CO-INFECTION RATES OF SMALL MAMMAL HOSTS IN CENTRAL WISCONSIN

By Bailey A. Bodeen

Lyme disease is the most common vector-borne illness in the United States. This disease is caused by the spirochete *Borrelia burgdorferi* and is transmitted by *Ixodes scapularis*, the black-legged tick or deer tick. Small mammals, particularly the white-footed mouse (*Peromyscus leucopus*), and some birds are important in the transmission cycle of *B. burgdorferi*, because they serve as reservoirs of the pathogen. These small, dark-colored ticks have a 2-year life cycle composed of four developmental stages: egg, larva, nymph, and adult. Larvae feed on small animals and can acquire *B. burgdorferi* infection at this stage. Nymphal and adult ticks are responsible for the transmission of the pathogen when feeding on new hosts, including humans and domestic animals. Other lesser-known infectious agents that cause disease in humans may co-occur in both hosts and vectors, thereby complicating transmission of the agents and diagnosis of disease in humans. These agents include *Anaplasma phagocytophilum*, various species of *Ehrlichia*, and *Babesia microti*. Both ticks and mammal hosts are primarily found in woody, brushy, or forest fragments. Recently, there has been concern that deer ticks are shifting and adapting to new reservoir hosts in more open habitats, which has major human health implications.

To examine co-infections by these agents in hosts, small mammals were live-trapped at Hartman Creek State Park in 2017 and 2018 to determine the influence of host species, sex, and weight of hosts on tick burdens, singular infection rates, and co-infection rates. *Peromyscus maniculatus* had the highest mean of 4.57 ticks per individual, while *P. leucopus* had 4.33 per individual and *Tamias striatus* had 3.86 per individual. Sex and weight characteristics did not influence tick burdens. Of 116 mammals screened, 34 (29.3%) were singularly infected, nine mammals (7.7%) were co-infected with *B. burgdorferi* and *A. phagocytophilum*, one (0.8%) was co-infected with *B. burgdorferi* and *B. microti*, and one (0.8%) was co-infected with all three pathogens. This study found no evidence of *Ehrlichia* infection. By understanding the ecology and behavior of mammal reservoir hosts, valuable information can be applied to interpret and predict the transmission dynamics of these poorly understood illnesses, with the ultimate goal of decreasing the likelihood of human exposure to infected ticks.

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For my amazing parents and loving sister.

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Introduction

Lyme disease is the most common vector-borne illness of humans in the United States. The causative agent is the spirochete *Borrelia burgdorferi*, which is transmitted by *Ixodes scapularis* (the black-legged or deer tick). Small mammals, particularly *Peromyscus leucopus* (the white-footed mouse), and some birds are important in the transmission cycle of *B. burgdorferi*, because they serve as reservoirs of the pathogen. Lesser-known infectious agents can share mammalian hosts, including *Anaplasma phagocytophilum, Ehrlichia* spp., and *Babesia microti*, thereby producing co-infection within an individual. Deer ticks can acquire and transmit these infectious agents, along with *B. burgdorferi*. Such co-infections are poorly known but are likely to have important implications for transmission dynamics of each infectious agent and ultimately human health.

Both ticks and mammal hosts are primarily found in woody, brushy, or forest fragments. Recently, there has been concern that deer ticks are exploiting new reservoir hosts in more open habitats. This expanding use of hosts has major human health implications; thus, people living in a variety of locations, not just those living in or near wooded areas, will be increasingly susceptible to tick infestations and subsequent infection by pathogenic agents.

This study examines the influence of mammal host species, sex, and weight of hosts on tick burdens, infection rates, and the potential for co-infection. This study is the

first of its kind in Central Wisconsin and one of the first to evaluate the infection rates of all four infectious agents in small mammals; most previous work has only included one or two agents. By understanding the ecology and behavior of mammal reservoir hosts, valuable information can be used to interpret and predict the transmission dynamics of these agents, with the goal of decreasing the likelihood of human exposure to infected ticks.

Infectious Agents and Diseases

Vector-borne diseases are illnesses caused by viruses, bacteria, or protozoans that are transmitted by a vector. Vectors are living organisms that transmit disease-causing agents from one animal (including humans) to another (CDC 2015). Some of the most common vectors are mosquitoes, ticks, mites, flies, lice, and fleas. Vector-borne diseases account for 17% of all infectious diseases and are responsible for more than 700,000 deaths annually worldwide (WHO 2017). In the United States, Lyme disease is the most common vector-borne disease, concentrated in the Midwest, along the Pacific Coast, and in the Northeast. Researchers estimate that over 300,000 cases of Lyme disease occur annually, with 20,000-30,000 of those cases being reported in Wisconsin each year (CDC 2015). However, many cases go unreported in Wisconsin, rendering this number a likely underestimate of the actual incidence of the disease.

Lyme Disease (borreliosis). Lyme disease is caused by the bacterium *B. burgdorferi* and is transmitted via the bite of an infected tick. The deer tick transmits the disease throughout the Midwest, mid-Atlantic, and northeastern United States, whereas

the western deer tick, *Ixodes pacificus*, transmits the disease along the Pacific Coast (CDC 2015). In most cases, ticks must be attached for 36 to 48 h before the bacterium can be transmitted. Reservoirs for the bacterium include birds and small mammals, which also function as hosts for the ticks. These reservoirs do not acquire the disease and therefore, remain asymptomatic. *Borrelia burgdorferi* is a bacterium of the spirochete class and is found in North America and Europe. It is a double-membrane bacterium, or diderm, due to the presence of an outer and inner membrane with a layer of peptidoglycans between. Its large number of flagellae enable it to move in low and high-viscosity environments, thereby contributing to its high virulence (Motaleb et al. 2000).

Progression of the disease occurs in three stages. The first stage occurs from 3 to 30 d after the tick bite, referred to as the early or localized stage. Common symptoms during this stage include the characteristic rash or skin lesion erythema migrans (80% of patients) at the site of the tick bite, fever, chills, muscle and joint pain, malaise, and headache. The second stage occurs from days to weeks after the initial bite. If the infection is left untreated, it is at this stage that the bacteria will disseminate and spread from the site of the bite to other areas of the body. Symptoms during this early, disseminated stage include erythema migrans rashes in other areas of the body, loss of muscle tone in the face, severe headaches, neck stiffness, swelling and pain in large joints, dizziness, or heart palpitations. Finally, the third stage can occur months to years after the initial bite (CDC 2015). At this stage, patients with untreated Lyme disease will have intermittent and lingering bouts of arthritis and may develop chronic neurological or cardiac complaints (Marques 2008). Ten to twenty percent of patients who have received

antibiotic treatment may experience persistent or recurrent symptoms that last months or years, a condition referred to as post-treatment Lyme disease syndrome (PTLDS) (Bratton et al. 2008). However, most early stages of Lyme disease can be treated effectively with oral antibiotics. Antibiotic intervention is necessary for treating this disease (Steere et al. 2004).

Anaplasmosis. The second most commonly-reported tick-borne disease in Wisconsin is anaplasmosis. In Wisconsin, the number of cases reported to the CDC has increased from ca. 300 cases in 2000 to almost 2000 cases in 2010, with a 52% increase between 2009 and 2010 alone (CDC 2016). Anaplasmosis, previously known as human granulocytic ehrlichiosis (HGE) and later renamed to human granulocytic anaplasmosis (HGA), is caused by the gram-negative, obligate intracellular bacterium *Anaplasma phagocytophilum* (Dahlgren et al. 2011). It is found in North America, Europe, and Asia (CDC 2016). This bacterium can only reproduce inside living cells and undergoes development within granulocytic white blood cells (Chapman et al. 2006). It is transmitted to humans through the bite of an infected *Ixodes* tick, primarily *I. scapularis* and *I. pacificus*. Typically, the tick must be attached for 36 to 48 h to transmit the bacterium (Dumler et al. 2005). Illness usually occurs within 7 to 14 d after the initial bite, and common symptoms include fever, headache, chills, and muscle aches. These symptoms are almost identical to those of Lyme disease and can also be effectively treated with antibiotics. If left untreated, anaplasmosis can lead to serious illness and fatality (CDC 2016).

Ehrlichiosis. Human ehrlichiosis is a general term used to describe a disease that is caused by at least three different ehrlichial species in the United States: *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, and *Ehrlichia muris-like* (EML). In Wisconsin, these gram-negative, obligate intracellular bacteria are transmitted to humans by the bite of an infected deer tick (Heitman et al. 2016). Since 2008, there has been an increase in the number of reported cases of ehrlichiosis in Wisconsin, from less than 200 in 2000 to more than 900 in 2008. There are no published data available for the attachment duration required for *I. scapularis* to transmit *Ehrlichia* spp. (Eisen 2017). Symptoms occur within 7 to 14 d after the initial bite (CDC 2016). Symptoms are similar to Lyme disease and anaplasmosis and can be effectively treated with a regimen of antibiotics.

Babesiosis. Although uncommon, human babesiosis is an emerging disease in the Midwest, northeastern United States, and parts of Europe. This disease is caused by a single-celled, protozoal parasite that infects and destroys red blood cells (Herwaldt et al. 2012). Most human cases of babesiosis are caused by *Babesia microti* and are acquired through the bite of an infected deer tick (Vannier and Krause 2012). Disease transmission time is ca. 36 h and symptoms include fever, chills, sweats, headache and body aches, fatigue, and anemia. Symptoms can last from several days to several months, whereas some infected individuals remain asymptomatic. This disease is treatable with antibiotics but is life threatening if left untreated (CDC 2012).

Co-infection

Incidences of co-infections in small mammal hosts are poorly known. When a human host is incidentally co-infected, these pathogens may act synergistically and often lead to a worsening of symptoms when compared to a single infection. Co-infected human hosts acquire a greater range of secondary symptoms and often display a more severe case of Lyme disease (Swanson et al. 2006). Unfortunately, these lesser-known infectious agents are poorly understood and often overlooked, leading to the frequent misdiagnosis of human cases. This study will shed light on the co-infection rates at Hartman Creek State Park and provide more knowledge on the concept of co-infections.

Vector

Borrelia burgdorferi, together with the other infectious agents described above, circulates between *Ixodes* ticks and vertebrate hosts in an enzootic cycle. *Ixodes scapularis* is a hard-bodied tick with a two-year life cycle consisting of four developmental stages: egg, larva, nymph, and adult. Ticks must take a blood meal at each stage after hatching before maturing to the next stage. Eggs are laid in spring and will hatch into larvae during mid to late summer. Infection in a living tick cannot be passed to its offspring, so eggs are uninfected (CDC 2015). Larvae feed on small mammals and birds, and it is at this stage that they can become infected with *B. burgdorferi* (Anderson and Magnarelli 1993). The bacterium migrates to the midgut of the tick, where it will survive and persist as the larva molts into a nymph and as the nymph overwinters (Skotarczak 2009). Nymphs will feed the following spring or early summer and may

transmit the infection to a new vertebrate host, including humans and domesticated animals (Ostfeld and Keesing 2000). During this feeding, the spirochete will migrate from the tick's gut to its salivary glands and penetrate the vertebrate host's tissues. However, the mechanisms behind this migration are not fully understood (Skotarczak 2009). In October and early November, nymphs will molt into adult ticks (Falco and Fish 1989). Adult female ticks will feed again, mainly on larger mammals, such as deer, that are not able to support the survival of *B. burgdorferi*. Although deer are not competent reservoirs for *B. burgdorferi*, they are the most important host of the tick; because they provide most adult female ticks with a bloodmeal that is necessary for a female to lay eggs. In addition, deer are usually abundant where *I. scapularis* ticks are found. After females are engorged, they will detach from the deer, overwinter in the leaf litter, and lay thousands of eggs the following spring (CDC 2015).

Most humans are infected through the bites of infected nymphs, which are <2 mm in length and difficult to see, given that nymph peak activity overlaps with human peak summer activity (CDC 2015). In spring, nymphs emerge before larvae. This temporal difference is a crucial component to the cycle of disease (Ostfeld and Keesing 2000). Nymphs are responsible for infecting small mammal hosts upon which emerging larvae will feed and from which they can acquire infection, enabling the enzootic cycle to continue. Adult ticks are much larger and are more likely to be discovered and removed before they can transmit infection (Barbour and Fish 1993). In addition, adults are most active during the cooler months of the year, whereas nymphs are most active during the spring and summer months. This summer activity coincides with greater human

recreational use of the outdoors (Falco and Fish 1989). Ticks wait for a host on the tips of grasses and shrubs with their upper pair of legs outstretched, in a behavior known as questing. When a potential host brushes by a questing tick, it latches on and finds a suitable feeding spot. The tick grasps the skin, cuts into the surface, and inserts its feeding tube. Ticks will also secrete small amounts of saliva with anesthetic properties to remain unnoticed, where they can feed for several days before detaching (CDC 2015).

Reservoir Hosts

This study focuses on eight small mammal species that were live-trapped at Hartman Creek State Park (Table 1).

Table 1
Latin and common names of the mammal species found at Hartman Creek State Park

Latin name	Common name
Sorex cinereus	Masked Shrew
Blarina brevicauda	Northern short-tailed shrew
Peromyscus leucopus	White-footed mouse
Peromyscus maniculatus	North American deer mouse
Tamias striatus	Eastern chipmunk
Ictidomys tridecemlineatus	Thirteen-lined ground squirrel
Microtus pennsylvanicus	Meadow vole
Myodes gapperi	Southern red-backed vole

Rodents. In eastern North America, including Wisconsin, the vector of these infectious agents is the deer tick, and the most important host is typically the white-footed mouse, *P. leucopus* (Gray et al. 2002). *Peromyscus leucopus* is found throughout the United States east of the Rocky Mountains, except in parts of the Southeast. This

rodent is widely distributed but prefers wooded or brushy areas, while sometimes being found in open areas. This mouse spends most of its time on the ground but also climbs into bushes and trees. (Marsh and Howard 1990).

Although the white-footed mouse is currently the most important host, it is not the only host. Many small mammals, including the deer mouse, *Peromyscus maniculatus*, are also known reservoirs. This species is a competent reservoir host in both laboratory and sylvatic settings (Rand et al. 1993). *Peromyscus maniculatus* is found throughout most of North America and occupies nearly every type of habitat within its range, including forests and grasslands. It is the most abundant and widely-distributed mammal in North America (Marsh and Howard 1990).

Both species of mice are largely granivorous but will feed on other items, such as insects, that are available (Jackson 1961). *Peromyscus maniculatus* nests are often built underground in cavities beneath tree roots or shrubs, beneath logs, or in burrows made by other rodents (Schwartz and Schwartz 1981). Abandoned birds' nests are often roofed and converted into nests by *P. leucopus*. Both species are mostly nocturnal, with a home range of 0.13 per ha to 1.6 per ha or larger (Jackson 1961). A typical, non-summer population has a density of about 8 or 10 adults per ha, whereas summer population density may reach a high of 38 to 50 mice per ha (Marsh and Howard 1990).

The Eastern chipmunk, *Tamias striatus*, is another known reservoir. Like *P. maniculatus*, this species is a competent reservoir host for *B. burgdorferi* in both laboratory and sylvatic settings (McLean et al. 1993, Slajchert et al. 1997). This species is a small, ground-dwelling rodent that is found across eastern North America. It lives in

deciduous woodlands and urban parks, preferring locations with brush, shrubs, or log piles to provide cover. These animals are active during the day and spend most of their time foraging or constructing their underground nests with elaborate tunnel systems (Schwartz and Schwartz 1981). They climb trees but do not jump from limb to limb like tree squirrels. Their diet consists of nuts, seeds, berries, and occasional small animals. In favorable habitat, the home range averages less than 0.16 ha, and population densities vary from 10 to 38 individuals per ha (Cassola 2016). Population densities are often higher in residential areas (Schulze et al. 2005).

The thirteen-lined ground squirrel, *Ictidomys tridecemlineatus*, has not yet been determined to be a competent reservoir of these infectious agents, either in laboratory or sylvatic settings. However, deer ticks are common ectoparasites of this species. *Ictidomys tridecemlineatus* is a small, slender mammal widely distributed across grasslands, prairies, and open areas of North America. Although active during the day, individuals spend most of their time underground in burrows. Their diet consists of plant and animal foods, including seeds, fruits, insects, and small vertebrates. Individuals enter hibernation in October and emerge in April or May. Although they are not colonial, most live in loosely-constituted families. Densities are estimated to be about 8 individuals per ha in spring and ca. 30 per ha following the emergence of young. Home ranges can be anywhere from less than an 0.4 ha to 5 ha, with males typically having a larger range (Cassola 2016).

Insectivores. The masked shrew, *Sorex cinereus*, is the most common shrew in North America, spanning across much of Canada, Alaska, and the northern United States.

It is a competent reservoir of infectious agents and a host of ticks (Brisson et al. 2007). Individuals occupy most terrestrial habitats, particularly those with dense vegetation or thick leaf litter in which to hide (Schwartz and Schwartz 1981). Depending on the weather, they can be either diurnal or nocturnal and are active year-round. This activity level is pertinent for securing sufficient food to maintain their high metabolic rate; they must eat almost constantly, consuming more than their own weight in food daily. These animals are opportunistic generalists, eating primarily insects, other invertebrates, seeds, carrion, and small vertebrates (Whitaker 2004). Individuals excavate tunnels but also use tunnels made by other mammals. Dry grass is used to make nests within these tunnels. Densities range from 3 to 30 shrews per ha, with a home range of ca. 0.04 ha (Cassola 2016).

The northern short-tailed shrew, *Blarina brevicauda*, also is an important host for *B. burgdorferi* (Telford et al.1990). This species is found throughout the more northern parts of central and eastern North America. One of the most common mammals in the Great Lakes region, *B. brevicauda* is most abundant in hardwood forests with thick leaf litter; it avoids areas with temperature and moisture extremes and little vegetation. Like the masked shrew, individuals also excavate tunnels or use existing tunnels, where they construct elaborate underground nests (Cassola 2016). They spend most of their time belowground and are only active aboveground 16% percent daily (Whitaker 2004). This species is mostly carnivorous, consuming vertebrates more often than other species of shrew. Additionally, this species is one of the few venomous mammals. The home range of *B. brevicauda* can be twice as large as other shrew species, with a mean of ca. 2.4 ha.

Densities are ca. 8 individuals per ha in winter and 13 to 25 per ha in summer and fall. These animals remain solitary until the breeding season (Schwartz and Schwartz 1981).

The meadow vole, *Microtus pennsylvanicus*, is a known competent reservoir host (Markowski et al. 1998). This species is widespread across North America and is found in a variety of habitats, including grasslands, wooded areas, and other areas with substantial amounts of leaf litter and loose soil for tunneling (Cassola 2016). It has the widest distribution of any North American *Microtus* species. Like shrews, voles build elaborate underground tunnel systems in which their nests are also located. This animal is active year-round and is mostly nocturnal. The diet mainly consists of plant material; such as grasses, seeds, and roots (Schwartz and Schwartz 1981). Although these animals live close together, they tend to be aggressive towards each other. Home range is ca. 0.1 ha with mean densities ca. 20 to 25 individuals per ha (Cassola 2016).

The southern red-backed vole, *Myodes gapperi*, is a small vole found in Canada and the northern United States. This species' competency as a host for infectious agents is unknown; however, *Ixodes* ticks infest these animals. *Myodes gapperi* prefers woodlands and forests, including coniferous, deciduous, and mixed forests, often near wetlands. They do not form colonies and are not gregarious. They use runways through surface vegetation in warm weather and tunnel through snow in the winter. Individuals are active year-round, mostly at night, and use underground burrows constructed by other animals. Their diet consists primarily of vegetative parts of plants, but they will also eat seeds, berries, fungi, insects, and snails (Cassola 2016). Within favorable habitats,

populations often reach 10 or 12 individuals per ha. Home ranges vary from 0.04 to 0.09 ha (Jackson 1961).

Chapter II

Co-infection Rates of Small Mammal Hosts in Central Wisconsin

Introduction

Ixodes scapularis, or deer ticks, can acquire numerous infectious agents that cause human disease and potentially co-transmit multiple infectious agents within a single bite, resulting in what is referred to as a co-infection. Aside from *Borrelia burgdorferi,* the causative agent of Lyme disease, *I. scapularis* ticks are known to acquire and transmit *Anaplasma phagocytophilum, Ehrlichia* spp., and *Babesia microti*. Small mammals, particularly the white-footed mouse (*Peromyscus leucopus*), and some birds are important in the transmission cycle of these infectious agents because they serve as reservoirs of the pathogens. The seasonality of feeding larvae and nymphs is critical for transmission of these infectious agents and ultimately permits the agents to persist in both reservoir hosts and the vector. Recently, there has been concern that deer ticks are exploiting new reservoir hosts in more open habitats. Thus, people living in a variety of locations, not just those living in or near wooded areas, may be susceptible to tick infestations and subsequent infection and co-infection by pathogenic agents. Unfortunately, co-infections are poorly known and often overlooked, leading to frequent misdiagnosis of human cases.

In several areas of Wisconsin, singular infection rates for *B. burgdorferi, B. microti*. *A. phagocytophilum,* and *Ehrlichia* spp. have been determined by collecting and testing *I. scapularis* from mammals within those sites. However, those studies do not

include the singular infection or co-infection rates of the hosts. This study examines singular and co-infection rates within eight different mammal species that are distributed across a variety of habitats in Central Wisconsin. This study provides a better understanding of the infection rates of these infectious agents in reservoir hosts within this area of Wisconsin and will elucidate the concept of co-infection of hosts.

This study specifically examines the influence of host species, sex, and weight of hosts on tick burdens, infection rates of these infectious agents, and co-infection rates. This study is the first of its kind in Central Wisconsin and one of the first to evaluate the infection rates of all four infectious agents transmitted by *I. scapularis* ticks. By understanding the ecology and behavior of mammal reservoir hosts, valuable information can be applied to predict the transmission dynamics of these disease-causing agents, with the goal of decreasing the likelihood of human exposure to infected ticks. This study provides novel information on these infection rates and also provides data on reservoir hosts and tick burdens. These results have important implications for human health.

Methods

Study site. The study was conducted at Hartman Creek State Park, which is located on the border of Portage and Waupaca counties in Central Wisconsin. This 600-ha park includes the upper Waupaca Chain O'Lakes and contains a variety of habitats, including deciduous woodlands, open grasslands, marshes, and red pine plantations. This site was selected based on the known presence of infectious agents, the vector (deer ticks), and vertebrate hosts (small mammals).

Live-trapping. Small mammals were live-trapped at Hartman Creek State Park in Central Wisconsin during two field seasons (May through September 2017 and April through July 2018), with Institutional Animal Care and Use Committee (IACUC) and Wisconsin DNR approval. Approximately 50 Sherman live-traps (H.B. Sherman Co., Tallahassee, FL) were set along different transects (of varying length) that passed through or were adjacent to a variety of habitats, including woodlands, prairies, marshes, and red pine plantations. Transects were selected based on accessibility, and two traps were set at each of approximately 25 sampling stations along each transect and then checked the following three days before being removed and placed along a new transect. I placed sunflower seeds and polyfill in each trap to provide food and bedding for the small mammals. I collected tissue samples from putative hosts (various species of small mammals, particularly rodents) and removed any ticks. The right ear of each captured mammal was tagged with a serially-numbered metal tag (National Tag and Band Co., Newport, KY), and a small piece of left ear tissue was excised and placed in 70% ethanol for preservation purposes. Ticks that were collected from small mammals were also preserved in 70% ethanol. The sex and weight of the captured mammal, using a Pesola scale, were also recorded. Methods described by Jackson (1961) and Schwartz and Schwartz (1981) were used to differentiate between *P. leucopus* and *P. maniculatus*.

Tick identification. Larval and nymphal ticks that were collected from small mammals were identified as *I. scapularis* using an Olympus SZ60 dissecting microscope (at 60x magnification) and dichotomous identification keys described by Clifford et al. (1961), Durden and Keirans (1996), and Coley (2015). (Appendix B). All materials used

in the identification process were sterilized after handling each tick to prevent contamination. Once identified, ticks were transferred to a new Eppendorf tube containing 70% ethanol for permanent storage. Only *I. scapularis* were included in the tick burden counts, which were determined by counting the number of ticks (*I. scapularis* only) removed from each individual mammal. Polymerase chain reaction (PCR) and species-specific primers, IScapF and IScapR, designed to amplify the small subunit 16S rRNA gene of *I. scapularis*, were used to confirm tick identity (Table 2).

Table 2
Forward and reverse primers for each infectious agent

Primer Name	Sequence (5' to 3')	Microbe Target	Target Gene	Product Size
Bab1F	5' CTTAGTATAAGCTTTTATACAGC 3 '		16S-like small	
Bab4R	5' ATAGGTCAGAAACTTGAATGATACA 3'	*B. microti*	subunit rRNA gene	238 bp
MSP3F	5' CCAGCGTTTAGCAAGATAAGAG 3'			
MSP3R	5' GCCCAGTAACAACATCATAAGC 3'	*A. phagocytophilum*	p44 gene	334 bp
OspA1F	5' AATAGGTCTAATATTAGCCTTAATAGC 3'			
OspA2R	5' TCAAGTCTGGTTCCGTCTGCTC 3'	*B. burgdorferi*	ospA gene	417 bp
SodBF	5' TTTAATAATGCTGGTCAAGTATGGAATCAT 3'			
SodBR	5' AAGCGTGTTCCCATACATCCATAG 3'	*Ehrlichia sp.*	sodB gene	304 bp
IScapF	5' TAAACAATTAAAAGCTTTCTT 3'		Small subunit 16S	
IScapR	5' AATCGCTAAAAACGGAACTTA 3'	*I. scapularis*	rRNA gene	460 bp

Sequences and target genes for *B. microti*, *A. phagocytophilum*, and *B. burgdorferi* were described by Prusinski et al. (2014). *Ehrlichia* spp. and *I. scapularis* sequences and target genes were described by Black and Piesman (1994) and Qurollo et al. (2014) respectively.

Sample analysis. Samples were returned to a laboratory at UW-Oshkosh for screening using standard molecular techniques to determine which infectious agents were present within small mammal hosts.

Extracting DNA from mammal tissue. DNA from the mammal tissue samples was extracted, purified, and concentrated using the DNeasy blood and tissue kit by Qiagen (Appendix C; 2011). All extractions were performed under a Hamilton SafeAire fume hood. Centrifugation was performed using an Eppendorf 5415C centrifuge and all vortexing was performed using a Fisher Scientific Vortex Genie 2. Gilson pipetman micropipettors were used for all pipetting steps. The final volume of extracted DNA ca. 30 µL was stored at -20°C in a Whirlpool chest freezer.

PCR screening of mammal tissue. DNA was amplified using Polymerase Chain Reaction (PCR) with a Bullseye brand kit, containing all required reagents and enzymes. A NuAire biological safety cabinet was used to establish all end-point single-target PCR reactions, using a protocol derived from Kogut et al. (2015), MidSci (2017) and Thermo Scientific (2017) (Appendix E). In addition to the kit, primers were used to target specific sequences of the microbial DNA of the infectious agents for which I was testing (Table 2). Sequences of primer sets, as described by Black and Piesman (1994), Prusinski et al. (2014), Qurollo et al. (2014), were the basis for primer synthesis (Table 2). Eurofins Genomics used salt-free purification to manufacture the primers.

A 10% bleach solution and UV light were used to sterilize the safety cabinet before and after each use. Using 0.2 mL Fisherbrand flat-capped PCR tubes, 20-µL reactions were established on ice and mixed thoroughly before use. PCR tubes contained

10 µL of hot start master mix (either Bullseye (2x) from MidSci or Thermo Scientific DreamTag (2x) from Fisher Scientific), 5 µL of sterile molecular-grade water, 2 µL of 10-µM combined forward and reverse primers (final concentration of 1 µM), and 3 µL of template DNA. I also conducted positive and negative controls to ensure precision of the test method. One positive control and two negative controls, one containing molecular grade water and the other containing DNA extracted from a lab-raised *Ictidomys tridecemlineatus* (courtesy of University-Wisconsin Oshkosh animal lab), were used for each run. Using manufacturer guidelines, Bio-Rad T100 or C1000 Thermal Cycler were programmed for 40 cycles (MidSci 2017, Thermo Scientific 2017; Appendix C).

Gel electrophoresis. Once the DNA was amplified, gel electrophoresis was performed to identify which infectious agents were present. PCR reactions were combined with 4 µL of Thermo Scientific 6X DNA loading dye. Approximately 5 µL of this solution was pipetted into a 1.5% Gibco BRL agarose gel in 1x Quality Biological TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) using a Gilson pipetman micropipettor. To achieve a final concentration of 0.5 µg/mL, Fisherbrand ethidium bromide was added to the agarose. A constant voltage of 75 volts was used, for a total of twenty minutes, to electrophorese the gel. Gels were imaged using a Bio-Rad molecular imager Gel Doc XR system. See Appendix C for a sample gel image.

Statistical analysis. For statistical analysis, I used a Fisher's exact test to determine whether the observed small mammal co-infection rates of *B. burgdorferi* and *A. phagocytophilum*, *B. burgdorferi* and *B. microti*, and *A. phagocytophilum* and *B. microti* were different from the expected rates based on singular infection rates of each

infectious agent. I performed a t-test to determine if there were differences between mean tick burdens of male and female hosts. I used a simple linear regression analysis to determine if there was a relationship between tick burdens and host weight.

Results

Tick burdens. Of the eight mammal species that I sampled, ticks were collected from only five (Table 3). The number of ticks removed per infested individual ranged from 1 to 22 ticks, with a mean of ca. 3 ticks per infested mammal overall. *Peromyscus maniculatus* had the overall highest tick burden mean of 4.57 ticks per individual, while *P. leucopus* had 4.33 ticks per individual, and *T. striatus* had 3.86 per individual. *Tamias striatus* had the highest burden (22 ticks) from one individual and also the second highest burden (15) from one individual. A *P. leucopus* individual also had 15 ticks removed, whereas the greatest number of ticks removed from a *P. maniculatus* individual was 12.

Table 3
I. scapularis tick burdens of small mammal species

Species	# of individuals from which ticks were removed	# of ticks removed	# of nymphs removed	Mean # of ticks per individual
P. leucopus	15/26	65	1	4.33
P. maniculatus	14/20	64	2	4.57
T. striatus	29/49	112	54	3.86
I. tridecemlineatus	3/5	5	1	1.66
M. pennsylvanicus	1/2	1	1	1

Nymphal ticks were pulled predominantly from *T. striatus* (23 out of 29 individuals that harbored ticks had nymphs, for a total of 54 nymphs). The remaining ticks were predominantly larvae.

Host sex. Tick burdens, by sex, within each species are represented in Table 4. The number of males and females within and amongst species varied.

Table 4
Total number of infested males and females across mammal species. N represents the total number of individuals sampled within that species

Mammal species	# of males with ticks	N	# of females with ticks	N
S. cinereus	-	-	0	3
B. brevicauda	0	1	0	3
P. leucopus	9	13	6	10
P. maniculatus	9	12	5	7
T. striatus	16	25	12	23
I. tridecemlineatus	2	2	1	3
M. pennsylvanicus	1	1	0	1
M. gapperi	0	1	0	1

Across species, tick burdens did not vary between males and females ($t = 0.0489$, $P > 0.05$, $df = 59$) (Table 4). Within species, *P. leucopus* females had the highest overall mean tick burden of 7.33 ticks per female (Table 5). *Ictidomys tridecemlineatus* and *M. pennsylvanicus* males had the lowest overall mean tick burden of one (Table 5).

However, there were no differences between mean male and female tick burdens amongst species ($t= 0.7321$, $P > 0.05$, $df=7$).

Table 5
Mean tick burdens of infested male and female mammals

Species	Mean # of ticks per male	Mean # of ticks per female
P. leucopus	4.77	7.33
P. maniculatus	5.33	3.2
T. striatus	3.43	4.75
I. tridecemlineatus	1	3
M. pennsylvanicus	1	-

Out of 116 mammals, 57 were males, 52 were females, and 7 were unknown. Across mammal species, 13 males and 13 females were positive for *B. burgdorferi*, 11 males, 16 females, and one unknown were positive for *A. phagocytophilum*, and the three individuals positive for *B. microti* were all males. Four males and seven females were co-infected.

Weight. *Tamias striatus* had the highest mean weight, whereas *S. cinereus* had the smallest mean weight. Mean weights across and within species is represented in Table 6. For the individuals whose sex could not be determined, their weight was not included in the calculations, and they were excluded from the sample size (N). *Blarina brevicauda* had one unknown individual, *P. leucopus* had three unknown individuals, *P. maniculatus*

had one unknown individual, *T. striatus* had one unknown individual, and *M. gapperi* had one unknown individual.

Table 6
Mean weights of male and female mammals across and within species that were sampled. N represents the total number of individuals sampled within that species

Mammal species	**Male mean weight (g)**	**N**	**Female mean weight (g)**	**N**	**Overall mean weight (g)**	**Overall N**
S. cinereus	-	0	1.5	3	1.16	3
B. brevicauda	12	1	11	3	11.4	5
P. leucopus	17.15	13	19.1	10	18	26
P. maniculatus	17.51	12	22.2	7	19.2	20
T. striatus	106.36	25	84.7	23	94.1	49
I. tridecemlineatus	40	2	68.33	3	57	5
M. pennsylvanicus	15	1	22	1	18.5	2
M. gapperi	18	1	15	1	16.5	3

There was no relationship between tick burden and mammal weight (R^2= 0.0338, P=0.156, N=61, Figure 1).

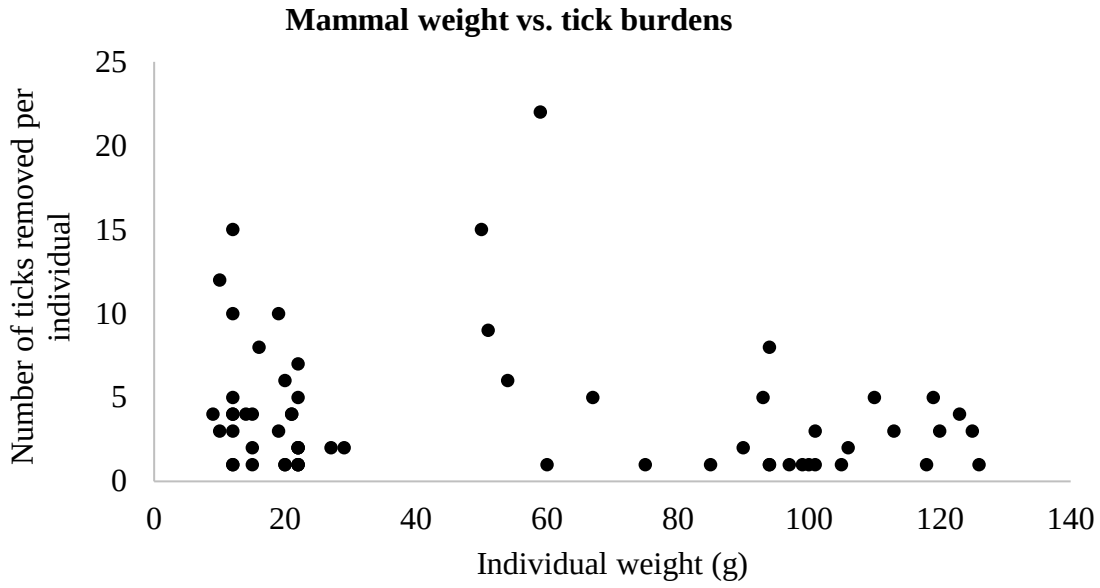


Figure 1. Mammal weight plotted against tick burdens.

Singular and co-infection rates. For singular infection rates, 26 out of 116 mammals (22.4%) were positive for *B. burgdorferi*, 28 (24.1%) were positive for *A. phagocytophilum*, 3 (2.6%) were positive for *B. microti*, and 0 were positive for *Ehrlichia* spp. Infection rates by host species are given in Table 7. Overall, *P. maniculatus* had the highest infection prevalence for *B. burgdorferi* with 45% of individuals infected, *I. tridecemlineatus* had the highest infection prevalence for *A. phagocytophilum* with 60% of individuals infected, and *T. striatus* had the highest infection prevalence for *B. microti*, with 6.1% of individuals infected.

Table 7
Infection distribution across the mammal species collected from Hartman Creek State Park

Mammal species	***B. burgdorferi* infected individuals (%)**	***A. phagocytophilum* infected individuals (%)**	***B. microti* infected individuals (%)**	**N**
S. cinereus	0	33	0	3
B. brevicauda	0	20	0	5
P. leucopus	7.6	7.6	0	26
P. maniculatus	45	10	0	20
T. striatus	28.5	38.7	6.1	49
I. tridecemlineatus	20	60	0	5
M. pennsylvanicus	0	0	0	2
M. gapperi	0	0	0	3

10 mammals were co-infected with *B. burgdorferi* and *A. phagocytophilum* (BB + AP), 2 were co-infected with *B. burgdorferi* and *B. microti* (BB + BM), 1 was co-infected with *A. phagocytophilum* and *B. microti* (AP + BM), and 1 mammal was co-infected with all three pathogens. Overall, 11 mammals (9.4%) were co-infected with two or more pathogens. Aside from two *P. maniculatus* individuals co-infected with BB + AP and; BB+ BM, co-infected hosts were predominantly *T. striatus*. Observed infection rates did not differ from random expectations (Table 8).

Table 8
Statistical values for co-infection prevalence rates

Pathogens	Observed prevalence	Fisher's exact P value
BB + AP	10	0.12
BB + BM	2	0.59
AP + BM	1	1

Overall, 71 mammals were not infected with any of the infectious agents, 34 were infected with a single pathogen, and 11 were infected with 2 or more pathogens (Figure 2).

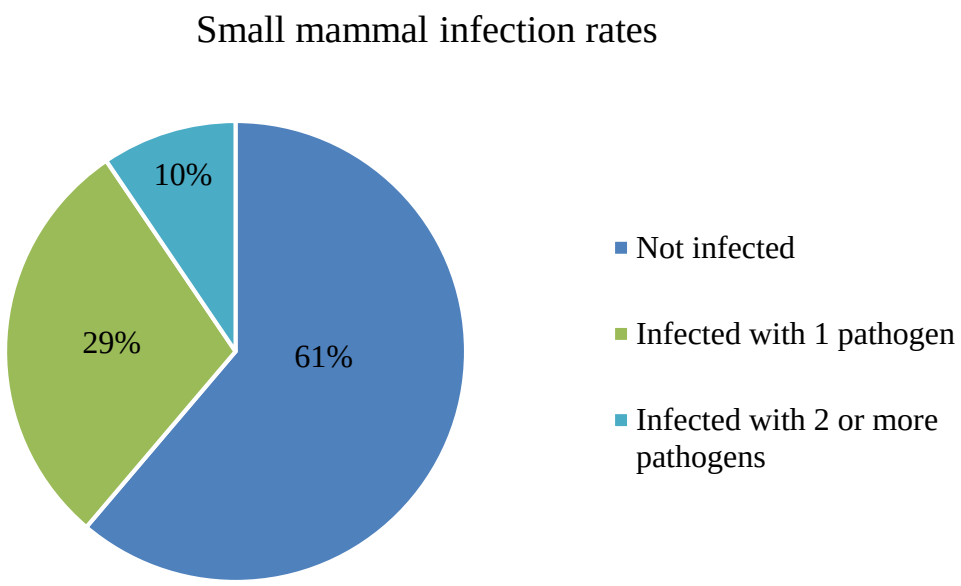


Figure 2. A graphical representation of small mammal infection rates.

Discussion

One of the most important elements in understanding and controlling the transmission of infectious agents is identifying the hosts of the vector and agents. In the present case, if ticks are aggregated on individual hosts, then a small proportion of hosts may be responsible for the majority of transmission events (LoGiudice et al. 2003). Brunner and Ostfeld (2008) found that tick burdens are a function of many intrinsic and extrinsic factors, including seasonality. They found that mice and chipmunk tick burdens increased with densities of host-seeking ticks, suggesting that hosts become saturated; chipmunks may draw larval ticks away from mice, and mice may draw nymphs away from chipmunks, which are key nymphal hosts (Brunner and Ostfeld 2008). Brunner and Ostfeld (2008) concluded that no group (species) or correlate (age, weight, or sex) could explain which individuals hosted a disproportionate number of ticks. Intrinsically, an unknown quality of individual hosts was responsible for the aggregation, given that ticks were strongly aggregated on hosts within and across species. My results corroborate that suggestion because no differences in tick burdens with respect to sex or weight within and among species were found.

I removed ticks were removed from five different species of small mammals, which exhibited a range of tick burdens. Across species, *Tamias striatus* harbored the greatest number of nymphal ticks. Aside from one individual from each species that hosted a nymph, both *P. leucopus* and *P. maniculatus* hosted predominantly larvae. This observation is comparable to a study by Shaw et al. (2003) that showed that mice have

greater larval tick burdens because mice are more likely to use larval tick-infested microhabitats and to attract questing larvae than are chipmunks.

There was variation concerning mean mammal weight within and among host species, in addition to variation in tick burdens of males and females within and among species. This coincides with the results of Brunner and Ostfeld's (2008) study given that they found variation among mammal weight within and across species and that tick burdens are a function of many intrinsic and extrinsic factors. The variable tick burdens in my study are likely the result of a complex combination of seasonality, questing tick densities, densities of focal and non-focal hosts and individual host characteristics. Although a single-factor explanation is simpler, the multiple levels of interacting factors must be addressed to understand the causes and consequences of variable tick burdens on small mammal hosts (Brunner and Ostfeld 2008).

In my study, *T. striatus* harbored more nymphs while also maintaining the highest singular infection rate for *B. microti* and the highest overall co-infection rate. Given that individuals that harbor more nymphs are more likely to be infected, this may be a plausible explanation as to why this species had the highest co-infection rate (Brunner and Ostfeld 2008). My study's small sample size may not be providing an accurate representation of the patterns or relationships occurring, if any, which may also explain the observed singular infection and co-infection rates for this species.

Because there are no past studies on small mammal infection rates in Central Wisconsin, I am only able to compare my study's rates to those of infected *I. scapularis* and *Peromyscus* species collected from other areas of Wisconsin. My study's observed

infection rate for *B. burgdorferi* of 22.4% is close to the state's mean of 22%, which is based on *I. scapularis* infection rates (CDC 2018). The infection rates for *A. phagocytophilum* ranged from 3% to 19%, with a rate of 4% in the areas surrounding Waupaca and Portage counties (CDC 2018). My study's infection rate of 24.1% is higher; however, this could be attributed to small sample size or the possibility that mammal infection rates differ from those of sampled ticks due to differences in location, pathogen exposure, pathogen prevalence, and individuals sampled. In addition, *T. striatus* individuals maintain moderate to high levels of *A. phagocytophilum*, which could explain the higher observed infection rate, given that more *T. striatus* individuals were sampled overall within my study (Foley et al. 2008).

My study's infection rate of 2.6% for *B. microti* is within the range of 2% to 5% in other Wisconsin areas, also based on *I. scapularis* infection rates (Wisconsin Department of Health Services 2017). The areas near Waupaca and Portage counties, however, were 0% (CDC 2018), which could explain the few instances of *B. microti* infection and co-infection that were found within my study. I found no instances of *Ehrlichia* infection, which was also found for the areas surrounding Hartman Creek State Park. *Ehrlichia* is found elsewhere in the state, with *I. scapularis* infection rates ranging from 2% to 9% (Wisconsin Department of Health Services 2017). Castillo et al. (2015) found only two instances (1.4%) of *Ehrlichia* in *P. leucopus* throughout Minnesota and Wisconsin, reaffirming its rarity and possibly explaining the lack of *Ehrlichial* infection in my study.

I found that *P. maniculatus* had a 45% *B. burgdorferi* infection rate and a 10% *A. phagocytophilum* infection rate. *Peromyscus leucopus* had a 7.69% infection rate for both pathogens. In comparison, Larson et al. (2018) studied singular infection and co-infection rates of *P. leucopus* and *P. maniculatus* in northern Wisconsin. They found that 24% of *P. leucopus* individuals were infected with *B. burgdorferi,* and 1.69% were infected with *A. phagocytophilum*, while *P. maniculatus* had a lower *B. burgdorferi* infection rate of 16.8% and a higher *A. phagocytophilum* infection rate of 4.73%. Of 10 co-infected individuals, Larson et al. (2018) found that nine were *P. maniculatus*; there were more co-infections than expected by chance. By contrast, only two of 11 co-infected individuals in my study were *P. maniculatus*. Again, my study's small sample size may be a misrepresentation of what is occurring. Differences in the biology of these two mammal species, such as behavior, habits, habitats, home range, pathogen exposure, etc., may have contributed to the observed variation of abundance of host ticks and pathogen prevalence. Geographic location and small sample sizes may explain the differences in infection rates between these two studies.

In my study, six species of small mammals were infected with at least one of the agents. *Peromyscus leucopus* and *T. striatus* are among the most heavily parasitized tick hosts within areas endemic for Lyme disease, given that these two species overlap extensively in micro- and macrohabitat preferences, diet, and behavior (Shaw et al. 2003). *Peromyscus leucopus* maintains population densities about twice those of *T. striatus*; however, in my study both *T. striatus* and *P. maniculatus* individuals exhibited greater infection rates than *P. leucopus* individuals (although not statistically). These

observations suggest that species other than *P. leucopus* are becoming key players in the transmission of these vector-borne pathogens, thereby rivaling *P. leucopus* for the role as primary reservoir. The species that were infected in my study were from a variety of habitats, including deciduous broad-leaved forests, open grasslands, and near marshes. Overall, these observations highlight the notion that these infectious agents, particularly that of Lyme disease, may no longer be limited to wooded areas.

Co-infection rates did not differ from random expectations. However, my study's sample sizes may have been too small to detect a statistical difference I found more instances of *B. burgdorferi* and *A. phagocytophilum* co-infection than either *B. burgdorferi* and *B. microti* and *A. phagocytophilum* and *B. microti* co-infections. In the United States, *B. burgdorferi* and *B. microti* co-infection within *I. scapularis* has a higher rate than any other combination. Hersh et al. (2014) in New York found 83% more co-infection with *B. burgdorferi* and *B. microti* than predicted by chance alone, as compared to my study's co-infection rate of 9%. Hersh et al. (2014) also found that co-infections involving *A. phagocytophilum* were less common than predicted by chance, whereas I found a co-infection rate with *B. burgdorferi* of 81% (9/11 instances of co-infection). Again, small sample size could be a plausible explanation. Furthermore, *T. striatus* exhibits moderate to high levels of anaplasmosis (Foley et al. 2008), which could explain my study's high co-infection rate because co-infected individuals were predominantly of this host. The Midwest has seen a dramatic increase in the prevalence of these pathogens, possibly resulting in the observed variation and increase in infection rates within

Wisconsin (CDC 2018). Unfortunately, the concepts of co-infected ticks and hosts are still not well understood (Hersh et al. 2014).

Chapter III

Conclusions

Ixodes scapularis may acquire numerous infectious agents that cause human disease and may also transmit multiple agents in a single bite. The seasonality of feeding larvae and nymphs is critical for transmitting these infectious agents and ultimately permits the agents to persist in both reservoir hosts and the vector. Co-infections are poorly known and often overlooked, leading to frequent misdiagnosis of human cases. Within a co-infected host, these agents may act synergistically and lead to more severe or persistent symptoms compared to a single infection. In addition, co-infected hosts acquire a greater range of secondary symptoms and often display a more severe case of Lyme disease (Swanson et al. 2006). My study's data from Hartman Creek State Park in Central Wisconsin shows that 11 of 116 (9.4%) of small mammals collected were co-infected with two or more pathogens. One of the most important elements in understanding and controlling the transmission of these infectious agents is identifying the hosts of the ticks. The species that were infected were from a variety of habitats, including deciduous broad-leaved forests, open grasslands, and near marshes. These results highlight the suggestion that these infectious agents may no longer be limited to wooded areas.

These observations also suggest that tick burdens are the result of a complex combination of seasonality, questing tick densities, densities of focal and non-focal hosts, and individual host characteristics. Thus, the multiple levels of interacting factors must be identified to understand the causes and consequences of variable tick burdens on small

mammal hosts (Brunner and Ostfeld 2008). My study provides a foundation for future work concerning infection and co-infection rates for these infectious agents in Central Wisconsin. Future studies should continue to address deficiencies in our knowledge of these agents to identify more patterns and processes relating to both the vector and hosts. By understanding the ecology and behavior of mammalian reservoir hosts, this information may be used to interpret and predict the dynamics of these agents, with the ultimate goal of decreasing the likelihood of human exposure to infected ticks.

APPENDIX A

Research Proposal Form

GRADUATE STUDIES

DEC 12 2017

UW Oshkosh

Research Proposal Form

University of Wisconsin Oshkosh
Office of Graduate Studies, Oshkosh, WI 54901-8621
Dempsey 337 (920) 424-1223

1. Completed by student (Please print or type)

Student Name (Last Name, First Name, Middle Initial)	Student ID Number
Bodeen, Bailey A	0737229
Address	**Telephone#**
520 W Irving Ave Oshkosh, WI 54901	541-589-0622
Degree Program / **Date admitted to candidacy** (semester/year)	
MS in Biology	

Type of Research Project (check one):

☑ Thesis ☐ Field Project ☐ Clinical Paper ☐ Other: ____ No. of credits: 6

Project Title: Small mammal reservoir hosts and infection prevalence of Borrelia burgdorferi

Name(s) of proposed reader(s)/committee (list chairperson first): Greg Adler, Bob Stelzer, Misty McPhee

Answer both — IRB approval needed? Circle one: Y / N IACUC approval needed? Circle one: Y / N

It is University policy and federal regulation (FR Title 45 Part 46, rev. 6/18/91) that all research conducted with humans or animals must comply with guidelines regarding the Use of Human Participants or Animal Care. By signing below, you certify that you will obtain the necessary IRB or IACUC approvals, as appropriate, for the research described herein.

Student Signature	Date
Bailey Bodeen	11/29/2017

Attach a brief proposal (approximately 250 words) that identifies the plan and purpose of your research. As applicable attach verification of your IRB or IACUC approval. Note that if IRB/IACUC approval is required for your research, this proposal will not be approved without the IRB/IACUC approval attached.

THIS IS NOT A REGISTRATION DOCUMENT. YOU MUST REGISTER SEPARATELY FOR THE APPROPRIATE COURSE CREDITS.

2. Completed by Research Committee

We certify that the student has obtained the necessary institutional approval(s) to use Human Participants or Animal Subjects for the research described herein. Committee Chairperson/Instructor/Reader – Name & Signature	Date	Department
Gregory H. Adler	DEC 2017	Biology
Second Committee Member – Name & Signature (if applicable) Robert Stelzer	12/7/17	Biology
Third Committee Member – Name & Signature (if applicable) M.E. McPhee	12-11-17	Biol/Envt St.

3. Program Coordinator Approval: Robert Stelzer Date: 12/7/17

4. Graduate Studies Approval: Peggy B. Cuffman Date: DEC 27 2017

Copies:
- ☐ Graduate Studies
- ☐ Graduate Program Coordinator
- ☐ Student
- ☐ Research Committee Chair/Instructor/Reader

ver. 0616

APPENDIX B

Dichotomous Identification Keys

Dichotomous identification key used for identifying *I. scapularis* larvae (Coley 2015).

1A. Anal groove not extending anteriorly around anus.....……..……..……....…7
1B. Anal groove extending anteriorly around anus (genus *Ixodes*)……..…....…..……2
2A. Palpal segment 2 extending anteriorly and posteriorly……*Ixodes angustus*
2B. Palpal segment 2 not extending anteriorly and posteriorly …….....…..…..…….……3
3A. Palps broad and relatively short ……..……..……..…..4
3B. Palps narrow and long ……....…….5
4A. Dorsal basis capituli almost triangular; small internal spur coxa I.....…. *Ixodes cookei*
4B. Dorsal basis capituli squarish; no coxal spurs.……..…..……...….*Ixodes texanus*
5A. Tip of hypostome pointed; tiny extensions (auriculae) present on ventral basis capituli……..…..…...*Ixodes scapularis*

Dichotomous identification key used for identifying larval Ixodid ticks (Clifford et al. (1961).

1. Sensilla sagittiformia absent. With 2 pairs post-hypostomal setae. Anal groove present ……..…….....….*Ixodes* 2
2(1). With 7 pairs marginal dorsal setae. Supplementary setae present. Dentition 3/3 in anterior third of hypostome (count does not include few small denticles in corona)..……3
3(2). With 2 triangular spurs on all coxae. Coxal setae short. Palpi fusiform.....….…… *Ixodes scapularis*

Dichotomous identification key used for identifying nymphs of the genus *Ixodes* (Durden and Keirans 1996).

1b. Spurs present on one or more coxae ……...……6
6b. Spurs absent on trochanters I-IV; auriculae present or absent; Hosts may include reptiles or birds, but usually small mammals.....……9
9b. External spurs present or absent on coxae I-IV, internal spur present on one or more coxae. Hosts various, but not seabirds….……..10
10a. Palpal segment I ventrally without an anterior or posterior process……...…..20
20b. Lacking this combination of characters: hypostomal dentition 4/4, scutum subcircular.....21
21a. Palpi elongate and slender; length width radio 3.5.1. or greater; hypostome borne on an anterior extension of the basis capitula, apex pointed or narrowly rounded dentition 4/4 or 3/3 apically then 2/2…….....…….24
24a. Lateral carinae absent…… ...…….....25
25a. Posterior margin of basis capitula dorsally sinuous, hypostome pointed.....….*Ixodes scapularis*

APPENDIX C

DNA Extraction and PCR Protocols

Protocol for the preparation and extraction of mammal DNA as described by Qiagen manufacturer guidelines (2011).

All centrifugation steps were performed at room temperature. Ethanol was added to Buffer AW1 and AW2 concentrates to bring them into solution.

1a. Tissue: Cut tissue (≤ 25 mg) into small pieces and place in a 1.5 mL microcentrifuge tube. For rodent tails, use a single 0.4 – 0.6 cm lengths of tail. Add 180 µL Buffer ATL. Add 20 µL proteinase K, mix by vortexing, and incubate at 56°C until completely lysed. Vortex every five minutes during incubation. Vortex for 15 seconds directly before proceeding to step 2.
2. Add 200 µL Buffer AL. Mix thoroughly by vortexing.
3. Add 200 µL ethanol (96-100%). Mix thoroughly by vortexing.
4. Pipet the mixture into a DNeasy Mini spin column placed in a 2 mL collection tube. Centrifuge at ≥ 6000 x g (8000 rpm) for 1 minute. Discard the flow-through and collection tube.
5. Place the spin column in a new 2 mL collection tube. Add 500 µL Buffer AW1. Centrifuge for 1 minute at ≥ 6000 x g. Discard the flow-through and collection tube.
6. Place the spin column in a new 2 mL collection tube. Add 500 µL Buffer AW2. Centrifuge for 3 minutes at ≥ 20,000 x g (14,000 rpm). Discard the flow-through and collection tube.
7. Transfer the spin column to a new 1.5 mL or 2 mL microcentrifuge tube.
8. Elute the DNA by adding 200 µL Buffer AE to the center of the spin column membrane. Incubate for 1 minute at room temperature. Centrifuge for 1 minute at ≥ 6000 x g.

PCR protocol

Component	**Volume**
Thermo Scientific DreamTaq hot start master mix (2x) from Fisher Scientific or Bullseye hot start master mix (2x) from MidSci	10 µL
10 µM primer (forward and reverse combined, for a final concentration of 1 µM)	2 µL
Sterile molecular grade water	5 µL
Template DNA	3 µL
Total volume	**20 µL**

For control reactions, the following was substituted in place of template DNA:

Target	**Positive Control**	**Source**
Babesia microti	BM1	Extracted DNA from infected *Mus musculus* blood, courtesy of New York State Department of Health
Anaplasma phagocytophilum	42	Extracted DNA from infected female *Ixodes scapularis*, courtesy of New York State Department of Health
Borrelia burgdorferi	B31	Extracted DNA from pure culture, courtesy of New York State Department of Health
Ehrlichia spp.	MM1	Extracted DNA from infected *Mus musculus* blood, courtesy of Centers for Disease Control and Prevention
Ixodes scapularis	IS	Extracted DNA from lab raised *Ixodes scapularis*, courtesy of Centers for Disease Control and Prevention

Target	**Negative Control**	**Source**
Babesia microti	IT	Extracted DNA from lab raised *Ictidomys tridecemlineatus* from the UW-Oshkosh animal lab
Anaplasma phagocytophilum	IT	Extracted DNA from lab raised *Ictidomys tridecemlineatus* from the UW-Oshkosh animal lab
Borrelia burgdorferi	IT	Extracted DNA from lab raised *Ictidomys tridecemlineatus* from the UW-Oshkosh animal lab
Ehrlichia spp.	IT	Extracted DNA from lab raised *Ictidomys tridecemlineatus* from the UW-Oshkosh animal lab
Ixodes scapularis	DV	Extracted DNA from *Dermacentor variabilis*, collected in Omro
*In addition to the negative controls listed, a negative control of molecular grade water in place of template was also performed.		

Protocol for Thermo Scientific DreamTaq hot start master mix and Bullseye hot start master mix.

For Thermo Scientific DreamTaq hot start master mix (2x) from Fisher Scientific:

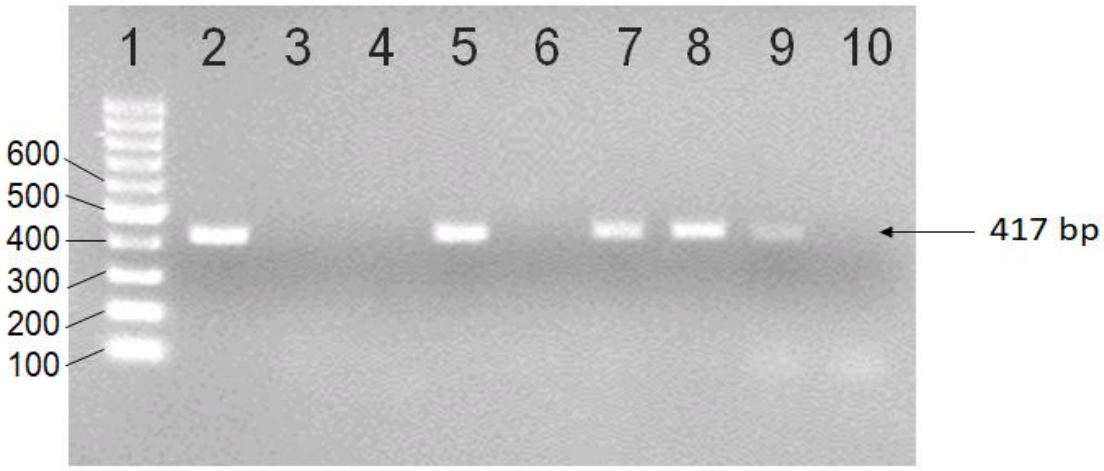
Step	**Temperature**	**Time**	**Function**
1	95°C	3 minutes	Initial denaturation
2	95°C	30 seconds	Denaturation
3	See below	30 seconds	Annealing
4	72°C	1 minute	Extension
5	Repeat steps 2-4 39 times, for a total of 40 cycles		
6	72°C	10 minutes	Final extension
7	12°C	Hold	Preservation

For Bullseye hot start master mix (2x) from MidSci:

Step	**Temperature**	**Time**	**Function**
1	95°C	15 minutes	Initial denaturation
2	95°C	20 seconds	Denaturation
3	See below	30 seconds	Annealing
4	72°C	30 seconds	Extension
5	Repeat steps 2-4 39 times, for a total of 40 cycles		
6	72°C	5 minutes	Final extension
7	12°C	Hold	Preservation

Target	**Primer**	**Anneal temperature**
Babesia microti	Bab1F/Bab4R	58°C
Anaplasma phagocytophilum	MSP3F/MSP3R	62°C
Borrelia burgdorferi	OspA1F/OspA2R	62°C
Ehrlichia spp.	SodBF/SodBR	62°C
Ixodes scapularis	IScapF/IScapR	54°C

Image of an agarose gel electrophoresis of PCR product screenings for *B. burgdorferi*, including a DNA ladder and positive and negative quality control lanes. The size of this product is 417 base pairs. Lane 1 is DNA ladder, lane 2 is extracted DNA from a pure culture of *B. burgdorferi*, lane 3 is a negative control (molecular water), lane 4 is a negative control (DNA extracted from a lab raised *I. tridecemlineatus* from the UW-Oshkosh animal lab), lanes 5, 7, 8, and 9 are mammals from Hartman Creek State Park that are positive for *B. burgdorferi*, and lanes 6 and 10 are mammals negative for *B. burgdorferi*.



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