CO-INFECTION RATES OF BLACK-LEGGED TICKS (*IXODES SCAPULARIS*) IN CENTRAL WISCONSIN

By Amanda L. Prigan

In the United States, reported cases of Lyme disease increased over two-fold between 2001-2016. The causative agent is the bacterium *Borrelia burgdorferi*, which is transmitted by the black-legged tick (*Ixodes scapularis*). This vector also transmits at least six additional pathogenic agents. My study focused on *B. burgdorferi*, *Anaplasma phagocytophilum*, *Babesia microti*, and *Ehrlichia* species. If the tick previously fed on a host that was co-infected, hosts may acquire multiple agents during a single blood meal. Such co-infections are poorly understood but likely to have important implications for the transmission dynamics of each infectious agent.

My objective was to determine if co-infection rates of *I. scapularis* occur at frequencies other than predicted by infection rates of individual agents. My research focused on quantifying infection and co-infection rates at Hartman Creek State Park in central Wisconsin. This site has the vector (black-legged tick) and competent hosts (deer and multiple small mammal species) for these infectious agents. I collected nymphal and adult black-legged ticks from small mammal hosts and by flagging vegetation. Ticks were then screened using end-point PCR for the four agents.

My data showed 103/253 (40.7%) of questing ticks infected with *B. burgdorferi*, 87/253 (34.4%) infected with *A. phagocytophilum*, and 24/253 (9.5%) infected with *B. microti*. My data also showed 50/253 (19.8%) of questing ticks were co-infected with ≥2 agents. I also found less co-infection of questing ticks with *A. phagocytophilum* and *B. microti* than predicted by chance, which suggests a negative population-level interaction between these two agents in the vector. The research outcomes will increase the knowledge of co-infection rates at this site and contribute to the growing body of knowledge on this subject, thereby positively impacting human health.

CO-INFECTION RATES OF BLACK-LEGGED TICKS (*IXODES SCAPULARIS*) IN CENTRAL WISCONSIN

By Amanda L. Prigan

A Thesis Submitted
In Partial Fulfillment of the Requirements
For the Degree of

Master of Science

Biology - Microbiology

at

The University of Wisconsin Oshkosh
Oshkosh WI 54901-8621

August 2018

COMMITTEE APPROVAL

_____ Advisor

3 AUG. 2018 Date Approved

_____ Member

3 AUG 2018 Date Approved

_____ Member

Aug. 3, 2018 Date Approved

PROVOST & VICE CHANCELLOR

_____

8/3/18
Date Approved

FORMAT APPROVAL

_____

8/3/18
Date Approved

ACKNOWLEDGEMENTS

First and foremost, I would like to acknowledge my advisors for their guidance and support. Dr. Teri Shors provided me with a lab, led me through developing this project, and pushed me to never give up. Dr. Greg Adler took me under his wing, gave me all the field experience I could have hoped for, opened my eyes to ecology and evolution, and taught me numerous life hacks. Dr. Eric Matson taught me how to understand and interpret scientific journal articles and isolate cellulose degrading bacteria from our biodigester on campus.

My fellow students, most importantly Bailey Bodeen and Konrad Fondrie, were also vital to this project. I would also like to thank Alix Mccullough, Jordan Borchardt, Steve Schaar, Tom Perzentka, the Centers for Disease Control and Prevention, the New York State Department of Health, the Mayo Clinic, the Office of Student Research and Creative Activity, and the Office of Grants and Faculty Development. Without the combined support of all these resources, I would not have succeeded in completing this project.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS iii

LIST OF TABLES v

LIST OF FIGURES vi

CHAPTER I: INTRODUCTION 1
- Infectious Agents 2
- *Ixodes Scapularis* 6
  - Life cycle 8
- Research Site 10
- Hosts of Infectious Agents 10

CHAPTER II: CO-INFECTION RATES OF BLACK-LEGGED TICKS (*IXODES SCAPULARIS*) IN CENTRAL WISCONSIN 11
- Introduction 11
- Materials and Methods 13
  - Sample Collection 13
  - Tick Identification 19
  - Tick DNA Extraction 21
  - PCR Screening of Tick DNA for Infectious Agents 22
  - Data Analysis 25
- Results 27
- Discussion 32
  - Singular Infection Rates 33
  - Co-infection Rates 34

CHAPTER III: CONCLUSIONS 38

APPENDIX A: PCR Protocol 40

REFERENCES 45

LIST OF TABLES

Page

Table 1. Incubation period, transmission time, and reportable status to the local and national health departments for each infectious agent 5

Table 2. Most recent data on the number of reported cases of four infectious diseases in the United States and Wisconsin 5

Table 3. Small mammal species captured at Hartman Creek State Park 17

Table 4. Primer sequences 20

Table 5. Numbers of *I. scapularis* collected in 2017 and 2018 from Hartman Creek State Park..... 27

Table 6. Singular infection rates of *I. scapularis* 29

Table 7. Co-infection rates of *I. scapularis* 30

Table 8. Statistical analysis of co-infection rates of *I. scapularis* 31

Table 9. Published co-infection of questing *I. scapularis* in Wisconsin..... 35

Table 10. Published co-infection of questing *I. scapularis* in the eastern United States 37

LIST OF FIGURES

		Page
Figure 1.	Composite image representing a co-infected tick	2
Figure 2.	SEM photo of *I. scapularis*	7
Figure 3.	Life cycle of *I. scapularis*	9
Figure 4.	Hartman Creek State Park map	14-15
Figure 5.	(a) Forest edge in Hartman Creek State Park. (b) Temperate broad leaf deciduous forest in Hartman Creek State Park	16
Figure 6.	Small and large H.B. Sherman traps set up in Hartman Creek State Park to trap small mammals	18
Figure 7.	Agarose gel electrophoresis of PCR products screening for *B. burgdorferi* DNA present in ticks	26

CHAPTER I

INTRODUCTION

Each year, there are over one billion cases and more than one million deaths from vector-borne diseases worldwide (WHO 2014). In the contiguous United States, 95% of vector-borne diseases are transmitted by ticks (Eisen and Eisen 2017).

A vector is a living organism that transmits an infectious agent from one individual to another (WHO 2014). It is possible for agents to be transmitted from nonhuman animals to other nonhuman animals, from humans to humans, from nonhuman animals to humans, or from humans to other animals (WHO 2014). In the United States, Lyme disease is the most commonly-reported vector-borne disease (CDC 2018). The Centers for Disease Control and Prevention (CDC) (2018) estimate that there are approximately 300,000 people in the United States diagnosed with Lyme disease every year.

In the United States, the vector *Ixodes scapularis* (black-legged or deer tick) can transmit at least seven agents that cause human disease: five bacteria (*Anaplasma phagocytophilum, Borrelia burgdorferi, Borrelia mayonii, Borrelia miyamotoi,* and *Ehrlichia muris eauclairensis*), one protozoan (*Babesia microti*), and one virus (Powassan virus) (Eisen and Eisen 2017). Ticks can potentially be co-infected (i.e. simultaneously harbor) with multiple infectious agents such as bacteria, viruses, and protozoa and co-transmit them in a single feeding (**Figure 1**; Diuk-Wasser et al. 2016). Such co-infections are poorly known but likely to have important implications for the

transmission dynamics and incidence of each infectious agent and diagnosis by medical personnel (Diuk-Wasser et al. 2016). There are documented cases of co-infection in human hosts, and such infections may manifest with more persistent and severe symptoms (Hersh et al. 2014).

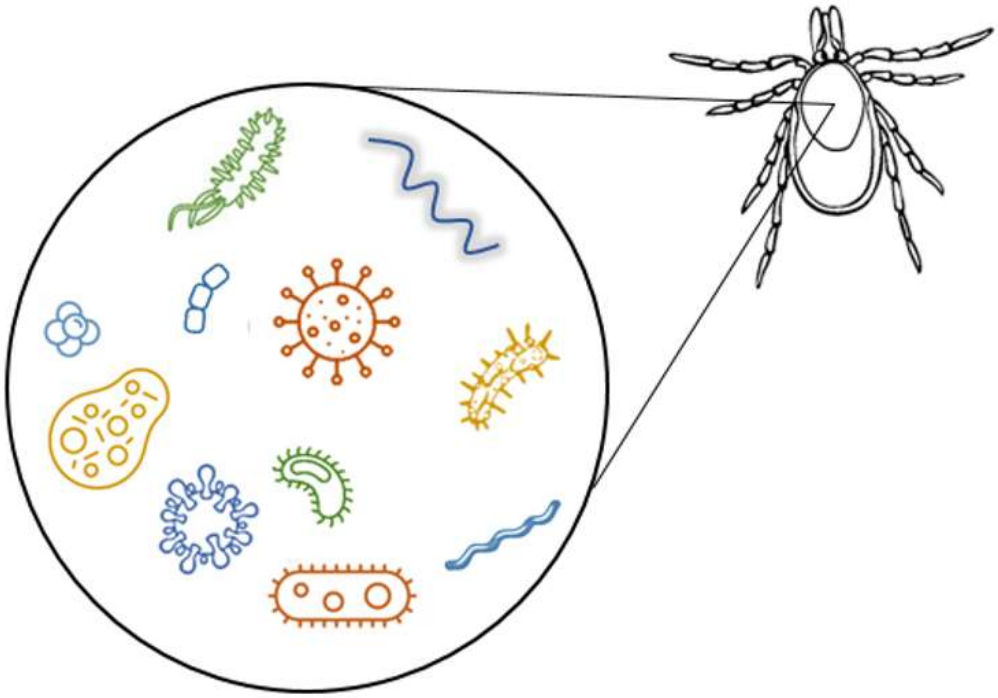


Figure 1. Composite image representing a co-infected tick. ©Amanda Prigan.

Infectious Agents

Lyme disease was first described in 1975 in Old Lyme, Connecticut. In 1981, Willy Burgdorfer first identified the causative agent of Lyme disease, the bacterium *B. burgdorferi* (Ostfeld 2012). This bacterium is in the family Spirochaetaceae, which was named for the characteristic spiral shape of the bacterium. Lyme disease typically

presents with erythema migrans (the classic bulls-eye lesion), fever, headache, myalgia, and malaise (Ostfeld 2012). If left untreated, Lyme disease can progress into chronic states that include arthritis, neuropathy, encephalitis, and cardiac damage (Ostfeld 2012).

Anaplasma phagocytophilum is a bacterium in the family Anaplasmataceae. This agent is obligately intracellular and resides inside the granulocytic line of host white blood cells (Prusinski et al. 2014). It causes human granulocytic anaplasmosis, which presents with fever, headache, myalgia, and malaise (Prusinski et al. 2014).

Ehrlichia is a genus of bacteria also in the family Anaplasmataceae. *Ehrlichia* and *Anaplasma* are closely related and were placed in the same genus until 2001 (Dumler et al. 2001). They are also obligately intracellular and reside inside the monocytic line of host white blood cells (Dumler et al. 2001). *Ehrlichia* causes human monocytic ehrlichiosis, which presents with fever, headache, myalgia, and malaise (Dumler et al. 2001). There are documented cases of human disease resulting from three different species of *Ehrlichia: E. muris eauclairensis, Ehrlichia ewingii,* and *Ehrlichia chaffeensis* (CDC 2018). *Ehrlichia ewingii,* and *E. chaffeensis* are usually transmitted by the lone star tick (*Amblyomma americanum*); however, *Ehrlichia* infection in humans is poorly documented and not well understood (CDC 2018). Furthermore, there were four cases (three in Wisconsin and one in Minnesota) of *E. muris eauclairensis* documented in 2009 that were putatively transmitted by *I. scapularis* (CDC 2018).

Unlike the other three infectious agents, *B. microti* is a protozoan (phylum Apicomplexa, Kogut et al. 2005). It is an obligate intraerythrocytic endoparasite, thereby

residing inside host red blood cells (Kogut et al. 2005). This protozoan causes babesiosis, which presents with fever, headache, myalgia, and malaise (Kogut et al. 2005).

Each agent has a unique incubation period and transmission time (**Table 1**). Infections with all four agents are reportable on local and national levels. **Table 2** contains the most recent data on the number of reported cases of these infectious diseases in the United States and Wisconsin.

There were 36,426 cases of Lyme disease reported in the United states in 2016 (CDC 2018). However, the CDC (2018) estimates that there are actually around 300,000 people in the United States diagnosed with Lyme disease every year. This discrepancy is due to the difficulty of definitive diagnosis with Lyme disease. In order to report a case, there must be a positive antibody test performed by medical personnel. Antibodies against *B. burgdorferi* take at least 30 days to develop (CDC 2018). If a patient comes into a physician's office with a history of a tick bite and/or erythema migrans, they may get antibiotics prescribed without ever being tested. These are probable cases of Lyme disease, and are not reported.

Table 1.

Incubation period and transmission time for each infectious agent. The incubation period is the period between exposure to an infectious agent and the appearance of the first symptoms. The transmission time is the amount of time the tick needs to be attached to the host before the infectious agent is passed between them.

Infectious Agent	Incubation Period (CDC 2018)	Transmission Time (Eisen 2018)
B. burgdorferi	3-30 days	24 hours
A. phagocytophilum	7-14 days	48 hours
B. microti	7-28 days	36 hours
Ehrlichia species	7-15 days	Unknown

Table 2.

Most recent data on the number of reported cases of four infectious diseases in the United States and Wisconsin. There are no published data from the CDC or Wisconsin Department of Health Services on co-infection incidences.

Infectious Disease	Reported cases in the US (CDC 2018)	Reported cases in WI in 2016 (WDHS 2017)
Lyme disease	36,429 (2016)	1,491
Anaplasmosis	4,151 (2016)	615
Babesiosis	1,744 (2014)	51
Ehrlichiosis	1,345 (2016)	39

Co-infection is complicated by many factors. Infectious agents may share the vector (*I. scapularis*), which gives hosts the potential to be infected by two or more agents in a single tick bite. Alternatively, a host may be infected by single agents from multiple tick bites (Hersh et al. 2014). The four agents have very similar symptoms that are nonspecific, thereby complicating diagnosis and treatment in humans (Hersh et al. 2014). Co-infection of ticks and hosts could lead to population-level interactions between these agents, such as competition or mutualism, which could result in increased replication of one or both agents in the vector and host (Diuk-Wasser et al. 2016). This interaction could have implications for disease severity, incidence, and transmission. Interactions between agents in the vector and hosts are currently being investigated to fully understand these relationships (Eisen and Eisen 2017).

Ixodes scapularis

There are three families of ticks: Ixodidae (hard ticks), Argasidae (soft ticks), and Nuttalliellidae (Latif et al. 2012). All ticks are obligate hematophagous ectoparasites (Black and Piesman 1994). Hard and soft ticks have different life cycles, are morphologically different, have different feeding lengths and strategies, and different geographical distributions (Latif et al. 2012). The scutum, a hard dorsal shield, is present in hard ticks but absent in soft ticks (Latif et al. 2012). There are at least seven species of hard ticks in the United States; the most relevant to human disease are *I. scapularis* (**Figure 2**), *Ixodes pacificus* (Western black-legged tick), *Dermacentor variabilis* (dog or wood tick), and *A. americanum* (CDC 2018).

One genus of soft ticks, *Ornithodoros*, is present in the United States (CDC 2018). There are three species that transmit tick-borne relapsing fever (TBRF) to humans: *Ornithodoros hermsi* is the most common, but *Ornithodoros parkeri* and *Ornithodoros turicata* can also transmit TBRF (CDC 2018). When transmitted by *Ornithodoros* spp., TBRF is usually caused by *Borrelia hermsii* or *Borrelia turicatae*, spirochete bacteria closely related to *B. burgdorferi* (CDC 2018).

The third family, Nuttalliellidae, consists of a single species: *Nuttalliella namaqua* (Latif et al. 2012). This family shares characteristics with both hard and soft ticks and is found exclusively in southern Africa (Latif et al. 2012).

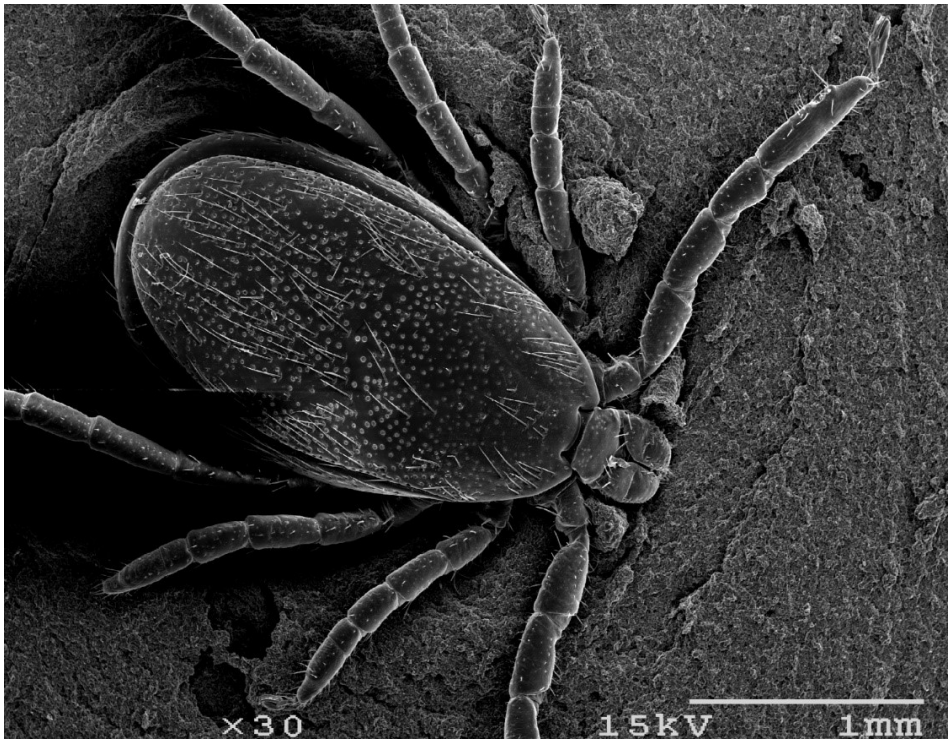


Figure 2. SEM photo of *I. scapularis*, 30x. ©Amanda Prigan.

Life cycle. The life cycle of *I. scapularis* is an important factor in the spread of human disease (**Figure 3**). *I. scapularis* is a three-host tick in which each stage feeds on a different vertebrate host (CDC 2018). Two agents, *B. miyamotoi* and Powassan virus, may be transmitted transovarially, from an infected adult female to her eggs (Eisen and Eisen 2017). The remaining agents are not transmitted transovarially and are only acquired by a tick when feeding upon an infected host (Eisen and Eisen 2017). Therefore, larval ticks can only transmit the two infectious agents with transovarial transmission (Eisen and Eisen 2017).

The eggs are laid by adult female ticks in spring, and larvae emerge in summer. They generally choose a small mammal host such as mice and squirrels but also choose other small hosts such as lizards (CDC 2018). The following spring, ticks molt and become nymphs. They feed on small to medium-sized hosts such as rodents, birds, domestic animals, and humans (CDC 2018). Due to the smaller size (relative to adults), and active period in spring, nymphs are usually the stage that transmits disease to humans (CDC 2018). They molt again, and in fall the adults feed on larger mammals such as deer and humans. Adult females always take a blood meal, but adult males usually do not (CDC 2018). The males and females copulate, and the female lays eggs. This cycle usually takes two years.

Black-legged ticks are semelparous (CDC 2018). Each year, nymphs are active before larvae. This activity pattern is crucial for transmission of infectious agents because if the nymphs are infected when they take their second blood meal in the spring, they will likely infect their second host (CDC 2018). The larvae become active in the

summer and take their first blood meal from individuals in the same population of small mammals and likely become infected (CDC 2018). Seasonality is therefore very important in disease transmission and drives agents to persist in the hosts and the vector (CDC 2018). Such seasonality can also cause a dramatic increase in infection rates over a short period of time (CDC 2018).

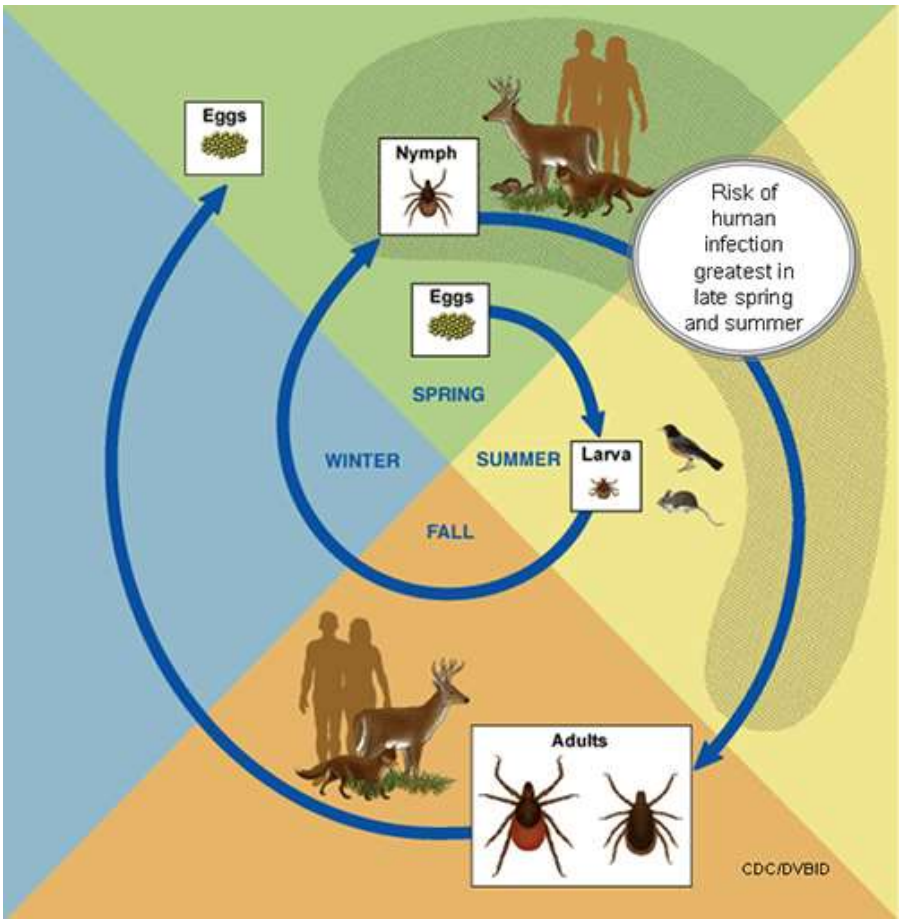


Figure 3. Life cycle of *I. scapularis* (CDC 2018).

Research Site

This study was conducted at Hartman Creek State Park, a 610-ha protected area in central Wisconsin (Waupaca and Portage counties; WI DNR 2018). It has various terrestrial habitats, including temperate broad-leaved deciduous forest, successional grasslands, and red pine plantations. These habitats putatively support the reservoirs and vector.

Hosts of Infectious Agents

A reservoir is an organism within which an infectious agent can survive and reproduce, for some period of time, and that contributes to enzootic maintenance (a stable relationship between the infectious agents and the animal hosts) (Eisen and Eisen 2017). Humans are incidental, or dead end, hosts of infectious agents transmitted by *I. scapularis,* and are not necessary for the survival of ticks or the transmission of infectious agents (Eisen and Eisen 2017). Potential reservoirs in Hartman Creek State Park include rodents and other mammals such as shrews, raccoons, and white-tailed deer.

CHAPTER II

CO-INFECTION RATES OF BLACK-LEGGED TICKS (*IXODES SCAPULARIS*) IN CENTRAL WISCONSIN

Introduction

The black-legged tick, *I. scapularis*, is a major public health concern in the United States because it can transmit at least seven infectious agents that cause human disease. Such agents include five bacteria (*A. phagocytophilum, B. burgdorferi, B. mayonii, B. miyamotoi,* and *E. muris eauclairensis*), one protozoan (*B. microti*), and one virus (Powassan virus) (Eisen and Eisen 2017). These ticks may potentially be co-infected with multiple infectious agents and co-transmit them in a single feeding (Diuk-Wasser et al. 2016). Such co-infections are likely to have important implications for the transmission dynamics and incidence of each infectious agent (Diuk-Wasser et al. 2016).

Co-infection in ticks, hosts, and humans is complicated and not well understood. Because infectious agents can share one vector (*I. scapularis*), hosts have the potential to be infected by two or more agents in a single tick bite. It is also possible to become a co-infected host by receiving unique single agents from multiple tick bites (Hersh et al. 2014). Most of these infectious agents have similar disease presentations and nonspecific symptoms in humans. This similarity makes diagnosis and treatment in humans difficult (Hersh et al. 2014). Co-infection in human hosts may manifest with more persistent and severe symptoms compared to a single infection (Hersh et al. 2014). Co-infection of ticks and hosts could lead to population-level interactions between pairs of these agents,

such as competition or mutualism, and these interactions could result in either decreased or increased replication of one or both agents in the vector and host (Diuk-Wasser et al. 2016). The influence on replication could have implications for disease severity, incidence, and transmission. Interactions between agents in the vector and in hosts are currently being investigated by other researchers to more fully understand these relationships (Eisen and Eisen 2017).

Co-infections in *I. scapularis* are not well studied in Wisconsin. Most studies focus on a pair of infectious agents. There is one recent study in northern Wisconsin that tested for three infectious agents in *I. scapularis* (Westwood 2017). However, in the northeastern United States, the primary epicenter of *I. scapularis*-borne disease, there are numerous recent studies testing for multiple agents (Tokarz et al. 2017; Johnson et al. 2017; Hutchinson et al. 2015; Feldman et al. 2015; Hersh et al. 2014; Xu et al. 2016).

This study examines co-infection rates of four infectious agents in a population of questing and feeding *I. scapularis* collected from Hartman Creek State Park in central Wisconsin. A study looking for multiple agents in the vector has not yet been performed in this location. This study also determined if co-infection rates of *I. scapularis* occur at frequencies other than predicted by infection rates of individual agents. The research outcomes will increase the knowledge of co-infection rates at this site and contribute to the growing body of knowledge on this subject, thereby positively impacting human health.

Materials and Methods

Sample collection. Hartman Creek State Park is a 610-ha protected area located in central Wisconsin (Waupaca and Portage counties; **Figure 4.** WI DNR 2018). It has a mosaic of habitats, including temperate broad-leaved deciduous forest, successional grasslands, red pine plantations, and forest edge (**Figure 5**). These habitats support the reservoir hosts of *I. scapularis*. All ticks were collected in Hartman Creek State Park.

Questing (host-seeking) ticks were collected between June and September of 2017 and April and June of 2018 using a standard flag method described by Carroll and Schmidtmann (1992). This method uses a 1-m^2 cloth attached to a dowel or pole. For a flag, this study used white faux fur Sherpa fabric (Joann Fabrics, Hudson, Ohio) attached to a meter stick with 3.2-cm (1.25-in) office supply clips. While walking transects, the flag was swept through leaf litter and vegetation. Every five minutes, the cloth was examined for ticks. If any ticks were present, they were removed with forceps and placed into 1.5-ml snap cap microfuge tubes (Invitrogen, Carlsbad, California) containing 500 µl of 70% ethanol. This flagging method was effective for collecting nymphal and adult ticks. All questing ticks were *I. scapularis* or *D. variabilis*. Wood ticks were reserved for negative control DNA when confirming the taxonomic identification of black-legged ticks by PCR.

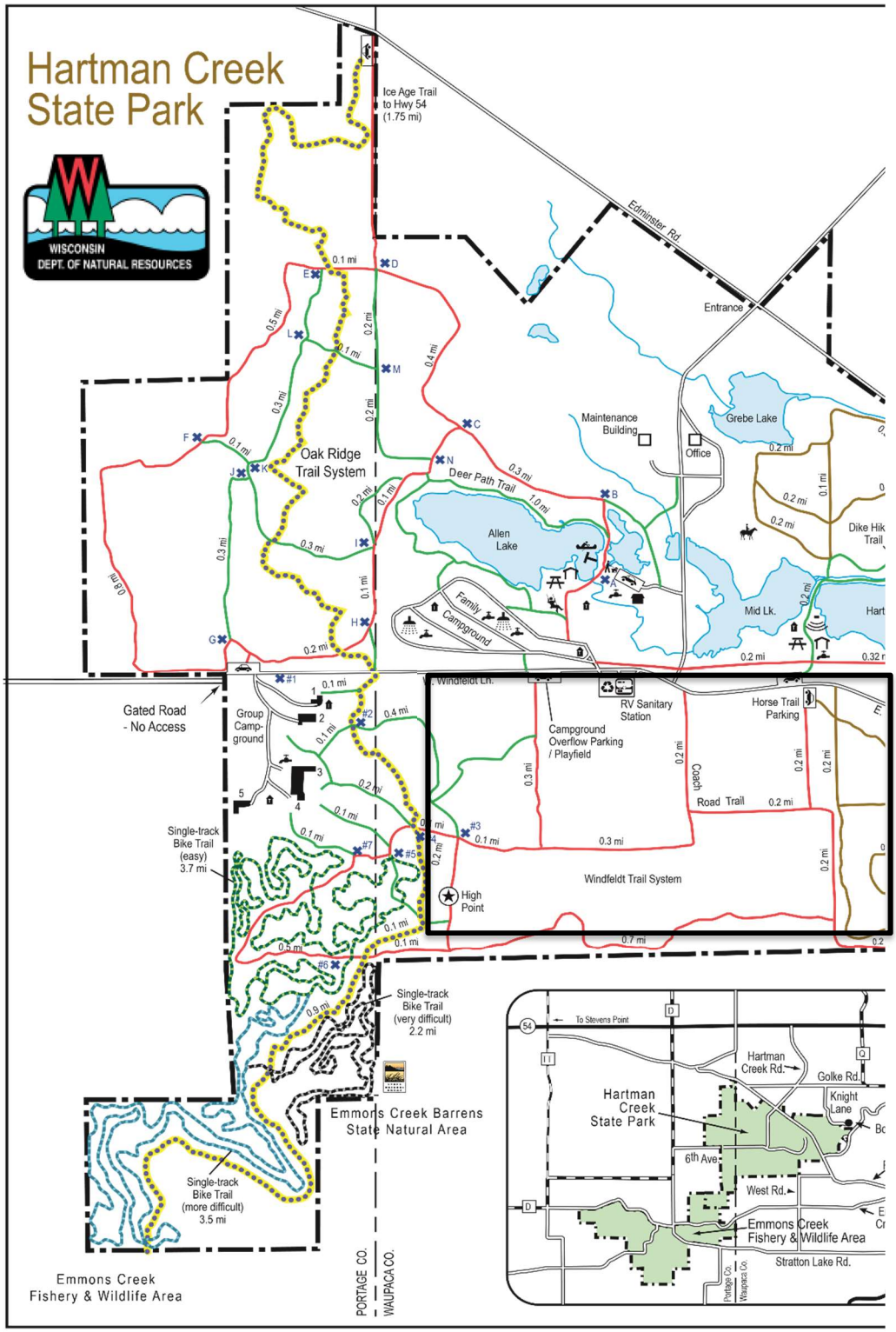


Figure 4. Hartman Creek State Park map. Source: Wisconsin Department of Natural Resources (WI DNR 2018). The majority of ticks were collected from the area inside the black box.

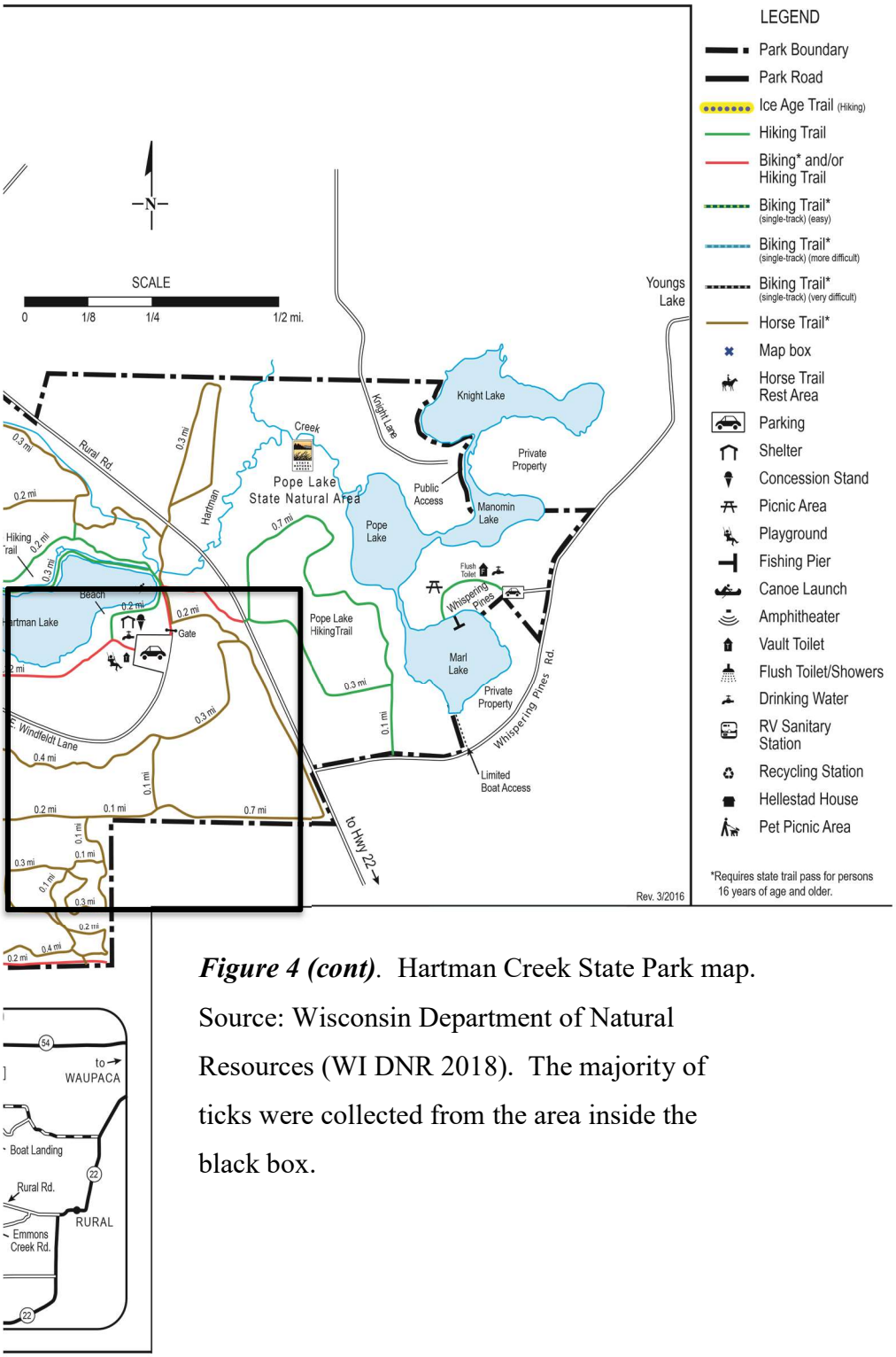


Figure 4 (cont). Hartman Creek State Park map. Source: Wisconsin Department of Natural Resources (WI DNR 2018). The majority of ticks were collected from the area inside the black box.

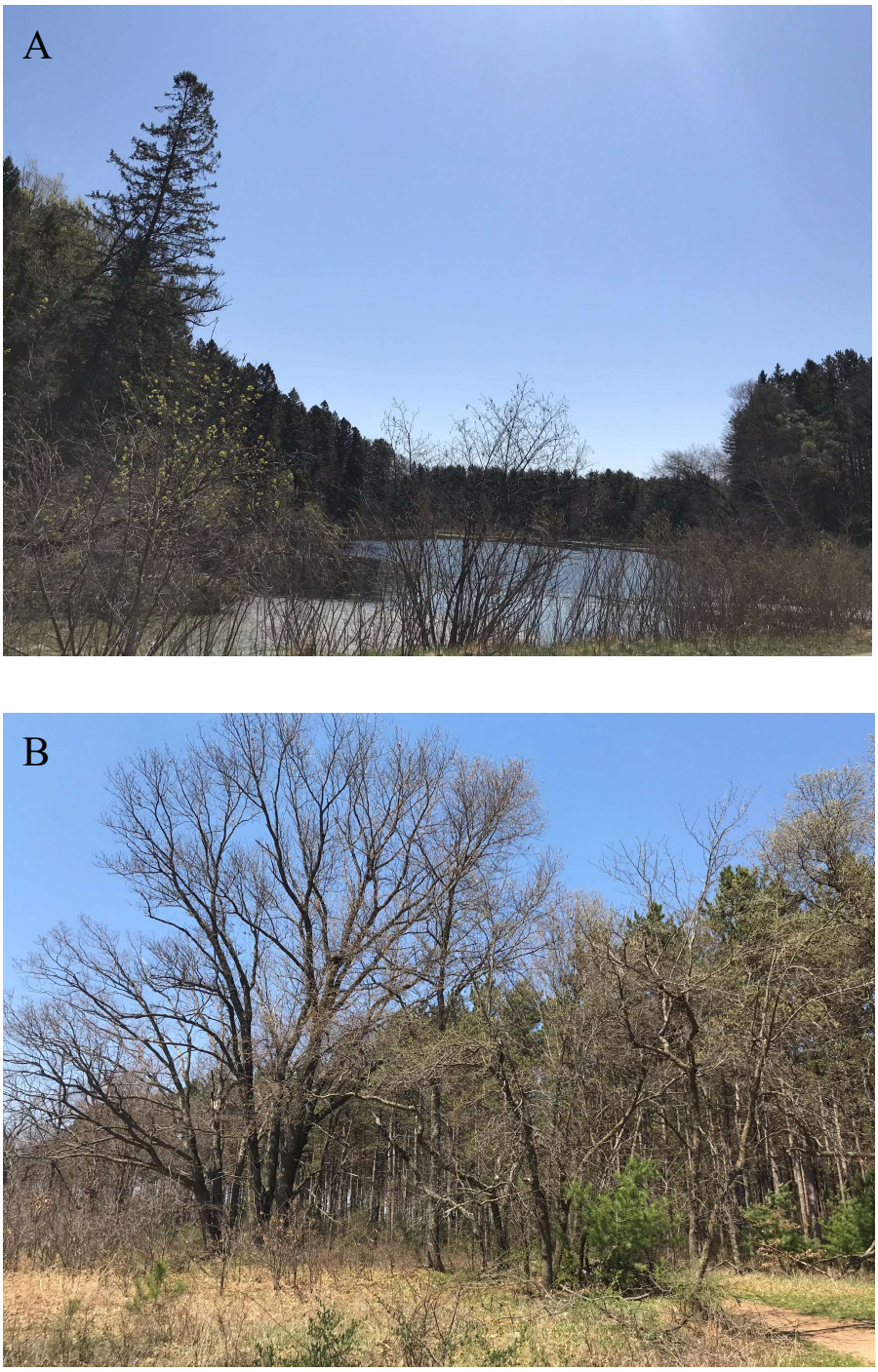


Figure 5. (a) Forest edge in Hartman Creek State Park. ©Amanda Prigan

(b) Temperate broad leaf deciduous forest in Hartman Creek State Park. ©Amanda Prigan

Replete (feeding) ticks were removed directly from small mammals that were captured in small (5.5x17.2x5.4 cm) and large (9x23.5x8.5 cm) H.B. Sherman traps (H.B. Sherman Traps, Inc., Tallahassee, Florida) (**Table 3; Figure 6**). Mammals were inspected for ticks, which were removed with forceps and placed into 1.5-ml snap cap microfuge tubes (Invitrogen, Carlsbad, California) containing 500 µl of 70% ethanol. Between 0-22 ticks were removed from each mammal, with a mean of 3 ticks per mammal. Replete nymphs and questing ticks were screened by PCR for 3 bacterial agents (*B. burgdorferi, A. phagocytophilum,* and *Ehrlichia* species) and one protozoan (*B. microti*).

Table 3.

Small mammal species captured at Hartman Creek State Park.

Scientific Name of Small Mammal	**Common Name of Small Mammal**
Blarina brevicauda	Northern short-tailed shrew
Sorex cinereus	Masked shrew
Ictidomys tridecemlineatus	Thirteen-lined ground squirrel
Microtus pennsylvanicus	Meadow vole
Peromyscus leucopus	White-footed mouse
Peromyscus maniculatus	North American deer mouse
Tamias striatus	Eastern chipmunk



Figure 6. Small and large H.B. Sherman traps set up in Hartman Creek State Park to trap small mammals. ©Amanda Prigan

Tick identification. Adult ticks were macroscopically identified as *I. scapularis* in the laboratory. Nymphs were microscopically identified as *I. scapularis* using a published key (Durden and Keirans 1996) and an Olympus (Shinjuku, Tokyo, Japan) SZ80 dissecting microscope at 60x magnification. Distinguishing characteristics used to putatively identify ticks were the scutum, mouthparts, genital pore, and the anal groove. The identification of all ticks was confirmed by PCR analysis of extracted tick DNA (See PCR screening of tick DNA for infectious agents) with a set of species-specific primers designed to amplify the mitochondrial 16S rRNA gene of *I. scapularis* (**Table 4**). Only ticks that were visually identified and confirmed by PCR screening to be *I. scapularis* were further analyzed for the presence of infectious agents.

Table 4.

Primer sequences

Primer Designation	Sequence (5' to 3')	Pathogenic Agent Targeted	Product size	Reference
Bab1F	5' CTTAGTATAAGCTTTTATACAGC 3'	*Babesia microti*	238 bp	Prusinski et al. 2014
Bab4R	5' ATAGGTCAGAAACTTGAATGATACA 3'			
MSP3F	5' CCAGCGTTTAGCAAGATAAGAG 3'	*Anaplasma phagocytophilum*	334 bp	Prusinski et al. 2014
MSP3R	5' GCCCAGTAACAACATCATAAGC 3'			
OspA1F	5' AATAGGTCTAATATTAGCCTTAATAGC 3'	*Borrelia burgdorferi*	417 bp	Prusinski et al. 2014
OspA2R	5' TCAAGTCTGGTTCCGTCTGCTC 3'			
SodBF	5' TTTAATAATGCTGGTCAAGTATGGAATCAT 3'	*Ehrlichia* species	304 bp	Qurollo et al. 2014
SodBR	5' AAGCGTGTTCCCATACATCCATAG 3'			
IScapF	5' TAAACAATTAAAAGCTTTCTT 3'	*Ixodes scapularis*	215 bp	Adelson et al. 2004
IScapR	5' AATCGCTAAAAACGGAACTTA 3'			

Tick DNA Extraction. DNA was extracted from questing and replete ticks using a crude ammonium hydroxide precipitation method derived from Ammazzalorso et al. (2015). This ammonium hydroxide (NH_4OH) extraction method is an inexpensive alternative to commercially-available kits. Ammonium hydroxide effectively disrupts the hard, chitinous exoskeleton of hard ticks, thereby facilitating DNA extraction.

To extract the DNA, ticks were placed on filter paper in sterile petri dishes until all ethanol evaporated. Depending on the tick size and life stage, one to three days were required for ethanol evaporation. Subsequently, each tick was placed into a sterile 1.5-ml snap cap microfuge tube (Invitrogen, Carlsbad, California), crushed with a sterile 1000-µl pipette tip, and 150 µl of 0.7M NH_4OH solution was added to each tube. The microfuge tubes were heated at 100°C for 15 minutes to rupture the tick and release its DNA. This step was performed in a Fisherbrand (Fisher Scientific, Hampton, New Hampshire) dry bath placed in a Hamilton (Manitowoc, Wisconsin) SafeAire laboratory chemical fume hood. Before and after use, the inside of the fume hood and micropipetters were disinfected with a 10% bleach solution.

Lid locks (Dot Scientific, Burton, Michigan) were used to prevent the caps from bursting open at the high temperature. After the heating step, all tubes were microfuged for 2 seconds to draw condensation down. All microfuge steps were performed in an Eppendorf (Hauppauge, New York) 5415 C microcentrifuge at 14,000 rpm. The heating step at 100°C was repeated with the caps open to allow the ammonium hydroxide to evaporate. The remaining sample volume at this point was approximately 70 µl of extracted DNA in water.

The tubes were microfuged again for 10 minutes at 14,000 rpm to remove tick debris. The supernatant (containing the tick DNA) was removed using a sterile Gilson (Middleton, Wisconsin) Pipetman micropipetter and sterile 200-µl pipette tips, avoiding any pelleted tick debris. The microfuge tube containing the pelleted tick debris was discarded and the tubes were microfuged for 2 minutes. The supernatant (DNA) was removed using a micropipette tip, using care to avoid the pelleted tick debris. The tube containing tick debris was discarded. The final volume of the tick DNA was approximately 30 µl. The extracted tick DNA was stored at -20°C in a frost-free Whirlpool (Benton Harbor, Michigan) chest freezer.

PCR screening of tick DNA for infectious agents. Single-target PCR reactions were performed using a protocol derived from Kogut et al. (2005), MidSci (2017), and Thermo Scientific (2017). Primers were manufactured by Eurofins Genomics (Louisville, Kentucky) using salt-free purification. The primers were synthesized based on published sequences (**Table 4**).

A highly-conserved region of the *sodB* gene in *Ehrlichia* species was targeted for amplification. Qurollo et al. (2014) developed this primer set to detect existing and new *Ehrlichia* spp. and to circumvent difficulties encountered by previous targets. Previous studies amplified regions on the 16S rRNA gene, which can result in nonspecific amplification of *Anaplasma* spp. (Qurollo et al. 2014). The *sodB* gene in *Ehrlichia* spp. encodes iron oxide dismutase, an enzyme responsible for protecting the bacterial cell from damage caused by free oxygen radicals (Qurollo et al. 2014).

To confirm identity of *I. scapularis* ticks, the small subunit mitochondrial 16S rRNA gene was targeted. Adelson et al. (2004) derived primers based on uniquely-conserved mitochondrial rRNA sequences present in *I. scapularis*. Ribosomal RNA is essential for translating cellular proteins.

The *ospA* gene of *B. burgdorferi* was targeted for amplification. This gene sequence is specific to *B. burgdorferi* DNA and has been used successfully to screen for its presence in numerous research studies (Prusinski et al. 2014). The *ospA* gene encodes for an outer surface lipoprotein that is recognized as an antigen in human hosts (Morrison et al. 1997).

The *p44* gene of *A. phagocytophilum* was targeted for amplification. This gene sequence is specific to *A. phagocytophilum* and has been used successfully in numerous studies (Prusinski et al. 2014). The *p44* gene encodes for a surface exposed outer membrane protein that is recognized as an antigen in human hosts (Rikihisa et al. 2003)

The 16S-like small subunit rRNA gene was targeted for amplification of *B. microti* DNA. The primer sequence is highly conserved and specific. Ribosomal RNA is essential for synthesis and function of a range of proteins.

All PCR reactions were set up in a NuAire (Plymouth, Minnesota) biological safety cabinet. Before and after use, the inside of the cabinet and micropipetters were disinfected with a 10% bleach solution, and the UV light was turned on for 10 minutes to ensure a sterile workspace. All reactions were prepared on ice, taking care to mix all components completely before placing them in the thermocycler. Reactions were

prepared in 0.2-mL Fisherbrand (Fisher Scientific, Hampton New Hampshire) flat capped PCR tubes. Each PCR reaction contained a total volume of 20 µl: 10 µL of Thermo Scientific DreamTaq 2X hot start master mix (Fisher Scientific, Hampton New Hampshire) or 10 µl of 2X Bullseye hot start master mix from MidSci (Midwest Scientific, Valley Park Montana), 2 µL of each primer set (10 µM), 5 µL of sterile molecular biology grade water (Research Genetics, Huntsville, Alabama), and 3 µL of extracted tick DNA. Two negative controls were performed with each run in which molecular biology grade water and DNA extracted from lab-raised *I. scapularis* ticks (courtesy of the Centers for Disease Control and Prevention) that did not harbor the pathogens of interest were substituted for template DNA. A positive control was also performed with each run in which DNA from *B. burgdorferi, A. phagocytophilum, Ehrlichia* spp., or *B. microti* was substituted for template DNA of the agent being tested for. See **Appendix A** for complete procedure and details. A Bio-Rad (Hercules, California) T100 or C1000 Thermal Cycler was programed according to manufacturer guidelines for 40 cycles of amplification.

After thermocycling was completed, 4 µL of Thermo Scientific (Fisher Scientific, Hampton, New Hampshire) 6X DNA loading dye was added to each 20 µl PCR reaction. Agarose gel electrophoresis was used to separate PCR products by preparing a 1.5% Gibco BRL (Fisher Scientific, Hampton, New Hampshire) agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; Fisher Scientific, Hampton, New Hampshire). The agarose was melted in 1-minute intervals on medium power in a General Electric (Boston, Massachusetts) Spacemaker III microwave until the solution

boiled and became clear. Before the gel was poured, ethidium bromide (Fisher Scientific, Hampton, New Hampshire) was added to the solution to a final concentration of 0.5 µg/ml. Wells were loaded with 5 µL of PCR reactions containing loading dye. The gel was electrophoresed for 20 minutes at constant voltage (75 v). Gels were imaged using a Bio-Rad (Hercules, California) Gel Doc XR+ Gel Documentation System. This system uses ultraviolet transillumination (302 nm) to detect the ethidium bromide-stained DNA bands. **Figure 7** represents PCR screening for the presence of *B. burgdorferi* DNA in *I. scapularis* ticks.

Data analysis. All statistical analyses were performed using RStudio (RStudio Inc., Boston, Massachusetts). Chi-square values were performed separately with one degree of freedom. Because some cell values were less than five, this study used the Fisher's exact test where appropriate (Zar 1999).

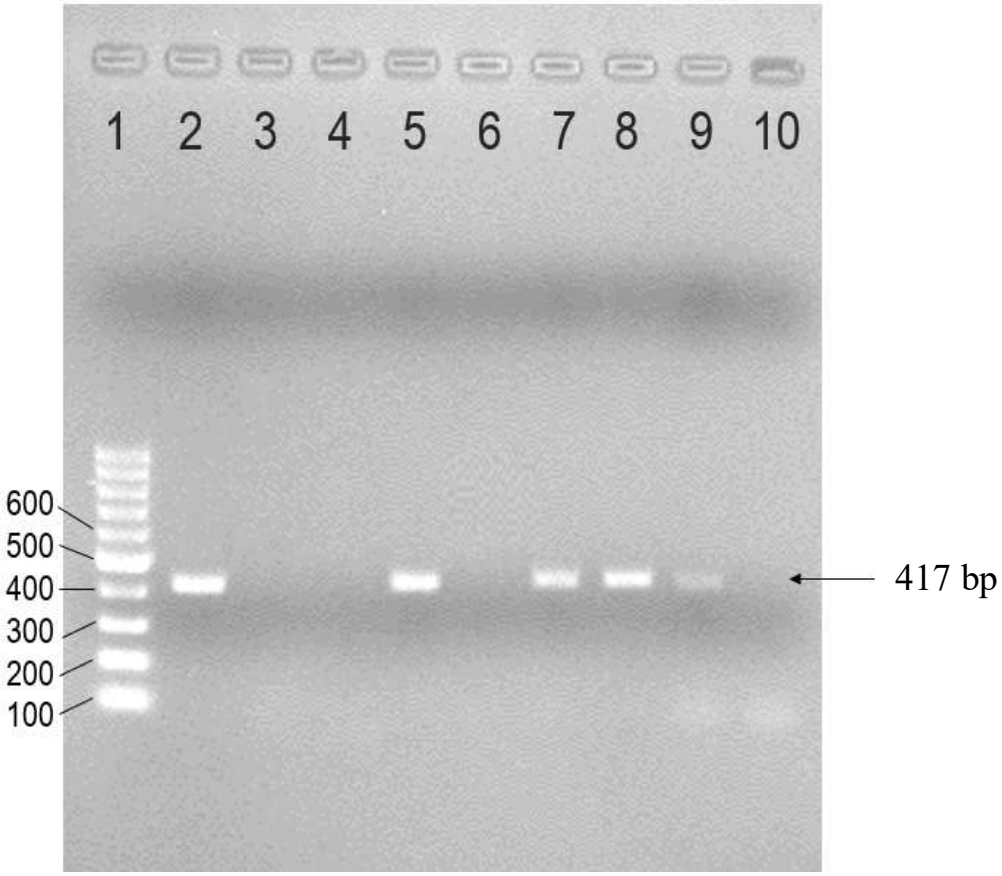


Figure 7. Agarose gel electrophoresis of PCR products screening for *B. burgdorferi* DNA present in ticks. The *OspA* gene of *B. burgdorferi* is the target for DNA amplification. The size of the amplified PCR product is 417 base pairs (bp). Lane 1 contains a 100 bp DNA ladder (GeneRuler; Fisher Scientific, Hampton, New Hampshire); Lane 2 represents a positive control in which extracted DNA from a pure culture of *B. burgdorferi* was kindly provided by the New York State Department of Health as the source of template DNA for this reaction; Lane 3 represents a negative control in which molecular biology grade water was substituted for template DNA whereas Lane 4 is a negative control in which DNA extracted from a lab-raised *I. scapularis* that was not infected with *B. burgdorferi* (courtesy of the Centers for Disease Control and Prevention) was substituted for template DNA. Lanes 5, 7, 8, and 9 represent PCR products amplifying *B. burgdorferi* DNA extracted from questing ticks collected at Hartman Creek State Park. No *B. burgdorferi* DNA was amplified from the DNA extracted from questing ticks in Lanes 6 and 10.

Results

I collected 314 *I. scapularis* over two field seasons. Of these, 253 were questing and 61 were replete. Of the questing ticks, 10 were nymphs, 121 were adult females, and 122 were adult males. All 61 replete ticks were nymphs. In 2017, 46 questing ticks and 41 replete ticks were collected. In 2018, 207 questing and 61 replete ticks were collected (**Table 5**). Identities of all 314 ticks were confirmed by PCR analysis of extracted DNA.

Table 5.

Numbers of I. scapularis collected in 2017 and 2018 from Hartman Creek State Park.

		2017	2018	Total
Questing	Adult Female	26	95	121
	Adult Male	18	104	122
	Nymph	2	8	10
Replete	Nymph	41	20	61
				314

The 61 replete nymphs were removed from 29 individual mammals: 24 *Tamias striatus* (eastern chipmunk), 2 *Peromyscus leucopus* (white-footed mouse), 1 *Peromyscus maniculatus* (deer mouse), 1 *Ictidomys tridecemlineatus* (thirteen-lined ground squirrel), and 1 *Microtus pennsylvanicus* (meadow vole). Most of the replete ticks (55) were collected from *T. striatus*.

Singular infection rates of questing and replete *I. scapularis* are detailed in **Table 6**. None of the questing or replete ticks collected in 2017 amplified the target for *Ehrlichia* species. To save resources and time, none of the ticks collected in 2018 were screened for *Ehrlichia* species.

Table 6.

Singular infection rates of questing and replete I. scapularis.

NT= not tested

		Uninfected	*Borrelia burgdorferi*	*Anaplasma phagocytophilum*	*Babesia microti*	*Ehrlichia* species
Questing	2017	16/46 (34.8%)	27/46 (58.7%)	6/46 (13.0%)	9/46 (19.6%)	0/46 (0%)
	2018	75/207 (36.2%)	76/207 (36.7%)	81/207 (39.1%)	15/207 (7.2%)	NT
	Combined	91/253 (36.0%)	103/253 (40.7%)	87/253 (34.4%)	24/253 (9.5%)	0/46 (0%)
Replete	2017	13/41 (31.7%)	18/41 (43.9%)	22/41 (53.7%)	5/41 (12.2%)	0/41 (0%)
	2018	10/20 (50.0%)	2/20 (10.0%)	8/20 (40.0%)	2/20 (10.0%)	NT
	Combined	23/61 (37.7%)	20/61 (32.8%)	30/61 (49.2%)	7/61 (11.5%)	0/41 (0%)

Table 7.

Co-infection rates of questing and replete I. scapularis.

Bb- Borrelia burgdorferi, Ap- Anaplasma phagocytophilum, Bm- Babesia microti

		Bb + Ap	Ap + Bm	Bb + Bm	Bb + Ap + Bm
Questing	2017	5/46 (10.9%)	0/46 (0%)	5/46 (10.9%)	1/46 (2.2%)
	2018	30/207 (14.5%)	3/207 (1.4%)	5/207 (2.4%)	1/207 (0.5%)
	Combined	35/253 (13.8%)	3/253 (1.2%)	10/253 (4.0%)	2/253 (0.8%)
Replete	2017	9/41 (22.0%)	2/41 (4.9%)	1/41 (2.4%)	3/41 (7.3%)
	2018	0/20 (0%)	2/20 (10%)	0/20 (0%)	0/20 (0%)
	Combined	9/61 (14.8%)	4/61 (6.6%)	1/61 (1.6%)	3/61 (4.9%)

Co-infection rates of questing and replete *I. scapularis* are given in **Table 7**. Statistical analysis of co-infection is given in **Table 8**. None of the co-infection pairs were found to be statistically different in the chi-square test. This indicates the observed co-infection prevalence is not statistically different than the expected co-infection prevalence based on individual infection rates. The Fisher's exact analysis of co-infection rates yielded one p-value <0.05, indicating significantly less co-infection of questing ticks with *A. phagocytophilum* and *B. microti* than predicted by chance.

Table 8.

Statistical analysis of co-infection rates of questing and replete I. scapularis. Values at p <0.05 are in bold.

Bb- Borrelia burgdorferi, Ap- Anaplasma phagocytophilum, Bm- Babesia microti

		Observed	Expected	χ^2	Fisher's Exact p-value
Questing	Bb + Ap	35/253 (13.8%)	35.4/253 (14.0%)	0.01	
	Ap + Bm	3/253 (1.2%)	8.3/253 (3.3%)		**0.02216**
	Bb + Bm	10/253 (4.0%)	9.8/253 (3.9%)	0.01	
Replete	Bb + Ap	9/61 (14.8%)	9.8/61 (16.1%)	0.08	
	Ap + Bm	4/61 (6.6%)	3.4/61 (5.6%)		0.7072
	Bb + Bm	1/61 (1.6%)	2.3/61 (3.8%)		0.409

Discussion

Major factors that affect the survival and proliferation of *I. scapularis* include hosts, predators of hosts and ticks, landscape, habitat type, and abiotic conditions (Ostfeld 2011). Due to changing climate, including warmer winters, higher humidity, and increased rainfall, suitable habitat for *I. scapularis* is expanding (Estrada-Peña 2002). According to remote sensing data collected by Estrada-Peña (2002), Waupaca county in central Wisconsin transitioned from unsuitable to suitable habitat for *I. scapularis* in 1994. Range expansion of this vector has fundamental implications for human disease transmission.

Co-infection of hosts and vectors also may play an integral role in this system. There is potential for population-level interactions between agents, such as competition or mutualism. Competition could result in decreased prevalence of one agent and increased prevalence of another, while mutualism could result in increased prevalence of both agents in the vector or host (Diuk-Wasser et al. 2016). There are numerous hypotheses for the mechanism, but it is currently not known what drives these population-level interactions. These possible interactions are currently being investigated to fully understand these relationships (Eisen and Eisen 2017). Numerous studies have identified the presence of co-infection patterns that could shed light on this confounding system (Steiner et al. 2008; Hersh et al. 2014; Prusinski et al. 2014).

Singular infection rates. The singular infection rate of questing *I. scapularis* for *B. burgdorferi* in Hartman Creek State Park was 103/253 (40.7%). These data are comparable to other studies performed in central Wisconsin. Turtinen et al. (2015) found 79/203 (38.9%) questing *I. scapularis* infected with *B. burgdorferi* in central Wisconsin (Chippewa, Clark, Marathon, and Eau Claire counties).

The incidence of *A. phagocytophilum* that was found in questing *I. scapularis* was substantially greater than other published studies at 87/253 (34.4%). Lee et al. (2014) found 11/365 (3.0%) of questing *I. scapularis* infected with *A. phagocytophilum* in Black River State Forest (Jackson County). Michalski et al. (2006) found 9/78 (11.5%) of questing *I. scapularis* infected with *A. phagocytophilum* in Waupaca County. The increase in prevalence of this agent in this area could be attributed to many factors, including (but not limited to) expansion of *I. scapularis* populations and increasing numbers of competent hosts.

The singular infection rate of questing *I. scapularis* that was found for *B. microti* was 24/253 (9.5%). There are limited data available on the infection rate of *I. scapularis* with *B. microti* in central Wisconsin. Westwood (2017) found an infection rate of 66/461 (14%) of *B. microti* in questing *I. scapularis* from Vilas County in northern Wisconsin. Steiner et al. (2008) tested 100 questing *I. scapularis* collected in Fort McCoy (Monroe County) and found none infected with *B. microti.*

There were 46 questing *I. scapularis* screened for *Ehrlichia* species and none were infected. There are limited data available on the infection rate of *I. scapularis* with

Ehrlichia species in Wisconsin. Murphy et al. (2017) found 10/1150 (0.9%) of questing *I. scapularis* collected in 19 counties throughout Wisconsin infected with *Ehrlichia muris*-like agent.

Co-infection rates. Published co-infection of questing *I. scapularis* with *B. burgdorferi* and *A. phagocytophilum* ranged from 2.0% to 8.2% in Wisconsin and 0.1% to 15.9% in the eastern United States (**Tables 9 and 10**). This study found 13.8% of questing *I. scapularis* co-infected with these two agents, which is greater than other studies in Wisconsin. This could be attributed to the increasing infection rates of questing *I. scapularis* with *A. phagocytophilum* in this location.

Published co-infection of questing *I. scapularis* with *B. burgdorferi* and *B. microti* ranged from 0% to 3.5% in Wisconsin and 1.0% to 16.7% in the eastern United States (**Tables 9 and 10**). This study found 4.0% of questing *I. scapularis* co-infected with these two agents, which is slightly higher than other studies in Wisconsin.

Published co-infection of questing *I. scapularis* with *B. microti* and *A. phagocytophilum* ranged from 0% to 4.3% in Wisconsin and 0.1% to 2.0% in the eastern United States (**Tables 9 and 10**). This study found 1.2% of questing *I. scapularis* co-infected with these two agents, which is comparable to other studies in Wisconsin.

Published co-infection of questing *I. scapularis* with all three infectious agents ranged from 0% to 1.7% in Wisconsin and 0.1% to 4.9% in the eastern United States (**Tables 9 and 10**). This study found 1.2% of questing *I. scapularis* co-infected with all three agents, which is comparable to other studies in Wisconsin.

Two of the three studies that examined co-infection of questing *I. scapularis* in Wisconsin (**Table 9**) tested statistically for deviations from random expectations. Westwood (2017) found no differences, while Steiner et al. (2008) found higher levels of co-infection of *B. burgdorferi* and *A. phagocytophilum* than predicted by chance. My results conformed to those of Westwood (2017).

Bb + Ap	Bb + Bm	Bm + Ap	Bb+ Ap + Bm	Location	Collection year(s)	Source
38/461 8.2%	16/461 3.5%	20/461 4.3%	8/461 1.7%	Vilas County	2016	Westwood 2017
9/365 2%				Black River State Forest	2009-2013	Lee et al. 2014
8/100 8%	0/100 0%	0/100 0%	0/100 0%	Fort McCoy	2006	Steiner et al. 2008

Table 9.

Published co-infection of questing I. scapularis in Wisconsin.

Three of the studies in the eastern United States **(Table 10)** tested statistically for deviations from random expectations. Steiner et al. (2008) and Hutchinson et al. (2015) found no differences. Hersh et al. (2014) found 84% higher levels of co-infection of *B. microti* and *B. burgdorferi* than predicted by chance and less co-infection of *A. phagocytophilum* and *B. microti* than predicted by chance. My results conformed to the latter co-infection pattern, which could indicate a population-level interaction, such as competition, between these two agents in questing ticks.

Table 10.

Published co-infection of questing I. scapularis in the eastern United States.

Bb + Ap	Bb + Bm	Bm + Ap	Bb+ Ap + Bm	Location	Collection year(s)	Source
20/318 6.3%	36/318 11.3%	3/318 0.3%	8/318 2.5%	Suffolk County, NY Tolland and Fairfield Counties, CT	2015-2016	Tokarz et al. 2017
15/961 1.6%	17/961 1.8%	2/961 0.2%	1/961 0.01%	6 National Parks in the Eastern US	2014-2015	Johnson et al. 2017
28/1,855 1.5%	32/1,855 1.7%	2/1,855 0.1%	1/1,855 0.05%	Pennsylvania	2012-2014	Hutchinson et al. 2015
115/4368 2.6%	261/4368 6.0%	26/4368 0.6%	17/4368 0.4%	Dutchess County, NY	2011-2012	Hersh et al. 2014
1/845 0.1%	22/845 2.6%	0/845 0%	0/845 0%	CT, NY, MD	2011-2012	Feldman et al. 2015
38/2088 1.8%	21/2088 1.0%	8/2088 0.4%	6/2088 0.3%	Massachusetts	2006-2012	Xu et al. 2016
48/466 10.3%	5/466 1.0%	0/466 0%	7/466 1.5%	Putnam and Dutchess counties, NY	2011	Aliota et al. 2014
45/286 15.7%	48/286 16.7%	0/286 0%	14/286 4.9%	Westchester and Suffolk Counties, NY	2008	Tokarz et al. 2010
9/100 9%	11/100 11%	2/100 2%	0/100 0%	Wells, ME	2003	Steiner et al. 2008

CHAPTER III

CONCLUSIONS

Vector-borne disease is a growing public health concern. Each year, there are over one billion cases and more than one million deaths from vector-borne diseases worldwide (WHO 2014). In the contiguous United States, 95% of vector-borne diseases are transmitted by ticks (Eisen and Eisen 2017). Due to changing climate, including warmer winters, higher humidity, and increased rainfall, suitable habitat for the tick *I. scapularis* is expanding (Estrada-Peña 2002).

In the United States, the tick *I. scapularis* can serve as a vector for at least seven agents that cause human disease: five bacteria (*A. phagocytophilum, B. burgdorferi, B. mayonii, B. miyamotoi,* and *E. muris eauclairensis*), one protozoan (*B. microti*), and one virus (Powassan virus) (Eisen and Eisen 2017). Incidence of these diseases, especially Lyme disease, are increasing at alarming rates. At Hartman Creek State Park in central Wisconsin, this study screened for four of these agents: *A. phagocytophilum, B. burgdorferi, B. microti,* and *Ehrlichia* species. My data showed 112/253 (44.3%) of questing black-legged ticks were infected with a single agent and an additional 50/253 (19.8%) were co-infected with either two or three agents.

Co-infection of hosts and vectors could result in population-level interactions between agents, such as competition or mutualism which could result in increased replication of one or both agents in the vector and host (Diuk-Wasser et al. 2016).

Published studies have identified enhanced co-infection rates that could shed light on this confounding system. My data show less co-infection of questing ticks with *A. phagocytophilum* and *B. microti* than predicted by chance which was also found by Hersh et al. (2014) in New York. This lower than expected probability of co-infection suggests a negative interaction, such as competition, is occurring between these two agents in the vector.

Documented cases of co-infection in human hosts may manifest with more persistent and severe symptoms (Hersh et al. 2014). Such co-infections are poorly known but likely to have important implications for the transmission dynamics and incidence of each infectious agent (Diuk-Wasser et al. 2016). My study, in concert with others, illustrate the need for public health workers and medical personnel to use diagnostic tests specific to each infectious agent when presented with human subjects who display the suite of nonspecific symptoms.

APPENDIX A

PCR Protocol

Each PCR reaction was set up in 0.2 mL Fisherbrand flat capped PCR tubes with four components.

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

Target	Positive Control DNA Designation	Source
B. microti	BM1	Extracted DNA from infected *Mus musculus* blood, courtesy of New York State Department of Health
A. phagocytophilum	42	Extracted DNA from infected female *I. scapularis,* courtesy of New York State Department of Health
B. burgdorferi	B31	Extracted DNA from pure culture, courtesy of New York State Department of Health
Ehrlichia species	MM1	Extracted DNA from infected *M. musculus* blood, courtesy of Centers for Disease Control and Prevention
I. scapularis	IS	Extracted DNA from lab raised *I. scapularis*, courtesy of Centers for Disease Control and Prevention

Appendix B (cont.)

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

Target	Negative Control Designation	Source
B. microti	IS	Extracted DNA from lab raised *I. scapularis* from the CDC
A. phagocytophilum	IS	Extracted DNA from lab raised *I. scapularis* from the CDC
B. burgdorferi	IS	Extracted DNA from lab raised *I. scapularis* from the CDC
Ehrlichia species	IS	Extracted DNA from lab raised *I. scapularis* from the CDC
I. scapularis	DV	Extracted DNA from *D. variabilis,* collected in Omro, WI
In addition to the negative controls listed, a negative control of molecular-grade water in place of template was also performed		

Appendix B (cont.)

Bio-Rad T100 or C1000 Thermal Cycler PCR Reaction Settings for Thermo Scientific DreamTaq hot start master mix (2x) (Fisher Scientific, Hampton, New Hampshire; Thermo Scientific 2017).

Step	Temperature	Time	Function
1	95°C	3 minutes	Initial denaturation
2	95°C	30 seconds	Denaturation
3	See next page	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

Appendix B (cont.)

Bio-Rad T100 or C1000 Thermal Cycler PCR Reaction Settings for Bullseye hot start master mix (2x) (Midwest Scientific, Valley Park, Montana; MidSci 2017).

Step	Temperature	Time	Function
1	95°C	15 minutes	Initial denaturation
2	95°C	20 seconds	Denaturation
3	See next page	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

Appendix B (cont.)

Annealing temperature and DNA target for each primer set.

Target	Primer Designation	DNA target	Annealing Temperature
B. microti	Bab1F/Bab4R	16S-like small subunit rRNA gene	58°C
A. phagocytophilum	MSP3F/MSP3R	*p44* gene	62°C
B. burgdorferi	OspA1F/OspA2R	*ospA* gene	62°C
Ehrlichia species	SodBF/SodBR	*sodB* gene	62°C
I. scapularis	IScapF/IScapR	Small subunit mitochondrial 16S rRNA gene	54°C

References

Adelson ME, Rao R-VS, Tilton RC, Cabets K, Eskow E, Fein L, Occi JL, Mordechai E. Prevalence of Borrelia burgdorferi, Bartonella spp., Babesia microti, and Anaplasma phagocytophila in Ixodes scapularis Ticks Collected in Northern New Jersey. Journal of Clinical Microbiology. 2004;42(6):2799–2801.

Aliota MT, Dupuis AP, Wilczek MP, Peters RJ, Ostfeld RS, Kramer LD. The Prevalence of Zoonotic Tick-Borne Pathogens in Ixodes Scapularis Collected in the Hudson Valley, New York State. Vector-Borne and Zoonotic Diseases. 2014;14(4):245–250.

Ammazzalorso AD, Zolnik CP, Daniels TJ, Kolokotronis SO. To beat or not to beat a tick: comparison of DNA extraction methods for ticks (*Ixodes scapularis*). PeerJ. 2015;3.

Black WC, Piesman J. Phylogeny of hard- and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences. Proceedings of the National Academy of Sciences. 1994;91(21):10034–10038.

Caporale DA, Rich SM, Spielman A, Telford SR, Kocher TD. Discriminating between Ixodes Ticks by Means of Mitochondrial DNA Sequences. Molecular Phylogenetics and Evolution. 1995;4(4):361–365.

Carroll JF, Schmidtmann ET. Tick Sweep: Modification of the Tick Drag-Flag Method for Sampling Nymphs of the Deer Tick (Acari: Ixodidae). Journal of Medical Entomology. 1992;29(2):352–355.

Centers for Disease Control and Prevention, 26 Apr. 2018, www.cdc.gov.

Davis S, Bent SJ. Loop analysis for pathogens: niche partitioning in the transmission graph for pathogens of the North American tick Ixodes scapularis. Journal of Theoretical Biology. 2011;269(1):96–103.

Diuk-Wasser MA, Vannier E, Krause PJ. Coinfection by Ixodes Tick-Borne Pathogens: Ecological, Epidemiological, and Clinical Consequences. Trends in Parasitology. 2016;32(1):30–42.

Dumler JS, Barbet AF, Bekker C, Dasch GA, Palmer GH, Ray SC, Rikihisa Y, Rurangirwa FR. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of Ehrlichia with Anaplasma, Cowdria with Ehrlichia and Ehrlichia with Neorickettsia, descriptions of six new species combinations and designation of Ehrlichia equi and HGE agent as subjective synonyms of Ehrlichia phagocytophila. International Journal of Systematic and Evolutionary Microbiology. 2001;51(6):2145–2165.

Dunn JM, Krause PJ, Davis S, Vannier EG, Fitzpatrick MC, Rollend L, Belperron AA, States SL, Stacey A, Bockenstedt LK, et al. Borrelia burgdorferi Promotes the Establishment of Babesia microti in the Northeastern United States. PLoS ONE. 2014;9(12).

Durden LA, Keirans JE. Nymphs of the Genus Ixodes (Acari: Ixodidae) of the United States: taxonomy, identification key, distribution, host, and medical veterinary importance. Lanham, MD: Published by the Entomological Society of America; 1996.

Egizi A, Roegner V, Faraji A, Healy SP, Schulze TL, Jordan RA. A historical snapshot of Ixodes scapularis-borne pathogens in New Jersey ticks reflects a changing disease landscape. Ticks and Tick-borne Diseases. 2018;9(2):418–426.

Eisen L. Pathogen transmission in relation to duration of attachment by Ixodes scapularis ticks. Ticks and Tick-borne Diseases. 2018;9(3):535–542.

Eisen RJ, Eisen L. The Blacklegged Tick, Ixodes scapularis: An increasing public health concern. Trends in Parasitology. 2018;34(4):295–309.

Estrada-Peña A. Increasing Habitat Suitability in the United States for the Tick that Transmits Lyme Disease: A remote sensing approach. Environmental Health Perspectives. 2002;110(7):635–640.

Feldman KA, Connally NP, Hojgaard A, Jones EH, White JL, Hinckley AF. Abundance and infection rates of Ixodes scapularis nymphs collected from residential properties in Lyme disease-endemic areas of Connecticut, Maryland, and New York. Journal of Vector Ecology. 2015;40(1):198–201.

Hahn MB, Bjork JK, Neitzel DF, Dorr FM, Whitemarsh T, Boegler KA, Graham CB, Johnson TL, Maes SE, Eisen RJ. Evaluating acarological risk for exposure to *Ixodes scapularis* and Ixodes scapularis -borne pathogens in recreational and residential settings in Washington County, Minnesota. Ticks and Tick-borne Diseases. 2018;9(2). 340-348.

Hartman Creek State Park. Wisconsin State Park System. 2018 Feb [accessed 2018 Apr]. dnr.wi.gov

Hersh MH, Ostfeld RS, Mchenry DJ, Tibbetts M, Brunner JL, Killilea ME, Logiudice K, Schmidt KA, Keesing F. Co-Infection of Blacklegged Ticks with Babesia microti and Borrelia burgdorferi Is Higher than Expected and Acquired from Small Mammal Hosts. PLoS ONE. 2014;9(6).

Hutchinson ML, Strohecker MD, Simmons TW, Kyle AD, Helwig MW. Prevalence Rates of Borrelia burgdorferi (Spirochaetales: Spirochaetaceae), Anaplasma phagocytophilum (Rickettsiales: Anaplasmataceae), and Babesia microti (Piroplasmida: Babesiidae) in Host-Seeking Ixodes scapularis (Acari: Ixodidae) from Pennsylvania. Journal of Medical Entomology. 2015;52(4):693–698.

Johnson TL, Graham CB, Boegler KA, Cherry CC, Maes SE, Pilgard MA, Hojgaard A, Buttke DE, Eisen RJ. Prevalence and Diversity of Tick-Borne Pathogens in Nymphal Ixodes scapularis (Acari: Ixodidae) in Eastern National Parks. Journal of Medical Entomology. 2016.

Keirans JE, Hutcheson HJ, Durden LA, Klompen J. Ixodes (Ixodes) scapularis (Acari: Ixodidae): Redescription of all Active Stages, Distribution, Hosts, Geographical Variation, and Medical and Veterinary Importance. Journal of Medical Entomology. 1996;33(3):297–318.

Keirans JE, Litwak TR. Pictorial Key to the Adults of Hard Ticks, Family Ixodidae (Ixodida: Ixodoidea), East of the Mississippi River. Journal of Medical Entomology. 1989;26(5):435–448.

Kogut SJ, Thill CD, Prusinski MA, Lee J-H, Backenson PB, Coleman JL, Anand M, White DJ. Babesia microti, Upstate New York. Emerging Infectious Diseases. 2005;11(3):476–478.

Latif AA, Putterill JF, Klerk DGD, Pienaar R, Mans BJ. Nuttalliella namaqua (Ixodoidea: Nuttalliellidae): First Description of the Male, Immature Stages and Re-Description of the Female. PLoS ONE. 2012;7(7).

Lee X, Coyle DR, Johnson DKH, Murphy MW, Mcgeehin MA, Murphy RJ, Raffa KF, Paskewitz SM. Prevalence of Borrelia burgdorferi and Anaplasma phagocytophilum in Ixodes scapularis (Acari: Ixodidae) Nymphs Collected in Managed Red Pine Forests in Wisconsin. Journal of Medical Entomology. 2014;51(3):694–701.

Lin Q, Rikihisa Y, Ohashi N, Zhi N. Mechanisms of Variable p44 Expression by Anaplasma phagocytophilum. Infection and Immunity. 2003;71(10):5650–5661.

Lovrich SD, Jobe DA, Kowalski TJ, Policepatil SM, Callister SM. Expansion of the Midwestern Focus for Human Granulocytic Anaplasmosis into the Region Surrounding La Crosse, Wisconsin. Journal of Clinical Microbiology. 2011;49(11):3855–3859.

Michalski M, Rosenfield C, Erickson M, Selle R, Bates K, Essar D, Massung R. Anaplasma phagocytophilum in central and western Wisconsin: a molecular survey. Parasitology Research. 2006;99(6):694–699.

MidSci (2017). Bullseye HS Taq Polymerase, 2x Master Mix Blue package insert. Qiagen.

Murphy DS, Lee X, Larson SR, Johnson DKH, Loo T, Paskewitz SM. Prevalence and Distribution of Human and Tick Infections with the Ehrlichia muris-Like Agent and Anaplasma phagocytophilum in Wisconsin, 2009–2015. Vector-Borne and Zoonotic Diseases. 2017;17(4):229–236.

Nelder MP, Russell CB, Sheehan NJ, Sander B, Moore S, Li Y, Johnson S, Patel SN, Sider D. Human pathogens associated with the blacklegged tick Ixodes scapularis: a systematic review. Parasites & Vectors. 2016;9(1).

Ostfeld RS. Lyme disease: the ecology of a complex system. New York: Oxford University Press; 2012.

Paskewitz SM, Vandermause M, Belongia EA, Kazmierczak JJ. Ixodes scapularis (Acari: Ixodidae): Abundance and Rate of Infection with Borrelia burgdorferi in Four State Parks in Wisconsin. Journal of Medical Entomology. 2001;38(1):33–38.

Persing DH, Mathiesen D, Marshall WF, et al. Detection of Babesia microti by polymerase chain reaction. Journal of Clinical Microbiology. 1992;30(8):2097-2103.

Pritt BS, Sloan LM, Johnson DKH, Munderloh UG, Paskewitz SM, Mcelroy KM, Mcfadden JD, Binnicker MJ, Neitzel DF, Liu G, et al. Emergence of a New Pathogenic Ehrlichia Species, Wisconsin and Minnesota, 2009. New England Journal of Medicine. 2011;365(5):422–429.

Prusinski MA, Kokas JE, Hukey KT, Kogut SJ, Lee J, Backenson PB. Prevalence of Borrelia burgdorferi (Spirochaetales: Spirochaetaceae), Anaplasma phagocytophilum (Rickettsiales: Anaplasmataceae), and Babesia microti (Piroplasmida: Babesiidae) in Ixodes scapularis (Acari: Ixodidae) Collected from Recreational Lands in the Hudson Valley Region, New York State. Journal of Medical Entomology. 2014;51(1):226–236.

Qurollo BA, Riggins D, Comyn A, Zewde MT, Breitschwerdt EB. Development and Validation of a Sensitive and Specific sodB-Based Quantitative PCR Assay for Molecular Detection of Ehrlichia Species. Journal of Clinical Microbiology. 2014;52(11):4030–4032.

Rudenko N, Golovchenko M, Grubhoffer L, Oliver JH. Updates on Borrelia burgdorferi sensu lato complex with respect to public health. Ticks and Tick-borne Diseases. 2011;2(3):123–128.

Rulison EL, Kuczaj I, Pang G, Hickling GJ, Tsao JI, Ginsberg HS. Flagging versus dragging as sampling methods for nymphal Ixodes scapularis (Acari: Ixodidae). Journal of Vector Ecology. 2013;38(1):163–167.

Schouls LM, Van De Pol I, Rijpkema SGT, Schot CS. Detection and Identification of Ehrlichia, Borrelia burgdorferi Sensu Lato, and Bartonella Species in Dutch Ixodes ricinus Ticks. Journal of Clinical Microbiology. 1999;37(7):2215-2222.

Shapiro ED. Lyme Disease. New England Journal of Medicine. 2014;370(18):1724–1731.

Steiner FE, Pinger RR, Vann CN, Grindle N, Civitello D, Clay K, Fuqua C. Infection and Co-infection Rates of Anaplasma phagocytophilum Variants, Babesia spp., Borrelia burgdorferi, and the Rickettsial Endosymbiont in Ixodes scapularis (Acari: Ixodidae) from Sites in Indiana, Maine, Pennsylvania, and Wisconsin. Journal of Medical Entomology. 2008;45(2):289–297.

Swanson SJ, Neitzel D, Reed KD, Belongia EA. Coinfections Acquired from Ixodes Ticks. Clinical Microbiology Reviews. 2006;19(4):708–727.

Telford SR III, Goethert HJ, Cunningham JK. Prevalence of Ehrlichia muris in Wisconsin Deer Ticks Collected During the Mid-1990s. The Open Microbiology Journal. 2011;5(1):18–20.

Tokarz R, Jain K, Bennett A, Briese T, Lipkin WI. Assessment of Polymicrobial Infections in Ticks in New York State. Vector-Borne and Zoonotic Diseases. 2010;10(3):217–221.

Tokarz R, Tagliafierro T, Cucura DM, Rochlin I, Sameroff S, Lipkin WI. Detection of Anaplasma phagocytophilum, Babesia microti, Borrelia burgdorferi, Borrelia miyamotoi, and Powassan Virus in Ticks by a Multiplex Real-Time Reverse Transcription-PCR Assay. mSphere. 2017;2(2).

Thermo Scientific (2017). DreamTaq Hot Start Master Mix (2x) package insert. Thermo Scientific.

Turtinen LW, Kruger AN, Hacker MM. Prevalence of Borrelia burgdorferi in adult female ticks (Ixodes scapularis), Wisconsin 2010-2013. Journal of Vector Ecology. 2015;40(1):195–197.

Westwood ML. Infection Prevalence in a Novel Ixodes scapularis Population in Northern Wisconsin. Wright State University Graduate Thesis. 2017 Aug 25.

WHO. World Health Organization. 2014 Mar [accessed 2018 Feb 20]. http://www.who.int/en

Wisconsin Department of Health Services, [accessed 2018 Apr 20]. www.dhs.wisconsin.gov.

Xu G, Mather TN, Hollingsworth CS, Rich SM. Passive Surveillance of Ixodes scapularis (Say), Their Biting Activity, and Associated Pathogens in Massachusetts. Vector-Borne and Zoonotic Diseases. 2016;16(8):520–527.

Zar JH. Biostatistical analysis. Upper Saddle River, NJ: Prentice Hall; 1999.