North America, Lyme disease is the most common arthropod borne illness, and the incidence of Lyme disease has recently increased both in range and rate of infection most notably in the Midwestern United States (CDC 2014). Data of this kind coincides with recent reports regarding the range expansion of the tick *Ixodes scapularis*, the Lyme disease vector in eastern North American, and observations of both the causative Lyme disease agent (the bacteria *Borrelia burgdorferi*) and *I. scapularis* in newly defined geographic regions (Diuk-Wasser et al. 2012; Russart et al. 2014). The following chapter will serve as an introduction to the biology of ticks and tick-borne diseases, with particular emphasis placed on the vector of Lyme disease in eastern North America, *Ixodes scapularis*, and the potential for expansion and increased tick-borne diseases in North Dakota. Analysis of human risk factors related to *I. scapularis* and Lyme disease in this region may have implications for the future spread of tick borne diseases throughout the Midwest.

## Classification

Ticks and mites belong to the subclass Acari, the largest subclass of the Class Arachnida which includes other chelicerate arthropods such as scorpions and spiders. Within the subclass Acari, ticks are grouped together with the mesostigmatid mites in the Superorder Parasitiformes. Ticks (Order Ixodida) are segregated among three families: the Ixodidae (hard ticks, ca. 660 species), the Argaside (soft ticks, ca. 170 species), and the Nutellidae which possesses features of both hard and soft ticks and is represented by a single rarely-encountered species (*Nuttelliella namaqua*) found only in arid, rocky regions of Tanzania and South Africa (Oliver 1989). In this section I will briefly discuss the distinguishing characteristics of the hard ticks, with particular emphasis on the genus *Ixodes* and those characteristics important to understanding the life history of these species.

Ixodidae (hard ticks) is the dominant tick family with respect not only to number of species represented, but also its economic and medical importance. Depending on the source cited, there are over 660 species arranged in 12 to 14 families. Present day ixodid families are thought to be composed of two major lineages, the more basal Prostriata represented by the large genus *Ixodes*, and the Metastriata which includes all the rest of the genera. A listing of the genera, together with general information on geographic distribution and life history characteristics is given in Table 1.

**Table 1.** Summary of ixodid tick genera of the world, From Kolonin 2009

| Genus | No# Species | Characteristics |
|---|---|---|
| *Ixodes* | 232 | Most often nidicolous, while some species are non-nidicolous and inhabit temperate wooded or grassy environments. Include the major vectors of tick-borne diseases in North America and Europe (*I. scapularis*, *I. pacificus*, and *I. ricinus*) responsible for transmission of Lyme disease, tick-borne encephalitis, babesiosis, anaplasmosis, and ehrlichiosis. |
| *Haemaphysalis* | 155 | One of the most widely distributed ticks occupying a wide range of habitats in the Americas, Europe, and Asia. Major reservoir of *Rickettsia rickettsia* in South America, and often a significant veterinary pest of small mammals. |
| *Amblyomma* | 105 | Large ticks adapted to hot climates and occupy a wide range of habitats. Usually one-host ticks which feed on large ungulates. Known to transmit various diseases to man and domesticated animals including: Rocky Mountain spotted fever, ehrlichiosis, and anaplasmosis. *A. gemma* recently identified as a major reservoir of viral livestock diseases. |
| *Rhipicephalis* | 75 | Mostly restricted to the Old World, particularly Africa, with one species (*R. sanguineus*) distributed worldwide. Mainly occupy savanna and open woodland habitats and only feed on mammals. |
| *Dermacentor* | 33 | Nearctic distribution with representatives on all continents except Australia. Major vectors of human and veterinary pathogens causing Rocky Mountain spotted fever, Q fever, anaplasmosis, encephalitis, tick paralysis, cattle tularemia, and equine piroplasmosis. |
| *Hyalomma* | 27 | Adapted to arid biomes of Africa, Middle East & Central Asia. Parasitize livestock and transmit many livestock diseases. Some species transmit Crimean-Congo haemorrhagic virus to humans. |

Table 1 Cont.

| | | |
|---|---|---|
| *Aponomma* (now included as a subgenus of Amblyomma) | | Mostly tropical in distribution, most species parasitize snakes & lizards, although a few parasitize marsupial and monotreme mammals in Australia; Considered as 3-host ticks although the biology of *Aponomma* ticks is poorly known. |
| *Boophilus* (now included as a subgenus of *Rhiphicephalis*) | | Widespread 1-host ticks of cattle and other ungulates; High tick burden and transmission of livestock diseases make this genus a pest to livestock industry, rarely if ever attacks humans. |
| *Anomalohimalya* | 3 | Central Asia, montane regions; Parasitize rodents |
| *Margaropus* | 3 | Africa, 1-host ticks, giraffes, zebra, horses |
| *Rhipicentor* | 2 | Africa, adults parasitize wild carnivores |
| *Cosmiomma* | 1 | Africa, dry savannah, adults collected from hippos, rhinos |
| *Dermacentonomma* | 1 | Indochina, single male on sambar (deer) |
| *Nosomma* | 1 | SE Asia, adults parasitize buffaloes, immatures feed on rodents |

## Anatomy & Morphology

Members of the family Ixodidae, or hard ticks, have an elongated body bearing two clear segments: the capitulum, which bears mouthparts including chelicerae, palps, and hypostome, and the idiosoma, bearing walking legs, stigmata (=spiracle), and the genital and anal pores (figure 1). The idiosoma can be further subdivided by the podosoma (bearing walking legs and genital pore) and the opisthosoma (the region posterior to the hind coxa bearing the anal aperture).

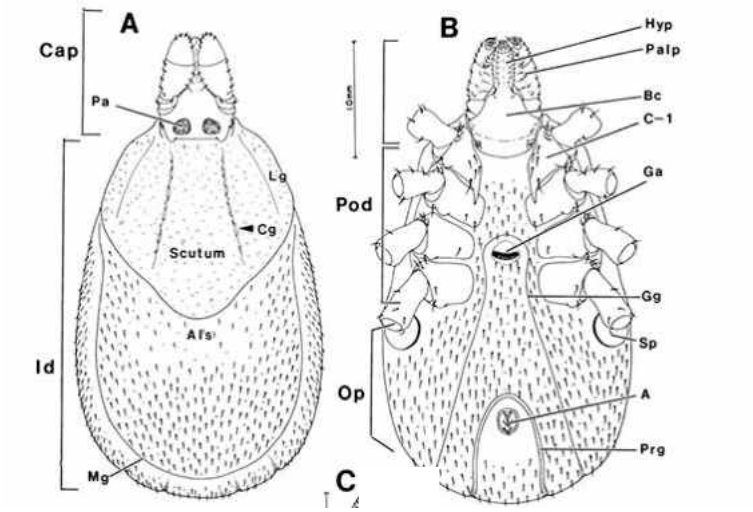


**Fig 1.** Generalized body plan of an adult ixodid tick female. A) Dorsal view, capitulum (Cap), idiosoma (Id), scutum, and alloscutum (Als) visible. B) Ventral view, genital pore (Ga) anal aperture (A), podosoma (Pod), opisthosoma (Op), preanal groove (Prg). Image reprinted from Sonenshine 1991.

The capitulum is made up of three unique appendages as well as the basis capituli which anchors the mouthparts to the idiosoma by a flexible membrane. The chelicerae are sheathed appendages armed with sharp digits bearing sensilla and powerful musculature enabling the tick to penetrate host skin. The hypostome is a structure composed of various canals to facilitate the transfer of essential fluids involved in blood feeding to and from the host. The hypostome is armed with recurved denticles which aid in prolonged attachment to vertebrate hosts. In the ixodid ticks, the paired chelicerae are located dorsally with respect to the hypostome, and the two structures form the opening to the oral cavity through which saliva is secreted and blood is ingested. In the ixodid ticks the mouthparts protrude anteriorally and are summarized in Figure 2. Located on either side of the capitulum are the four-segmented palps. The palps of ixodid ticks are made up of a two small immobile segments (I and IV) conjoined by two larger mobile segments (II and III). Segment IV is greatly modified and bears a large cluster of sensory cells on the

extensible terminal segment which are essential to host identification and questing behavior.

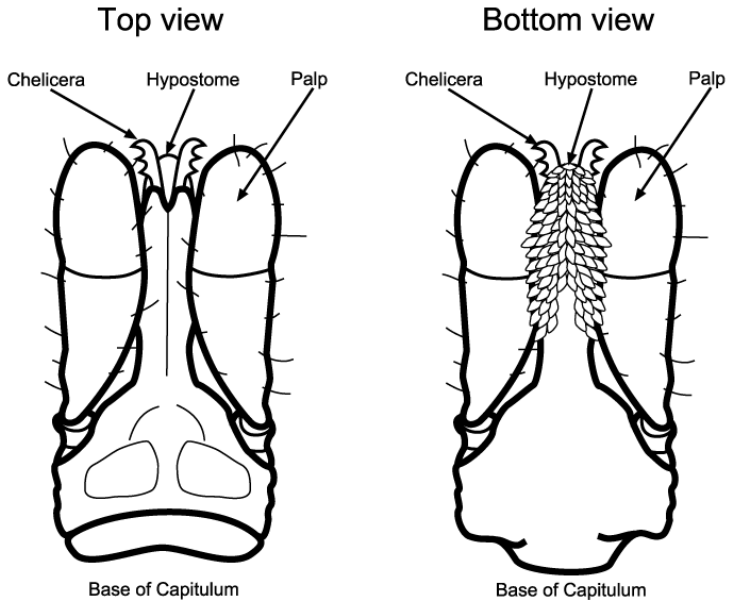


**Fig. 2.** Mouthparts of the blacklegged tick, *Ixodes scapularis*. Image provided by Purdue University (http://extension.entm.purdue.edu/publichealth/insects/tick.html)

The podosomal region is distinguished dorsally by the presence of a hardened scutum, a generally smooth hardened structure bearing sensory organs including the sensilla auriformia (propioreceptive organs; Mullen & Durden 2002) and ocelli (simple photoreceptive organs; Parry 1947). In the genus *Ixodes* the scutum lacks ornamentation and ocelli. Ventrally, this region also bears four paired legs, as well as a medially located genital pore. The alloscutum refers to the flexible dorsal region posterior to the scutum, and outlines the opisthosomal body region in ticks. In adult male ixodid ticks, the alloscutum is often greatly reduced. In the larval, nymphal, and adult female life stages the alloscutum is enlarged to accommodate a large increase in body size associated with large blood meals necessary to molt or nourish developing eggs. Ventrally, the opisthosomal region houses the anal aperture as well as the anal groove. In the genus

*Ixodes*, this anal groove extends past the body margin forming a loop anterior to the anus (figure 1, Prg).

Like all arthropods, the tick integument consists of a cellular layer, the *epidermis*, and an acellular layer, the *cuticle*, which consists of layer upon layer of chitin polymers secreted by the epidermis. The cuticle can be further divided between the thin outer epicuticle and the thick inner procuticle. Soon after molting, large regions of the relatively flexible procuticle harden into plates or sclerites during a process known as sclerotization. In these regions, the long polymers of chitin become cross-linked by proteins which results in a rigid cuticle. In intervening areas, chitin is not "sclerotized" and the cuticle remains flexible, allowing for hardened sclerites to articulate and for the animal to move. Penetrating the tick cuticle are various pore canals utilized by the tick for the passage of important chemicals produced by the animal through the impenetrable cuticle. Lipids produced by the tick are transported via these canals to the epicuticle to form a waxy layer of sterol/steryl esters which aid in water retention; during feeding (and thus an influx of aqueous nutrients) production and lipid composition of the cuticle is can increase up to four fold (Sonenshine 1991). Water retention is an important limiting factor in the distribution of ixodid ticks, particularly of the genus *Ixodes,* which is more sensitive to desiccation than many other tick species (Sonenshine 1991). Slight changes in relative humidity can greatly alter the survivability of *Ixodes spp.* and alter suitable range distribution (Berger et al. 2014).

Ticks interact with their environment through the cuticle using generalized sensory organs found in abundance throughout the body, most commonly in the form of specialized setae or pits capable of detecting vapor or particular molecules and

stimulating the transmission of nerve impulses to a centralized brain or synganglion. Of particular note are several sensory organs unique to ticks. Haller's organ, located on the tarsi of leg I is a specialized structure housing large clusters of diverse sensory receptors which respond to very small amounts of chemicals present in the natural odor of mammalian hosts, such as phenols and lactones (Leonovich 2004; Duo et al. 2013). While similar primitive structures are present in mites, they lack the uniformity and specialized organization observed in Haller's organ of the ticks. Similarly, an eversible cluster of specialized setae present on the terminal segment of the palp is hypothesized to aid in host recognition and feeding site attachment (Sonenshine 1991). Finally, specialized sensory structures on the digits of the chelicerae serve an important role in mate recognition and copulatory rituals (Kiszewski et al. 2001).

## Life History & Ecology

Most argasid or soft ticks spend their lives within the burrows or nests of their hosts, a strategy referred to as 'nidicolous', and thus do not actively hunt or quest for hosts out in the open. Many prostriate ticks (=genus *Ixodes*) are also nidicolous, however some are not, including the species that is the topic of this thesis – *Ixodes scapularis*. All metastriate ticks are non-nidicolous and quest for hosts.

### *Life Cycle of Non-nidicolous ticks*

Ixodid ticks have three life stages: the six-legged larval stage, the eight-legged nymphal stage, and the eight-legged adult stage. For most tick species, each life stage seeks a vertebrate host (questing behavior), attaches and feeds on the host, and then drops off the host to develop or, in the case of engorged female ticks, lay eggs. This type of

feeding and reproductive behavior in ticks is known as a 3-host life cycle and is the most common type of life cycle seen in ixodid ticks. Typically, the host species used by larval and nymphal ticks are smaller in size (e.g., rodents) than host species used by the adult tick (e.g., dog, ungulate, etc.) Some tick species that specialize on parasitizing large mammals exhibit a 1-host life cycle where the larvae, nymphs and adults stay on the same host throughout the entire life cycle, feeding and molting, and only drop off as engorged females to lay eggs. A few tick species exhibit a 2-host life cycle whereby the larvae and nymphs parasitize the same host before dropping off as engorged nymphs and then, after molting to adults, seek a second host on which to feed. Without exception, all ixodid tick species drop off the host as engorged females and after oogenesis is complete, lay their eggs in a single large cluster (thousands) and then die. Under optimal climatic conditions, the 3-host life cycle in most tick species can be completed within a single year (Sonenshine 1991). In most cases, the cycle takes two years to complete; in the winter when climatic conditions become unfavorable, ticks overwinter in the adult or larval stage. In cases of extreme cold, this life cycle can become lengthened to three or more years, a behavior observed in the European vector of Lyme disease *Ixodes ricinus* (Arthur 1962). Ogden (2004) describes temperature-dependent lengthening of *I. scapularis* development in southern Canada, and remarks: "the longer the tick's life cycle, the greater proportion of ticks that will die before reaching adulthood." In other words, populations of *I. scapularis* in Northernly latitudes may exhibit a lengthened, three-year life cycle which may limit population establishment in regions like North Dakota.

The typical three-host life cycle of ixodid ticks is reviewed by Sonenshine (1991) and is summarized here. The eggs are laid and undergo embryogenesis in the natural environment. Larvae hatch and begin host-seeking behavior or, under unfavorable environmental conditions, enter diapause and overwinter. Upon recognition of an appropriate host, the tick will engage in a sequential series of behaviors: attachment to host, feeding, engorgement (satiation following several days of feeding), and detachment. At this point the blood meal will be digested and the larvae will undergo ecdysis emerging as nymphs, only to complete the cycle of attachment, feeding, and detachment a second time. Fed nymphs again undergo ecdysis, this time emerging as either male or female adult ticks. During these immature phases, the ideal host for *I. scapularis* are small mammals, most notably various mouse and vole species. Mature ticks engage in host-seeking behavior and complete the attachment and feeding cycle for a third time. In metastriate ticks, bloodfeeding is required for initiation of the gonotrophic cycle and mating occurs on the host. In *Ixodes* the gonotrophic cycle may be initiated without a blood meal and mating often occurs not on the host but in the natural environment prior to feeding. In *I. scapularis*, the successful "off-host insemination" of an unfed female tick by a male tick renders her "unattractive" to other males and hence inhibits subsequent copulations. However, the re-mating inhibition degrades progressively once a mated female attaches to a host and engorges with blood, allowing for re-mating to ensue. (Kiszewski and Spielman 2002). Replete females will drop off the host and may begin oogenesis directly or may enter diapause and initiate oviposition under more favorable climatic conditions. Males take relatively small blood meals, and may feed and mate multiple times over the course of their lives. Female ixodid ticks swell greatly during the

blood meal, "in excess of 100 times their unfed body weight… [and] more than 50% of the engorged, mated female body weight is converted to eggs during this process" (Sonenshine 1991). The female tick lays numerous (often thousands) eggs and then dies.

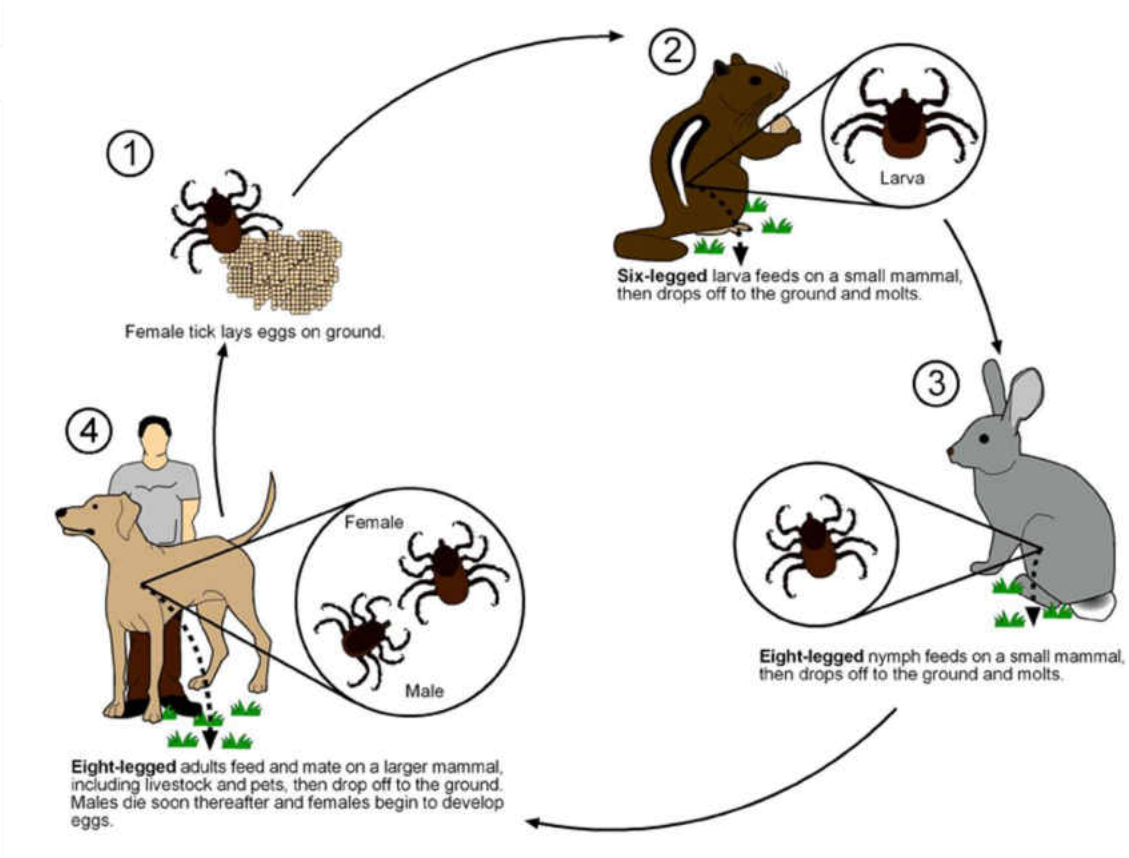


**Fig. 3**. Life cycle of the *Ixodes scapularis* tick. Larval ticks emerge in the spring and initiated questing behavior, most often attaching and feeding on small mammals such as mice or voles. Following successful feeding, larval ticks undergo ecdysis and emerge as nymphs or enter diapause. Nymphal ticks repeat this process and undergo ecdysis resulting in male or female adult ticks. Adult *Ixodes scapularis* mate in the natural environment and then feed on a large mammal, most often deer, before detaching and beginning oviposition. Replete females may enter diapause until climatic conditions are more favorable for oviposition. Image modified from Purdue University (http://extension.entm.purdue.edu/publichealth/images/tick/popups/tick03.gif).

*Habitat & Distribution*

Ixodid ticks are found in a wide array of habitats (see Table 1), but individual species are often restricted to a single optimum habitat type. For the purposes of this research, I will focus on the optimal habitats associated with non-nidicolous prostriate

ticks of the *Ixodes ricinus* complex, including the North American Lyme disease vector *Ixodes scapularis*.

*Ixodes scapularis* is associated with densely forested areas maintaining high populations of white-tailed deer. Adult *Ixodes scapularis* often congregate along trails made by humans or other large mammals and in high shrub environments, while immature *Ixodes spp.* are often found in grassy low-shrub or woodland communities (Sonenshine 1991). Differences in habitat utilization may be a result of differing host preferences among life stages, but remains poorly understood. Similarly, feeding rhythms in non-nidicolous ticks are timed to facilitate population distribution throughout the environment. Detachment in *Ixodes spp.* is often associated with the mammalian scotophase (the period just before dusk); in this way ticks detach while their mostly nocturnal mammalian hosts are foraging, increasing potential future host contact (Sonenshine 1991). Macroenvironments (e.g. large forested areas) are further subdivided by microclimates which are utilized by ticks. Within environments a vertical humidity and temperature gradient exists from the macro- (shrub, leaf, etc.) to micro- (soil, mat, leaf litter) environments, with the latter being particularly important to tick survival. Analysis of microenvironments suggests ticks spend a relatively small portion of their lives engaged in host-seeking behavior. When analyzing tick abundances of the closely-related European species, *I. ricinus,* in such microenvironments Daniel et al. (1977) found only approximately 5% of the active tick population were found in questing zones, while approximately 90% were collected from dense vegetative mats. Similarly, *I. scapularis* survival rates were greatly diminished when ticks were prevented from freely moving among microclimates (Bertrand & Wilson 1996).

Traditionally, *I. scapularis* populations have been limited to the northeastern United States and small isolated areas in Wisconsin (Spielman et al. 1984). More recently, blacklegged ticks have been discovered as far west as North Dakota in habitats previously deemed unsuitable for the species (Russart et al. 2014). Models using environmental metrics and predicted temperature shifts associated with climate change suggest the northern front of *I. scapularis* range distribution is expanding as ticks establish populations in regions which were once too cold (Brownstein et al. 2003; Ogden et al. 2006; figure 4.).

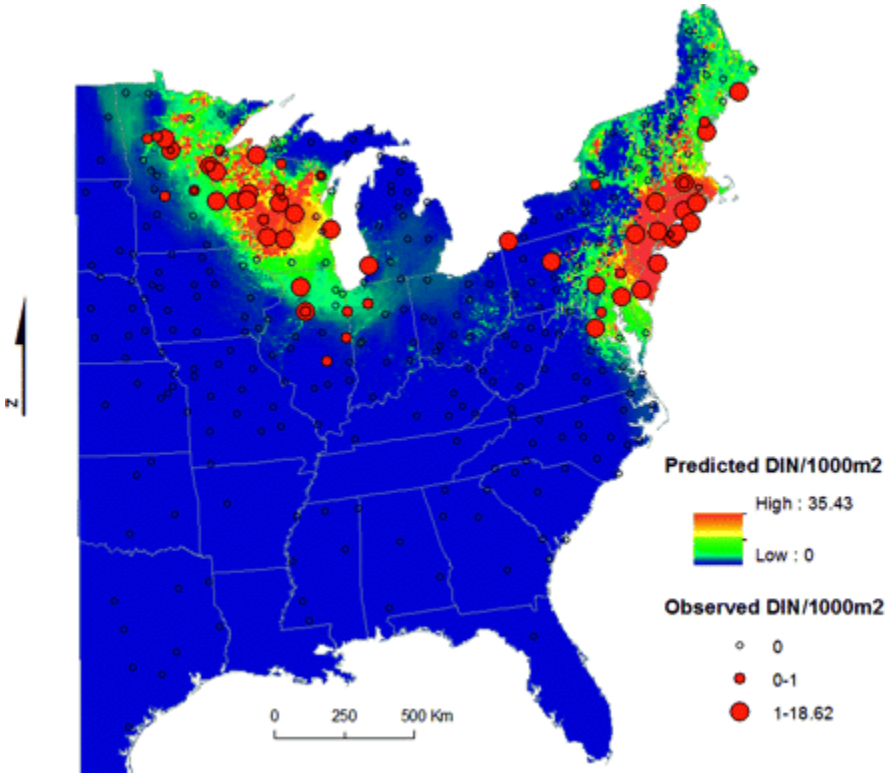


**Fig. 4.** Most recent Lyme disease risk map as predicted by models of climate change (Diuk-Wasser et al 2012). Areas of eastern North Dakota (primarily the Red River Valley) identified as at-risk for *Ixodes scapularis* population establishment and Lyme disease incidence.

Levy (2014) hypothesizes that migratory birds bring small clusters of founder ticks to new regions in central North America, and as temperatures rise these ticks are

capable of founding new populations. In this way, the spread of *I. scapularis* to new regions paves the way for pathogenic organisms reliant on the vector (e.g. *B. burgdorferi*) to follow.

## Ticks as Vectors of Bacterial Pathogens

Ticks are the most significant vector of arthropod borne diseases in North America, and transmit the most diverse array of arthropod borne diseases worldwide (Sonenshine 1991). On a global scale, ticks are "considered to be second only to mosquitoes as vectors of human infectious diseases" (Parola & Raoult 2001). In North America, tick-borne bacterial pathogens are responsible for the most common vector-borne diseases of humans, including Lyme disease (*Borrelia burgdorferi)* transmitted by *I. scapularis* (east of Rocky Mountains) and *I. pacificus* (west of Rocky Mountains), Rocky Mountain fever (*Rickettsia rickettsia)* transmitted by *Dermacentor.variabilis* (east of Rocky Mountains) and *D. andersoni* (Rocky Mountains westward), human anaplamosis transmitted by *I. scapularis*, and ehrilichosis (*Ehrlichia chaffeensis*, *Ehrlichia ewingii)* transmitted by *Amblyomma americanum.* This section will focus on *B. burgdorferi*, and use this species as a model for bacterial transmission.

Ticks may become infected with bacteria during feeding on a bacteremic reservoir host. In order to transmit bacteria to a new vertebrate host, two conditions must be met by the tick vector: 1) the bacteria must multiply in the gut of the tick, leave the gut and enter the tick salivary glands (facilitating transmission during the next blood meal) or tick ovaries (facilitating transovarial transmission) and 2) the bacteria must persist through the molting process, as ticks feed only once per life stage (reviewed by Parola & Raoult 2001). If the bacteria is passed both transtadially and transovarially, tick species

themselves can serve as disease reservoirs. This is the case in many *Rickettsia spp.* which multiply in prolific numbers in almost all organs and tissues of the tick vector. In some cases bacterial pathogens are not transmitted transovarially, and thus mammalian reservoirs and continued cycling of the pathogen in the tick vector is essential to disease maintenance. This is the case in the Lyme disease agent, *B. burgdorferi*, in which transovarial transmission is rare.

Tick-borne bacterial diseases are zoonotic diseases, and maintained in transmission cycles involving ticks and wild animals (Parola & Raoult 2001). Zoonotic diseases rely on various wild mammalian bacterial 'reservoirs', which may involve multiple species of ticks or hosts (Parola & Raoult 2001). Maintenance of pathogen populations in the zoonotic cycle is dependent on a wide range of factors, including: infectivity of host species, distribution of hosts, tick preference, tick infestation rate, host immunity, and tick attachment duration on reservoir hosts (Lane et al. 1991; Mather & Ginsberg 1994). Understanding dynamics of disease reservoirs in this way is essential to understanding epidemiology of bacterial diseases, and are of particular importance in species such as *B. burgdorferi* in which the tick vector does not serve as a major pathogen reservoir. Maintenance and persistence of zoonotic diseases is important to human health, as humans entering these environments may serve as incidental hosts for bacterial pathogens after being parasitized by bacteremic adult or nymphal ticks. The transmission cycle of *B. burgdorferi* and incidental human infection is given in figure 5.

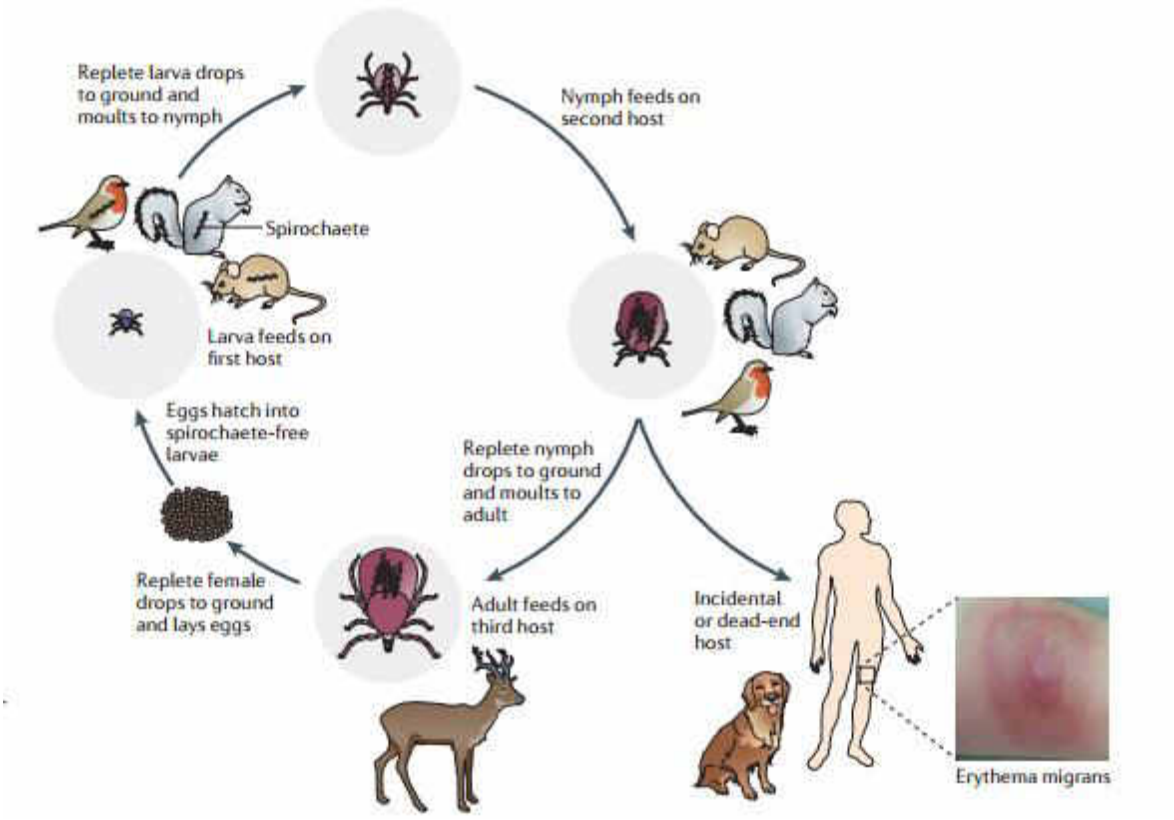


**Fig. 5.** The enzootic cycle of *Borrelia burgdorferi*. Transmission depends on larvae feeding on bacteremic mammalian reservoirs and subsequent feeding by infected nymphal ticks on novel mammalian hosts. In this manner the bacteria is cycled among novel reservoirs and newly hatched ticks. Humans and domesticated animals serve as 'dead-end' hosts for the pathogen, as larval ticks will not feed on these hosts, and thus the bacteria will fail to be cycled throughout the natural environment. Image reprinted from Radolf et al. 2012.

Transmission of Lyme disease by ticks occurs by contamination of the feeding site by infected salivary secretions. During feeding, ticks insert the immobile hypostome into the host skin and secrete various cement-like substances. During the first 24 hours a feeding pit is established at the bite site; at this point very little blood has entered the midgut and the spirochete has not yet begun replication. By 24-48 hours post-attachment, the tick begins to consume host blood, and the bacterial spirochete undergoes massive replication in the midgut and subsequent migration to the tick salivary glands (Radolf et al. 2012). Tick saliva contains a wide array of bioactive proteins which the bacteria exploits in order to avoid host immune defenses including: inhibition of complement

cascade, impairment of various antigen presenting cells, repression of cytokine production and cell-signaling, and the blocking of T-lymphocyte proliferation (Hovius 1999; Figure 6). Dissemination and survival of the bacteria in mammals relies on various outer-surface proteins involved in evasion of both the innate and adaptive immune system. Genetic variation in these proteins may be associated with the rate of dissemination and severity of disease symptoms in humans (Tilly et al. 2008).

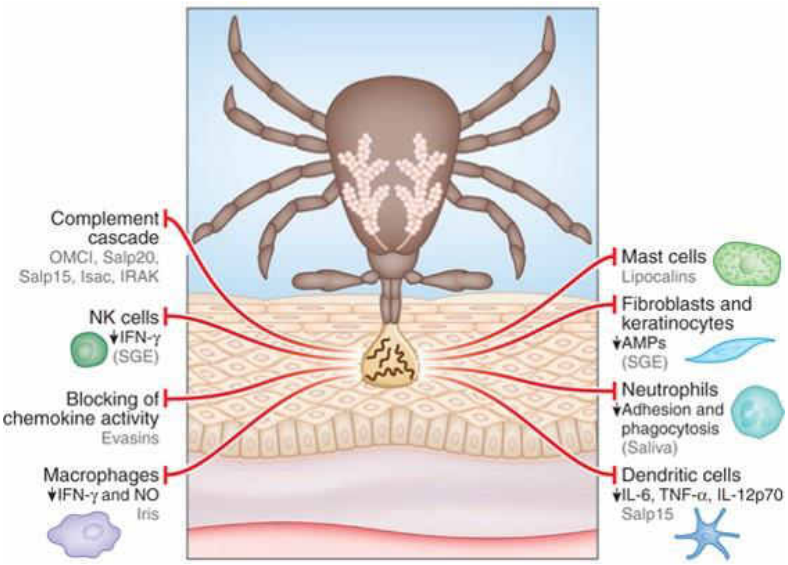


**Fig. 6.** Diagrammatic representation of the formation of the tick feeding site. The hypostome is inserted into host skin to create a feeding pool, after 24 hours the tick begins to produce salivary proteins used to facilitate feeding which are exploited by the spirochete to enter the parasitized host. Image reprinted from Hovius 2009.

Lyme disease diagnosis poses a significant challenge to physicians. The disease is often difficult to detect in the early stages; individuals are often unaware that they have been bitten by a tick and present only vague symptoms such fever, headache and fatigue. Erythema migrans is the most notable early symptom of the disease, and is described as a 'bullseye rash' emanating from the location of the infective tick bite (Wormser et al. 2006). However, early symptoms may also include more serious complications such as Lyme meningitis resulting from early dissemination of spirochetes into the blood stream

and subsequently the brain. During the early infection phase, antimicrobial drugs may be administered for 14-21 days with a high rate of success. Late stages of the disease are often characterized by recurring arthritis or severe neurological complications and often require extensive antibacterial treatment lasting four or more weeks (Wormser et al. 2006). Clinically, early identification of Lyme disease is essential to avoid the more severe symptoms associated with chronic infection. Development of effective rapid early diagnostic tests for the *B. burgdorferi* spirochete has proven difficult with a low rate of successful diagnosis (Smit et al. 2015). Smit et al. (2015) demonstrate that rapid diagnostic testing often requires high antibody levels and serum (as opposed to whole blood) samples in order to be reliably sensitive.

## Summary

Ticks are the most common vectors of arthropod borne diseases in North America, and significant vectors of a wide variety of pathogens worldwide. In North America, *Ixodes spp.* are responsible for transmission of the causative Lyme disease agent, *Borrelia burgdorferi*, and human incidence of Lyme disease in the Midwest has been rising consistently since 2001, particularly in the North. This phenomenon may be explained by the expanding range of the disease vector in this region, *Ixodes scapularis*. Range expansion of this nature may be the result of climate change as determined by predictive models, and vector populations may continue to expand in both range and abundance in the future. In this thesis, I will analyze newly established *I. scapularis* populations at the northwestern edge of their current range distribution in North Dakota. Studies presented here will analyze: 1) phenology of tick vectors in this unique climatic region, 2) genetic structure of isolated blacklegged tick populations and their relation to

the broader continental distribution, and 3) the role of an abundant regional host, *Myodes gapperi* (the red-backed vole), as potential disease reservoir.

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CHAPTER II

# SURVEY OF TICKS AND TICK-BORNE PATHOGENS IN NORTH DAKOTA

## Introduction

Lyme disease is caused by infection with the spirochete, *Borrelia burgdorferi*, and transmitted by the tick species *Ixodes scapularis* in the eastern and midwestern United States, *I. pacificus* in the western United States, and *I. ricinus* in Europe (Lane et al. 1991). Lyme disease is the most common vector-borne disease in the United States, with approximately 20,000 new cases reported each year (CDC 2007). Historically, an overwhelming majority of Lyme disease cases (92%) have been reported from eight northeastern states and two north central states (Minnesota and Wisconsin; Orloski 2000). In recent years the incidence of Lyme disease in North Dakota has increased from zero (ca. 2004) to 3.2 (ca. 2010-2014; CDC 2015). Similarly, field surveys throughout North Dakota have revealed several established populations of the tick vector *I. scapularis* and confirmed the presence of *B. burgdorferi* (Russart et al. 2014). While historical data suggests the region is inhospitable for population establishment by *I. scapularis*, meteorological models suggest climate change may lead to the establishment of populations in areas of North Dakota previously deemed unsuitable (Diuk-Wasser et al. 2012; Ogden 2006). Genetic analyses of *I. scapularis* from this region suggest these populations are recently established and currently undergoing expansion, implicating this region as a potentially significant source of future Lyme disease infections (see chapter 3).

Preventative public health initiatives concerning Lyme disease rely largely on the identification of *I. scapularis* populations both geographically and temporally with regards to human contact with the vector; Lyme disease incidence is frequently correlated with abundance of the tick vector (Falco et al. 1999). In the United States, tick bites and reported cases of Lyme disease are most common in the June and July (CDC 2015), coinciding with peaks in nymphal abundance throughout the United States (Diuk-Wasser 2006). This pattern arises from the commonly observed two-year life cycle observed in blacklegged ticks, however, in extreme climates exhibiting extreme periods of cold this life-cycle may be extended to encompass three years as observed in the related species *I. ricinus* and will result in a radically different phenology (Arthur 1962). Additionally, year-to-year seasonal abundance of *I. scapularis* and human Lyme incidence can vary significantly (Schultze & Jordan 1996). Meteorological models suggest temporal shifts in *I. scapularis* emergence may be the result of climate change, with warmer winter/spring temperatures resulting in earlier average onset of Lyme disease cases annually (Monaghan 2015). The phenology of *I. scapularis* in North Dakota and consequently seasonal Lyme disease risk remains unexamined.

## Materials and Methods

Tick collections were conducted at two field sites in Grand Forks County, North Dakota: Forest River Wildlife Management Area (48°12'54.76"N, 97°28'5.30"W) and Turtle River State Park (47°56'19.35"N, 97°29'27.63"W). Questing adult and nymphal ticks were collected by dragging with a $1.0m^2x0.5m^2$ white cloth and from the clothes of field collectors. Collection sites were sampled in this way for four hours on a weekly basis between the dates of May 4 - July 5. Immature ticks were also collected from small

mammals (UND IACUC protocol 1304-3). Mammals were collected using Sherman live traps baited with peanut butter and oats, and set at the site in the evening. The following morning, mammals were anesthetized in the field using Isoflurane, identified to species, and attached ticks were removed using forceps and stored in 70% ethanol as previously described. Ethanol-preserved ticks were counted, identified to species, sex and lifestage in the laboratory (Clifford et al. 1961, Durden and Keirans 1996). For analyses, tick count data were normalized to reflect a consistent effort where necessary (flagging = 4 hours per week, trapping = 10 mammals per week). Tick collections and small mammal trapping was conducted under North Dakota Game & Fish scientific collecting permits GNF03566675 and GNF03100040.

For pathogen detection, *I. scapularis* ticks were bisected longitudinally and DNA was extracted using a previously described guanidine thiocyanate method (Tkach and Pawlowski 1999). Adult *I. scapularis* from individual study sites were screened for *Borrelia burgdorferi* using previously described PCR primers (FL6: 5'-TTCAGGGTCTCAAGCGTCTTGGACT-3'; FL7: 5'-GCATTTTCAATTTTAGCAAGTGATC-3'; Picken 1992). Positive PCR products were sequenced using the ABI PRIMS 3100 Genetic Analyzer and the previously described primers. Sequence fragments were compared with the GenBank database to confirm the presence of *B. burgdorferi*.

## Results

Flagging collections are summarized in Table 2. A total of 1,271 ticks were collected by flagging and consisted of two species; *Ixodes scapularis* and *Dermacentor variabilis*. Both species were collected at each site. A much larger quantity of ticks was

collected from Forest River (n=1,139) compared to Turtle River State Park (n=132) despite similar collection effort at both sites. *Dermacentor variabilis* constituted a majority of ticks collected (n=974) compared to *I. scapularis* (n=297). However, the tick species composition differed significantly between Forest River (21% of total collection was *I. scapularis*) and Turtle River (42% *I. scapularis*) (Pearson's chi-square: $\chi^2$=27.5, p<0.0001). While only adult *D. variabilis* were collected by flagging, *I. scapularis* nymphs accounted for a small portion of individuals (n=33). *Borrelia burgdorferi* was detected in *I. scapularis* collected from Forest River (n=242, infection rate = 3.31%) but not in *I. scapularis* collected from Turtle River State Park (n=55).

**Table 2.** Adult and nymphal ticks collected by flagging during the summer of 2013. Surveys conducted at two study sites: Forest River and Turtle River, Grand Forks County, ND.

| Site | Sampling Effort (hours) | *Dermacentor variabilis* | | | *Ixodes scapularis* | | |
|---|---|---|---|---|---|---|---|
| | | Adults | Nymphs | Total | Adults | Nymphs | Total |
| Forest River | 28 | 897 | 0 | 897 (79%)[A] | 209 | 33 | 242 (21%)[A] |
| Turtle River | 26 | 77 | 0 | 77 (58%)[B] | 45 | 10 | 55 (42%)[B] |
| Total | | 974 | 0 | 974 (77%)[C] | 252 | 43 | 297 (23%)[C] |

[A] = Percentage of Forest River tick collection
[B] = Percentage of Turtle River tick collection
[C] = Percentage of total tick collection

Four species of mammals were trapped over the course of 320 trap nights; 33 *Peromyscus spp*. 33 *Myodes gapperi*, 5 *Tamias striatus*, and 1 *Zapus hudsonias*. No ticks were found on *Tamias striatus* or *Zapus hudsonias*. Two species, *Peromyscus spp.* and *Myodes gapperi* accounted for >90% of all mammals collected. For all immature tick lifestages and species combined, there were no significant differences between mice and

voles in prevalence of tick infestation (70 and 73%, respectively) or average tick burdens (7 ticks/mouse versus 12 ticks/vole) (Wilcoxon rank sum, p=0.145; Table 3).

With respect to *D. variabilis* larvae, there was no significant difference in the infestation prevalence between *Peromyscus* mice (0.45) versus *Myodes* voles (0.61) ($\chi^2$=1.52, p=0.217). However, the infestation prevalence of *I. scapularis* larvae was significantly greater on *Peromyscus* mice (0.48) than on *Myodes* voles (0.15) ($\chi^2$=8.45, p=0.004). For nymphs, the infestation prevalence of *I. scapularis* and *D. variabilis* on mice and voles ranged from 0.12 to 0.30 but did not differ significantly from each other for either host species (Pearson's chi-square, p>0.05) (Table 3). Infested *M. gapperi* harbored significantly more *D. variabilis* larvae (11.4 $\pm$ 13.0 larvae/vole) than *I. scapularis* larvae (2.4 $\pm$ 1.7 larvae/vole) (Wilcoxon rank sum test, U=325, p=0.044). For infested *Peromyscus*, there was no difference in the larval tick burdens between the two tick species (3.2 $\pm$ 3.7 *I. scapularis*/mouse; 5.6 $\pm$ 8.2 *D. variabilis*/mouse)(Wilcoxon rank sum test, p>0.05). For nymphal ticks, there were no significant difference in tick burdens between *Peromyscus* and *M. gapperi* (Wilcoxon rank sum test, p>0.05). In general, there tended to be more larval than nymphal ticks on infested mammals.

In both tick species, larval ticks were unevenly distributed among hosts, as determined by calculating variance-to-mean ratios (VMR) as indices of dispersion (*I. scapularis*: VMR=3.7; *D. variabilis*: VMR=14.61). Nymphal ticks were more uniformly distribution among hosts (*I. scapularis*: VMR=0.38; *D. variabilis*: VMR=2.79).

**Table 3**. Species and lifestages of ticks collected from small mammals in Grand Forks County, ND.

| | Larval Ticks | | Nymphal Ticks | | All Ticks |
|---|---|---|---|---|---|
| | *Dermacentor* (n=312) | *Ixodes* (n=63) | *Dermacentor* (n=36) | *Ixodes* (n=21) | (n=432) |
| *Peromyscus* (n=33) | | | | | |
| Prevalence | 0.45 | 0.48 | 0.12 | 0.21 | 0.70 |
| Average Tick Burden | 5.60 | 3.19 | 1.75 | 1.43 | 7.00 |
| Uniformity of tick burden (VMR)* | 11.98 | 4.36 | 0.52 | 0.43 | 9.23 |
| *Myodes* (n=33) | | | | | |
| Prevalence | 0.61 | 0.15 | 0.30 | 0.21 | 0.73 |
| Average Tick Burden | 11.40 | 2.40 | 2.90 | 1.57 | 11.67 |
| Uniformity of tick burden (VMR)* | 14.78 | 1.17 | 3.33 | 0.39 | 13.00 |

* VMR = variance-to-mean ratio. Low values indicate uniform distribution, high values indicate aggregated distribution of ticks.

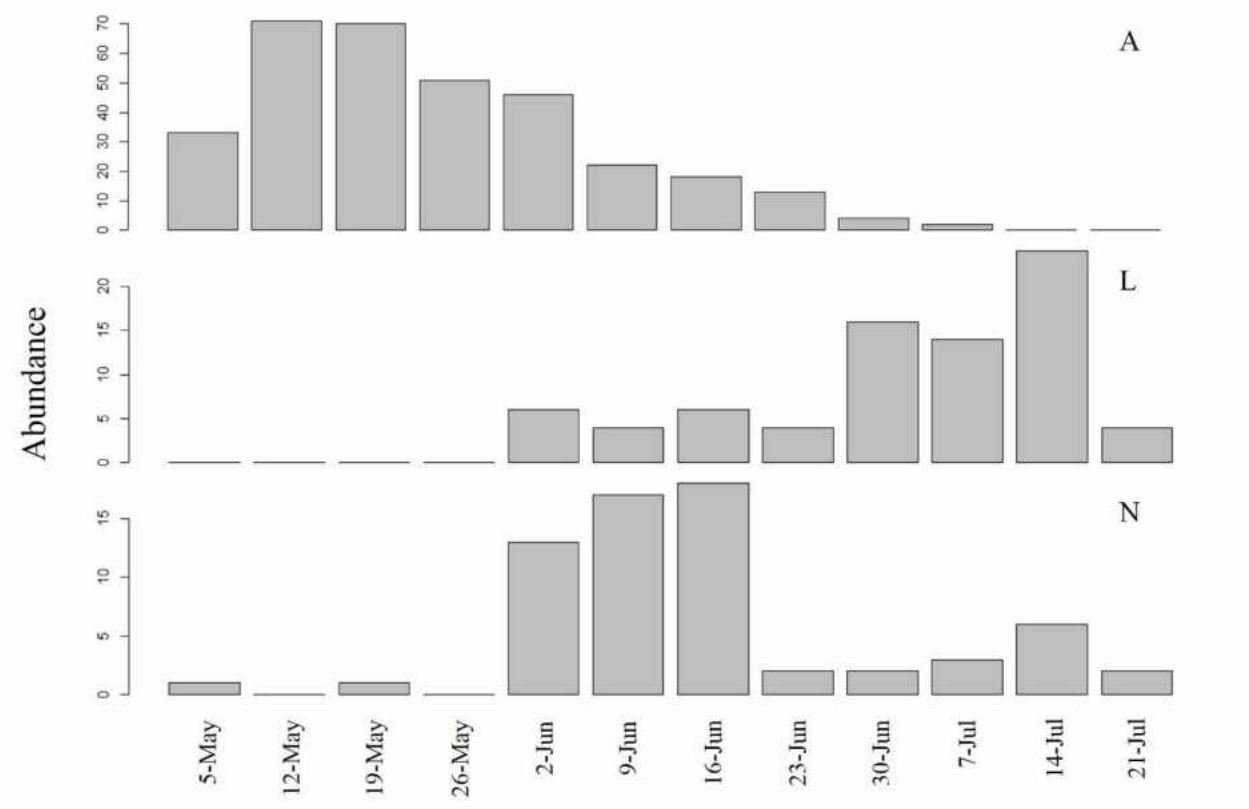


**Fig. 7.** *Ixodes scapularis* phenology in eastern North Dakota. Abundances based on weekly surveys of two established populations. Life stages represented by: A = adult ticks, L = larval ticks, N = nymphal ticks. Adult ticks were collected by dragging, larval ticks collected from small mammals, and nymphal ticks were collected using both methods. Abundances refer to the number of individuals collected during surveying, and data normalized to a uniform survey effort per week (flagging = 4 hours per week, trapping = 10 mammals per week).

Trends in seasonal abundance of *I. scapularis* by life stage are given in Figure 7. *I. scapularis* adults exhibited peak seasonal abundance during the late spring season (mid-May to early-June), and gradually declined in abundance until early July. Shortly after the decline of adult *I. scapularis* abundance, nymphal ticks reached peak abundance in early- to mid-June, with a second less pronounced peak in mid-July. Nymphal ticks were present in low quantities throughout the duration of this study. Larval *I. scapularis* were not collected until early June, and reached peak abundance from late-June to late-July.

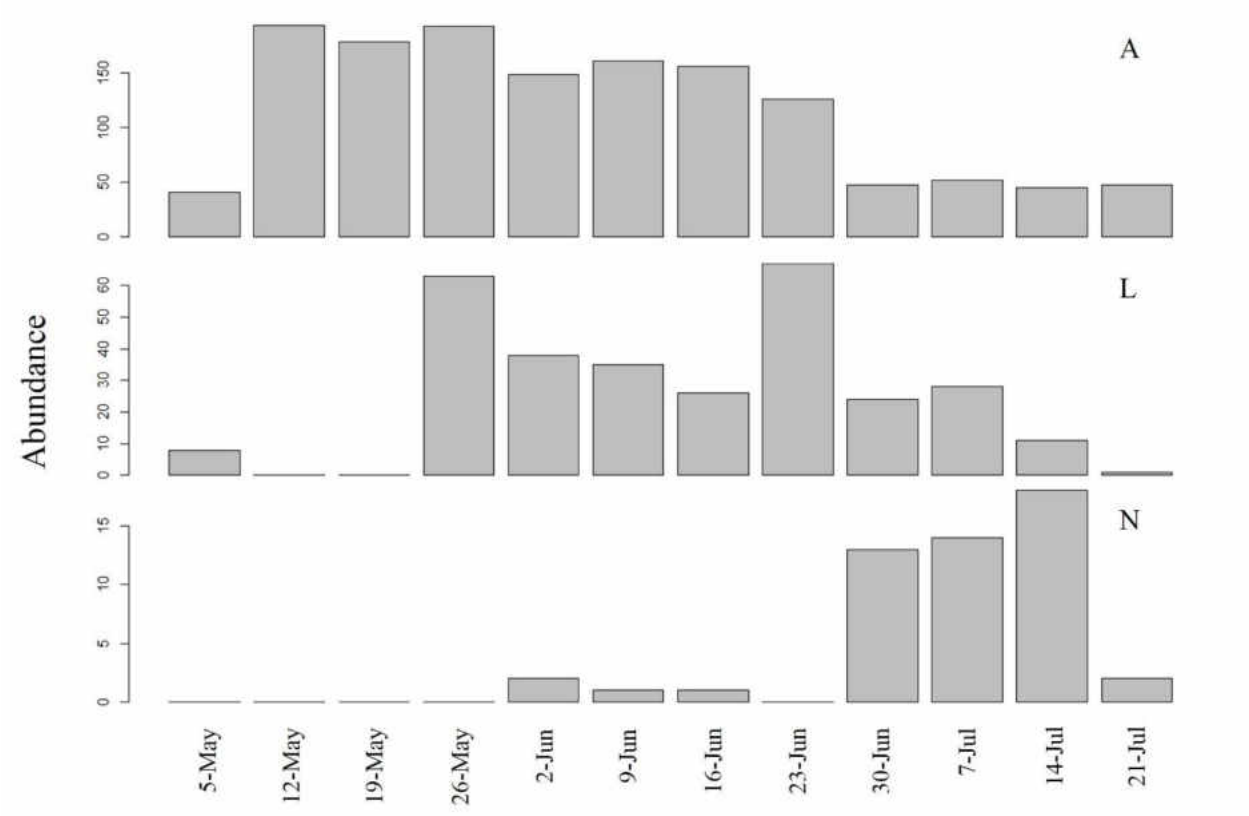


**Fig. 8.** *Dermacentor variabilis* phenology in eastern North Dakota. Abundances based on weekly surveys of two established populations. Life stages represented by: A = adult ticks, L = larval ticks, N = nymphal ticks. Adult ticks were collected by dragging, larval ticks collected from small mammals, and nymphal ticks were collected using both methods. Abundances refer to the number of individuals collected during surveying, and data normalized to a uniform survey effort per week (flagging = 4 hours per week, trapping = 10 mammals per week).

Trends in seasonal abundance of *D. variabilis* by life stage are given in Figure 8. *Dermacentor variabilis* adults were collected throughout the entire survey period in much higher abundance than *I. scapularis*. *Dermacentor variabilis* adults reached peak abundance in May and June, and the peak questing period for adult ticks lasted 6 to 7 weeks and was of longer duration than for adult *I. scapularis* (ca. 4 weeks, Figure 7). Similarly, larvae were collected from small mammals throughout the entire survey period

with peak abundances lasting up to 7 weeks. Nymphs were most common during early- to mid-July and collected only from small mammal trapping

## Discussion

Two tick species were collected in this study: the American dog tick, *Dermacentor variabilis*, and the blacklegged tick, *Ixodes scapularis*. Both of these species are known to vector diseases of significant public health concern: Rocky Mountain spotted fever (*Rickettsia rickettsii*) and Lyme disease (*Borrelia burgdorferi*), by *D. variabilis* and *I. scapularis* respectively. Previous studies have shown that *B. burgdorferi* (as well as other *Ixodes*-borne diseases) are present in eastern North Dakota, although at low infection rates (ca. 3-5%) consistent with the infection rates presented in this study (Russart et al. 2014). *D. variabilis* is the dominant tick species in the region, and diseases associated with this tick species have been rarely observed in the Midwest despite the presence of non-pathogenic rickettsial bacteria in ticks (Russart et al. 2014). Although a majority of ticks collected in this study were adults, it is suspected that this is the result of sampling bias; a much higher quantity of ticks was collected by flagging when compared to mammal trapping, resulting in an over-representation of adult and nymphal ticks (obtained by flagging) when compared to larval ticks (obtained by mammal trapping).

*I. scapularis* in the region exhibited a relatively low *B. burgdorferi* infection rate compared with previous studies in this region or elsewhere within the species' range (Russart et al. 2014; Burgdorfer et al. 1982; Nedler et al. 2014). Prior survey of Midwestern *I. scapularis* populations has shown a high rate of seasonal *B. burgdorferi*

infection prevalence (Sharon et al. 1992). The low prevalence observed in this study may be due to a series of extreme winter climates; *I. scapularis* experience high mortality in extreme cold, and it is suggested that depletion of essential chemicals for *B. burgdorferi* survival is exacerbated during the overwintering and molting processes leading to higher mortality in infected ticks (VanDyk et al. 1996; Piesman et al. 1990).

The uniformity of immature tick infestation on small mammals differs dramatically by life stage, as indicated by the large differences in indices of dispersion between larval and nymphal ticks. Most likely, this is a product of their respective life histories. Replete female ticks lay single large batches of eggs (often thousands) from which the larvae emerge, resulting in a highly aggregated distribution of questing larvae; conversely, nymphal ticks are detach from hosts asynchronously and are dispersed randomly throughout the environment. Thus, larvae are often found in large quantities on individual animals or are absent (i.e. high VMR), whereas nymphal ticks are found in consistent quantities on many individual mammals (i.e. low VMR). Anecdotally, adult *I. scapularis* were found in higher quantities along trails frequented by deer and humans while nymphal *I. scapularis* collected by flagging were found in areas dominated by shrubs and/or grasses, consistent with previous studies regarding questing *I. ricinus* (Daniel et al. 1977; Sonenshine 1991). Statistically significant differences in host-preference by larval *I. scapularis* ticks shows a higher abundance of larval blacklegged ticks infesting *Peromyscus spp.* compared to *M. gapperi* despite a relatively similar prevalence of each mammalian host in the environment. This may be the result of temporal or environmental differences in host behavior, and may have result in a lowered ability of *I. scapularis* to establish populations in regions in which *M. gapperi* is present

in much higher quantities than *Peromyscus spp.* Additionally, the effect of heavy *I. scapularis* infestation on *Peromyscus spp.* is shown to have no effect on fitness, while the effect on *M. gapperi* is unknown (Ostfeld et al. 1996).

Seasonal abundance of *I. scapularis* suggests distinct, brief periods of peak abundance which vary by life stage. Adults were most common in late spring/early summer, while nymphs were prevalent during a single brief period in mid-summer, and larvae prevalent during the later summer. These trends are consistent with surveys conducted in both the northeastern United States as well as the midwestern United States (Diuk-Wasser et al. 2006; Hamer et al. 2012). In all of these scenarios ticks are hypothesized to exhibit a two year life cycle, and a second peak in questing adult abundance is observed in the fall (e.g. Hamer et al. 2012 describe a high number of questing adults in October and November as well as May at a site recently colonized by blacklegged ticks). Ogden (2004) hypothesizes that climactic conditions in southern Canada make it difficult for ticks to quest during this time, and thus lengthen the life-cycle to encompass three years. While the abundance of adult *I. scapularis* in this region is consistent with that of a two year life cycle, we hypothesize that frequent snow and extreme cold during the fall months may impede adult *I. scapularis* from questing during this time. Due to these factors, nymphs obtaining blood meals during the mid-summer would not be able to reproduce in the fall. The end product would be a combination of ticks undergoing a two- and three-year life cycle; this asynchronicity and potentially higher mortality in some *I. scapularis* cohorts questing in colder climates may account for the lower prevalence of blacklegged ticks in North Dakota. However, altered climactic conditions may lead to a higher rate of establishment by *I. scapularis* in the

future (Ogden 2006). This study suggests residents of eastern North Dakota should be most cautious of ticks in June and July, when nymphal *I. scapularis* which most commonly transmit Lyme disease to humans, are most active.

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CHAPTER III

# POPULATION GENETICS OF *IXODES SCAPULARIS* IN NORTH DAKOTA

## Introduction

The blacklegged tick, *Ixodes scapularis*, is the most common vector of arthropod borne-diseases in North America, including *Borrelia burgdorferi*, the causative agent of Lyme disease. Tick surveillance in eastern North Dakota has revealed the presence of established *I. scapularis* populations in habitats previously regarded as unsuitable for this species, and implicates the region as the Northwestern frontier of population expansion in the United States (Russart et al. 2014). Understanding the population structure of this vector is essential to predicting the possible expansion of blacklegged tick populations and the associated pathogens. Highly variable mitochondrial DNA (mtDNA) sequences have proven useful in elucidating population structure of *I. scapularis* through North America (Norris et al. 1996; Ogden et al. 2006; Sakomoto et al. 2014; Kelly et al. 2014). Significant variation in mtDNA sequences suggests distinctive northern and southern *I. scapularis* clades hypothesized to be the result of migration during the recession of Pleistocene ice sheets (Sakomoto et al. 2014; Kelly et al. 2014). Low rates of genetic variation among blacklegged tick populations and the presence of largely ubiquitous haplotypes throughout North America have made clarifying routes of migration throughout the continent difficult (Krakowetz et al. 2014). However, studies have shown variation in the frequency of closely related haplotypes to be significantly different even

on relatively small geographic scales (Ogden et al. 2006). Haplotype networking and frequency analysis often provides a useful tool in distinguishing patterns of relatedness among closely related samples analyzed in large quantities (Bandelt et al. 1999). Identifying areas of high *Ixodes scapularis* abundance is critical to public health and risk assessment for exposure to a number of arthropod-borne diseases, including: Lyme disease, human granulocytic anaplasmosis, and babesiosis (Wormser et al. 2006). Incidence of Lyme disease in the Midwest has been increasing in recent years, possibly driven by anthropogenic factors such as climate change and increased range expansion by the tick vector (Piesman & Eisen 2008; Diuk-Wasser et al. 2012). This study provides insight into the source of founders for newly established populations. This knowledge, coupled with increased understanding of the severity of ecological constraints placed on these populations, will help define the future distribution and epidemiology of tick-borne diseases in the upper Great Plains.

## Materials and Methods

**Tick Collection**. Questing *I. scapularis* ticks were collected from five study sites in North Dakota during the summer of 2013 and 2014: Turtle River State Park (TRSP; 47°56'19.35"N, 97°29'27.63"W), Lake Metigoshe State Park (LMSP; 48°59'2.04"N, 100°20'2.40"W), Fort Ransom State Park (FRSP; 46°32'39.19"N, 97°55'31.85"W), Icelandic State Park (ISP; 48°44'28.70"N, 97°43'58.52"W), and the Forest River Wildlife Management Area (FR; 48°12'54.76"N, 97°28'5.30"W), and Roseau County, Minnesota (48°41'31.97"N, 95°58'56.37"W). Collections were conducted during the months of May-July during the period of highest tick abundance (see chapter 2). Tick samples were collected by dragging with a $1.0m^2x0.5m^2$ white cloth and from the clothes of field

collectors. Samples were stored in 70% ethanol and identified to species, sex and lifestage in the laboratory (Clifford et al. 1961, Durden and Keirans 1996).

**DNA Extraction/Molecular Methods.** Adult *I. scapularis* were bisected longitudinally and DNA was extracted using a guanidine thiocyanate method (Tkach and Pawlowski 1999). The DNA barcoding region of the mitochondrial Cytochrome C oxidase subunit one (COI) was amplified using previously described primers LCOI490 5'- GGTCAACAAATCATAAAGATATTGG-3' and HCO2019 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer et al. 1994). PCR products were bi-directionally sequenced using the ABI PRIMS 3100 Genetic Analyzer. Bi-directional sequences were trimmed and aligned using Sequencher software.

**Genetic Analysis.** The PCR amplicons from 178 individual ticks were sequenced. All *I. scapularis* collected from sites exhibiting low tick presence (LMSP, ISP, FRSP) were included in analysis. Bi-directional sequences with trimmed lengths > 500 bp were aligned with their analogous pair. Minimal (<1%) mismatch disagreements (n=36) were edited manually using Sequencher, while high (>1%) mismatch disagreements (n=42) were discarded.

All sequences obtained for the COI gene were aligned using the ClustalW2.1 function and BioEdit software (Hall 1999). Haplotypes were identified using DnaSP v5.10.1 (Librado and Rozas 2009) and were aligned as previously described for phylogenetic analysis.

In order to determine how *I. scapularis* in the region related to the broader species distribution, 136 sequences from this study were aligned to an overlapping 306-bp region

of 137 databased samples obtained from GenBank (Mechai et. al.2013; Sakamoto et al. 2014). A median joining network tree was then calculated using Network software. The median joining method was chosen as the most appropriate phylogenetic method of distinguishing relationships among *I. scapularis* populations due to the large sample size and small genetic distances between individuals (Bandelt et al. 1999).

All calculations of genetic diversity were conducted using DnaSP v5.10.1 software. All tests were conducted for each study site as well as all *I. scapularis* collected from North Dakota and Minnesota. The following summary statistics were calculated: haplotype number (h), haplotype diversity (Hd), nucleotide diversity (Pi), and average nucleotide difference (k). Statistical deviation from population equilibrium was calculated using three test statistics: Tajima's D, Fu and Li's D, and Fu and Li's F (Tajima 1989, Fu and Li 1993). Demographic evolutionary history was obtained by graphing nucleotide pairwise number of differences (mismatch distribution) in relation to models of population growth and neutrality, respectively (Rogers and Harpending 1992, Tajima 1989).

## Results

136 usable 306 bp sequences were obtained and used for further analysis. A total of 31 haplotypes were identified using the COI gene. Haplotype diversity at all sites was high (total Hd=0.80; table 4) while the average nucleotide difference over 306bp was relatively low (k=1.74; table 4), evident of a high number of single nucleotide mutations. Regionally, populations showed evidence of population expansion using three increasingly stringent test statistics ($D=-2.01$, $p<0.05$; $D^*=-3.68$, $p<0.01$; $F^*=-3.60$, $p<0.01$). Individually, two study sites showed evidence of population expansion using

Tajima's D (FR, TR) while one site showed significant evidence of expansion by all three (TR). Base pair composition of all North Dakota haplotypes is summarized in table 5.

**Table 4.** Genetic Diversity and standard tests of deviations from population equilibrium in *Ixodes scapularis* from North Dakota and Minnesota.

| Population | n | h | Hd | Pi | k | D | D* | F* |
|---|---|---|---|---|---|---|---|---|
| Forest River | 52 | 13 | 0.81 | 0.006 | 1.74 | -1.18* | -0.55 | -0.9 |
| Turtle River | 49 | 13 | 0.80 | 0.005 | 1.67 | -1.54* | -2.32* | -2.44* |
| Lake Metigoshe | 10 | 2 | 0.20 | 0.001 | 0.20 | -1.11 | -1.24 | -1.34 |
| Icelandic | 18 | 9 | 0.82 | 0.005 | 1.56 | -1.43 | -1.61 | -1.80 |
| Minnesota | 7 | 6 | 0.95 | 0.005 | 1.62 | -0.04 | -0.07 | -0.07 |
| Total | 136 | 32 | 0.80 | 0.006 | 1.69 | -2.01* | -3.68** | -3.60** |

*$p<0.05$ **$p<0.01$

h = number of haplotypes

Hd = haplotype diversity

Pi = nucleotide diversity

k = average number of nucleotide differences

D = Tajima's D

D* = Fu and Li's D

F* = Fu and Li's F

When all North Dakotan ticks were analyzed, the pairwise mismatch distribution fit the model of sudden population expansion as defined by Tajima (Figure 9; model values: $\Theta_0$=1.36, $\Theta_F$=1000, tau=1.77; Tajima 1989).

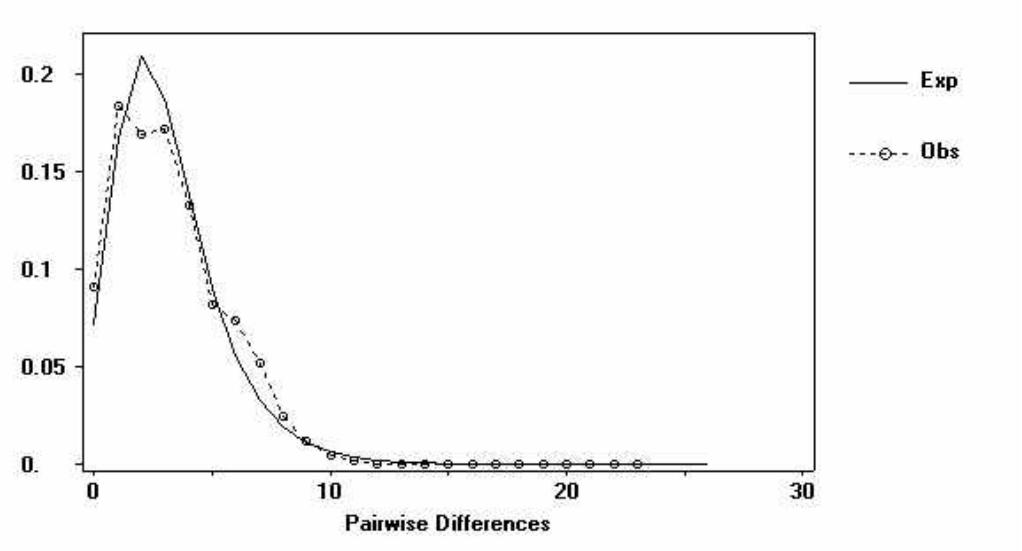


**Fig. 9.** Observed nucleotide mismatch distribution in North Dakota *Ixodes scapularis* populations assuming a model of population change.

**Table 5.** Base pair composition of haplotypes collected from North Dakota in this study.

| Ht | N | Nucleotide Position | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 13 | 18 | 19 | 29 | 44 | 47 | 50 | 65 | 101 | 110 | 113 | 116 | 134 | 173 | 174 | 176 | 194 | 216 | 224 | 243 | 248 | 255 | 263 | 278 | 279 | 283 |
| Hap_1 | 4 | G | G | C | C | C | A | T | C | C | A | A | T | A | T | C | T | C | G | C | C | T | G | T | G | T | T |
| Hap_2 | 52 | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | C | . |
| Hap_3 | 2 | . | . | T | . | . | . | . | . | . | . | . | . | G | . | . | . | . | A | . | . | . | . | . | . | C | . |
| Hap_4 | 2 | . | . | T | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | C | . |
| Hap_5 | 2 | . | . | . | T | . | . | C | T | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | C | . |
| Hap_6 | 30 | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Hap_7 | 4 | . | . | T | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | C | . |
| Hap_8 | 5 | . | . | . | . | . | . | . | T | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | C | . |
| Hap_9 | 3 | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | T | . | . | T | . | . | . | . | . | . |
| Hap_10 | 1 | . | . | . | T | . | . | . | . | . | G | . | . | G | . | . | . | . | . | . | . | . | . | . | . | C | . |
| Hap_11 | 1 | . | . | . | . | . | . | . | . | T | . | . | . | G | . | . | . | T | . | . | . | . | . | . | . | . | . |
| Hap_12 | 7 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | . | . | . | . | . |
| Hap_13 | 1 | . | . | . | . | . | . | . | . | . | . | . | . | G | . | T | . | . | . | . | . | . | . | . | . | . | . |
| Hap_14 | 1 | . | . | . | G | . | . | . | . | . | . | . | . | G | . | . | . | A | . | . | . | . | . | . | . | . | . |
| Hap_15 | 1 | A | . | . | T | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | C | . |
| Hap_16 | 1 | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | T | . | . | . | . | . | . | . |
| Hap_17 | 1 | . | . | . | . | . | G | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | C | . | C | . |
| Hap_18 | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | . | . | . | . | A |
| Hap_19 | 1 | . | . | . | . | G | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | C | . |
| Hap_20 | 2 | . | . | . | T | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Hap_21 | 1 | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | A | . | . | . | . | . | . | . | C | . |
| Hap_22 | 1 | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | G | . | . | . | . | . | . | . | . | C | . |
| Hap_23 | 1 | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | T | . | . | . | . | C | . |
| Hap_24 | 1 | . | A | . | . | . | . | . | . | . | . | . | A | G | . | . | . | . | . | . | T | . | . | . | . | . | . |
| Hap_25 | 2 | . | . | . | . | . | . | . | . | . | . | . | A | G | . | . | . | . | . | . | T | . | . | . | . | . | . |
| Hap_26 | 2 | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | A | . | . | . | . | . | . | . | . | . |
| Hap_27 | 1 | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | C | . | . | . | C | . |
| Hap_28 | 1 | . | . | . | . | . | . | . | . | . | . | . | . | G | C | . | . | . | . | . | . | . | . | . | . | . | . |
| Hap_29 | 1 | . | . | . | . | T | . | . | . | . | . | . | . | G | . | . | . | A | . | . | . | . | . | . | . | . | . |
| Hap_30 | 1 | . | . | . | . | . | . | . | . | T | . | G | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Hap_31 | 1 | . | . | . | . | . | . | . | . | . | . | . | A | G | . | . | . | . | . | . | T | . | A | . | . | . | . |
| Hap_32 | 1 | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | A | . | . |

Hap_2 was the most abundant haplotype and accounted for 38% of ticks collected (n=52). Hap_6 was the second most abundant haplotype, accounting for 22% of ticks collected (n=30). These dominant haplotypes were present and accounted for a large majority of ticks at all study sites except Lake Metigoshe, where Hap_6 was absent (Figure 10).

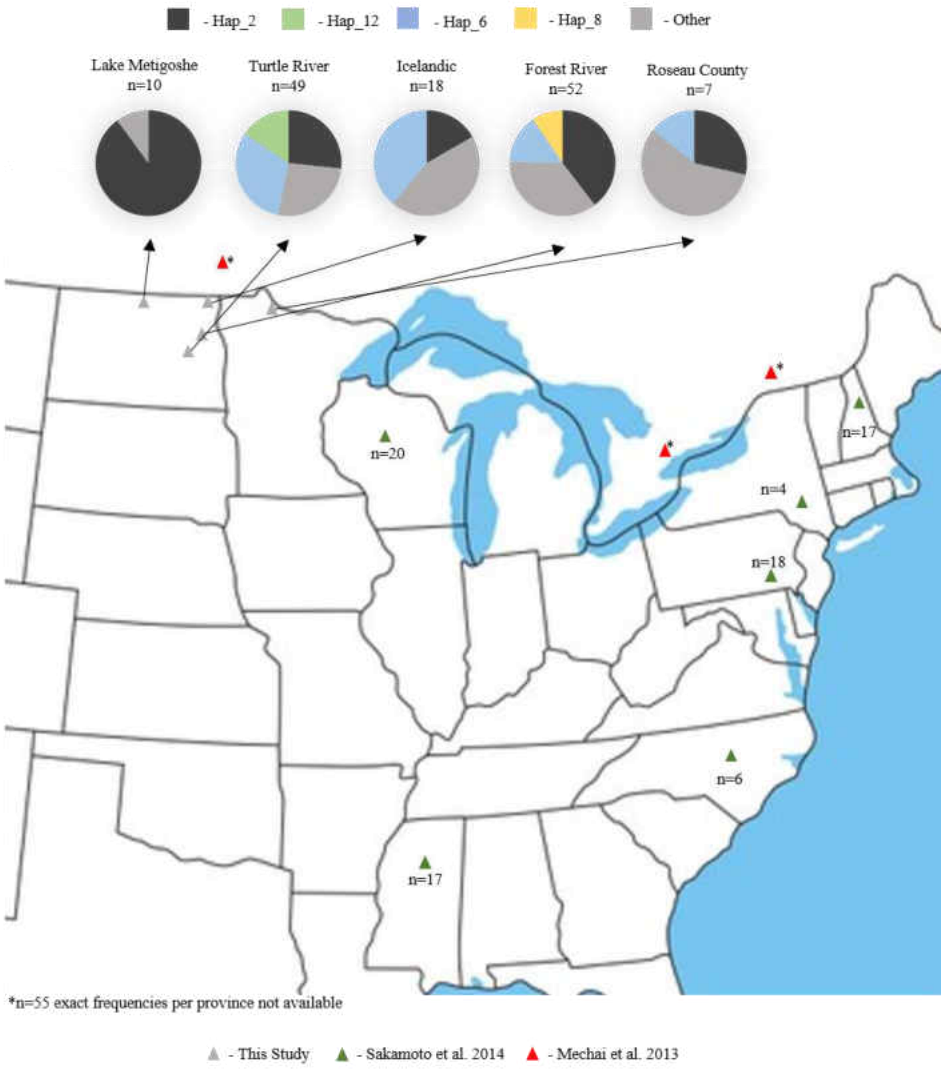


**Fig. 10.** Source locations and haplotype frequencies for collected ticks. Haplotype frequencies for collection sites from this study are compared; haplotypes frequent in less than four individuals are identified in the other category. Green and red triangles represent generalized source locations for GenBank sequences obtained for analysis to the state or provincial level.

31 haplotypes were included in the median joining network and summarized in Figure 11. Four extreme outliers were omitted from final tree construction due to extremely high genetic divergence; these samples are representatives from the *I. scapularis* 'Southern clade' from Mississippi as defined by various studies (Norris et al. 1996, Qiu et al. 2002). *I. scapularis* from the upper Midwest predominately fell into two large closely related taxonomic groups (Hap_2 n=62, Hap_6 n=75), and both dominant taxa were represented by all sites sampled throughout the study. Less frequent haplotypes present consist mostly of single nucleotide mutations from one of these two ancestral haplotypes. These haplotypes (Hap_2 and Hap_6) are the basis for the most basal tree bifurcation and identified as Northern and Southern groupings (with respect to westward migration across the Great Lakes) by the source locations and frequency of related ticks present in each. Hap_2 was characterized by high relative abundance of *I. scapularis* from Manitoba (n=3), Eastern Canada (n=13) and the Northeastern United States (n=8) as well as ticks from this study (n=30). Conversely, the taxon Hap_6 was characterized by high relative abundance of *I. scapularis* from the Northeastern United States (n=13), a low abundance of ticks from Eastern Canada (n=4), no representatives from Manitoba, and ticks from this study (n=52). Geographic frequency of individuals representing these two major haplotypes is summarized in Figure 12. The presence of two haplotype lineages in the region thus corresponds to the relation of these haplotypes to their respective founder populations to the north Dakota are more closely related to *I. scapularis* populations in Canada (north and south of the Great Lakes. A much higher proportion of haplotypes unique to Nor 11; white circles denote haplotypes observed only in Midwestern tick populations).

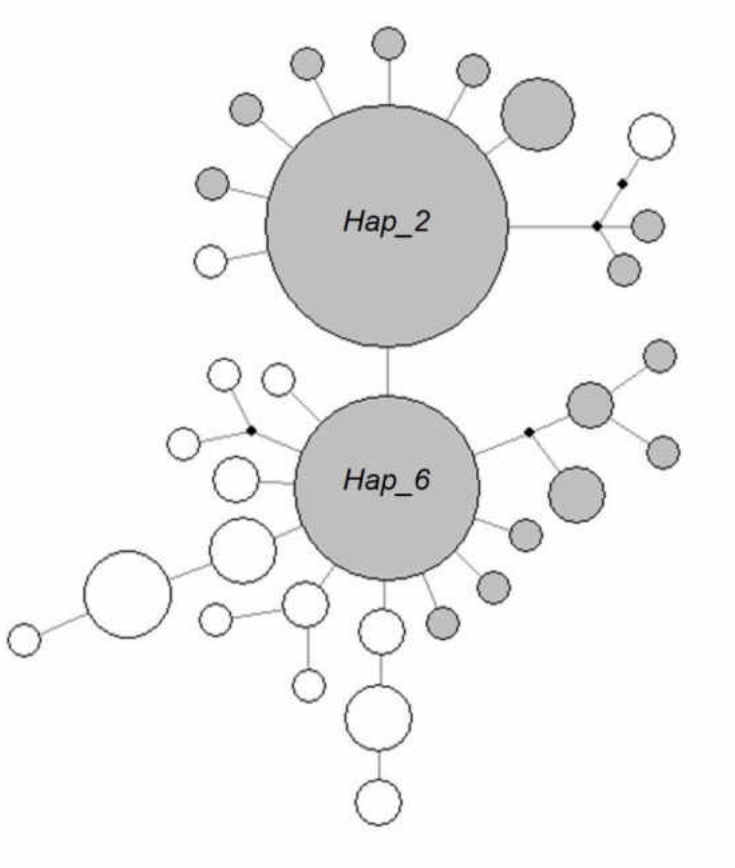


**Fig. 11.** Median-joining haplotype network from this thesis and databased sequences. Shortest tree calculated using maximum parsimony. Node and slice sizes scaled to the number of representatives collected in this study per haplotype. Branches represent single nucleotide mutations. Node size represents the number of individuals collected from this study per haplotype. White nodes represent haplotypes unique to the Midwest, while grey nodes represent haplotypes shared distributed across broad geographic regions.

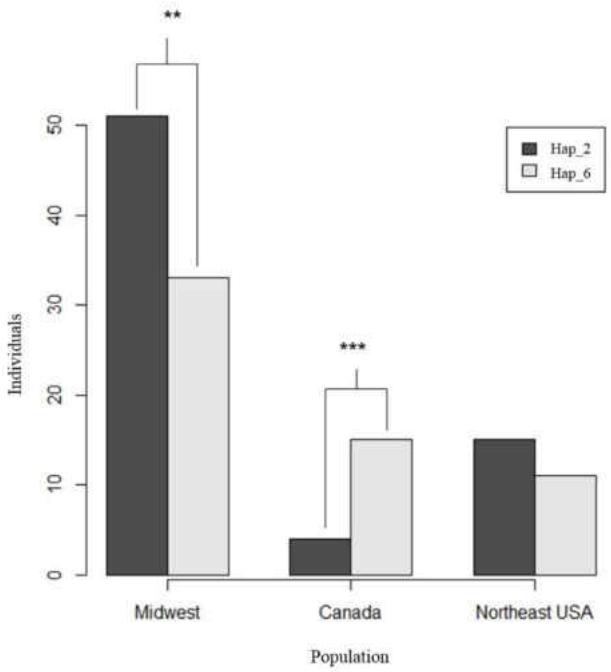


**Fig 12.** Frequency of the two most common haplotypes in various geographic regions. Pearson's Chi-Square **p<0.01, ***p<0.001.

## Discussion

Blacklegged tick populations in North Dakota are fragmented by large areas of inhospitable grasslands isolating unique forested sites. For this reason, we hypothesize drastic differences in tick abundance and structure can be observed on relatively small geographic scales. Bayesian analysis suggests some preliminary striation among isolated forest patches. Clades distinguished by the highly variable COI gene show clear divergence among two sites in particular (ISP and TRSP; not shown). However, the population largely represents one homogeneous group. These results are consistent with the observations of similar studies (Mechai et al. 2013; Sakamoto et al. 2014).

Evidence of highly significant population expansion in this region suggest that human risk of exposure to *I. scapularis* and Lyme disease may increase in the future.

High prevalence of individuals belonging to two major clades seem to implicate the region as a site of convergent migration from blacklegged tick populations to both the north and the south. Evidence presented here suggests historical migration beginning in the Northeastern United States, with populations moving west and diverging as they are geographically isolated by the Great Lakes.

The Great Lakes have proven to be a significant barrier to *I. scapularis* expansion. Migratory birds cross the great lakes in large numbers and may provide low rates of gene flow among tick populations, but prevalence of *I. scapularis* infestations on birds in the region has been shown to be extremely low (Ogden et al. 2008). Evidence of this nature coupled with the historical genetic evidence assessed here suggests migration in the region to occur over large periods of time and on a small geographic scale. This may imply that forest patches in historically low-risk Lyme endemic areas in the Midwest may be colonized over time by converging migratory pathways.

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CHAPTER IV

# RESERVOIR COMPETENCY OF THE RED-BACKED VOLE (*MYODES GAPPERI*) FOR THE LYME DISEASE AGENT *BORRELIA BURGDORFERI*

## Introduction

In nature, the spirochete bacteria and causative agent of Lyme disease, *Borrelia burgdorferi*, has a complex life cycle involving horizontal transmission between competent tick vectors and vertebrate reservoirs (Radolf et al. 2012). Transovarial transmission of spirochetes in ticks is rare (ca. 0.7%) (Piesman et al. 1986). Larval ticks acquire *B. burgdorferi* when feeding on rodents and songbirds that have spirochetes in their blood and skin. After dropping off and molting to the nymphal stage, infected nymphs can infect naïve animals (including humans) during nymphal feeding (Radolf et al. 2012). A highly diverse array of vertebrates can serve as hosts for immature *I. scapularis* and reservoirs of *B. burgdorferi,* including chipmunks (*Tamias spp.*), mice (*Peromyscus spp.* and *Mus musculus*), or voles (*Microtus spp.*) (Gern et al. 1998).

Recently, the Bank vole *Myodes glareolus* has been implicated as a reservoir of *Borrelia* and other tick-borne bacteria in Europe, and human risk of Lyme disease has been linked to the presence of biotopes harboring abundant vertebrate reservoirs such as *M. glareolus* (Jaenson et al. 2009; Marsot et al. 2011; Burri et al. 2014). In North America, the genus *Myodes* is represented most prominently by the Southern red-backed vole, *Myodes gapperi.* Little is known about the southern red-backed vole's ability to transmit *B. burgdorferi,* although previous studies have shown *M. gapperi* are capable of

harboring spirochetes for extended periods of time when infected via needle injection (Bey et al. 1995).

Surveys in western North America suggest *M. gapperi* is capable of sustaining large populations of both tick vectors and their associated pathogens in the absence of various *Peromyscus spp.* ( Anstead & Chilton 2013; Russart et al. 2014). Xenodiagnosis performed Russart et al. (2014) suggests that in eastern North Dakota, the southern red-backed vole harbors large quantities of immature *I. scapularis* and *B. burgdorferi* spirochetes have been obtained from voles collected in the region (Stone et al. 2014). Furthermore, the southern red-backed vole is the most common rodent species in coniferous forests of the Midwest (Hazard 1982).

In this study, I colonized a local strain of *M. gapperi* voles and examined the ability of their offspring to become infected and to transmit a locally acquired strain of *B. burgdorferi* to the tick vector *I. scapularis* ticks. This study elucidates the potential role for the Southern red-backed vole as a competent disease reservoir in Grand Forks County, North Dakota.

## Materials and Methods

**Breeding Voles.** A breeding colony of Southern Red-backed Voles (*M. gapperi*) was established in the laboratory (UND IACUC protocol: 1306-3). Voles were captured from Forest River Biological Station, Grand Forks County, ND using live Sherman traps baited with peanut butter and oats. Traps were set in the evening and recovered the next

morning. Voles were transported to the laboratory prior to being moved into the main animal housing facility in Starcher Hall, voles were dusted with 2% carbaryl insecticide in order to kill fleas, ticks and mites in their fur. Voles were housed in standard plastic mouse cages and maintained on a diet of commercial laboratory rodent chow supplemented occasionally with fresh celery and/or lettuce. Gender was determined and breeding pairs were housed together for five days, then separated. Offspring (F1 voles) were housed individually 14 days after birth.

***B. burgdorferi* Cultures.** *B. burgdorferi* cultures were collected using methods described by Stone et al. (2014). Briefly, *M. gapperi* were captured by live traps in the Forest River Biological Station, Grand Forks, ND. Captured voles were euthanized in the field with isoflurane overdose and hearts were excised and placed immediately into modified Barbour-Stoenner-Kelly (BSK-II) medium containing 6% rabbit serum and 50 μg/mL rifampin (Barbour 1984). Surgical tools were sterilized with 95-99% ethanol between each field necropsy. Three days later, uncontaminated cultures were passed into modified BSK-II with 6% rabbit serum but without rifampin, incubated for three additional days, then examined for spirochetes via dark field microscopy. Twenty viewing fields were examined, and infection determined by the presence of one or more spirochetes.

***M. gapperi* Infection and Tick Collection.** A total of 20 F1 voles were infected with a North Dakota strain of *B. burgdorferi* that was recovered and cultured from the

heart of an infected *M. gapperi* captured at Forest River Biological Station. A small needle (27 gauge, ½ inch) was used to subcutaneously inject 0.1cc of culture media containing approx. $10^7$ spirochetes in the occipital skin fold of each vole. At 10, 20 and 40 days after injection (DPI), voles were experimentally infested with larval *Ixodes scapularis* ticks (obtained from the tick rearing facility at Oklahoma State University, Stillwater, OK). Injection and subsequent nymphal tick infestations were conducted twice using six voles for each experiment. Voles were lightly anesthetized using Isoflourane, and placed individually into small plastic ventilated cages (approximately 1 1/2 by 5 inches). Approximately 40 to 100 larval *I. scapularis* ticks were emptied from their containment vials onto the head, nose and back of anesthetized voles. Voles were confined to the small cages and wrapped loosely in paper towel for 3 to 8 hours to facilitate successful tick attachment. These small cages were placed inside large pans to guard against the escape of larval ticks that did not attach. Afterwards, each vole was released from its confinement and placed individually into a standard size mouse cage that had been modified such that the solid bottom had been cut out and replaced with hardware cloth with a 0.5 inch mesh. The wire-bottom cage was then placed inside a larger, standard rat cage that contained two to three inches of slightly soapy water (Figure 13). This facilitated the recovery of engorged larvae that dropped off into the water. Voles were monitored daily for tick drop-off. Tick drop-off typically occurred 4 to 6 days after attachment. Experiments were approved by UND IACUC protocol 1403-2 and UND IBC protocol 201404-002.

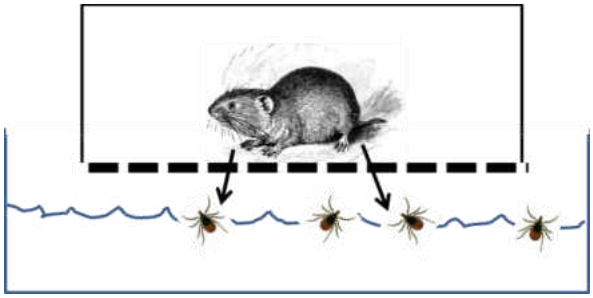

**Figure 13**. Housing device for infested voles & mice in wire-bottom cages to facilitate tick collection. Rodents were exposed to ticks and housed for one week in wire-bottomed cages suspended over water to confine detaching ticks. Water was replenished and checked for ticks daily.

**Nymphal Infection Rates.** Engorged larvae were maintained and molted to nymphs inside 25 ml plastic screw-top tubes modified such that there was a screened ventilation hole in the cap. Tubes containing potentially infected ticks were placed within sealed ZipLoc bags containing a moist paper towel. ZipLoc bags were then placed within sealed Tupperware containers and placed inside standard rat cages lined along the top with Vaseline. These bio-containment procedures were done to prevent escape of potentially infective ticks. Ticks were maintained at 15 C at high humidity for ca. 5 months. For handling and sorting, nymphs were emptied out onto a 'chill table', constructed simply of a white porcelain-coated pan set in a Styrofoam cooler filled with crushed ice. Nymphs were macerated individually into plastic microfuge tubes using disposable plastic pestles. The DNA was extracted and assayed for the presence of *B. burgdorferi* DNA by polymerase chain reaction (PCR) techniques (see below).

To determine how long infected voles remained infective to ticks, larval infestations were conducted at 10, 20 and 40 days after voles had been injected with *B.*

*burgdorferi* spirochetes (DPI=days post-infection). From each infestation, engorged larvae were allowed to molt and representative samples of nymphs were assayed. The sample sizes were; 10 DPI (n=100 ticks), 20 DPI (n=8 ticks) and 40 DPI (n=31 ticks).

**Transmission to Mice**. Batches of nymphs infected on voles at 20 and 40 DPI were used in transmission studies. These nymphs were placed on uninfected BALB/c mice and allowed to attach and feed, as described above for vole infestations. Engorged nymphs dropped off and were collected at 3-7 days post-feeding. Engorged nymphs were assayed for *B. burgdorferi* DNA using real-time PCR (see below). Seven days after exposure to infected nymphs, mice were killed by carbon dioxide inhalation and decapitation. One tibiotarsal joint, ear punch, heart, and bladder were collected and placed in BSK-II medium. Cultures were examined daily for spirochetes after 4-7 days of adding tissue from experimental mice to BSK-II culture medium using dark field microscopy as previously described.

**Real-time PCR.** Ticks were assayed for *B. burgdorferi* using real-time PCR. The DNA was extracted from ticks using the QIAGEN DNeasy Blood & Tissue Kit (Valencia, CA) and manufacturer's instructions. Real-time PCR reactions were run using SYBR Green master mix (Life Technologies, Grand Island NY) and primers previously described by Jiang et al. (2003) to amplify the *B. burgdorferi* flagellin gene: Brec442F 5'-GCTGAAGAGCTTGGAATGCAAC-3' and Brec551R 5'-GCTTCATCCTGATTTGCACCAAC -3'.

## Results

Five of seven breeding pairs successfully produced F1 offspring. Voles from each collection site (Turtle River and Forest River) successfully mated with individuals from within and outside their own population. Mating pairs were successful within five days of initial pairing, and litters were produced ca. 9 days after separation. Two females produced multiple litters, and the average litter size was 6 ± 2.65 offspring. Breeding pairs produced offspring at a sex-ratio nearing 50% male to female (4 females, 5 males).

Over half (64 ± 8%) of 139 larval *I. scapularis* fed on experimentally-infected voles acquired and maintained spirochete infections through the molt to the nymphal stage. Nymphal infection rates ranged from 56 to 75% and did not differ significantly over the 40 day post-infection period (Pearson's chi-square, $\chi^2$=0.18, p=0.67 Table 6). Nymphal ticks successfully transmitted *B. burgdorferi* to laboratory mice (Table 6). There was no significant difference in the transmission rates between nymphs infected on 20 DPI (78% transmission rate) versus 40 DPI (67% transmission rate) (Fisher's exact test, p=0.56)

**Table 6.** Ability of experimentally-infected Southern Red-backed voles (*Myodes gapperi*) to infect larval *Ixodes scapularis* ticks with *Borrelia burgdorferi*.

| Duration of infection in voles at time of larval feeding | Nymphal infection rate (no. infected nymphs / no. nymphs tested) | Nymphal transmission rate * (no. infected mice / no. mice exposed to infected nymphs) |
|---|---|---|
| 10 days | 56 / 100 | Not determined |
| 20 days | 6 / 8 | 7 / 9 |
| 40 days | 20 / 31 | 6 / 9 |

*No significant difference (Fisher's exact test, p=0.56)

Most (61.2%) of the 67 engorged nymphs recovered from 19 mice were infected with *B. burgdorferi* (Table 7), however spirochete transmission was variable and not always predictable. For example, several mice became infected after being bitten by a single infected tick (*e.g.,* mice 7, 14, 18) whereas other mice did not (*e.g.,* mice 6, 10, 15). Two mice remained uninfected despite being bitten by multiple infected ticks (*e.g.,* mice 4, 19). Mouse 12 did not get bitten by an infected tick and did not become infected.

Successful culturing of spirochetes from infected mice varied significantly by tissue ($\chi^2 = 10.44$, $p<0.05$) (Table 7). Spirochetes were recovered at significantly higher rates in the bladder ($\chi^2=9.33$, $p<0.05$) and the tibiotarsal joint ($\chi^2 = 3.85$, $p<0.05$) than in other tissues (ear, heart).

**Table 7.** Mouse exposure to *Borrelia burgdorferi*-infected *Ixodes scapularis* nymphs and culture of spirochetes from various mouse tissues seven days after detachment of nymphal ticks.

| DPI – Mouse No. | Tick Exposure and Infection Status | Ear | Joint | Bladder | Heart | No. infected ticks / Total no. ticks fed |
|---|---|---|---|---|---|---|
| 20DPI -1 | Infected | + | + | + | | 4 / 7 |
| 20DPI -2 | Infected | | | + | + | 2 / 5 |
| 20DPI -3 | Infected | + | + | + | | 5 / 6 |
| 20DPI -4 | Exposed but not infected | | | | | 3 / 4 |
| 20DPI -5 | Infected | | + | + | | 4 / 5 |
| 20DPI -6 | Exposed but not infected | | | | | 1 / 5 |
| 20DPI -7 | Infected | + | + | + | | 1 / 3 |
| 20DPI -8 | Infected | | + | + | | 2 / 4 |
| 20DPI -9 | Infected | + | + | + | | 4 / 4 |
| 40DPI -10 | Exposed but not infected | | | | | 1 / 2 |
| 40DPI -11 | Infected | | | + | + | 2 / 3 |
| 40DPI -12 | Not Exposed | | | | | 0 / 1 |
| 40DPI -13 | Infected | | | + | + | 3 / 3 |
| 40DPI -14 | Infected | + | + | + | + | 1 / 1 |
| 40DPI -15 | Exposed but not infected | | | | | 1 / 3 |
| 40DPI -16 | Infected | | + | + | | 3 / 3 |
| 40DPI -17 | Infected | + | + | + | | 2 / 3 |
| 40DPI -18 | Infected | | + | + | | 1 / 1 |
| 40DPI -19 | Exposed but not infected | | | | | 2 / 2 |
| Totals | | 32% | 53%* | 68%* | 21% | 41 / 67 |

*Pearson's Chi-square, $p<0.05$

## Discussion

*M. gapperi* proved to be competent Lyme disease reservoirs. As previous reports suggest, *I. scapularis* populations appear to be expanding in the upper Midwest (see chapter 3; Bey et al. 1996) and interactions between Southern red-backed voles, blacklegged ticks, and *B. burgdorferi* seem to be occurring at a higher rate here than in other geographic regions (Bouchard et al. 2011). While *I. scapularis* abundance is highly correlated with deer abundance and various abiotic factors (Diuk-Wasser et al. 2012), the presence of appropriate rodent hosts is essential for long-term establishment of tick/disease populations. Here we provide evidence that *M. gapperi* become infective rapidly (within 10 days of exposure to spirochetes) and remain competent reservoirs for at least 40 days. Thus, *M. gapperi* are capable of infecting questing larvae at a high rate for the complete duration of seasonal questing behavior observed by field studies in the region (Russart 2014). Furthermore, vole-infected *I. scapularis* are capable of transstadial transmission and, as questing nymphs, are capable of infecting naïve rodents with a local strain of *B. burgdorferi*. The short prepatent period (<10 days) and high percentage of infected nymphs (ca. 60-70%) produced by experimentally-infected *M. gapperi* were nearly identical to that described for experimentally-infected *P. leucopus,* the main reservoir implicated in Lyme disease epizootiology within the eastern USA, P. leucopus (Donahue et al. 1987). The reservoir competence of southern red-backed voles rivals that of white-footed mice.

Evidence presented here also suggests that local strains of *B. burgdorferi* disseminate preferentially to the bladder and the tibiotarsal joint (in comparison to the heart and ear tissues). These results are consistent with previous studies on Lyme dissemination in mice (Barthold et al. 1991). However, as Barthold et al. (1991) demonstrate, given a long enough time period (ca. 21 days) it is possible that the spirochetes will have disseminated to a majority of tissue systems; only a single time point (7 days post-feeding) was examined in this study.

Collectively, data presented here suggest there is the potential for establishment of transmissible *B. burgdorferi* spirochetes in local tick/mammal populations in which *M. gapperi* is the dominant rodent species.

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