The Complex Eco-Epidemiology of Tick Borne Disease: Ticks, Hosts and Pathobiomes in an Urbanizing Environment

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Steve Woodrow Bennett

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Dr. Craig Hedberg (Adviser), Dr. Katey Pelican (Co-Adviser)

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Dedication

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Abstract

Emerging infectious diseases have become a rapidly increasing public health threat over the past 4 decades with the majority of pathogens being zoonotic or vector borne. Ticks are the second most important vector of disease globally, behind mosquitoes, and transmit a more diverse group of pathogens of medical importance than any other vector. Tick-borne diseases (TBDs) represent the most common vector-borne disease threats in North America, impacting both human and animal health. In Minnesota, the Lyme disease agent, *Borrelia burgdorferi,* emerged in 1980 and over the past two decades, incidence has risen over 700%. In order to better understand the local eco-epidemiology of these pathogens in the Twin Cities metro area a holistic approach to their ecology must be taken into account.

The pathobiome concept has been introduced to ecological research to move beyond the 'one pathogen= one disease' principle. Pathogens must be evaluated within the framework of the microbial communities in which they exists. The impact of these communities can significantly drive disease transmission and vector competence. There are many gaps in data that currently exist in regards to the *Ixodes scapularis* pathobiome.

To fill existing knowledge gaps, data were collected from the Metropolitan Mosquito Control District which had been trapping rodents and collecting ticks in the metro area since 1991. First, the pathobiome of *Ixodes scapularis* ticks was described. The analysis revealed significant changes depending upon the year ticks were collected. Next, tick and mammal population changes from 1993-2013 were analyzed to determine host vector relationships as well as vector range expansion. *Ixodes scapularis* have greatly expanded their range around the twin cities; however, urban development may

have impacted that expansion. Additionally, the role of the Eastern chipmunk was shown to be important in local TBD ecology, and there is evidence of possible competition between tick species. Lastly, the impact of host blood meal and pathogen acquisition on the pathobiome of *I. scapularis* was examined. Both pathogens and host meal had a significant impact on tick pathobiome. The results from these studies are a step towards better understanding tick and TBD ecology and hopefully predicting and mitigating future transmission risk.

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Introduction:

Tick Borne Disease and the Pathobiome

Ticks are obligate hematophagous ectoparasites, or blood feeding arthropods, that feed on multiple vertebrates including birds, reptiles and mammals including humans. Globally, ticks are second only to mosquitoes as vectors of public health importance with the ability to transmit pathogens including viruses, fungi, bacteria and protozoans (Parola & Raoult, 2001; Brites-Neto, Duarte, & Martins, 2015). Multiple species of ticks, hard (Ixodidae) and soft (Argasidae), can transmit human pathogens, and each exist in their own complex eco-epidemiology involving a variety of animal hosts (McCoy, Léger, & Dietrich, 2013). Ticks transmit multiple important pathogens worldwide including the agents of Lyme disease, Crimean Congo Hemorrhagic fever, Rocky Mountain spotted fever, and tularemia (Petersen, Mead, & Schriefer, 2008; Burgdorfer W. , 1975; Messina, et al., 2015). Tick borne disease (TBD) pathogens usually require an animal reservoir thus complicating the ecology of these pathogens even further. Understanding the complex eco-epidemiology of ticks, their hosts and the pathogens they carry is important for public health measures attempting to mitigate human disease risk.

Ixodes scapularis, local disease vector:

Ixodes scapularis, or black-legged ticks, are the primary vector of multiple pathogens of public health importance in the United States including *Borrelia burgdorferi* and *Borrelia mayonii* which cause Lyme disease, the most prevalent vector borne disease in the United States (Piesman & Eisen, 2008; Burgdorfer W. , 1984; CDC, 2013). While there are more than 30,000 confirmed cases of Lyme disease in the United States every year the CDC estimates that this number is grossly underreported and that the true number may be 3 to 12 times higher (CDC, 2013). Additionally, *I. scapularis* is a

vector of other human pathogens including those causing tick-borne relapsing fever (*Borrelia miyamotoi*), Powassan encephalitis, human granulocytic anaplasmosis (*Anaplasma phagocytophilum*), babesiosis (*Babesia microti*) and a newly defined ehrlichiosis (*Ehrlichia muris* subsp. *eauclairensis*, subsp. nov) (Pritt, et al., 2017; Pritt, et al., 2016; Lantos, et al., 2017; Oliver, Bennett, Beati,, & Bartholomay, 2017; Mitchell, Reed, & Hofkes, 1996; Krause, Fish, Narasimhan, & Barbour, 2015). *I. scapularis* ticks and the pathogens they transmit have recently increased their geographic range in the United States with main foci in the Northeastern United States and the Upper Midwest (Eisen, Eisen, & Beard, 2017; Oliver, Bennett, Beati,, & Bartholomay, 2017; Robinson, et al., 2015). While *I. scapularis* ticks in Minnesota were once confined close to the Wisconsin border, their range, as well as risk for TBD transmission, now extends across the state and into North Dakota (Eisen, Eisen, & Beard, 2017; Dennis, Nekomoto, Victor, Paul, & Piesman, 1998; Robinson, et al., 2015; KD Sanders, 2000) (Figure 1). The range expansion of *I. scapularis* has been tied to multiple variables including climate, land use, and host biodiversity and movement (Ostfeld & Brunner, 2015; Harrus & Baneth, 2005; Robinson, et al., 2015; Ogden, Mechai, & Margos, 2013).

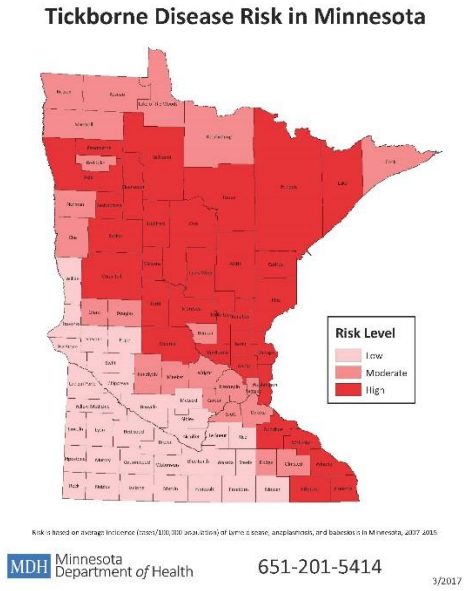

Figure 1: Map of Minnesota counties indicating TBD risk level by county (MDH, 2017)

In Minnesota, cases of TBD have been steadily increasing. Endemic *I. scapularis* transmitted TBDs in Minnesota include Lyme, anaplasmosis, babesiosis, ehrlichiosis and Powassan virus encephalitis (MDH, 2017; Neitzel & Kemperman, 2012; Birge & Sonnesyn, 2012). Although rarer, Rocky Mountain spotted fever (RMSF) which is transmitted by *Dermacentor variabilis* is also endemic to parts of the state (Neitzel & Kemperman, 2012). Between 1996 and 1999 there was an average of 263 confirmed cases of Lyme disease per year in Minnesota, and between 2012 and 2015 that number rose to an average of 1103.7 cases per year, an increase of 419% (MDH, Tick Borne Disease, 2017). Nationally, Minnesota also recorded the greatest increase in number of counties reporting at least 1 confirmed case of Lyme disease, from 33% of counties in 1992 to 74% in 2006 (Bacon, Kugeler, & Mead, 2008), Additionally, anaplasmosis and babesiosis have also been on the rise in Minnesota indicating the region has

increasingly become an important focal point of transmission (MDH, Tick Borne Disease, 2017) (Figure2).

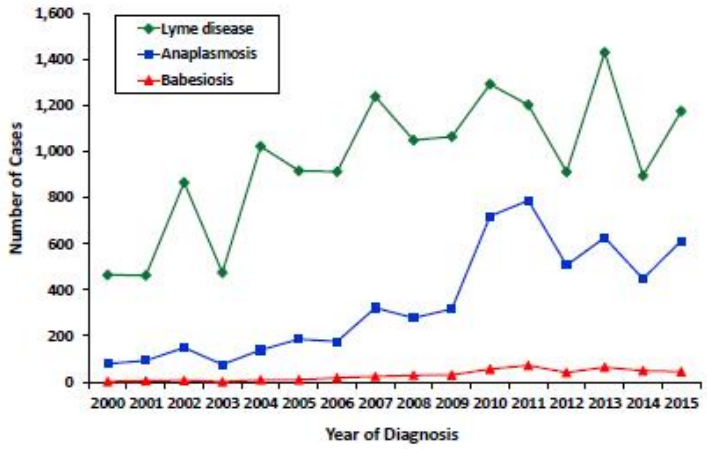

Figure 2: Minnesota Department of Health summary of diseases transmitted by *I. scapularis* showing increases in Lyme, anaplasmosis and babesiosis since 2000. (MDH, Annual Summary of Communicable Diseases Reported to the Minnesota Department of Health, 2015, 2017)

The microbiome of vectors:

The term microbiome was first used and defined in 1988 as a characteristic microbial community with-in a specific and well-defined habitat (Whipps, Lewis, & Cooke, 1988). The gut microbiota, or internal microbiome, has become increasingly regarded as having a pivotal role in host health, fitness, and behavior (Nicholson, et al., 2012; Ezenwa, Gerardo, Inouye, Medina, & Xavier, 2012). Inversely, loss of host health and fitness, such as through infection or disease, can impact the microbiome (Youmans, 2015; Ryan, 2013). The components of arthropod microbiomes are derived from a variety of sources including the environment, diet, hosts and vertical transmission (Yun, et al., 2014; Martinez, et al., 2014). Evidence has shown arthropods can be profoundly impacted by changes in their microbiome. For example, mosquito larval development can be impeded through alterations in their microbiome (Coon, Vogel, Brown, & Strand, 2014). Dysbiosis, caused by exposure to antibiotics or changes in nutrition, has a

deleterious systemic effect on honey bees, *Apis mellifera*, and can negatively impact colony health (Maes, Rodrigues, Oliver, Mott, & Anderson, 2016; Raymann, Shaffer, & Moran, 2017). Aging related issues in *Drosophilia* cause shifts in the microbiome that contribute to an immune response and subsequent death (Clark, et al., 2015). Interactions between bacteria and other microbes within the microbiome have been shown to increase or decrease host immunity and presence of certain bacteria may actually be important in pathogenesis (Chow, Tang, & Mazmanian, 2011; Lin & Zhang, 2017).

The arthropod microbiome may impact vector competence in pathogen acquisition and transmission (Hegde, Rasgon, & Hughes, 2015). The mid gut microbiota of vectors acts as a line of defense against infection by killing or inhibiting the development of parasites (Azambuja, Garcia, & Ratcliffe, 2005). The mosquito microbiome influences viral transmission through its impact on host immunity, resource competition and production of anti-viral activity through secondary metabolite production (Jupatanakul, Sim, & Dimopoulos, 2014). The microbiome plays a role in vector competence of *Culex spp*., as well as seasonality of transmission of West Nile virus, due to cold temperatures reducing *Wolbachia* abundance thereby increasing susceptibility to the virus (Novakova, et al., 2017). *Trypanasoma cruzi*, the protozoan parasite that causes Chagas disease, has been shown to signal a host immune response in its triatomine vector which decreases the vector microbiome density and diversity to facilitate parasite reproduction and transmission (Castro, et al., 2012). Vector competence for tsetse flies, *Glossina palpalis gambiensis*, is increased when the symbiont *Sodalis glossinidius* is present although these interactions are not yet understood (Welburn & Maudlin, 1999; Geiger, et al., 2007). Bacterial microbiome

communities have also been shown to impact reproduction, development and transmission of malaria species in *Anopheles* mosquito populations (Pumpuni, Demaio, Kent, Davis, & Beier, 1996; Cirimotich, et al., 2011; Gonzalez-Ceron, Santillan, Rodriguez, Mendez, & Hernandez-Avila, 2003).

The role of microorganisms, pathogenic and non-pathogenic, is important in the ecology of ticks and TBD (Bonnet, Binetruy, Hernández-Jarguín, & Duron, 2017). *Borrelia* may assist other pathogens, such as *Babesia*, with establishing themselves in an environment by increasing probability of transmission from host to tick when co-infected (Dunn, et al., 2014). *Anaplasma* has been shown to cause *I. scapularis* ticks to release a glycoprotein that increases the ability of the pathogen to reproduce and may assist with the tick's survival overwinter (Abraham, et al., 2017; Neelakanta, Sultana, Fish, Anderson, & Fikrig, 2010). Interactions between TBD pathogens and other microbes may alter transmission dynamics as *Borrelia burgdorferi* has been shown to be less able to establish an infection in ticks raised in a sterile environment that are never exposed to environmental bacterial influence (Narasimhan S., 2014).

Understanding the broader microbiome is important in tick and TBD ecology as many commensal bacteria which co-exist with TBD agents are of vital importance to tick survival (Bonnet, Binetruy, Hernández-Jarguín, & Duron, 2017). The removal of the symbiont Coxiella-LE through antibiotic treatment decreased the fitness and survival of the lone star tick, *Amblyomma americanum* (Zhong J., 2007). Increasing our understanding of the microbiome of ticks is imperative to understanding the complex relationships that exists between pathogen, host, vector and the environment. Overall, this knowledge may help with future endeavors to decrease human risk for TBD.

From Microbiome to Pathobiome:

The pathobiome is described as integrating a pathogen, it's host, and vector within all levels of its biotic and abiotic environment and environmental stressors (Vayssier-Taussat, et al., 2014). The pathobiome moves beyond Koch's postulate of "one pathogen = one disease" and takes into account that microbes, pathogenic or not, live together in communities that have complex interactions with varying outcomes for the host (Vayssier-Taussat, et al., 2014; Sweet & Bulling, 2017) The purpose of examining the pathobiome is to discover the complex interactions between pathogens and non-pathogenic microbes and how that drives disease processes including virulence and infectivity (Chow, Tang, & Mazmanian, 2011; Sweet & Bulling, 2017; Mosser, et al., 2015). For example, mosquitoes in the *Anopholese gambiae* species complex are impacted by the presence of skin bacteria on some humans which may increase the attractiveness of certain individuals to mosquitoes (Busula, Takken, DE Boer, & Mukabana, 2017; Verhulst, et al., 2011). Additionally, soil microbes have been shown to be a source of antibiotic resistance genes for human pathogens (Forsberg, et al., 2012).

Pathobiome analysis is gaining prominence in medical research as scientists study the impact pathogens have on the microbial community structure of surgical patients and use that information to guide care (Defazio, Fleming, Shakhsheer, Zaborina, & Alverdy, 2014; Krezalek, DeFazio, Zaborina, Zaborin, & Alverdy, 2016). The dysbiotic human gut microbiome has also been the focal point for many analyses of pathobiome microbial interaction (Lloyd-Price, Abu-Ali, & Huttenhower, 2016; DeFazio, et al., 2014) The pathobiome concept is just beginning to enter broader ecological disciplines as it is being applied to both coral and plant health (Wright, et al., 2017;

Jakuschkin, et al., 2016; Sweet & Bulling, 2017). Recently, it was proposed that ticks and TBD should be examined under the pathobiome paradigm (Vayssier-Taussat, et al., 2015).

Although microbiome studies of ticks have recently become more common, the majority focus solely on the internal microorganisms that ticks harbor. Often, ticks in these studies are surface sterilized to remove external microorganisms and, while it is important to understand and quantify the internal microbiome, these studies lack data or analysis on bacteria that ticks come into contact in their external environment (Ponnusamy, et al., 2014; Van Treuren, et al., 2015). Through their questing activities ticks come in direct contact with bacteria and other microbes on plants, in soil, and in some surface water. Additionally, being obligate blood feeders, ticks must take blood meals to reach adulthood. These feedings expose ticks to bacteria residing on the skin of hosts as well as bacteria that exist in the host blood meal. As mentioned, ticks reared in a sterile environment have lowered vector competence indicating an important role environmental microbes play in pathogen transmission (Narasimhan, 2014). These environmental and host bacterial exposures are part of the broader pathobiome of ticks and should be considered in an analysis of the microbial ecology ticks and TBD (Vayssier-Taussat, et al., 2015). Information on this broader pathobiome for *I. scapularis* ticks is extremely scant with only one study done on 7 individual ticks in Massachusetts, and there is no published information on pathobiome for ticks in the Midwest region of the United States (Benson, Gawronski, Eveleigh, & Benson, 2004).

The purpose of this thesis is to address some of the knowledge gaps that exists in our understanding of the ecology of TBD in Minnesota including issues related to tick and host populations as well as the pathobiome of *I. scapularis* ticks.

To this goal the specific aims of this thesis are to:

1. To describe and analyze the microbiome of *Ixodes scapularis* ticks collected in the Twin Cities Metro Area and to determine species diversity and richness over multiple years as well as presence of specific pathogenic bacteria and rickettsia (i.e. *Borrelia burgdorferi* and *Anaplasma phagocytophilum*). Additionally, to determine infection and co-infection rates in tick samples.
2. To detect, quantify, and investigate spatial and temporal trends in small mammal and tick population changes in the Twin Cities Metro area. We will investigate the eco-epidemiological relationship between multiple tick and small mammal populations over the course of 20 years.
3. To determine important differences in the microbiomes of ticks based on multiple variables and to link those differences to TBD ecology we will examine the impact of pathogen presence, time and rodent host on microbial community.

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Chapter 1:

The Pathobiome of *Ixodes scapularis* in the Twin Cities Seven County-Metropolitan Area

Introduction:

Emerging infectious diseases have become a rapidly increasing public health threat over the past 4 decades. Zoonotic and vector borne diseases make up the majority of emerging infectious diseases (Jones, et al., 2008). Ticks are the second most import vector of disease globally, behind mosquitoes, and transmit a more diverse group of pathogens of medical importance than any other vector (Parola & Raoult, 2001). Tick-borne pathogens represent the most common vector-borne disease threats in North America, impacting both human and animal health. In Minnesota, the Lyme disease agent, *Borrelia burgdorferi*, was first identified in 1980. Over the past two decades, incidence has risen over 700%, with 1,176 confirmed human cases reported in 2015. More recently, anaplasmosis and babesiosis have emerged, and their incidence is increasing rapidly (Robinson, et al., 2015; MDH, 2017)). The more recent detection of ehrlichiosis and Powassan encephalitis further emphasizes the importance of emerging tick-borne threats to public and veterinary health in Minnesota (Pritt, 2011; Hermance & Thangamani, 2017). The black- legged tick, *Ixodes scapularis*, is the primary vector of the causative agents of these diseases in Minnesota. The spread of these pathogens has correlated with the increased geographic range of the vector and the main animal reservoir, *Peromyscus leucopus,* across the United States, with *B. burgdorferi* spreading and establishing itself more rapidly than other TBD pathogens (Dunn, et al., 2014). Tick-borne diseases exhibit a complex ecology in which climate, landscape characteristics, and potential host populations are all known to influence tick populations and infection dynamics of TBD (Robinson, et al., 2015; Ostfeld, 1997; Larsen, MacDonald, & Plantinga, 2014; Walsh, 2013; LoGiudice, Ostfeld, Schmidt, 2003).

The recent advances and increase in widespread use of high through-put second generation sequencing technologies has dramatically increased our understanding of the microbiome and its importance. The term microbiome was first used and defined in 1988 as a characteristic microbial community with-in a specific and well-defined habitat (Whipps, Lewis, & Cooke, 1988). The gut microbiota, or internal microbiome, has become increasingly regarded as having a pivotal role in host health, fitness, and behavior (Nicholson, et al., 2012; Ezenwa, Gerardo, Inouye, Medina, & Xavier, 2012). Changes in host microbial community have been shown to alter over time and be affected by multiple external factors including host diet, temperature, and other microbes. Interactions between bacteria and other microbes within the microbiome has been shown to increase or decrease host immunity and presence of certain non-pathogenic bacteria may be important in pathogenesis (Chow, Tang, & Mazmanian, 2011; Lin & Zhang, 2017). Additionally, there is a growing body of evidence that the microbiome may impact vector competence in pathogen acquisition and transmission. Infection with *Wolbachia* may play a role in vector competence of *Culex spp*. in transmission of West Nile virus (Novakova, et al., 2017). *Trypanasoma cruzi*, the protozoan parasite that causes Chagas disease, has been shown to signal a host immune response in its triatomine vector which decreases the vector microbiome density and diversity to facilitate parasite reproduction and transmission (Castro, et al., 2012). Bacterial microbiome communities have also been shown to impact reproduction, development and transmission of malaria species in *Anopheles* mosquito populations (Pumpuni, Demaio, Kent, Davis, & Beier, 1996; Cirimotich, et al., 2011; Gonzalez-Ceron, Santillan, Rodriguez, Mendez, & Hernandez-Avila, 2003).

Beyond the internal microbiome, vectors and hosts are in constant contact with a variety of other microbial organisms including bacteria, fungi, protozoans and viruses. The expansion of our understanding of the microbiome is taking us from simply looking at the gut microbiome, or inner microbiome, to a broader view encomapassing the 'pathobiome' of the host or vector. The pathobiome is described as integrating a pathogen, it's host, and vector within all levels of its biotic and abiotic environment and environmental stressors (Vayssier-Taussat, et al., 2014) (Figure 3). This holistic approach to pathogenesis has an oppurtunity to better explain why certain pathogens are asymptomatic in one host while causing illness in another or to look at how a pathogen fits into its environment on multiple levels, from internal to external (Ryan, 2013; Vayssier-Taussat, et al., 2015). While historically microbiome studies have attempted to elucidate its effect on host organisms there is an increasing effort to understand that everything the host experiences and the environment of the host can impact the microbiome itself (Alverdy & Luo, 2017). This impact of external factors on host

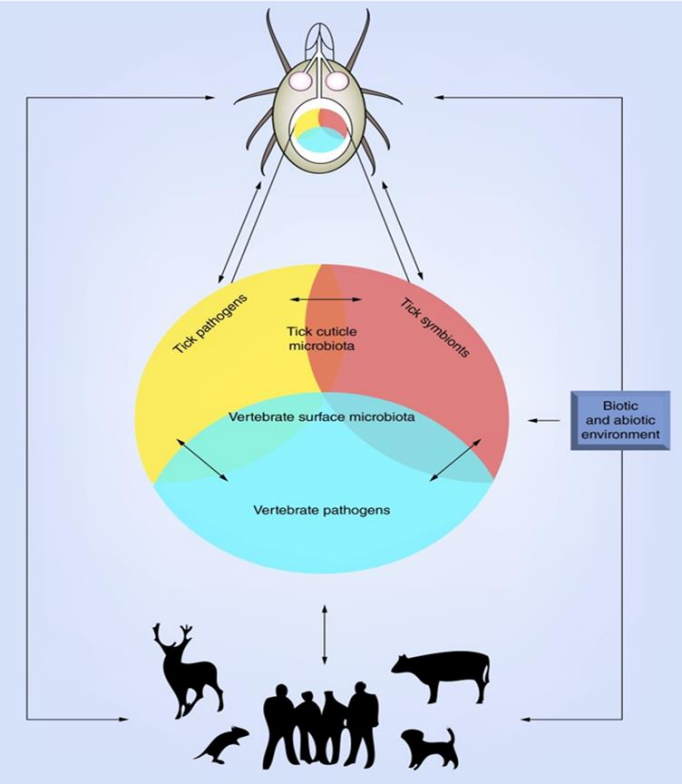

Figure 3: The tick pathobiome consists of microbes beyond the internal tick microbiome including those associated with the external biotic and abiotic envirnment as well as host associated microbes. Image from (Vayssier-Taussat, et al., 2015)

microbiome could effectively change pathogen transmission dynamics. For example, mosquitoes in the *Anopholese gambiae* species complex are impacted by the presence of skin bacteria on some humans which may increase the attractiveness of certain individuals to mosquitoes (Busula, Takken, DE Boer, & Mukabana, 2017; Verhulst, et al., 2011). Additionally, soil microbes have been shown to be a source of antibiotic resistance genes for human pathogens (Forsberg, et al., 2012). Due to these factors the external microbiome needs to be considered along with the internal microbiome as part of a larger pathobiome when attempting to examine pathogen ecology. The first step in understanding the pathobiome is having complete knowledge of the microbial

environment that defines it, which may include microbes in the gut and other tissues, as well as the external microbiome of the vector itself (Vayssier-Taussat, et al., 2014; Vayssier-Taussat, et al., 2015).

There is mounting evidence on the role of microorganisms, pathogenic and non-pathogenic, in the ecology of ticks and TBD (Bonnet, Binetruy, Hernández-Jarguín, & Duron, 2017). *Borrelia* may assist other pathogens, such as *Babesia*, with establishing themselves in an environment (Dunn, et al., 2014). *Anaplasma* has been shown to cause *I. scapularis* ticks to release a glycoprotein that increases the ability of the pathogen to reproduce and may assist with the tick's survival overwinter (Abraham, et al., 2017; Neelakanta, Sultana, Fish, Anderson, & Fikrig, 2010). *Borrelia burgdorferi* has been shown to be less able to establish an infection in ticks raised in a sterile environment, that are never exposed to external bacterial influence, thus pointing to the importance of the external microbiome (Narasimhan S., 2014). Additionally, many commensal bacteria are of vital importance to tick survival. The removal of the symbiont Coxiella-LE through antibiotic treatment decreased the fitness and survival of the lone star tick, *Amblyomma americanum* (Zhong J., 2007). Increasing our understanding of the pathobiome of ticks is imperative to understanding the complex relationships that exists between pathogen, host, vector and the environment. This knowledge may help with future endeavors to decrease human risk for TBD.

While microbiome studies of ticks have recently become more common, almost all focus solely on the internal microorganisms that ticks harbor. Ticks in these studies are surface sterilized to remove external microorganisms and, while it is important to understand and quantify the internal microbiome, these studies lack data on bacteria that ticks come into contact in their external environment (Ponnusamy, et al., 2014; Van

Treuren, et al., 2015).Through their questing activities ticks come in direct contact with bacteria on plants, in soil, and in surface water. Additionally, ticks are obligate blood feeders that must take blood meals to reach adulthood. These feedings expose ticks to bacteria residing on the skin of hosts as well as bacteria that exist in the host blood meal. These environmental and host bacterial exposures are part of the broader pathobiome of ticks and should be considered in an analysis of the microbial ecology ticks and TBD (Vayssier-Taussat, et al., 2015). Information on this broader pathobiome for *I. scapularis* ticks is extremely scant with only one study done on 7 individual ticks in Massachusetts, and there is no published information on pathobiome for ticks in the Midwest region of the United States (Benson, Gawronski, Eveleigh, & Benson, 2004).

In light of the risks to human health, the goal of this study is to describe the whole pathobiome of *I. scapularis* ticks in Minnesota. Documenting and quantifying the various microbes within the pathobiome and factors influencing the microbial community allows us to better understand microbe interactions and how those interactions impact pathogen spread and transmission. This holistic understanding of pathogen ecology could be vital in vector control methods as well as in disease prevention.

Methods:

Sample Collection:

In 1990, the Metropolitan Mosquito Control District initiated the Lyme Disease Tick Surveillance Program to determine the distribution and prevalence of *I. scapularis* and TBD agents and their rodent reservoirs within the Minneapolis-Saint Paul metropolitan area. Sampling is done through rodent trapping and ticks are collected from all trapped rodents. There are 100 collection sites around the Twin Cities metro area (Figure 4). Of these 100 repeat sites, 56 are located in Anoka (28 sites), Washington (25

sites), and Ramsey (3 sites) counties. 44 repeat sites are located south of the Mississippi River and are dispersed throughout Dakota (15 sites), Hennepin (14 sites), Scott (8 sites), and Carver counties (7 sites).

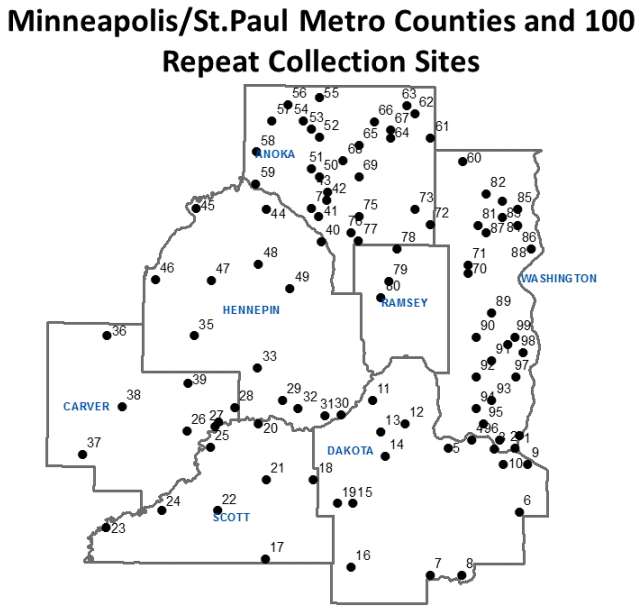

Figure 4. Map of the 7 Minneapolis/St. Paul Metropolitan area counties (Carver, Scott, Dakota, Washington, Ramsey, Anoka and Hennepin) encompassing the study area as well as the 100 repeatedly tested sites within those counties.

Sampling during each 27 week study period was divided into three 9-week periods and all sites were sampled for 21 trap nights (7 traps x 3 consecutive nights) per period. Weeks of site visitation were randomly selected within each sampling period. One three-hundred foot transect was established at each sampling location and Sherman live traps (H. B. Sherman Traps, Inc., Tallahassee, Fla.), baited with peanut butter and oats, were placed along these transects at fifty foot intervals. All small mammals caught in the traps were identified and euthanized through CO_2 inhalation following World Health Organization protocol. Any ticks found on trapped mammals were removed, identified and stored in 97% ethanol for later testing (Neitzel, Jarnefeld, & Sjorgren, 1993; MMCD, 2017).

DNA Extraction:

A subset of ticks were tested for pathobiome. DNA was extracted from ticks collected in 2000, 2004, 2008, 2012, and 2014 to provide a longitudinal analysis spanning 14 years. Extracted DNA was subjected to metagenomics testing to determine presence and makeup of the microbial. All nymph ticks from a chosen year were included to achieve a more comprehensive picture of pathogen prevalence and host relationships.

Extraction was completed at room temperature using a clean hood in a room where PCRs were not carried out to minimize contamination of reactions. All ticks were rinsed with 95% ethanol before extraction to remove debris and storage fluid in order to ensure the full pathobiome was tested rather than just the gut microbiome (Benson, Gawronski, Eveleigh, & Benson, 2004). DNA extraction was carried out with an adapted protocol using the DNeasy® Blood and Tissue Kit Quick-Start Protocol (Qiagen, Inc.). As per the Qiagen recommendations for tick DNA extraction for *Borrelia* testing, Carrier RNA (VX Carrier RNA, Qiagen, Inc.) was added to increase DNA volume. While there is some evidence that laboratory contamination may alter microbiome analysis and change microbe diversity, our study has followed rigorous procedure and our analysis is consistent with previously published studies (Salter, et al., 2014)

Primary/Secondary PCR Amplification:

The V4 region of 16S ribosomal RNA (rRNA) gene was amplified using a two-step PCR protocol. The primary amplification was done using an ABI7900 qPCR machine. The following reaction mixture was used: 3 µl template DNA, 0.48 µl nuclease-free water, 1.2 µl 5x KAPA HiFi buffer (Kapa Biosystems, Woburn, MA), 0.18 µl 10 mM dNTPs (Kapa Biosystems, Woburn, MA), 0.3 µl DMSO (Fisher Scientific, Waltham, MA),

0.12 µl ROX (25 µM) (Life Technologies, Carlsbad, CA), 0.003 µl 1000x SYBR Green, 0.12 µl KAPA HiFi Polymerase (Kapa Biosystems, Woburn, MA), 0.3 µl forward primer (10 µM), 0.3 µl reverse primer (10 µM). Cycling conditions were: 95°C for 5 minutes, followed by 20 cycles of 98°C for 20 seconds, 55°C for 15 seconds, and 72°C for 1 minute. The primers for the primary amplification contained both 16S-specific primers (V1_27F and V3_V34R), as well as adapter tails for adding indices and Illumina flow cell adapters in a secondary amplification. The following primers were used (16S-specific sequences in bold): Meta_V4_515F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GTGCCAGCMGCCGCGGTAA**) and Meta_V4_806R (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GGACTACHVGGGTWTCTAAT**).

The amplicons from the primary PCR were diluted 1:100 in sterile, nuclease-free water, and a second PCR reaction was set up to add the Illumina flow cell adapters and indices. The secondary amplification was done on a fixed block BioRad Tetrad PCR machine using the following recipe: 5 µl template DNA, 1 µl nuclease-free water, 2 µl 5x KAPA HiFi buffer (Kapa Biosystems, Woburn, MA), 0.3 µl 10 mM dNTPs (Kapa Biosystems, Woburn, MA), 0.5 µl DMSO (Fisher Scientific, Waltham, MA) 0.2 µl KAPA HiFi Polymerase (Kapa Biosystems, Woburn, MA), 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM). Cycling conditions were: 95°C for 5 minutes, followed by 10 cycles of 98°C for 20 seconds, 55°C for 15 seconds, 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes. The following indexing primers were used (X indicates the positions of the 8 bp indices): Forward indexing primer: AATGATACGGCGACCACCGAGATCTACACXXXXXXXXTCGTCGGCAGCGTC and Reverse indexing primer:

CAAGCAGAAGACGGCATACGAGATXXXXXXXXGTCTCGTGGGCTCGG (Gohl, et al., 2016).

Normalization and Sequencing:

The samples were normalized using a SequalPrep capture-resin bead plate (Life Technologies, Carlsbad, CA) and pooled using equal volumes. The final pools were quantified via PicoGreen dsDNA assay (Life Technologies, Carlsbad, CA) and diluted to 2nM. 10 µl of the 2 nM pool was denatured with 10 µl of 0.2 N NaOH, diluted to 8 pM in Illumina's HT1 buffer, spiked with 15% phiX, heat denatured at 96*C for 2 minutes, and sequenced using a HiSeq 500 cycle kit (Illumina, San Diego, CA)**.**

Sequence Processing:

Sequences were processed through the bioinformatics software Mothur (version 1.35.1). Quality control parameters were based on the Schloss MiSeq SOP pipeline (http://www.mothur.org/wiki/ MiSeq_SOP) (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013). Parameters for discarding sequences were unidentifiable barcode and/or reverse primer sequence. Sequences with greater than 2 errors in their barcode or primer sequence, homopolymer runs greater than 8 bp, any ambiguous bases, or less than 150 bp were filtered out of the dataset. SILVA SEED bacterial 16S database was utilized for sequence alignment (Schloss P. D., 2009) . Remaining high quality and chimera free sequences were placed into operational taxonomic units (OTUs) using 97% similarity. SILVA bacterial 16S database assigned genus level classification to all remaining high quality sequences. The top 30 most abundant OTUs were blasted using National Center of Biological Information (NCBI) Web blast to determine species similarity (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul, Gish, Miller, Myers, & Lipman, 1990).

A total of 609 individual ticks were sequenced from five separate years (2000, 2004, 2008, 2012, and 2014). Following sequence read quality trimming and chimera removal, the range of coverage per sample was >98%, and the average read length was >240 nt. Samples were rarefied to 1,000 sequences. Rarefication resulted in removal of 110 of the 609 ticks from analysis based on a low number of quality reads (<1000). A total of 491 ticks were included in the final analysis spread over 5 collection years (2000=177, 2004=50, 2008=80, 2012=122, 2014=61). The top 30 OTUs were then further analyzed to species level, when possible, using NCBI BLAST using Nucleotide collection (nt) and 16S rRNA gene (Bacteria and Archaea) library databases.

Statistics:

Analysis was performed on extracted DNA from 491 *I. scapularis* nymphs. Statistical analyses including Chi square, Fisher's exact test, Kruskal-Wallis Rank Sum, Wilcoxon Rank Sum and production of box and whisker plots was performed in R (R Development Core Team, 2012).

Mothur 1.35.1 was used to calculate Alpha diversity, α, or within-community diversity, using Chao1, S_{obs} (observed OTUS), and the inverse Simpson diversity index (1/D). Observed OTUs indicate total numbers of OTUs in samples while Chao1 estimates the actual number of species in a community, given the observed sample, while accounting for the relative distribution of species (Chao, 1984). Inverse Simpson combines richness with evenness to determine relevant diversity. Analysis of molecular variance (AMOVA) was performed in Mothur to determine whether variance of each microbial community between groups was greater than the variance within each group (Schloss P., 2008). Homogeneity of molecular variance (HOMOVA) was the performed to test if the variation in samples was different between groups (Schloss P., 2008).

Metastats was implemented in Mothur to analyze beta diversity, or within group, differences. Non parametric multidimensional scaling (nmds) was calculated in Mothur and visualized in R. Bonferroni p value adjustment methods were used to account for multiple comparisons. Maps were developed in ArcMap and ArcGIS 10.1 (Esri Inc). A p value of ≤ 0.05 was considered significant.

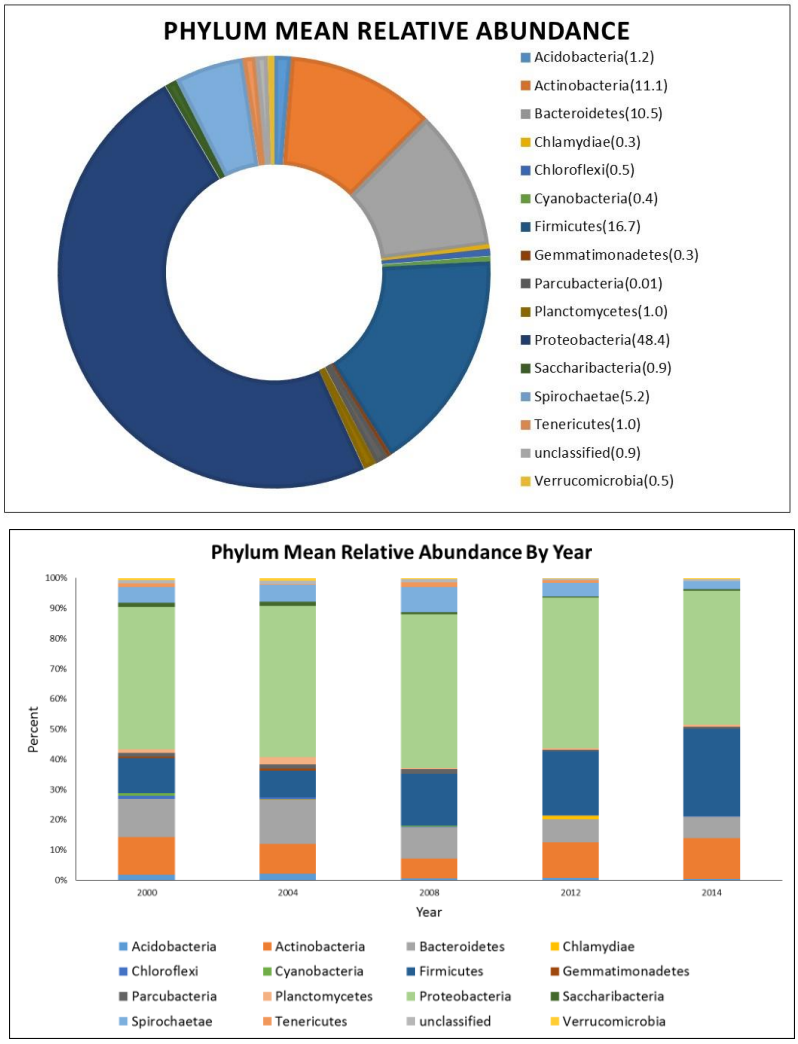

Figure 5a, 5b: Mean relative abundance of phylum in (a) all tested ticks and (b) stratified by year (n=491). Phylum names are followed by their relative abundance (%). Sequences that were high quality and chimera free were used to generate OUT's based on a 97% similarity. All OTUs were classified using the SILVA 16S database

Results

A total of 4700 individual operational taxonomic units (OTUs) were identified. Proteobacteria was the most abundant phylum among all samples. The other major phyla represented were Firmicutes (16.7%), Actinobacteria (11.1%), Bacteriodetes (10.5) and Spirochaetae (5.2%) (Figure 5a). All other phyla represented ~1% or less. The major phyla were consistent over the 5 separate years with minor variation (Figure 5b). For the 491 total samples, the mean Chao1 species richness estimate was 55.7 (±64.6, IQR 42.1) and the S_{obs} mean was 45.2 (±41.2, IQR 12.4). Year of collection was shown to be a significant factor when comparing both Chao1 (Kruskal-Wallis X^2= 30.0, df = 4, p<0.001) and S_{obs} (Kruskal-Wallis X^2= 23.2, df = 4, p = 0.001) species richness estimates When comparing both Chao1 and S_{obs} estimates by year, 2008 was significantly different from other years in community richness (Wilcoxon rank sum test p=0.03) (Figure 6). Inverse Simpson was measured to determine diversity based on the species evenness and richness between the years of the study.

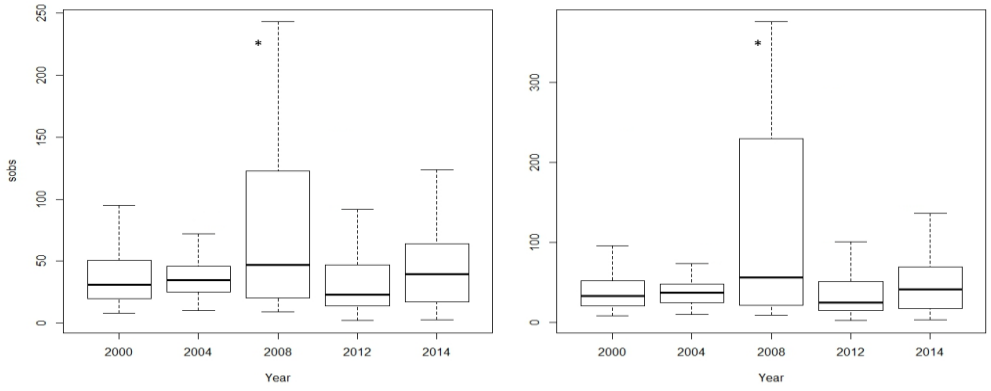

Figure 6: S_{OBS} (left) and Chao1 (right) analysis by year indicating a significant increase in species number or richness in 2008 compared to other years

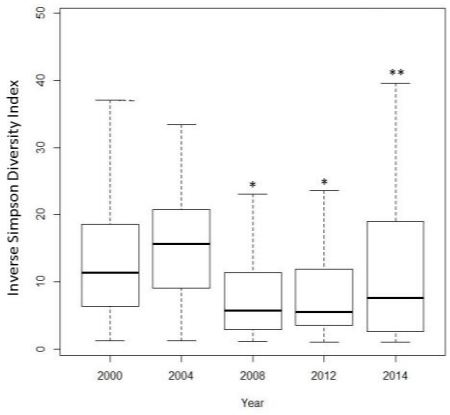

Figure 7: Inverse Simpson Diversity Index showing significant differences by year tick was collected. Note that 2008 (n=80) and 2012 (n=122), while similar to each other, were significantly different from 2000 (n=177) and 2004 (n=50). 2014 (n=62) was only significantly different from 2004.

The Inverse Simpson diversity index indicated year of tick collection was a significant driver of species diversity based on richness and evenness (Kruskal-Wallis X^2= 47.1, df = 4, p <0.001) (Figure 7). 2008 and 2012 had significantly lower species diversity based on the inverse Simpson diversity index than 2000 and 2004 (Wilcoxon rank sum test p< 0.001). 2014 was significantly different from only 2004 with a p value of 0.037 (Table 1). Differences in OTU prevalence was compared between years and the top 30 OTUs and their significant changes over the collection years recorded (Table 2). Analysis of molecular variance (AMOVA) was performed in Mothur to determine whether variance in each microbial community between years was greater than the variance within each year. AMOVA showed significant overall differences in microbial community between year of study in all years (Among 1.5, Within 0.4, Fs 3.7, p<0.001). Homogeneity of molecular variance (HOMOVA) was then performed in Mothur to test if the variation in samples in each year was different between years. The year 2000 had a greater variance within microbial community than both 2004 (0.42/0.39, p=0.003) and

2008 (0.42/.38, p=0.01). 2012 had higher variance within microbial community than 2004(0.43/0.39, p<0.0010) and 2008 (0.43/0.37, p<0.01).

Inverse Simpson analysis of microbial community by Year				
Year	2000	2004	2008	2012
2004	0.51065	-	-	-
2008	*4.70E-05	*0.00011	-	-
2012	*6.50E-06	*8.70E-06	1	-
2014	0.06312	*0.0373	1	1

Table1: Inverse Simpson diversity Index analysis by year with associated p-values. Simpson's diversity index was calculated using mother version 35.1. Statistical significance (*) was calculated with Pairwise comparisons using Wilcoxon rank sum in R with the Bonferroni p-value adjustment method.

23 bacterial families were represented in the top 30 OTUs and included bacteria from water, soil, symbionts of ticks as well as skin and internal microbes of the small mammal and rodent hosts (Table 3). The top 30 OTUs accounted for 72.8 percent of all reads with the largest (OTU1 *Rickettsia*) being 17.6 percent of reads and the smallest (OTU30 *Borrelia myamotoi*) making up only 0.39 percent of total reads. Percent of total reads combined with prevalence is used to determine not just how many ticks the microbe was in but also how comparatively dense the population of that microbe was within the sample group. OTU prevalence among samples ranged from 5 (OTU24 *Aeromonas* and OTU23 *Candidatus* Rhabdochlamydia) to 421 (OTU8 *Sphingomonas spp.*) with a mean of 131 OTUs and a standard deviation of 115(Figure 4). Lefse analysis in Mothur shows that OTUs 1 and 2 (*Rickettsia* and *Borrelia*) were significantly elevated in 2008 compared to all other years (*Rickettsia* LDA 4.8, p <0.001, *Borrelia* LDA 4.3 p=0.008). Additionally, OTUs 3 and 14 (*Staphylococcus* and *Luteibacter*) were elevated in 2014 (Staphylococcus LDA= 4.6, p=0.002, *Luteibacter* LDA=4.1, p<0.001) and OTU7 (*Sphingomonas*) was elevated in 2004 (LDA 4.3, p<0.001).

OTU	Genus	2000-mean(sd)	2004-mean	2008-mean	2012-mean	2014-mean	2000/2004	2000/2008	2000/2012	2000/2014	2004/2008	2004/2012	2004/2014	2008/2012	2008/2014	2012/2014
1	Rickettsia	0.071(0.01)	0.0839	0.1819	0.078033	0.06829	-	0.001998	-	-	0.005994	-	-	0.001998	0.000999	-
2	Borrelia	0.048(0.01)	0.04834	0.077525	0.042672	0.024742	-	-	-	-	-	-	-	-	0.043956	-
3	Staphylococcus	0.058(0.009)	0.02436	0.100225	0.069451	0.115984	0.030969	0.044955	-	-	0.001998	0.020979	0.005994	-	-	-
4	Anaplasma	0.0008(0.0007)	0.0131	0.024812	0.013836	0.000016	-	-	-	0	-	-	-	-	0.000999	-
5	Streptococcus	0.00058(0.0004)	0.00072	0.008513	0.033549	0.000177	-	0.025974	0.000999	0.000023	0.004995	0.000999	0.000011	0.011988	0.000999	0.000999
6	Lactobacillus	0.0009(0.0003)	0.00038	0.0081	0.036459	0.003387	0.000071	0.014985	0.000999	-	0.001998	0.000999	-	0.004995	-	0.000999
7	Flavobacterium	0.038(0.003)	0.0403	0.028362	0.014164	0.017855	-	-	0.000999	0.000999	-	0.001998	0.000999	0.008991	0.045954	-
8	Sphingomonas	0.058(0.005)	0.07136	0.05315	0.085549	0.038548	-	-	0.01998	0.028971	-	-	0.006993	0.021978	-	0.000999
9	Paenibacillus	0(0)	0.0011	0.000575	0.000074	0.077984	0.000999	-	0.000313	0.000999	-	-	0.001998	-	0.000999	0.000999
10	Sphingomonas	0.0126(0.003)	0.02032	0.0101	0.016205	0.012532	-	-	-	-	-	-	-	-	-	-
11	Variovorax	0.0316(0.005)	0.03178	0.021887	0.026549	0.034581	-	-	-	-	-	-	-	-	-	-
12	Paenibacillus	0.000096(0.000096)	0.0027	0.000363	0.000082	0.047339	-	0.000009	-	0.000999	-	-	0.008991	0.00002	0.000999	0.000999
13	Bacillus	0.000034(0.00034)	0.00008	0.0077	0.000016	0.015935	-	0.03996	-	-	0.028971	-	-	0.010989	-	-
14	Luteibacter	0.0106(0.002)	0.00244	0.00835	0.007148	0.017532	0.01998	-	-	-	0.042957	0.044955	0.001998	-	-	-
15	Pseudomonas	0.008(0.0014)	0.00396	0.006825	0.013311	0.008742	-	-	-	-	-	0.030969	-	-	-	-
16	Mycobacterium	0.007(0.0016)	0.00568	0.004913	0.00841	0.013048	-	-	-	0.048951	-	-	0.003996	-	0.003996	-
17	Lactobacillus	0.0005(0.003)	0.00054	0.002938	0.00982	0.001419	-	-	0.000999	-	-	0.003996	-	-	-	0.012987
18	Pseudomonas	0.005(0.001)	0.0046	0.0053	0.007689	0.006145	-	-	-	-	-	-	-	-	-	-
19	Candidatus_Rhodoluna	0.010576(0.0013)	0.0188	0.017325	0.002344	0.004484	-	-	0.000999	0.000999	-	0.000999	0.000999	0.000999	0.000999	0.071928
20	Mycoplasma	0.0098(0.004)	0.00008	0.009663	0.007975	0.000306	0.020979	-	-	0.031968	0.001998	0.040959	0.010324	-	0.013986	0.041958
21	Corynebacterium	0.009(0.004)	0.0005	0.001675	0.000459	0.015048	-	-	0.047952	-	-	-	-	-	-	-
22	Massilia	0.008(0.001)	0.00274	0.008363	0.006352	0.004371	0.016983	-	-	-	0.035964	-	-	-	-	-
23	Candidatus Rhabdochlamydia	0(0)	0	0	0.011598	0.000081	1	-	0.001998	0.001175	-	0.00999	-	0.025974	0.015866	-
24	Aeromonas	0.000011(0.000008)	0	0.003388	0.000016	0.000081	1	-	-	0.015189	-	-	-	-	-	0.047386
25	Pseudarcicella	0.007621(0.001)	0.0101	0.010038	0.002549	0.005065	-	-	0.001998	-	-	0.00999	-	0.000999	0.022977	-
26	Phyllobacterium	0.0071(0.0018)	0.00442	0.003425	0.008016	0.024097	-	-	-	-	-	-	0.002997	-	0.002997	-
27	klebsiella	0.0032(0.0018)	0.00008	0.002925	0.002631	0.002548	-	-	-	-	0.003996	-	0.002997	-	-	-
28	Propionibacterium	0.02(0.003)	0.01378	0.002625	0.024574	0.013774	-	0.000999	-	-	0.000999	-	-	0.000999	-	-
29	Wolbachia	0.0004(0.0004)	0	0.002887	0.00468	0.002032	0	-	-	-	0.035964	-	-	-	-	-
30	Borrelia	0.000006(0.000006)	0.0021	0.002387	0.001057	0.000016	-	-	-	-	-	-	-	-	-	-

Table 2. *Ixodes scapularis* microbiome comparison between collection years of top 30 OTUs. Mothur calculated the mean presence of each *I. scapularis* OTU was calculated in mother. P-values were then determined using variability seen between the OTUs calculated means. Only significant p-values are reported (<= 0.05).

OTU	Family	Genus	Species	# of samples	% of reads	Bacterial Association (Tick, Rodent, Soil, Water)
1	Rickettsiaceae	Rickettsia	monacensis , buchneri	292	17.61	Tick
2*	Spirochaetaceae*	Borrelia*	burgdorferi ss.*	162	14.2	Tick and Rodent
3*	Staphylococcaceae*	Staphylococcus*	pettenkoferi *	275	12.11	Rodent, Environment
4*	Anaplasmataceae*	Anaplasma*	phagocytophillum *	32	3.5	Tick, Rodent
5	Streptococcaceae	Streptococcus	spp. gallolyticus	67	2.8	Rodent
6	Lactobacillaceae	Lactobacillus	murinis, animalis	94	2.53	Rodent
7	Flavobacteriaceae	Flavobacterium	spp. collinsii	346	2.07	Rodent
8	Sphingomonadaceae	Sphingomonas	spp. leidyi	421	1.8	Tick
9	Paenibacillaceae	Paenibacillus	spp.	24	1.7	Soil, Environment
10	Sphingomonadaceae	Sphingomonas	spp. aerolata	182	1.5	Tick
11	Comamonadaceae	Variovorax	Spp.	336	1.4	Soil
12	Paenibacillaceae	Paenibacillus	spp glucanolyticus	28	1.02	Environment
13	Bacillaceae	Bacillus	spp.idriensis, indicus,	14	1.	Soil
14	Xanthomonadaceae	Luteibacter	spp rhizovicinus	129	0.9	Environment
15	Pseudomonadaceae	Pseudomonas	spp.	151	0.8	Soil
16	Mycobacteriaceae	Mycobacterium	Spp. arabiense, hodleri	180	0.7	Soil
17	Lactobacillaceae	Lactobacillus	Reuteri	56	0.62	Rodent
18	Pseudomonadaceae	Pseudomonas	sp fluorescens, azotoformans	116	0.62	Soil, water
19	Microbacteriaceae	Candidatus_Rhodoluna	spp. Limnophila	227	0.61	Water
20	Mycoplasmataceae	Mycoplasma	Candidatus mycoplasma haemomuris ratii/musculi	43	0.59	Rodent
21	Corynebacteriaceae	Corynebacterium	spp.	26	0.57	rodent
22	Oxalobacteraceae	Massilia	spp. aurea	130	0.54	Soil
23*	Simkaniaceae*	Candidatus Rhabdochlamydia*	spp.*	5	0.53	Tick
24*	Aeromonadaceae*	Aeromonas*	spp. enchelia	5	0.53	Soil, Water, Rodent
25	Cytophagaceae	Pseudarcicella	spp.	194	0.5	
26	Phyllobacteriaceae	Phyllobacterium	Spp. Ifrigiyense	99	0.42	
27*	Enterobacteriaceae*	Klebsiella*	spp.*	66	0.41	Rodent, Environment
28	Propionibacteriaceae	Propionibacterium	spp.	209	0.4	Skin, rodent
29	Rickettsiaceae	Wolbachia	spp.	11	0.39	Tick
30*	Spirochaetaceae*	Borrelia(100)*	Miyamotoi*	6	0.39	Tick, Rodent

Table 3: Top 30 OTU (operational taxonomic units) by Family, Genus and species, when applicable. Included are percent of total reads and number of samples containing the OTU. Likely source or associating is noted and those OTUs with an asterisk (*) are confirmed or possible threats to human health

Possible infection with known TBD agents was identified based on presence of OTU 1 (*Rickettsia*), OTU 2 (*Borrelia*) or OTU 4 (*Anaplasma*). *Rickettsia spp.* were identified in 292 of 491 samples (59.47%). *Borrelia burgdorferi ss*. was identified in 162 samples (33%) with the 100 top results from the NCBI BLAST having >99% identity with *Borrelia burgdorferi sensu stricto.* While *Borrelia bissettii* was also identified in the same BLAST, because it could not be differentiated based on the amplified portion of the 16s gene, the occurrence of *Borrelia* is almost certainly *B. burgdorferi ss.* as *B. bissettii* transmission is generally focused on the West Coast and in some Southern states (Margos, et al., 2010). *Anaplasma spp*. was found in 32 samples (6.52%) with all species identified NCBI returns with >99% match being *Anaplasma phagocytophilum* (non-species identified returns were listed as 'uncultured *Anaplasma* species). Co-infection of *Borrelia* and *Anaplasma* occurred in 17 samples (3.5%) with 16 of those samples testing positive for all three possible pathogens (*Rickettsia*, *Borrelia*, and *Anaplasma*). Co-infection of *Borrelia* and *Anaplasma* occurred in 17 samples. Analysis indicated co-infection was significantly different than expected by chance (X^2 =4.7787, df = 1, p = 0.02, Fisher's Exact Test p=0.04). Lefse analysis in Mothur showed a significant increase in *B. burgdorferi* density in ticks infected with *A. phagocytophilum* (p=0.009); however, there was no significant difference in *A. phagocytophilum* in ticks based on *B. burgdorferi* inection status. Additionally, 6 samples tested positive for OTU30, *Borrelia miyamotoi* (top 27 NCBI results with a match >99%).

Discussion:

Few studies exist analyzing the pathobiome, or even just the gut microbiome, of *I. scapularis* ticks. This study is the first to investigate the whole microbiome, or pathobiome, of *Ixodes spp.* ticks over multiple years. Previous studies have examined

lab-reared ticks, ticks collected from a single year or ticks whose microbiomes have been experimentally altered through feeding and other methods (Van Treuren, et al., 2015; Swei & Kwan, 2017; Zolnik, Prill, Falco, Daniels, & Kolokotronis, 2016; Abraham, et al., 2017). Additionally, this is the first microbiome study of wild ticks in Minnesota or the Midwest. The diversity of microbes reported from the tick pathobiome, beyond tick internal microbiome, is useful in being able to better understand host-vector-microbe interaction. Multiple species, with possible pathogenicity, were discovered using the pathobiome approach. Here we highlight the major findings of microbes in the tick pathobiome associated with environment (soil and water), rodent, and ticks with special focus on those microbes that may impact human health.

Soil and Water Microbes:

Both soil and water microbes were found within tick samples. *Aeromonas* (OTU 24) is a group of highly diverse bacteria that often reside in soil and freshwater environments and can cause infection in multiple warm and cold blooded species (Lye, Rodgers, Stelma, Vesper, & Hayes, 2007). Aeromonads, while omnipresent environmental bacteria, are also emergent human disease agents of public health concern (Igbinosa, Igumbor, Aghdasi, Tom, & Okoh, 2012). *Klebsiella spp.* are also known to be opportunistic pathogens in immunocompromised individuals (Podschun & Ullmann, 1998). Both of the Enterobacteriaceae detected, *Aeromonas* (OTU 24) and *Klebsiella* (OTU 27), have also been previously identified in the guts of rodent species (Coşkun, El-Namee, & Kaya, 2013). Other soil microbes included *Pseudomonas spp.* (OTU 15), *Massilia aurea* (OTU 22) and *Bacillus spp.* (OTU 13). *Candidatus Rhodoluna limnophila* (OTU 19) is a freshwater bacterioplankton residing in temperate lakes and streams (Hahn, 2009). The environmental species may have been present on the tick cuticle originating either from the tick environment or from the dermis of the rodent host

from which the ticks were collected. While many of these may serve no major role in tick, rodent or pathogen ecology, they may still compete with other bacteria for resources. Additionally, the presence of these known pathogenic environmental species could be an indication that ticks might be a viable source of transmission to humans through infected bites.

Rodent Associated microbes:

Two species of *Lactobacillus* were identified in the samples*. Lactobacillus reuteri* (OTU 17) is a known gut endosymbiont of many animals including human and rodents (Oh, et al., 2010). *Lactobacillus reuteri* is also a known 'probiotic', often shown to have health benefits for people when used as a dietary supplement or in bacteriotherapy (Sabatini, Lauritano, Candotto, Silvestre, & Nardi, 2017; Rasouli, Ghadimi-Darsajini, Nekouian, & Iragian, 2017). *Lactobacillus murinus* (OTU 6) was identified in samples in an even greater amount. *Lactobacillus murinus* is the dominant flora in the rodent gut microbiome (Hemme, et al., 1980). Another rodent associated microbe that was detected was *Candidatus* Mycoplasma *haemomuris* subsp. musculi (OTU 20) (Harasawa, Fujita, Kadosaka, Ando, & Rikihisa, 2015). This species is a rodent pathogen causing anemia and splenomegaly (Messick, 2004). *Flovobacterium* has been isolated from the lungs of rodents (Scheiermann & Klinman, 2017).

The presence of these rodent-associated bacterial species is a clear indication of how closely intertwined the biomes of the tick and rodent are. It is valuable to document and study the microbial biome communities and the possible interactions between those biomes which could be significant whether through antagonistic action or symbiosis (Vayssier-Taussat, et al., 2015).

Tick associated microbes:

As shown, the potential threats to human health from ticks are not limited to traditional TBD. An additional potential threat to human health identified in the samples is *Candidatus* Rhabdochlamydia*.* The *Chlamydiales* order is an increasingly diverse group of intracellular organisms of which numerous members are implicated as threats to human and animal health (Wheelhouse & Longbottom, 2012; O'Connell & Ferone, 2016). *Ca.* Rhabdochlamydia is one of four recently proposed families and has yet to be officially accepted (Burnard, et al., 2017). *Ca.* Rhabdochlamydia is known to mostly infect arthropods at this time, being originally identified in cockroaches and wood lice, although it has recently been detected in one species of snapper fish (Corsaro, et al., 2007; Corsaro & Work, 2012; Kostanjsek, Strus, Drobne, & Avgustin, 2004). Members of *Ca.* Rhabdochlamydia have also been identified in *Ixodes* tick species in Australia and Switzerland (Burnard, et al., 2017; Pilloux, et al., 2015). Although there is some evidence that *Ca.* Rhabdochamydia may impact the health of newborns and children, no clear pathogenic role has been determined (Lamoth, Jaton, Vaudaux, & Greub, 2011; Lamoth, Aeby, Schneider, Jaton-Ogay, & Greub, 2009). The discovery of this genus within Minnesotan ticks adds weight to the need for increased research on this, and other *Chlamydiales* species, their role in tick microbial ecology as well as possibly pathogenic importance for humans and animals.

Consistent with previous studies of *Ixodes spp*. ticks, the genus *Rickettsia*, which represents obligate intracellular Gram-negative bacteria, were the most prevalent bacterial microbe within the samples (Moreno, Moy, Daniels, Godfrey, & Cabello, 2006). Bacteria in the genus *Rickettsia* are assigned to the ancestral, transitional, typhus and spotted fever groups (SFG) (Gillespie, et al., 2008). Many *Rickettsia* species in the tick borne SFG group are known to be highly pathogenic such as *Rickettsia rickettsia,* the cause of Rocky Mountain spotted fever (Parola, et al., 2013). *Rickettsia* symbionts of

unknown pathogenicity have been previously identified in *I. scapularis* ticks (Benson, Gawronski, Eveleigh, & Benson, 2004; Ahantarig, Trinachartvanit, Baimai, & Grubhoffer, 2013). *Rickettsia* species in samples were identified through NCBI BLAST as possibly either *R. monacensis* or *R. buchneri.* While *I. scapularis* has been shown to be able to maintain *R. monacensis* in a laboratory setting these bacteria have not been previously shown to be present in tick populations in North America (Baldridge, et al., 2007; Oliver, et al., 2015). *Rickettsia buchneri* is the primary symbiont of *I. scapularis* (Kurtti, et al., 2015) and most likely the species represented in the samples as it has been shown to be highly prevalent among *I. scapularis* ticks in North America and the Midwest, while other *Rickettsia* species appear much more rarely (Oliver, Bennett, Beati,, & Bartholomay, 2017; Kurtti, et al., 2015; Gillespie, et al., 2012). *R. buchneri*, which resides in *I. scapularis* ovaries, is exclusively transmitted transovarially rather than through infection of feeding ticks (Noda, Munderloh, & Kurtti, 1997; Kurtti, et al., 2015).

Anaplasma phagocytophilum, the causative agent of human granulocytic anaplasmosis, appeared in slightly more than 6% of samples. In addition to its role in human health, *A. phagocytophilum* has been observed to play a significant role in tick biology and impact its microbial community (Abraham, et al., 2017). Along with possibly promoting tick survival overwinter, *A. phagocytophilum* inhibits tick cellular apoptosis to protect infected cells and promote the spread of infection (De la Fuente, et al., 2017; Neelakanta, Sultana, Fish, Anderson, & Fikrig, 2010; Abraham, et al., 2017). Additionally, previous studies indicated a primary infection of *A. phagocytophilum* may protect against co-infection with *Borrelia burgdorferi* and vice versa (Levin & Fish, 2004). While more than 50% of our ticks infected with *A. phagocytophilum* were also co-infected with *B. burgdorferi* a significantly smaller amount of *B. burgdorferi* infected ticks were co-infected with *A. phagocytophilum* (10.4%). Our data do not support the notion of

A. phagocytophilum having a protective effect against co-infection with *Borrelia burgdorferi* but could support the notion that *B. burgdorferi* may compete to slow the spread of *A. phagocytophilum;* this could also help to explain why *A. phagocytophilum* prevalence is generally lower than *B. burgdorferi* in tick populations (Johnson, Kodner, Jarnefeld, Eck, & Xu, 2011; Oliver, Bennett, Beati,, & Bartholomay, 2017).

Two species of *Borrelia* were detected in our samples, *B. burgdorferi ss*. and *B. miyamotoi*. *Borrelia burgdorferi*, the causative agent for Lyme disease, is the most prevalent vector borne disease in the United States. *Borrelia burgdorferi* causes between 30-40,000 confirmed or probable cases of Lyme disease every year (CDC, Lyme Disease, 2017). Cases, however, are most likely underreported and the CDC estimates the number may be closer to 300,000 cases per year (CDC, Newsroom, 2013). As *B. burgdorferi* has shown to both compete with and possibly assist other microbes to establish infection, it is worth examining further (Levin & Fish, 2004). The impact of *B. burgdorferi* on the pathobiome of the tick may be substantial as has been shown previously with *A. phagocytophilum* (Abraham, et al., 2017). *B. miyamotoi*, a pathogen of emerging importance, causes relapsing fever and was originally discovered in Japan (Fukunaga, et al., 1995). The pathogen group, *B. miyamotoi sensu latu*, is now known to be present in most areas where *B. burgdorferi* exists, including the United States (Scoles, Papero, Beati, & Fish, 2001; Krause, et al., 2014) (Krause, et al., 2014). The ecology, seroprevalence, and distribution of *B. miyamotoi* is currently of increasing importance as its role in human health becomes more apparent (Krause, Fish, Narasimhan, & Barbour, 2015).

Our results indicate that the pathobiome of *I. scapularis* ticks is variable between years. Multiple variables have been shown to have a significant impact on tick gut microbiome including geography, life stage, pathogen prevalence, sex and species

(Moreno, Moy, Daniels, Godfrey, & Cabello, 2006; Zolnik, Prill, Falco, Daniels, & Kolokotronis, 2016; Van Treuren, et al., 2015; Swei & Kwan, 2017; Abraham, et al., 2017). Changes in any of these variables over the years of the study may have led to significant differences. Additionally, the microbes themselves may play a role in changing community structure. Increases in the amounts of certain microbes, such as *A. phagocytophilum* and *B. burgdorferi*, during a particular year may have led to changes in the overall pathobiome discovered in that year. For example, *B. burgdorferi* levels were significantly higher in 2008 indicating that it may have been driver in the reduction of microbial diversity that year. The relationship between these pathogens and the pathobiome should be investigated further. Additionally, analysis of the impact of mammal host species, climate and geography in relation to year are warranted.

Future studies may benefit from greater specificity in microbiome analysis with increased resolution to determine species and sub species of bacterial strains. This specification may show with greater clarity which OTUs are of greater importance to human health. Further studies need to be done on ticks in the Midwest to understand their microbial ecology in comparison to ticks elsewhere in the United States to determine how difference may impact local TBD ecologies. Understanding the interplay between these important variable may open new doors into our understanding of tick and tick borne pathogen ecology and prevention.

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Chapter 2:

Changing Tick and Host Population Dynamics and its Impact on Pathogen Prevalence in an Urbanizing Environment

Introduction:

The majority of emerging infectious diseases that threaten human health are zoonotic and more than 70% of zoonotic diseases are linked to wildlife (Jones, et al., 2008). Zoonotic pathogen emergence and spread requires competent animal reservoirs and for some transmission requires a competent vector species as well. In the Upper Midwest of the United States, diseases transmitted by *Ixodes scapularis*, the black-legged tick, including Lyme disease (*Borrelia burgdorferi, Borrelia mayonii*), tick-borne relapsing fever (*Borrelia miyamotoi*), Powassan encephalitis, human granulocytic anaplasmosis (*Anaplasma phagocytophilum*), babesiosis (*Babesia microti*) and a newly defined ehrlichiosis (*Ehrlichia muris* subsp. *eauclairensis*, subsp. nov.) are all emerging diseases of public health importance (Pritt, et al., 2017; Pritt BS1, 2016; Lantos, et al., 2017; Oliver, Bennett, Beati,, & Bartholomay, 2017; Mitchell, Reed, & Hofkes, 1996). The emergence and geographic spread of the causative pathogens has been linked to multiple factors including migratory birds serving as a dispersal vehicle for the tick vector, climate change impacts on vector and host ecology, changes in land use that favor the spread of the tick vector (Ogden, et al., 2008; Robinson, et al., 2015; Ogden, et al., 2006; Estrada-Peña & de la Fuente, 2014). While multiple regions have endemic tick and pathogen populations, their eco-epidemiology and evolutionary histories are distinct thus illustrating the need for regionally specific studies of TBD ecology (Margos, et al., 2012; Humphrey, Caporale, & Brisson, 2011). These pathogens exist in a complex regional eco-epidemiological system involving the tick vector, *I. scapularis*, and small mammal host reservoir species of varying competence.

Although white- tailed deer, *Odocoileus virginianus*, are sometimes erroneously referred to as a main reservoir for tick borne disease (TBD), they are not effective

reservoirs for pathogens such as *B. burgdorferi* and may in fact remove the pathogen from feeding ticks (Telford SR, 1988; Roome, 2017). Deer serve mainly as hosts for ticks and are of great importance for tick population size and geographic spread, although the impact of deer on tick geographic spread has come under scrutiny (Ostfeld, Canham, Winchcombe, & Keesing, 2006; Wilson, Levine, & Spielman, 1984). Defining trends in host population patterns as well as vector ecology is vital to understanding and predicting disease emergence and spread. While *I. scapularis* feeds on a diversity of vertebrates including reptiles, birds and mammals, multiple small mammals have been implicated in tick and TBD ecology, with *Peromyscus spp.* often being identified as the most important reservoir for TBD (Lynn, Oliver, Cornax, O'Sullivan, & Munderloh, 2017; Magnarelli, Anderson,, Stafford, & Dumler, 1997; Mather, Wilson, SI, Ribeiro, & Spielman, 1989). Additional reservoirs of various regional importance may include meadow voles (*Microtus pennsylvanicus*), woodland jumping mice (*Napaeozapus insignis*), redwood chipmunks (*Tamias ochrogeys*), Eastern chipmunks (*Tamias striatus*) and Western gray squirrels (*Sciurus griseus*), among others (Anderson, 1989; Foley & Nieto, 2011; Slajchert, Kitron, Jones, & Mannelli, 1997; Salkeld, et al., 2008).

Tick borne disease ecology has been traditionally explored in rural and woodland environments, but the role and potential public health risk of urban and urbanizing environments for TBDs is of increasing importance (LaDeau, Allan, Leisnham, & Levy, 2015; Hansford, et al., 2017; Rizzoli, et al., 2014; Maetzel, Maier, & Kampen, 2005). The significance of various reservoirs for TBD may be distinctly different in urbanizing regions where population dynamics have been altered through changes in landscape and biodiversity (Jahfari, et al., 2017; Schulze, Jordan, & CJ., 2005). Additionally*, I. scapularis* and host populations may function as invasive species, altering local ecology,

as they may have been recently introduced or recently increased their population numbers, in some of these areas due to their geographic spread (Oliver, Bennett, Beati,, & Bartholomay, 2017; Robinson, et al., 2015; Peng Wang, 2014; Myers, Lundrigan, Hoffman, Haraminac, & Seto, 2009). In order to determine the specific regional eco-epidemiology of tick and rodent populations as well as TBD agents within an urbanizing region a retrospective study was carried out. Twenty years of rodent capture and tick collection data were used to study population, geographic, and temporal relationships among tick species and their small mammal hosts. Additionally a subset of collected ticks was tested for TBD pathogens to investigate the relationships pathogenic agents had with their vectors and hosts.

Methods:

SAMPLE COLLECTION:

In 1990 the Metropolitan Mosquito Control District initiated the Lyme Disease Tick Surveillance Program to determine the distribution and prevalence of tick species, TBD agents and their small mammal reservoirs within the Minneapolis-Saint Paul seven county- metropolitan area. Sampling involved rodent trapping and ticks were collected from all trapped rodents. There are 100 collection sites around the Twin Cities (Figure 8). Of the 100 site sampled repeatedly, 56 are located north of the Mississippi River in Anoka (28 sites), Washington (25 sites), and Ramsey (3 sites) counties. The 44 repeat sampling sites located south of the Mississippi are distributed throughout the counties of Dakota (15 sites), Hennepin (14 sites), Scott (8 sites), and Carver (7 sites). (Neitzel, Jarnefeld, & Sjorgren, 1993; MMCD, 2017)

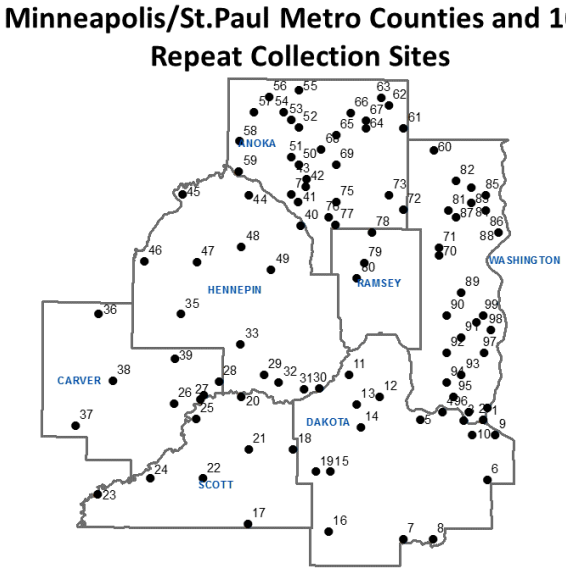

Figure 8. Map of the 7 Minneapolis/St. Paul Metro counties (Carver, Scott, Dakota, Washington, Ramsey, Anoka and Hennepin) encompassing the study area as well as the 100 repeatedly tested sites within those counties.

Sampling during each 27- week study period was divided into three 9-week sampling units, and all sites were sampled for 21 trap nights (7 traps x 3 consecutive nights) per period. Weeks of site visitation were randomly selected within each sampling period. One 300 foot transect was established at each sampling location and Sherman live traps (H. B. Sherman Traps, Inc., Tallahassee, Fla.), baited with peanut butter and oats, were placed along these transects at 50 foot intervals. All small mammals caught in the traps were identified and euthanized with CO2 according WHO protocol. Any ticks found on trapped mammals were removed, identified and stored in 97% ethanol for later testing (Neitzel, Jarnefeld, & Sjorgren, 1993; MMCD, 2017).

Pathogen Testing:

A subset of ticks were tested for pathogens. DNA was extracted from ticks collected in 2000, 2004, 2008, 2012, and 2014 to provide a longitudinal analysis spanning 14 years. Extracted DNA was subjected to metagenomics testing to determine presence of pathogenic microbes. All nymphs from each year were included to achieve a more comprehensive picture of pathogen prevalence and host relationships.

DNA Extraction:

Extraction was completed at room temperature in a separate room using a clean hood where PCRs were not carried out to minimize contamination of reactions. All ticks were rinsed with 95% ethanol before extraction to remove debris and storage fluid in order to ensure the full pathobiome was tested rather than just the internal microbiome (Benson, Gawronski, Eveleigh, & Benson, 2004). DNA extraction was carried out with an adapted protocol using the DNeasy® Blood and Tissue Kit Quick-Start Protocol (Qiagen, Inc.). As per the Qiagen recommendations for tick DNA extraction for *Borrelia* testing, carrier RNA (VX Carrier RNA, Qiagen, Inc.) was added to increase DNA volume.

Primary/Secondary PCR Amplification:

The V4 region of 16S rRNA was amplified using a two-step PCR protocol. The primary amplification was done using an ABI7900 qPCR machine. The following reaction mixture was used: 3 µl template DNA, 0.48 µl nuclease-free water, 1.2 µl 5x KAPA HiFi buffer (Kapa Biosystems, Woburn, MA), 0.18 µl 10 mM dNTPs (Kapa Biosystems, Woburn, MA), 0.3 µl DMSO (Fisher Scientific, Waltham, MA), 0.12 µl ROX (25 µM) (Life Technologies, Carlsbad, CA), 0.003 µl 1000x SYBR Green, 0.12 µl KAPA HiFi Polymerase (Kapa Biosystems, Woburn, MA), 0.3 µl forward primer (10 µM), 0.3 µl

reverse primer (10 µM). Cycling conditions were: 95°C for 5 minutes, followed by 20 cycles of 98°C for 20 seconds, 55°C for 15 seconds, and 72°C for 1 minute. The primers for the primary amplification contained both 16S-specific primers (V1_27F and V3_V34R), as well as adapter tails for adding indices and Illumina flow cell adapters in a secondary amplification. The following primers were used (16S-specific sequences in bold): Meta_V4_515F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GTGCCAGCMGCCGCGGTAA**) and Meta_V4_806R (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GGACTACHVGGGTWTCTAAT**).

The amplicons from the primary PCR were diluted 1:100 in sterile, nuclease-free water, and a second PCR reaction was set up to add the Illumina flow cell adapters and indices. The secondary amplification was done on a fixed block BioRad Tetrad PCR machine using the following reaction mixture: 5 µl template DNA, 1 µl nuclease-free water, 2 µl 5x KAPA HiFi buffer (Kapa Biosystems, Woburn, MA), 0.3 µl 10 mM dNTPs (Kapa Biosystems, Woburn, MA), 0.5 µl DMSO (Fisher Scientific, Waltham, MA) 0.2 µl KAPA HiFi Polymerase (Kapa Biosystems, Woburn, MA), 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM). Cycling conditions were: 95°C for 5 minutes, followed by 10 cycles of 98°C for 20 seconds, 55°C for 15 seconds, 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes. The following indexing primers were used (X indicates the positions of the 8 bp indices): Forward indexing primer: AATGATACGGCGACCACCGAGATCTACACXXXXXXXXTCGTCGGCAGCGTC and Reverse indexing primer: CAAGCAGAAGACGGCATACGAGATXXXXXXXXGTCTCGTGGGCTCGG (Gohl, et al., 2016).

Normalization and Sequencing:

The samples were normalized using a SequalPrep capture-resin bead plate (Life Technologies, Carlsbad, CA) and pooled using equal volumes. The final pools were quantified via PicoGreen dsDNA assay (Life Technologies, Carlsbad, CA) and diluted to 2nM total DNA. 10 µl of the 2 nM pool was denatured with 10 µl of 0.2 N NaOH, diluted to 8 pM in Illumina's HT1 buffer, spiked with 15% phiX, heat denatured at 96*C for 2 minutes, and sequenced using a HiSeq 500 cycle kit (Illumina, San Diego, CA)**.**

Sequence Processing

Sequences were processed through the bioinformatics software Mothur (version 1.35.1). Quality control parameters were based on the Schloss MiSeq SOP pipeline (http://www.mothur.org/wiki/ MiSeq_SOP) (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013) Parameters for discarding sequences were unidentifiable barcode and/or reverse primer sequence. Sequences with greater than 2 errors in their barcode or primer sequence, homopolymer runs greater than 8 bp, any ambiguous bases, or less than 150 bp were filtered out of the dataset. SILVA SEED bacterial 16S database was utilized for sequence alignment (Schloss, 2009) . Remaining high quality and chimera free sequences were placed into operational taxonomic units (OTUs) using 97% similarity. SILVA bacterial 16S database assigned genus level classification to all remaining high quality sequences. The top 30 most abundant OTUs were blasted using National Center of Biological Information (NCBI) Web blast to determine species similarity (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul, Gish, Miller, Myers, & Lipman, 1990).

A total of 609 individual ticks were sequenced from five separate years (2000, 2004, 2008, 2012, and 2014). Following sequence read quality trimming and chimera removal, the range of coverage per sample was >98%, and the average read length was >240 nt. Samples were rarefied to 1,000 sequences. Rarefication resulted in removal of 110 of the 609 ticks from analysis based on a low number of quality reads (<1000). 491 ticks were included in the final analysis spread over 5 collection years (2000=177, 2004=50, 2008=80, 2012=122, and 2014=61).

Statistics:

Analysis was performed on records from 23,590 individual trapped mammals from 1993 to 2013 and DNA from 491 *I. scapularis* nymphs. Statistical analyses were performed in R (R Development Core Team, 2012), Excel and Mothur version 1.35.1. Linear regression, t-tests and Pearson correlation tests were performed in Excel. Chi square tests were performed in R. Analysis was performed on 23,590 individual trapped mammals and DNA from 491 *I. scapularis* nymphs. A p value of ≤ 0.05 was considered significant.

Statistical Spatial Analysis:

Spatial analysis was done using ArcMap and ArcGIS 10.1 (Esri Inc). Space-Time Cluster analysis was performed using SaTScan v9.4.4 (SatScan.org). Spatial clustering occurs when more *I. scapularis* ticks are collected at a particular site than would be expected by chance. The clusters of *I. scapularis* were considered significant only after adjustment for spatial variations in the density of the total tick population. (Wu, 2010; Kulldorff, Rand, & Williams, 1996)

Directional space- time analysis was done with Cluster Seer version 2.5 using the Spatial-Temporal Direction method with adjacent parameters and 1,000 Monte Carlo randomization runs (BioMedware, Inc.). The Direction method tests for space-time interaction of retrospective, individual level data, and calculates the average direction of advance of a spread of *I. scapularis* based on collection year and site (Jacquez & Oden, 1994). A p value of ≤ 0.05 was considered significant.

Results:

From 1993 to 2013, a total of 23,590 (1123.3 ±326.6 per year) small mammals were trapped, of which 3,970 were infested with ticks (16.8%). *Peromyscus leucopus*, the white footed mouse, was the most abundant animal trapped with 19,493 (928.2 ± 282.4 per year) individuals being trapped accounting for 82.6% of mammals (Figure 9). *Tamias striatus*, the eastern chipmunk, accounted for 6.6% of total trapped small mammals (74.6 ±34.9 per year), and *Clethrionomys gapperi*, the southern red backed vole, accounted for 5% of mammals (56.8 ±23.5 per year). The northern short-tailed shrew, *Blarina brevicauda*, only made up 3.5% of total captures (39.8 ±28.0 per year). Other trapped species included *Microtus pennsylvanicus, Spermophilus tridecemlineatus, Zapus hudsonius, Mustela erminea, Tamiasciurus hudsonicus, Glaucomys volans, Sorex articus, Sorex cinereus,* and *Mus musculus*. These other species accounted for 2% of captures (23.9 ±9.9 per year).

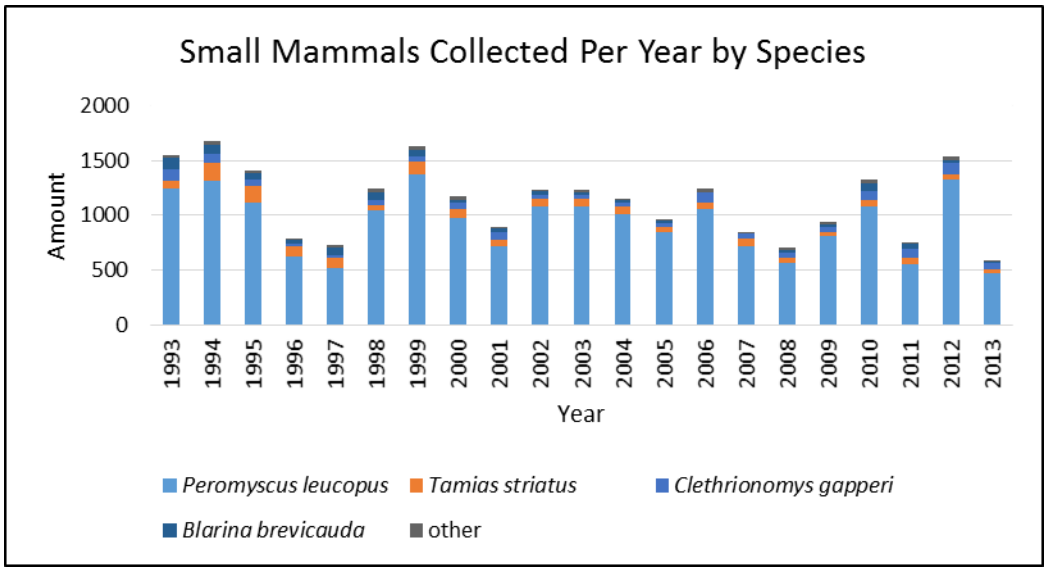

Figure 9: Total small mammals collected per year showing *P. leucopus* as the main mammal collected (82.6%) and *T. striatus* as the second most trapped animal (6.6%). Years were highly variable with1993, 1994, 1999, and 2013 were the most abundant years for mammal trapping all with levels over 1,500 individuals.

From 1993-2013 a total of 31,323 ticks were collected from trapped small mammals. Larval ticks were most abundant on small mammals with 14,029 *Dermacentor variabilis* (668.0 ± 261.7 per year) and 12,032 *I. scapularis* (573.0 ± 340.0 per year) larvae collected. Nymphs collected consisted of 3,076 *D. variabilis* (146.4 ± 61.3 per year) and 2,039 *I.scapularis* (97.1 ± 64.4 per year). *Dermacentor variabilis* accounted for 55% of ticks collected while *I. scapularis* made up the other 45% and 147 ticks were listed as 'other'. Total number of ticks collected was highly variable depending upon year (Figure 10). The lowest collection years were 1996 (n=724), 1997 (n=691), and 2013 (n=370), and the highest collection years were 2000 (n=3,152), 2002 (n=2,185) and 2012 (n=2,223).

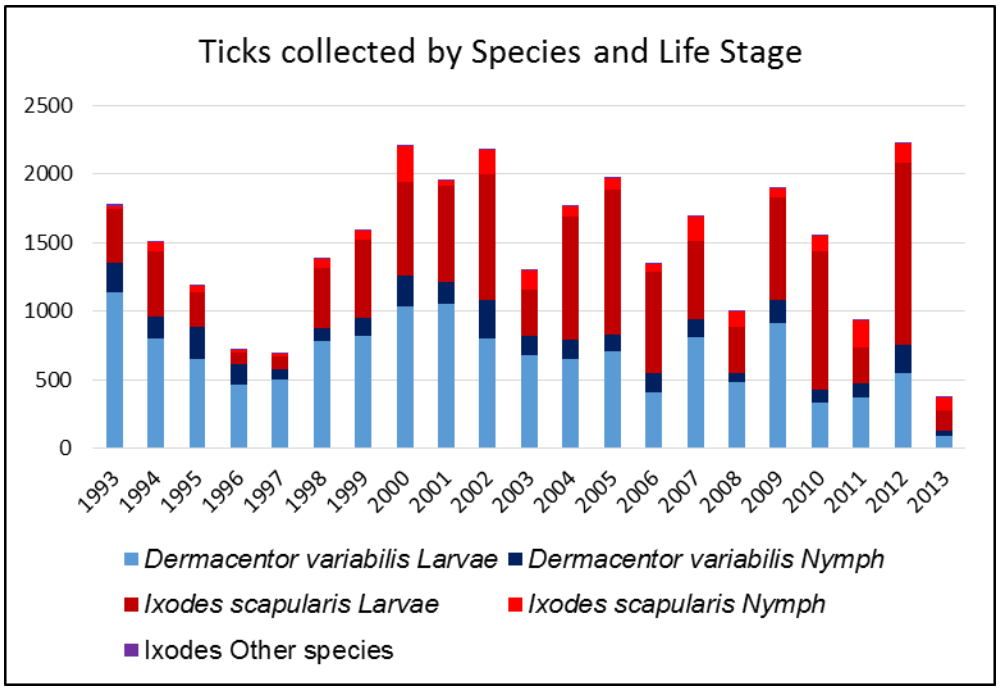

Figure 10 : Total ticks collected per year showing *species and life stage/* Years were highly variable with the lowest collection years were 1996 (n=724), 1997 (n=691), and 2013 (n=370) and the highest collection years were 2000 (n=3,152), 2002 (n=2,185) and 2012 (n=2,223).

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Variability of small mammal species by year was similar to that of ticks with 1996, 1997 and 2013 being the lowest trap years. 1993, 1994, 1999, and 2013 were the most abundant years for mammal trapping all with levels over 1,500 individuals. As expected, there was significant correlation between the number of ticks collected and the number of mammals caught (Pearson Coefficient= .51, P-value= .018).

Peromyscus leucopus was the greatest total carrier of ticks (n=29,181) followed by *C. gapperi* (n=2,513) and *T. striatus* (n=1,601). Considering *I. scapularis* alone, *C. gapperi* (n=284) was a much less important host compared to *P. leucopus* (n=13,726) and *T. striatus (*n=1,469). Nymphal tick counts that revealed that *P. leucopus* and *T. striatus* harbored the vast majority of ticks, 1,178 and 853 respectively, while captured *C. gapperi* individuals hosted only 55 *Ixodes* nymphs.

Species	Total *Ixodes*	Total tick Burden (SD,Var)	Total nymps	Nymph tick burden (SD, Var)
Peromyscus leucopus	13726	0.6637(2.9, 8.2)	1178	0.057 (0.4,0.16)
Blarina brevicauda	103	0.1202(1.3, 1.6)	2	0.0023(0.05, 0.002)
Clethrionomys gapperi	284	0.219(0.7, 0.6)	55	0.0424(0.28, 0.08)
Sorex cinereus	10	0.0279(0.2,0.04)	1	0.0028(0.05,0.003)
Tamias striatus	1469	*0.8974(4.2, 17.3)	853	*0.5211(2.6,6.72)
Microtus pennsylvanicus	2	0.0417(0.2, 0.04)	0	0
Zapus hudsonius	166	2.1282(10.6, 115.9)	21	0.2692(1.17,1.37)

Table 4: Total collected *I. scapularis* ticks and collected *I. scapularis* nymphs by small mammal host species with host tick burden (mean tick amount per rodent). Asterisk (*) indicating that *T. striatus* had a significantly ($p<0.03$) higher tick burden than other species excluding *Z. hudsonius* which had an elevated total tick burden but not a significant nymph burden.

The *I. scapularis* burden per individual differed among mammal species (Table 4). *Tamias striatus* carried the greatest *Ixodes* species tick burden among captured mammals except for *Zapus hudsonius*. The *I. scapularis* burden was significantly higher for *T. striatus* than *P. leucopus* in total *Ixodes* ticks (*P.l*=0.66, *T.s*.=0.89, Welch t-test p value=0.026) and for *Ixodes* nymphal burden (P.l.=0.057, T.s.=0.52, Welch t test p value<0.001). The meadow jumping mouse, *Zapus hudsonius*, had a comparatively high tick burden for *Ixodes* nymphs, 0.27, and an extremely high comparative tick burden for total *Ixode*s, 2.13; however, the rodent was rarely caught and the number of ticks on them was highly variable between individuals (±10.6). The average tick burden per mammal for *I. scapularis* has been on the rise (Linear regression, R^2 =0.37, p= 0.004) (Figure 11). Tick burden for *D. variabilis* has been trending down although not in a significant manner (p=0.25).

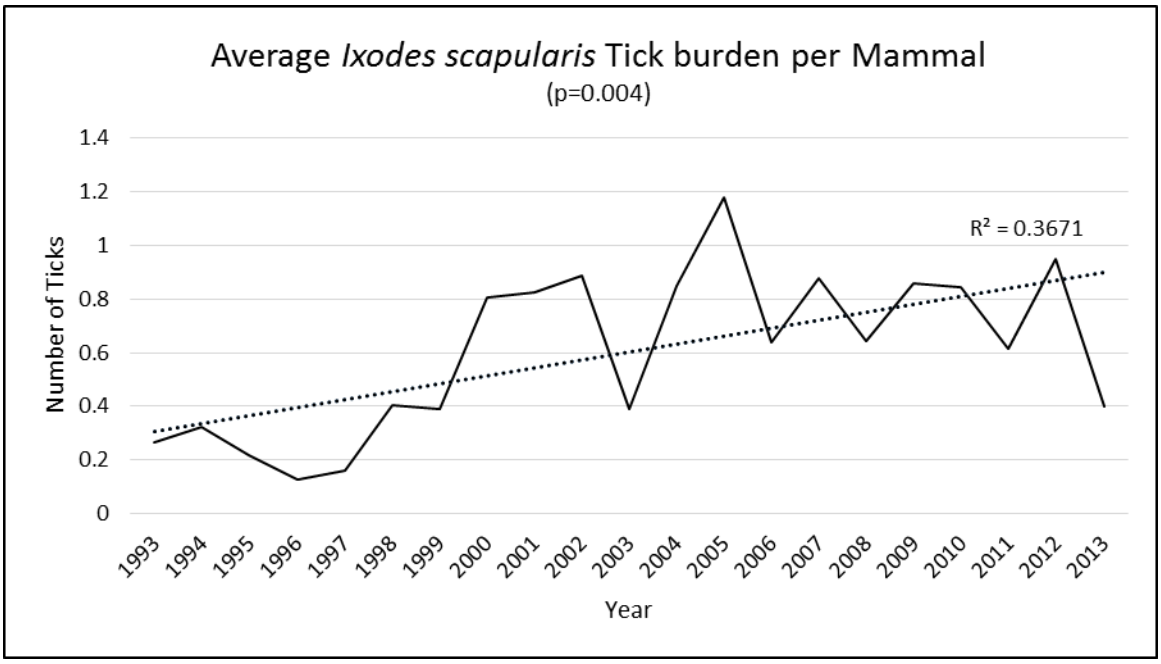

Figure 11: When considering all trapped small mammals together, there was a significant increase in total *I. scapularis* tick burden over the course of the study. Linear regression shows significance (p=0.004).

Tick population numbers have shifted significantly over the course of the study. In 1990, *D. variabilis* accounted for 93% of all ticks collected. The proportion of *I. scapularis* among ticks collected has been steadily growing, replacing *D. variabilis*. *Ixodes scapularis* became the most common species identified on rodents in 2005 (55%), and by 2010 made up 77% of ticks collected. The increase in *I. scapularis* collections followed a significant linear trend (Linear regression, R^2 =0.78, P=value<0.001) showing their establishment as the dominant tick species parasitizing small mammal populations over the past two decades (Figure 12). Total numbers of ticks collected have not followed a significant trend upwards and do not explain the increase in percentage of *I. scapularis* collected each year. Additionally, total number of *D. variabilis* collected, while highly variable, has trended lower (Linear regression, p

=0.01) while *I. scapularis* numbers, though also highly variable, have trended higher (Linear regression, p =0.05).

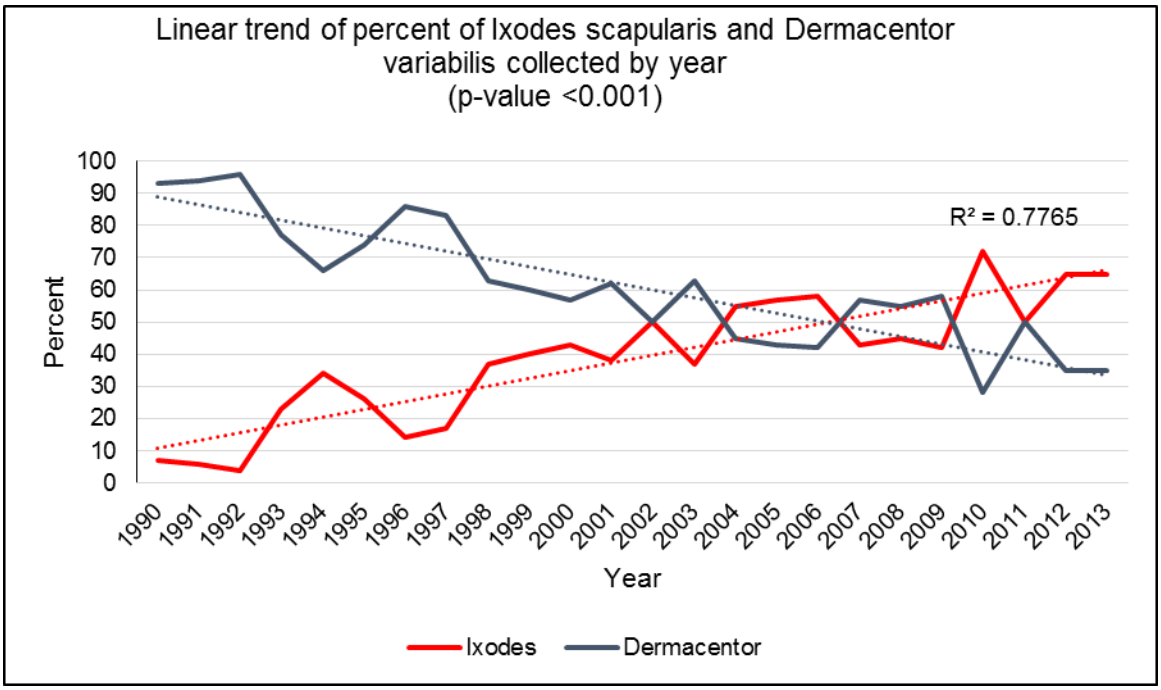

Figure 12: The increasing proportion of *I. scapularis* among ticks collected as they replace *D. variabilis* in the numbers of ticks collected. *Ixodes scapularis* made up more than ¾ (77%) of ticks collected by 2010. The increase in *I. scapularis* collections follows a significant linear trend (Linear regression, R^2 =0.78, P=value<0.001)

Spatial analysis of the *I. scapularis* range shows it expanding through the Twin Cities metro area from the Northeast in Anoka and Washington counties and following a clear directional pattern through the duration of the study. Initial expansion of tick populations followed a South by Southwest course. Spatial analysis showed that between 2003 and 2008 populations expanded directly South before moving westward between 2009 and 2014 in a clockwise direction around the urban metro area. Overall,

Ixodes populations have taken more than two decades to circumnavigate the Twin Cities metro area with the western counties of Hennepin, Carver and Scott just recently reporting moderate *Ixodes* populations (Figure 13a).

Along with the trends in population movement, the first major significant space-time cluster of *Ixodes* ticks emerged in Anoka County from 2000-2010 (observed/expected ratio of 1.39, p-value <0.001) indicating this was the highest risk area during that time period. The second space-time cluster occurred from 2010-2014 where risk shifted South to Washington, Dakota and Ramsey Counties (observed/expected ration of 1.64, p-value<0.0001) (Figure 13b). After the shift in 2010 Anoka only had a few high risk sites remaining. This change points to an observed decrease in *Ixodes* captures from Anoka County after 2005 as well as an increase in Washington and Dakota County. The changing *Ixodes* population in these counties follows the directional movement trends observed as well (Figure 14). Neither Scott County nor Hennepin County showed significant populations of *I. scapularis* until 2009. Since then tick populations have been expanding into these counties in increasing numbers with the exception 2013 where trapping numbers were low for all counties.

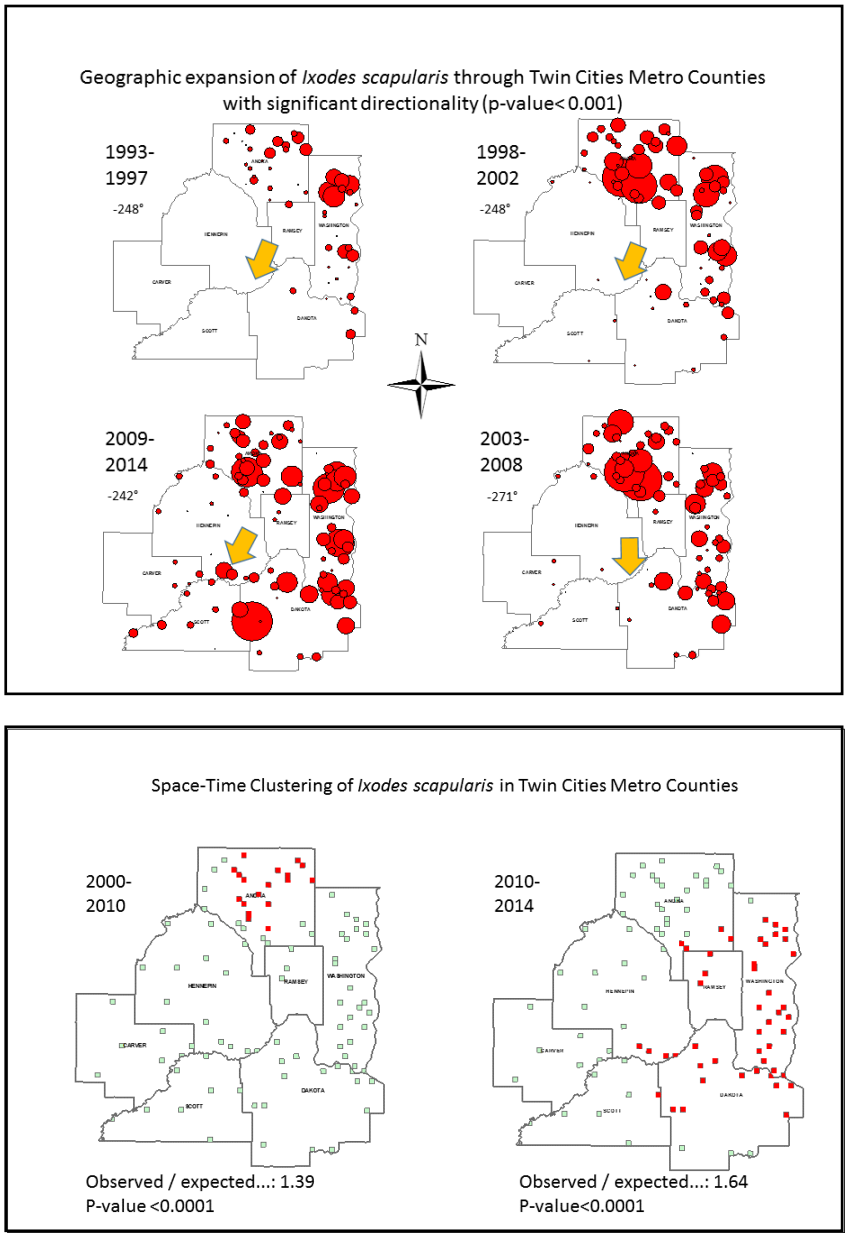

Figure 13a (top) & 13b (bottom): Figure 13a shows the relative *Ixodes* population growth, range expansion and directional movement by site over the course of the study period. There is statistically significant directional movement around the Twin Cities Metro area indicating urban areas may be impacting geographic spread of the vector. Figure 13b describes significant space time clustering of *I. scapularis* ticks indicating Anoka county was the original significant cluster of tick populations although that has now moved south to Washington, Dakota and Ramsey Counties.

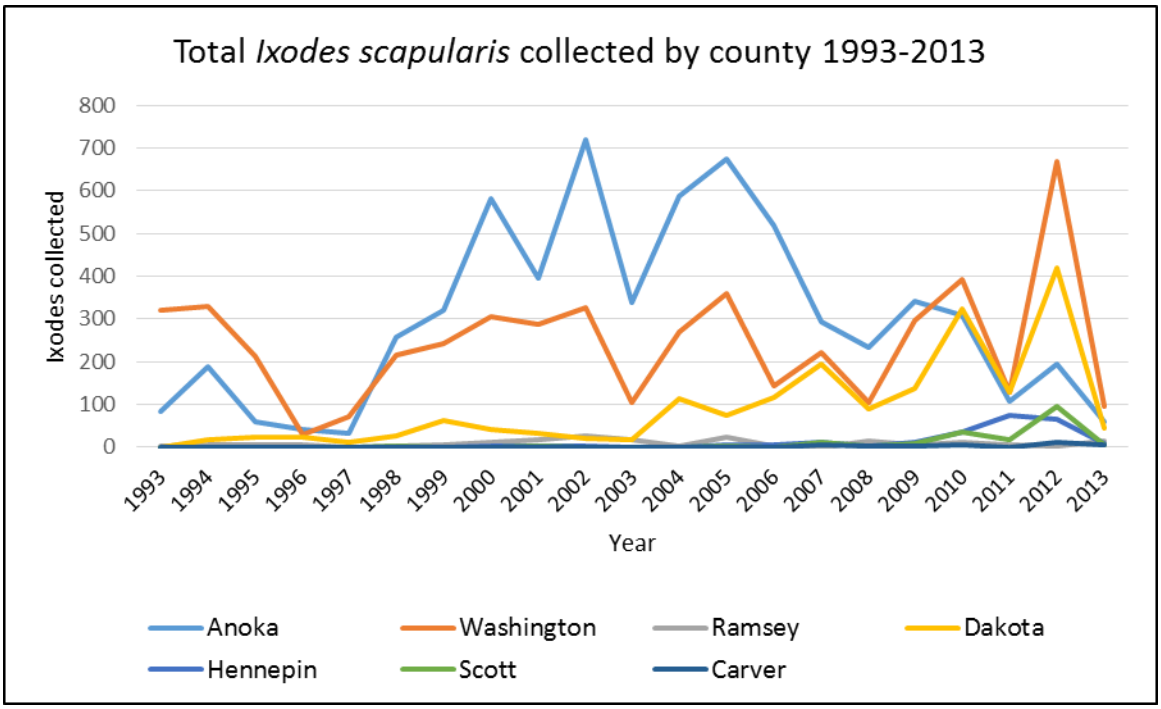

Figure 14: Total *I. scapularis* ticks collected by year indicating a fall in collections from Anoka County as there are greater numbers being collected from Daktoa and Washington counties. Note the increase in Scott and Hennepin counties after 2009 when the ticks began establishing themselves in the region.

Infection with known TBD agents was identified based on presence of OTU 2 (*Borrelia*), OTU 4 (*Anaplasma*) and OTU30 (*Borrelia miyamotoi*). *Rickettsia* spp. were identified in 292 of 491 samples (59.5%). *Borrelia burgdorferi ss*. was identified in 162 samples (33%) with the 100 top results from the NCBI BLAST having >99% identity with *Borrelia burgdorferi sensu stricto.* While *Borrelia bissettii* was also identified, because it could not be differentiated based on the amplified portion of the 16s gene, the occurrence of *Borrelia* is almost certainly *B. burgdorferi ss.* as *B. bissettii* transmission is generally focused on the West Coast and in some Southern states (Margos, et al., 2010). *Anaplasma spp.* was found in 32 samples (6.5%) of which all species identified in NCBI returns with >99% match were *Anaplasma phagocytophilum* (non-species identified returns were listed as 'uncultured *Anaplasma* species). Co-infection of *Borrelia*

and *Anaplasma* occurred in 17 samples (3.5%) with 16 of those samples testing positive for *Rickettsia*, *Borrelia*, and *Anaplasma*. While *P. leucopus* served as the main host for total number of ticks it was not significantly different from *T. striatus* as a host for nymphs. *T. striatus* was, however, significantly more important as a host for infected ticks. *T striatus* scored significantly higher in *B. burgdorferi* infection (X^2= 15.003, df = 1, p = 0.0001), as well as in *A. phagocytophilum* infection (X^2 = 8.618, df = 1, p = 0.003) and co-infection with *A. phagocytophilum* and *B. burgdorferi* (X^2 = 5.1068, df = 1, p = 0.024). *Borrelia miyamotoi* infection was not significantly correlated to any host species (Table 5).

	Number of *I. scapularis* nymphs	***Borrelia burgdorferi ss.***	***Anaplasma phagocytophilium***	**co-infected (Ap and Bb)**	***Borrelia miyamotoi***
Peromyscus leucopus	266	70(26.3)	10(3.8)	5(1.8)	2(0.7)
Clethrionomys gapperi	16	2(12.5)	0	0	1(6.3)
Tamias striatus	208	*90(43.3)	*22(10.6)	*12(17.3)	3(1.4)
Zappus hudsonius	1	0	0	0	0
Total	491	162(32.9)	32(6.5)	17(3.5)	6(1.2)

Table 5: Pathogen prevalence among I. scaularis nymphs collected from various small mammal species with total number followed by percent in parenthesis (%) . A significantly larger proportion of ticks collected from *T. striatus* were positive for *B. burgdorferi* (p=0.0001), *A. phagocytophilum* (p=0.003), or a co-infection of the two (p=0.024). Asterisk (*) indicates significantly higher infection prevalence.

Discussion

This study highlights important changes to local tick and rodent population dynamics that have been occurring over the past two decades in the Twin Cities metro. Tick populations were shown to be going through an ecological shift as *I. scapularis* has risen as the dominant species on small rodent hosts. Additionally, significant tick range

expansion and movement was described. While *P. leucopus* is still shown to be the primary rodent host for juvenile ticks, our data indicated a large and significant role for *T. striatus* in local tick and TBD ecology which needs to be investigated further. It is important for researchers and public health professionals to pay attention to areas in the path of *I. scapularis* range expansion, specifically Carver, Scott and Hennepin Counties, which are just beginning to see increasing in tick populations, as transmission is likely to increase in those areas.

***Ixodes scapularis*: an invasive species**

The range expansion of the black-legged tick, *I. scapularis* and its associated pathogens has been well documented (Oliver, Bennett, Beati,, & Bartholomay, 2017; Robinson, et al., 2015; Ogden, et al., 2006; Peng Wang, 2014). Our research describes the specific movement and expansion of *I. scapularis* around the Twin Cities Metro area. The first record of *I. scapularis* ticks in Minnesota is from 1980 when two ticks were collected from hikers, although there were anecdotal reports of ticks in regions 50-100 miles north of the Twin Cities during the 1970s (Williams, Vance, Hedberg, Foley, & Osterholm, 1982). A larger survey conducted between 1985 and 1986 showing populations of Ixodes scapularis concentrated approximately 50 miles north of the Twin Cities (Drew, Loken, Bey, & Swiggum, 1988). The expansion of *Ixodes* from Northeast of the Twin Cities supports evidence that, at some time before the study period, the tick vector may have entered Minnesota somewhere along the Wisconsin border north of the Twin Cities and is currently expanding westward (Dennis, Nekomoto, Victor, Paul, & Piesman, 1998; Eisen, Eisen, & Beard, 2017). While the state expansion, reaching into areas previously assumed to be unsuitable for tick populations, has moved Northwest into North Dakota, our data point out the tick population around the Twin Cities is moving

south and slightly west (Robinson, et al., 2015; Eisen, Eisen, & Beard, 2017; Russart, Dougherty, & Vaughan, 2014).

There is strong indication that the tick population expansion has been slowed around the Twin Cities metro with more than two decades passing before ticks circled the urban center. Across the United States ticks have expanded their geographic range at a much faster rate than they have around Twin Cities Metro area (Robinson, et al., 2015; Oliver, Bennett, Beati,, & Bartholomay, 2017; Eisen, Eisen, & Beard, 2017). The range for *I. scapularis* in Minnesota has extended far beyond the Twin Cities at a much faster rate with large portions of the state being covered before the few counties around the metro (Eisen, Eisen, & Beard, 2017; Robinson, et al., 2015). This slow progression through the region points to possible obstruction of movement in urban regions. This may be due to the dependence on deer for long distance movement, which may be highly reduced in the urban environment. With deer as a main dispersal agent, ticks would have spread much faster in the region due to the larger ranges of deer (Madhav, Brownstein, Tsao, & Fish, 2004). The larger population centers and urbanization of peri-urban areas may have impeded deer population size or movement and, therefore, their efficacy as a primary driver of geographic expansion of the *Ixodes* vector. The slow progression may point to the role of the small mammals and rodents or other hosts such as birds and dogs as the main agents of expansion in an urban environment.

Ixodes **and** ***Dermacentor*** **Competition**

Our evidence indicates an ecological shift in tick population dynamics between the two main tick species, *I. scapularis* and *D. variabilis*, with *I. scapularis* replacing *D. variabilis* on small mammal host species. To our knowledge, this apparent competition and replacement of tick species and its implication for host-pathogen relationships has

not been previously documented between these species. There is evidence *I. scapularis* will compete with *D. albipictus* for space on the same deer host showing that competition between tick species is possible as part of resource partitioning (Baer-Lehman, et al., 2012; Schoener, 1974). *Ixodes scapularis* and *D. variabilis* share similar small mammal host preference for their juvenile stages (Kollars, 1996). Despite favoring similar hosts, our data show comparative numbers for *D. variabilis* have decreased while *I scapualris* have increased on these preferred hosts, indicating that these host-vector interactions can drive an ecological shift. It is unclear if total *D. variabilis* populations have decreased with the influx of *I. scapularis* or if they have shifted to other hosts not focused on in this study, such as larger mammals like dogs, raccoons and skunks. Environmental and host community conditions may also play a role in *I. scapularis* showing apparent dominance within the ecological landscape. Understanding these possible ecological shifts is vital in employing productive vector control and for predicting vector habitat and geographic spread.

Rodent host- vector- pathogen dynamics

The results of this study provide further evidence that Eastern chipmunks, *T. striatus*, play a significant role in tick ecology. Historically, most studies on TBD have focused the ecology of pathogens, ticks and their hosts in the Northeast United States and have indicated that *P. leucopus*, the white footed mouse, is the primary reservoir for *B. burgdorferi* among other TBD (Schmidt, Ostfeld, & Schauber, 1999). The vital importance of *P. leucopus* to tick ecology is supported with our data as *P. leucopus* was the most abundant small mammal captured and the main source for collected *I. scapularis* ticks. However, while *P. leucopus* has been shown to be the most competent

reservoir for the disease in many areas, *T. striatus* are very competent reservoirs as well (McLean, Ubico, & Cooksey, 1993).

Multiple newer studies indicate regional and geographic differences in the eco-epidemiology of TBD and a need for specific regional and local analysis (Foley & Nieto, 2011; Margos, et al., 2012; Humphrey, Caporale, & Brisson, 2011; Nadolny, et al., 2015). In the Upper Midwest, eastern Chipmunks have a larger regional role in pathogen transmission and maintenance (Mannelli, Kitron, Jones, & Slajchert, 1993; Slajchert, Kitron, Jones, & Mannelli, 1997). One study from New Jersey indicated a larger role for chipmunks in urban and peri-urban environments (Schulze, Jordan, & Schulze, 2005). Our data add to current evidence that supports the position held by the Eastern chipmunk as a secondary host for *I. scapularis* juveniles and an important reservoir for TBD in the region (Johnson, Kodner, Jarnefeld, Eck, & Xu, 2011). Previous studies have shown high infection rates as well as high prevalence of TBD pathogen DNA in chipmunks, suggesting that they gain no immunity or resistance to pathogens of human importance (Johnson, Kodner, Jarnefeld, Eck, & Xu, 2011). *Peromyscus leucopus* very rarely survive past 1 year of age in the wild while chipmunks can regularly survive beyond 2-3 years (Schug, Vessey, & Korytko, 1991; Nowak, 1999). Coupled with the longer survival time and the greater tick burden shown in our study, chipmunks have the opportunity to feed high numbers and multiple generations or cohorts of ticks allowing for the increased transmission of TBD. This increase in potential transmission would be important in urban areas where there are possibly fewer mice and ticks may move to other functional hosts, in this case chipmunks (Fish & Dowland, 1989). Chipmunk and tick populations around the Twin Cities should be investigated in greater detail, especially in higher population centers where mouse populations may have declined.

The population of chipmunks may also be underestimated in our study due to the use of small Sherman traps which are better designed for catching mice rather than chipmunks (H.B. Sherman Traps, Inc., Tallahassee, FL, USA).

Our study supports the notion of focused regional and local analysis being important to understanding tick, rodent and pathogen ecology. Additionally, we show the influence that an urbanizing environment can have on the complex eco-epidemiology of this system. Additional study should be done on environmental and spatial characteristics of the urban landscape across the Twin Cities metro. It could be highly valuable in TBD control and surveillance to determine the impact that specific landscape features and host species have on expansion in an urbanizing environment in the Twin Cities Metro area. An assessment of the impact of population density, weather cycles, climate change and land cover should be incorporated in the analysis to determine their influence on tick and rodent populations. Additionally, more information on deer population and movements within the urban areas may help to determine why the progression of ticks has been comparatively slow in the region. Further studies are needed to clarify the relationship between *I. scapularis* and *D. variabilis* and their host community in Minnesota and elsewhere. As *I. scapularis* ticks continue to move westward around the Twin Cities, mouse and chipmunk population surveys could help to predict where TBD may affect human population and cause the greatest risk to public health.

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Chapter 3:

Impact of Pathogen Acquisition and Host Meal on *Ixodes scapularis* Pathobiome

Introduction:

Ixodes scapularis, or black-legged ticks, are the primary vector of multiple pathogens of public health importance including *Borrelia burgdorferi*, the cause of Lyme disease, the most prevalent vector borne disease in the United States (Piesman & Eisen, 2008; CDC, 2013). Additionally, *I. scapularis* is an important vector of tick-borne relapsing fever (*Borrelia miyamotoi*), Powassan encephalitis, human granulocytic anaplasmosis (*Anaplasma phagocytophilum*), babesiosis (*Babesia microti*) and a newly defined ehrlichiosis (*Ehrlichia muris* subsp. *eauclairensis*, subsp. nov) (Pritt, et al., 2017; Pritt, et al., 2016; Lantos, et al., 2017; Oliver, Bennett, Beati, & Bartholomay, 2017; Mitchell, Reed, & Hofkes, 1996; Krause, Fish, Narasimhan, & Barbour, 2015). *Ixodes scapularis* ticks and the pathogens they transmit have recently increased their geographic range in the United States with main foci in the Northeastern United States and the Upper Midwest (Eisen, Eisen, & Beard, 2017; Oliver, Bennett, Beati,, & Bartholomay, 2017; Robinson, et al., 2015).

The assembly of all microbes associated with ticks (referred to as the microbiome), whether pathogens or not, is now recognized to influence vector competence in several ways that are not completely understood (Weiss & Aksoy, 2011). Current widespread use and advances of high through-put second generation sequencing technologies have altered our understanding of the microbiome and its importance. With multiple species, the gut microbiota, or internal microbiome, is increasingly regarded as having a pivotal role in host health, fitness, and behavior

(Nicholson, et al., 2012; Ezenwa, Gerardo, Inouye, Medina, & Xavier, 2012; Charles., Joergermann & Aliprantis, 2015). Inversely, loss of host health and fitness, such as through infection or disease, can impact the microbiome (Youmans, 2015). Evidence has shown arthropods can be profoundly impacted by changes in their microbiome. Dysbiosis, caused by exposure to antibiotics or changes in nutrition, has a deleterious systemic effect on honey bees, *Apis mellifera*, and can negatively impact colony health (Maes PW1, 2016; Raymann, Shaffer, & Moran, 2017). Aging related issues in *Drosophilia* cause shifts in the microbiome that contribute to an immune response and subsequent death (Clark, et al., 2015). In addition to host related health impacts, the microbiome of arthropods vectors can impact their competence for pathogen transmission (Weiss & Aksoy, 2011). *Leishmania infantum*, a parasitic agent of leishmaniasis, relies on the specific gut microbiome of its vector, *Lutzomyia longipalpis*, to complete its life cycle in order to become infectious (Kelly, et al., 2017). The microbiome of *Anopheles* mosquitoes impacts multiple stages in the malaria-causing *Plasmodium* life cycle including reproduction, development and transmission (Pumpuni, Demaio, Kent, Davis, & Beier, 1996; Cirimotich, et al., 2011; Gonzalez-Ceron L1, 2003). The tick microbiome also of importance to the tick vector and pathogenic agents of TBD. Changes in the microbiome of *Dermacentor andersoni*, the Rocky Mountain wood tick can significantly impact the presence of pathogenic agents (Gall, 2016). Additionally, the gut microbiota of *Ixodes scapularis* plays an important role in infection of *B. burgdorferi* (Narasimhanm, et al., 2014).

The variables impacting the *Ixodes* microbiome have yet to be understood. External and internal variables impact microbial community (Vayssier-Taussat, et al., 2015). For example, tick life stage, sex and species are important factors in microbial

community (Will Van Treuren, 2015; Kurtti, et al., 2015; Zolnik, Prill, Falco, Daniels, & Kolokotronis, 2016). Additionally, geography, whether through distance or change in environment, plays a role, as well, although the distance required for changes in tick microbiome to be observed is variable (Will Van Treuren, 2015; Clay & Fuqua, 2010; Ponnusamy L, 2014).

Through their questing activities ticks come in direct contact with bacteria on plants, in soil, and in some surface water. Additionally, *I. scapularis*, being obligate blood feeders, must take blood meals from multiple hosts in order to reach adulthood. These feeding events expose ticks to bacteria residing on the skin of a variety of hosts as well as bacteria that exist in the host blood meal. These potentially important environmental and host bacterial exposures are part of the broader pathobiome of ticks and are considered in our analysis (Vayssier-Taussat, et al., 2015).

Previous data analysis indicated that year of collection was significant as a factor in microbial commuity diversity. In order to clarify and understand other variables that may be important to driving microbial diversity this study examined the impact that pathogenic microbe presence, *B. burgdorferi* and *A. phagocytophilum*, and host have on the pathobiome of *I. scapularis*. Examining the interactions between vectors, hosts, pathogens and the commensal microbial community will help to build a clearer picture of the complex eco-epidemiology of TBD.

Methods:

SAMPLE COLLECTION:

In 1990, the Metropolitan Mosquito Control District began the Lyme Disease Tick Surveillance Program to determine the distribution and prevalence of tick species, tick

borne disease (TBD) agents and their small mammal reservoirs within the Minneapolis-Saint Paul metropolitan area. Sampling was done through rodent trapping and ticks were collected from all trapped rodents. There were 100 collection sites in the Twin Cities metro area (Figure 15). Of the 100 repeated sites, 56 were located north of the Mississippi River in Anoka (28 sites), Washington (25 sites), and Ramsey (3 sites) counties. The 44 repeat sampling sites located south of the Mississippi were distributed throughout the counties of Dakota (15 sites), Hennepin (14 sites), Scott (8 sites), and Carver (7 sites).

Sampling during each 27- week study period was divided into three 9-week sampling units, and all sites were sampled for 21 trap nights (7 traps x 3 consecutive nights) per period. Weeks of site visitation were randomly selected within each sampling period. One 300 foot transect was established at each sampling location and Sherman live traps (H. B. Sherman Traps, Inc., Tallahassee, Fla.), baited with peanut butter and oats, were placed along these transects at 50 foot intervals. All small mammals caught in the traps were identified and euthanized with CO_2 according WHO protocol. Any ticks found on trapped mammals were removed, identified and stored in 97% ethanol for later testing. (Neitzel, Jarnefeld, & Sjorgren, 1993; MMCD, 2017)

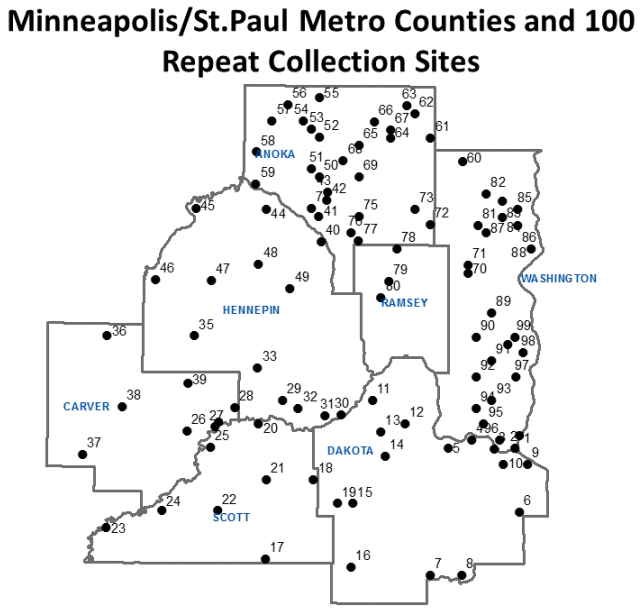

Figure 15. Map of the 7 Minneapolis/St. Paul Metro counties (Carver, Scott, Dakota, Washington, Ramsey, Anoka and Hennepin) encompassing the study area as well as the 100 repeatedly tested sites within those counties.

Pathogen Testing:

A subset of ticks were tested for pathogens. DNA was extracted from ticks collected in 2000, 2004, 2008, 2012, and 2014 to provide a longitudinal analysis spanning 14 years. Extracted DNA was subjected to metagenomics testing to determine presence of pathogenic microbes. All nymphs from each chosen year were included to achieve a more comprehensive picture of pathogen prevalence and host relationships.

DNA Extraction:

Extraction was completed at room temperature using a clean hood in a room where PCRs were not carried out to minimize contamination of reactions. All ticks were rinsed with 95% ethanol before extraction to remove debris and storage fluid in order to

ensure the full pathobiome was tested rather than solely the gut microbiome (Benson, Gawronski, Eveleigh, & Benson, 2004). DNA extraction was carried out with an adapted protocol using the DNeasy® Blood and Tissue Kit Quick-Start Protocol (Qiagen, Inc.). As per the Qiagen recommendations for tick DNA extraction for *Borrelia* testing, carrier RNA was added to increase DNA volume.

Primary/Secondary PCR Amplification:

The V4 region of 16S rRNA was amplified using a two-step PCR protocol. The primary amplification was done using an ABI7900 qPCR machine. The following reaction mixture was used: 3 µl template DNA, 0.48 µl nuclease-free water, 1.2 µl 5x KAPA HiFi buffer (Kapa Biosystems, Woburn, MA), 0.18 µl 10 mM dNTPs (Kapa Biosystems, Woburn, MA), 0.3 µl DMSO (Fisher Scientific, Waltham, MA), 0.12 µl ROX (25 µM) (Life Technologies, Carlsbad, CA), 0.003 µl 1000x SYBR Green, 0.12 µl KAPA HiFi Polymerase (Kapa Biosystems, Woburn, MA), 0.3 µl forward primer (10 µM), 0.3 µl reverse primer (10 µM). Cycling conditions were: 95°C for 5 minutes, followed by 20 cycles of 98°C for 20 seconds, 55°C for 15 seconds, and 72°C for 1 minute. The primers for the primary amplification contained both 16S-specific primers (V1_27F and V3_V34R), as well as adapter tails for adding indices and Illumina flow cell adapters in a secondary amplification. The following primers were used (16S-specific sequences in bold): Meta_V4_515F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GTGCCAGCMGCCGCGGTAA**) and Meta_V4_806R (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GGACTACHVGGGTWTCTAAT**).

The amplicons from the primary PCR were diluted 1:100 in sterile, nuclease-free water, and a second PCR reaction was set up to add the Illumina flow cell adapters and

indices. The secondary amplification was done on a fixed block BioRad Tetrad PCR machine using the following reaction mixture: 5 µl template DNA, 1 µl nuclease-free water, 2 µl 5x KAPA HiFi buffer (Kapa Biosystems, Woburn, MA), 0.3 µl 10 mM dNTPs (Kapa Biosystems, Woburn, MA), 0.5 µl DMSO (Fisher Scientific, Waltham, MA) 0.2 µl KAPA HiFi Polymerase (Kapa Biosystems, Woburn, MA), 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM). Cycling conditions were: 95°C for 5 minutes, followed by 10 cycles of 98°C for 20 seconds, 55°C for 15 seconds, 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes. The following indexing primers were used (X indicates the positions of the 8 bp indices): Forward indexing primer: AATGATACGGCGACCACCGAGATCTACACXXXXXXXXTCGTCGGCAGCGTC and Reverse indexing primer: CAAGCAGAAGACGGCATACGAGATXXXXXXXXGTCTCGTGGGCTCGG (Gohl, et al., 2016).

Normalization and Sequencing:

The samples were normalized using a SequalPrep capture-resin bead plate (Life Technologies, Carlsbad, CA) and pooled using equal volumes. The final pools were quantified via PicoGreen dsDNA assay (Life Technologies, Carlsbad, CA) and diluted to 2nM total DNA. 10 µl of the 2 nM pool was denatured with 10 µl of 0.2 N NaOH, diluted to 8 pM in Illumina's HT1 buffer, spiked with 15% phiX, heat denatured at 96*C for 2 minutes, and sequenced using a HiSeq 500 cycle kit (Illumina, San Diego, CA)**.**

Sequence Processing:

Sequences were processed through the bioinformatics software Mothur (version 1.35.1). Quality control parameters were based on the Schloss MiSeq SOP pipeline

(http://www.mothur.org/wiki/ MiSeq_SOP) (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013). Parameters for discarding sequences were unidentifiable barcode and/or reverse primer sequence. Sequences with greater than 2 errors in their barcode or primer sequence, homopolymer runs greater than 8 bp, any ambiguous bases, or less than 150 bp were filtered out of the dataset. SILVA SEED bacterial 16S database was utilized for sequence alignment (Schloss P. D., 2009) . Remaining high quality and chimera free sequences were placed into operational taxonomic units (OTUs) using 97% similarity. SILVA bacterial 16S database assigned genus level classification to all remaining high quality sequences. The top 30 most abundant OTUs were blasted using National Center of Biological Information (NCBI) Web blast to determine species similarity (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul, Gish, Miller, Myers, & Lipman, 1990).

A total of 609 individual ticks were sequenced from five separate years (2000, 2004, 2008, 2012, and 2014). Following sequence read quality trimming and chimera removal, the range of coverage per sample was >98%, and the average read length was >240 nt. Samples were rarefied to 1,000 sequences. Rarefication resulted in removal of 110 of the 609 ticks from analysis based on a low number of quality reads (<1000). 491 ticks were included in the final analysis spread over 5 collection years (2000 =177, 2004 =50, 2008 =80, 2012 =122, and 2014 =61).

Statistics:

Analysis was performed on records from 23,590 individual trapped mammals and DNA from 491 *I. scapularis* nymphs. Statistical analyses including Kruskal-Wallis Rank Sum, Wilcoxon Rank Sum and production of box and whisker plots was performed in R (R Development Core Team, 2012).

Mothur 1.35.1 was used to calculate Alpha diversity, α, or within-community diversity, using Chao1, Sobs (observed OTUS), and the inverse Simpson diversity index (1/D). Observed OTUs indicate total numbers of OTUs in samples while Chao1 estimates the actual number of species in a community, given the observed sample, while accounting for the relative distribution of species (Chao, 1984). Inverse Simpson combines richness with evenness to determine relevant diversity. Analysis of molecular variance (AMOVA) was performed in Mothur to determine whether variance of each microbial community between groups is greater than the variance within each group (Schloss P. , 2008). Homogeneity of molecular variance (HOMOVA) was the performed to test if the variation in samples was different between groups (Schloss P. , 2008). Additionally, metastats was implemented in Mothur to analyze beta diversity, or within group, differences. Non parametric multidimensional scaling (nmds) was calculated in Mothur and visualized in R. Bonferroni p value adjustment methods were used to account for multiple comparisons. A p value of ≤ 0.05 was considered significant.

Results:

Impact of Pathogen Presence on Microbial Community:

Borrelia burgdorferi sensu stricto was identified in 162 samples (33%) with the 100 top results from the NCBI BLAST having >99% identity with *Borrelia burgdorferi s.s.* While *Borrelia bissettii* was also identified, because it could not be differentiated from *B. burgdorferi* based on the amplified portion of the 16s gene, the occurrence of *Borrelia* is almost certainly *B. burgdorferi ss.* as *B. bissettii* transmission is generally focused on the West Coast and in some Southern states (Margos, et al., 2010). *Anaplasma spp.* was found in 32 samples (6.5%) of which all species identified in NCBI returns with >99% match were *Anaplasma phagocytophilum* (non-species identified returns were listed as ‘uncultured *Anaplasma* species’). Co-infection of *Borrelia* and *Anaplasma* occurred in 17

samples. Analysis showed co-infection was significantly different than expected by chance (X^2=4.7787, df = 1, p=0.02, Fisher's Exact Test p=0.04).

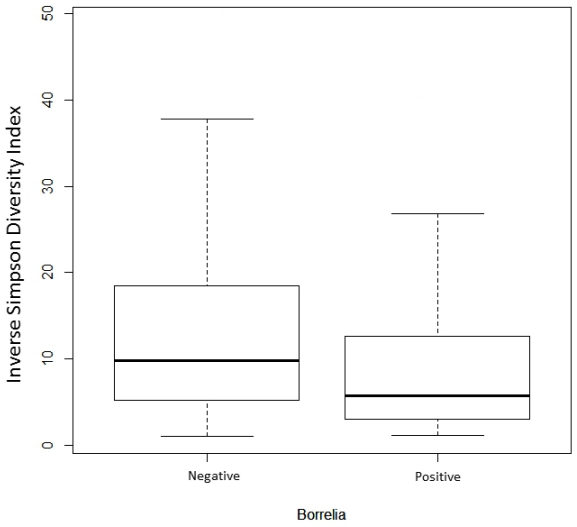

Figure 16: The Inverse Simpson diversity index score, analyzing diversity and evenness, showed lower diversity for *B. burgdorferi* infected *I. scapularis* ticks with a score of 9.1 (± 8.5, IQR 9.9) compared to uninfected ticks which was 12.9 (± 10.0, IQR 13.3).

Estimated observed taxonomic units (OTUs), using the S_{obs} calculator, for the162 samples positive for *B. burgdorferi* infected ticks was 42.4 (±40.9, IQR 31.8). Estimated OTUs for ticks uninfected with *B. burgdorferi* was 46.5 (±41.3, IQR 38.0) showing no significant difference (Kruskal-Wallis X^2 = 1.2, df = 1, p = 0.3). *Ixodes scapularis* ticks infected with *B. burgdorferi* had a mean Chao1 species richness estimate of 56.8 (± 66.3, IQR 38.1). The mean Chao1 for uninfected ticks was 55.0 (± 63.9, IQR 43.0) which showed no significant difference from infected ticks (Kruskal-Wallis X^2 = 0.03, df = 1, p = 0.9). While there was no significance in Chao1 or S_{obs}, the Inverse Simpson diversity index score, analyzing diversity and evenness, showed lower diversity for *B. burgdorferi* infected *I. scapularis* ticks with a score of 9.1 (± 8.5, IQR 9.9) compared to uninfected

ticks which was 12.9 (± 10.0, IQR 13.3) (Figure 16). This difference in Inverse Simpson score was highly significant (Kruskal-Wallis X^2 = 22.8, df = 1, p<0.0001). Visualization of Theta yc analysis showed some distinct clustering among infected and uninfected groups (Figure 17). AMOVA analysis for *B. burgdorferi* infected ticks, which tests whether genetic diversity within different populations is significantly different from those populations combined, was significant for differences in bacterial community between infected and non-infected ticks (Among 4.0, Within 0.4, Fs. 9.6, p<0.001) and HOMOVA confirmed a difference of within group variation between infected and uninfected ticks (SS within/ (Ni-1) 0.42/0.41, p=0.049).

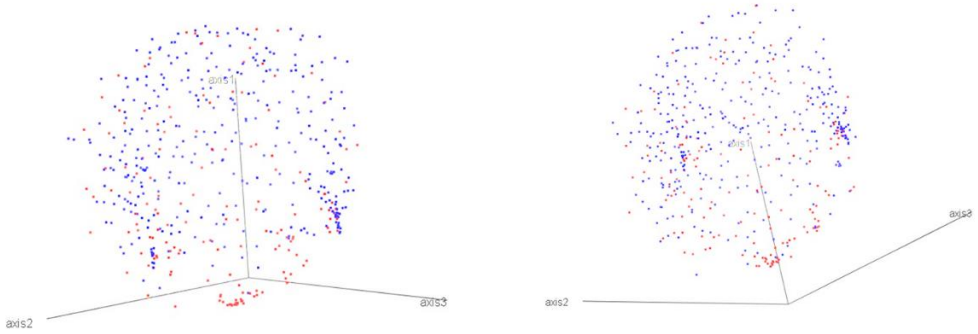

Figure 17: Differences in community structure between *Ixodes scapularis* ticks testing positive (red) and negative (blue) for *B. burgdorferi*. Non-metric multidimensional scaling was used to plot ThetaYC distances, representing bacterial community structures. Samples with similar community structures cluster closer together.

Metastats analysis identified 9 separate OTUs out of the top 30 which had significantly different mean relative abundance between infected and non-infected ticks (Table 6). *Streptococcus* (OTU 5) and *Lactobacillus* (OTU 6) were more abundant in infected ticks while all other sugnificant OTUs were more abundant in uninfected ticks. Of note is that the other pathogenic species of *Borrelia*, *B. miyamotoi*, did not appear in samples that were infected with *B. burgdorferi*. Lefse analysis showed no significant

OTUs, or metagenomic biomarkers, that could be used to define the infected tick microbiome in the sample population.

OTU	Classification	Positive	Negative	*p*
OTU 5	*Streptococcus*	0.02(0.006)	0.005(0.002)	0.03
OTU 6	*Lactobacillus*	0.02(0.006)	0.007(0.002)	0.05
OTU 7	*Flavobacterium*	0.02(0.003)	0.03(0.002)	0.01
OTU11	*Variovorax*	0.02(0.003)	0.03(0.003)	0.006
OTU15	*Pseudomonas*	0.003(0.0008)	0.01(0.002)	0.001
OTU16	*Mycobacterium*	0.004(0.0009)	0.01(0.001)	0.002
OTU 18	*Pseudomonas*	0.004(0.001)	0.007(0.001)	0.04
OTU 26	*Phyllobacterium*	0.004(0.001)	0.01(0.002)	0.005
OTU 30	*Borrelia miyamoti*	0	0.001(0.0007)	0.03

Table 6: Percent median relative abundance (±SD) of significantly different OTUs between *Ixodes scapularis* that were positive for *B. burgdorferi* and those that were negative.

The 32 *I. scapularis* ticks infected with *A. phagocytophilum* had a mean Chao1 species richness estimate of 94.1 (± 96.3, IQR109.3). The mean Chao1 for uninfected ticks was 53 (± 61.1, IQR 38) which was not significantlydifferent from infected ticks (Kruskal-Wallis X^2= 2.7, df = 1, p = 0.1). Similarly, there was an estimate of 56 (± 50.31, IQR 64.8) observed OTUs, using the S_{obs} calculator, in *A. phagocytophilum* infected ticks compared to uninfected ticks, which had a S_{obs} score of 44.4 (± 40.4, IQR 34.0). The difference in observed taxonomic units was not significant between infected and non-infected *I. scapularis* ticks (Kruskal-Wallis X^2= 0.93, df = 1, p = 0.34). The Inverse Simpson diversity index, which takes into account microbial diversity and community evenness, was calculated between infected and uninfected ticks. The Inverse Simpson index number for infected ticks was 6.73 (±8.1, IQR= 4.4) and uninfected ticks scored 12.05 (SD=9.7, IQR=12.9) indicating greater diversity in uninfected ticks(Kruskal-Wallis

X^2= 16.55, df = 1, p= 4.738e-05). The difference in the Inverse Simpson diversity index score between the two groups indicated that infection with *A. phagocytophilum* impacts *I. scapularis* bacterial community diversity (Figure 18). AMOVA analysis for *A. phagocytophilum* infected ticks revealed significant differences in bacterial community between infected and non-infected ticks (Among 1.3, Within 0.4, Fs 2.9, p=0.001). However, HOMOVA analysis showed no difference of within group variation between infected and uninfected ticks (SSwithin/(Ni-1) 0.42/0.41, p=0.2).

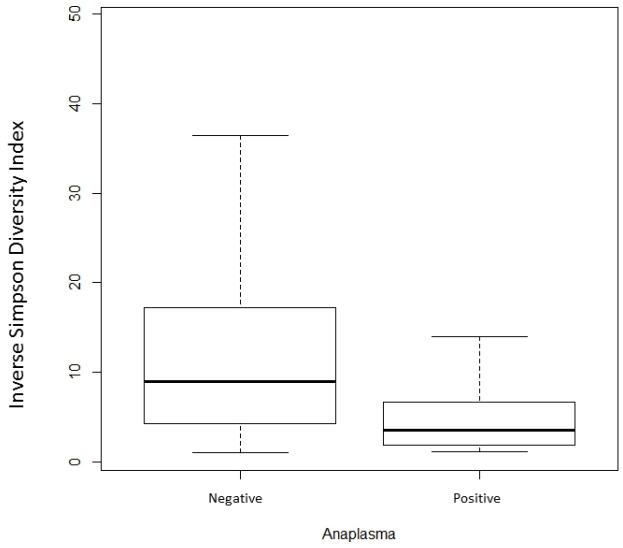

Figure 18: Inverse Simpson Diversity Index of ticks positive and negative for infection with *Anaplasma Phagocytophilum*. *Ixodes scapularis* tick that were infected with *A. phagocytophilum* showed lower (6.73 ±8.1, IQR= 4.4) community diversity compared to uninfected ticks (12.05, ±9.7, IQR=12.9) (p<0.001).

Metastats analysis identified 6 separate OTUs in the top 30 which had significantly different mean realtive abundance between *A. phagocytophilum* infected and non-infected ticks (Table 7). *Variovorax* (OTU 11) was the only OTU present in higher abundance in infected ticks. All other significant OTUs occured in greater abundance in uninfected ticks. *Pseudomonas* (OTU15) and *Mycobacterium* (OTU16)

were elevated in uninfected ticks compared to those infected with *A. phagocytophilum* which was similar to the result for *B. burgdorferi* infected ticks. Of note, *B. miyamotoi* also did not appear in samples that were infected with *A. phagocytophilum* althougn the result was not significant as sample size may have been too small. Lefse analysis identified two metagenomic biomarkers significantly raised in the bacterial communities of infected ticks. *Borrelia burgdorferi* (LDA 4.6, p= 0.01) and *Stapholococcus* (LDA 4.8, p= 0.03) were both identified as significantly increased in *A. phagocytophillum* infected ticks while *Sphingomonas* (4.5, p=0.005) were significantly decreased compared to unninfected ticks.

OTU	Classification	Positive	Negative	p
Otu9	*Paenibacillus*	0	0.01(0.004)	0.0001
Otu11	*Variovorax*	0.02(0.005)	0.03(0.003)	0.05
Otu12	*Paenibacillus*	0	0.007(0.002)	0.01
Otu15	*Pseudomonas*	0.001(0.0007)	0.01(0.001)	0.01
Otu16	*Mycobacterium*	0.003(0.001)	0.01(0.001)	0.02
Otu28	*Propionibacterium*	0.0007(0.0005)	0.02(0.002)	0.001

Table 7: Percent median relative abundance (±SD) of significantly different OTUs between *Ixodes scapularis* that were positive for *A. phagocytophilum* and those that were negative.

Impact of Host Species on Microbiome:

The tested subset of samples of *I. scapularis* were collected from four rodent host species. 266 *I. scapularis* ticks were collected from *Peromyscus leucopus* (the white footed mouse), 201 from *Tamias striatus* (Eastern chipmunk), 16 from *Clethrionomys gapperi* (Southern red-backed vole) and 1 from *Zapus hudsonius* (meadow jumping mouse). The Chao1 species richness estimate for *P. leucopus* fed nymphs was 42.1 (± 37.2, IQR 33.0). Ticks from *T. striatus* has a Chao1 score of 70.0 (± 81.0, IQR 58.0) and those from *C. gapperi* had a score of 98.5 (±116.0, IQR 139.0). The single sample collected from *Z. hudsonius* had the comparatively low score of 17.0

(Figure 19). Differences in Chao1 estimates between hosts were significant (Kruskal-Wallis X^2 = 12.92, df = 3, p= 0.005). Pairwise comparisons using Wilcoxon rank sum test showed that the Chao1 estimate for *T. striatus* was significantly higher, and therefor richer, than *P. leucopus* (p=0.004). Ticks collected from *C. gapperi* had higher indices but due to low capture rates and, therefore, low samples numbers, the result was not significant.

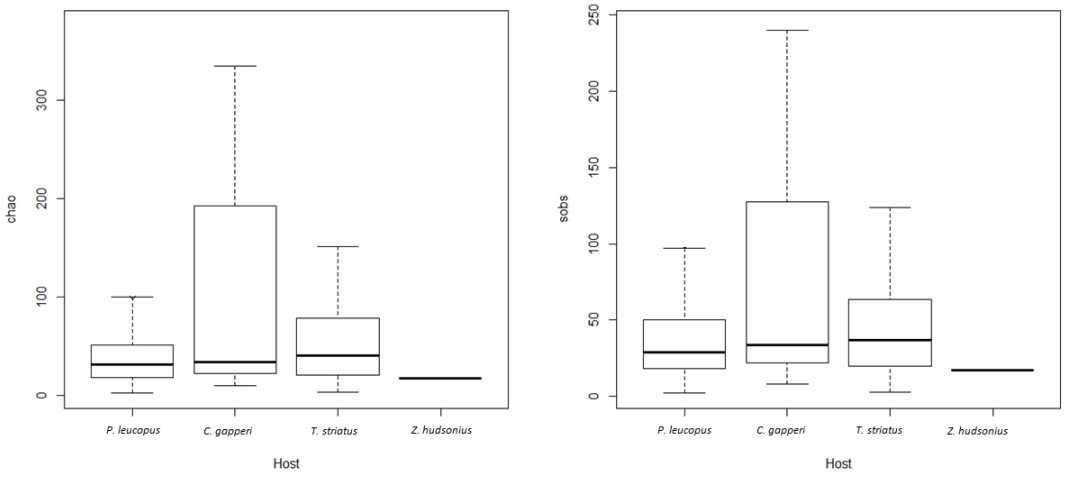

Figure 19: Chao (left) and Sobs (right) species diversity and richness estimates between host species. Both tests showed host as a significant variable in diversity (X^2, Chao p=0.005, Sobs p= 0.02). Additionally, *T. striatus* was shown to have the highest diversity in both tests (Wilcoxon rank sum, Chao p=0.004, Sobs p= 0.04)

The S_{obs} species richness estimate for observed OTUs for *P. leucopus* was 38.0 (± 28.2, IQR 32.0). *Tamias striatus* had a score of 53.0 (± 49.1, IQR 42.8) and *C. gapperi* had a score of 71.8 (±75.0, IQR 102.3). The sample collected from *Z. hudsonius* again had the comparatively low S_{obs} estimate of 17.0, as with Chao1 (Figure 19). Differences in observed taxonomical unit estimates between hosts were significant (Kruskal-Wallis X^2 = 9.0, df = 3, p-value = 0.02). Pairwise comparisons, using the

Wilcoxon rank sum test, again showed that *T. striatus* was significantly higher in diversity than *P. leucopus* (p=0.04). Again, *C. gapperi* results were not significant due to low sample numbers.

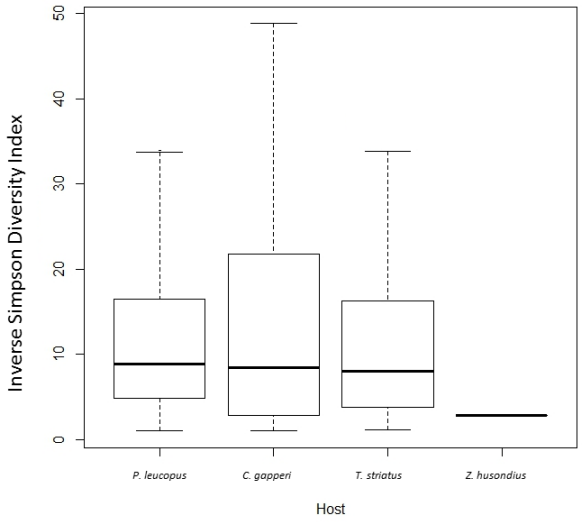

Figure 20: The Inverse Simpson diversity index score comparting *Ixodes scapularis* samples collected from various host species. No significant differences were found between species. (p=0.6) However, AMOVA analysis did show significant differences in community variance among hosts (p=0.001)

An inverse Simpson diversity index score was calculated for each rodent group. Samples collected from *P. leucopus* had a score of 11.8 (± 9.5, IQR 11.7) while ticks from *T. striatus* had a similar score of 11.4 (± 9.6, IQR 12.4). *Ixodes scapularis* ticks from *C. gapperi* showed higher diversity in this test with a score of 13.9 (± 14.2, IQR 18.0) and the tick from *Z. hudsonius* showed very low diversity with a score of 2.8 (Figure 20). There was no overall difference in Inverse Simpson score among ticks from the various rodent hosts (Kruskal-Wallis X^2= 2.0, df = 3, p= 0.6). AMOVA analysis showed significant variation differences between host species (Among 1.1, Within 0.4, Fs 2.5, p<0.001). HOMOVA analysis indicated that the variance within microbial communities of ticks from different hosts was not significant. Metastats analysis identified 9 OTUs that

were significantly different between the two top *Ixodes* hosts in the study, *P. leucopus* and *T. striatus* (Table 8). Important to note is that the percent median relative abundance of the pathogenic agent *B. burgdorferi* was much higher in ticks collected from *T. striatus* than in *P. leucopus* (p=0.001).

OTU	Classification	*T. striatus*	*P. leucopus*	p
Otu2	*Borrelia burgdorferi*	0.1(0.02)	0.01(0.004)	0.001
Otu5	*Streptococcus*	0.02(0.01)	0.0002(0.0001)	0.001
Otu6	*Lactobacillus murinis*	0.02(0.005)	0.004(0.002)	0.002
Otu8	*Sphingomonas*	0.05(0.005	0.08(0.01)	0.001
Otu13	*Bacillus*	0.003(0.002)	0.00002(0.00001)	0.01
Otu17	*Lactobacillus reuteri*	0.01(0.002)	0.0004(0.0001)	0.001
Otu20	*Mycoplsma*	0.01(0.004)	0.00001(0.00001)	0.001
Otu21	*Corynebacterium*	0.001(0.0006)	0.01(0.004)	0.02
Otu28	*Propionibacterium*	0.01(0.003)	0.02(0.003)	0.01

Table 8: Percent median relative abundance (±SD) of significantly different OTUs between the two main *Ixodes scapularis* hosts, *T. striatus* and *P. leucopus*.

Discussion:

This study illuminates the impact that pathogen infection and host selection have on the microbiome of *I. scapularis* ticks. Tick host and pathogenic agents for TBD were shown to be important variables in shaping the pathobiome of *I. scapularis* ticks in the Upper Midwest. Both pathogen species, *B. burgdorferi* and *A. phagocytophilum*, impacted the tick microbiome in distinct ways while also having some common effects. Host selection impacts from *P. leucopus* and *T. striatus* were significant and data indicated a larger and more significant role for *T. striatus* in tick and TBD ecology.

Impact of Pathogen Presence on Microbial Community:

Previous studies have indicated that pathogenic agents have an impact on tick microbiome diversity or size and vice versa that microbiome structure can impact pathogens viability and transmission success (Trout Fryxell & DeBruyn, 2016; Abraham, et al., 2017; Narasimhanm, et al., 2014). The arthropod microbiome is important in shaping vector competence (Kelly, et al., 2017; De la Fuente, et al., 2017; Castro, et al., 2012; Jupatanakul, Sim, & Dimopoulos, 2014). Additionally, there is evidence that the microbiome of ticks may attack or impede human pathogenic agents requiring those agents to remove competitive species or otherwise change the vector microbiome to their benefit. This study adds weight to the idea that agents of TBD may actively alter the microbiome of the ticks that they infect as both *A. phagocytophilum* and *B. burgdorferi* made significant impacts on tick microbiome by possibly removing or reducing some species and decreasing overall diversity. This alteration could be due to the inherent competition between bacterial species or other microorganisms such as viruses, fungi or protozoans that occurs in a microbiome population (Crotti, et al., 2012; Coyte, Schluter, & Foster, 2015).

The Inverse Simpson diversity index for ticks infected with either of the two pathogenic agents was lower than that of uninfected ticks indicating a significantly decreased amount of overall diversity in infected ticks. This reduction in diversity may be due to competition. These pathogens could be competing for resources and may reduce the population of other microorganisms that share the preferred niche for the TBD agent.

Infection with these pathogenic agents has also been shown to alter tick biological responses and behavior leading to an increase in survivability and resistance to weather (Neelakanta G. , Sultana, Fish, Anderson, & Fikrig, 2010; Herrmann & Gern, 2010). This increase in survivability may lead to higher transmission by increasing blood

meals and lifespan. Notably, laboratory experimentation has shown decreased microbial diversity hinders successful colonization with *B. burgdorferi* (Narasimhanm, et al., 2014). Laboratory experiments often do not reflect true wild ecology and our data do not seem to support these findings that increased diversity is beneficial for *B. burgdorferi*. While some bacterial species may be beneficial to *B. burgdorferi* infection in ticks it is apparent that the pathogen thrives when the overall community is lowered. Alternatively, *B. burgdorferi* may benefit from increased microbial diversity during acquisition but then reduce that diversity during colonization. Additionally, neither species of *Paenabacillus* appear in ticks harboring A*. phagocytophilum.* While the *Paenabacillus* are likely environmental species this is an indication that they may be involved in the broader pathobiome and the relationships between those species should be investigated further.

The interaction between TBD agents is also of ecological and public health interest. There is debate on how tick borne pathogens interact and whether their presence functions to aid or hinder the establishment of an infection with another pathogen (Levin & Fish, 2004; Dunn, et al., 2014). Our study indicates that an infection with *A. phagocytophilum* may increase the chance of an infection with *B. burgdorferi* as analysis indicated that relative mean amounts of *B. burgdorferi* were higher in individual ticks co-infected with *A. phagocytophilum* and co-infections were higher than expected by chance. At minimum, it is clear that *B. burgdorferi* is present in greater amounts when co-infected with *A. phagocytophilum* indicating the relationship is beneficial for *B. burgdorferi*. The inverse is not true, however, as no impact is shown on *A. phagocytophilum* numbers in ticks infected with *B. burgdorferi*. While no co-infections with *B. miyamotoi* were discovered in this study, they have been previously reported (Takano, et al., 2014; Barbour, et al., 2009).

Impact of Host Species on Microbiome:

Very few studies have analyzed the impact of host meal on tick microbiome. One study analyzed the differences in microbiome from *Ixodes pacificus* nymphs that fed on lizards versus mice and found significant differences (Swei & Kwan, 2017). One other study examined *I. scapularis* and *Dermacentor variabilis* microbiomes but could not find a significant impact from hosts on community structure (Rynkiewicz, Hemmerich, Rusch, Fuqua, & Clay, 2015). Our study is the first to compare the impact of various rodent hosts on the microbiome of *I. scapularis* ticks. Importantly, this study confirmed that host blood meal does have an impact on the *I. scapularis* microbiome.

Sample sizes for *Z. hudsonius* and *C. gapperi* were very small compared to *P. leucopus* and *T. striatus* inhibiting a full analysis of the impact those two species had on the microbiome of the tick. The focus for analysis was on *P. leucopus* and *T. striatus* as the most important hosts for *I. scapularis* larvae and nymphs and reservoirs for TBD in Minnesota (Johnson, Kodner, Jarnefeld, Eck, & Xu, 2011). While *P. leucopus* is often cited as the most important reservoir in TBD ecology there is a growing understanding of the importance of *T. striatus* in regional ecology (Mather, Wilson, SI, Ribeiro, & Spielman, 1989; Slajchert U. D., 2008; Slajchert, Kitron, Jones, & Mannelli, 1997; Lynn, Oliver, Cornax, O'Sullivan, & Munderloh, 2017; Magnarelli, Anderson,, Stafford, & Dumler, 1997). Again, our evidence does not support the idea that increased microbial diversity promotes colonization of *B. burgdorferi* (Swei & Kwan, 2017; Narasimhanm, et al., 2014). While according to the Inverse Simpson analyses there was no difference in diversity between ticks collected from the two species, there was significantly higher load of *B. burgdorferi* seen in ticks collected from *T. striatus* compared to *P. leucopus*. Additionally, previous analyses of this data showed a significantly larger proportion of

ticks collected from *T. striatus* were positive for *B. burdorferi* than those collected from *P. leucopus*. The higher mean relative abundance of *B. burgdorferi* in ticks collected from *T. striatus* may also indicate its ability to serve as a highly functional reservoir for TBD in the region.

This study shows that host community diversity significantly impacts the microbiome of tick populations through blood meals and therefore could impact pathogen transmission. Combined with higher tick burdens and longer life spans than *P. leucopus,* these data suggest a more important role for *T. striatus* in tick and TBD ecology than has often been assumed (Schug, Vessey, & Korytko, 1991; Nowak, 1999). The pathogenic agents themselves also play an important role in shaping the microbiome of the tick vector, possibly to promote their own reproduction and transmission. Furthermore, the relationships between host, tick microbiome and TBD pathogenic agents were shown to be highly complex and interconnected. Understanding these relationships may be of importance for control of TBD and disease risk mitigation.

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Conclusion

Ticks are the second most important vector of disease globally and exist within a complex and changing eco-epidemiology with various hosts and pathogenic microbes. To better understand the local eco-epidemiology of these pathogens in the Twin Cities metro area a holistic approach to their ecology must be taken into account. As noted, pathogens must be evaluated within the framework of the microbial communities in which they exists and these communities can be impacted by a variety of external factors. The focus of this dissertation was to address gaps in data that currently exist in regards to the local *Ixodes scapularis* eco-epidemiology and to introduce the pathobiome concept to local tick borne disease (TBD) ecological research.

Microbiome research has increased in usage recently although it is often limited to analyzing the internal microbiome of vectors rather than the broader pathobiome. In this study high through put sequencing methods were used to describe and quantify the tick pathobiome. The diverse pathobiome of *I. scapularis* includes multiple TBD pathogens as well as other commensal bacteria that could impact TBD ecology. Additionally, multiple other potential pathogens were documented that were associated with soil, water and mammal hosts. These microbes should be considered in future research concerning disease ecology and transmission. The pathobiome of ticks was highly variable, depending on the year that they were collected. Multiple variables could be impacting the tick pathobiome including geography, hosts, climate and presence of specific pathogenic agents like *B. burgdorferi*. Documenting and describing this pathobiome is the first step in better understand the ecological basis of TBD.

To build on the microbial information two decades of rodent and tick capture data was analyzed to begin to connect microbial ecology to the changing ecology of ticks and their hosts. Important changes have been occurring with tick and rodent populations in

the Twin Cities metro area. An ecological shift occurred over the past 23 years in which *I. scapularis* overtook *D. variabilis* as the dominant species on small mammal. This shift has important consequence to TBD ecology as increased vectors on primary reservoirs indicates a likely increase in risk for human transmission. Additionally, significant expansion around the urban environment was shown the urbanizing landscape may impact tick range expansion and movement. While *P. leucopus* was still shown to be the primary rodent host for juvenile ticks, our data indicated a large and significant role for *T. striatus* in local tick and TBD ecology which needs to be investigated further. This information will be useful in future work that attempts to investigate, model, and predict changing TBD risk in this urban environment. Further analysis should be undertaken to look at how these populations have shifted in the 100 repeatedly sampled sites and to examine the fine scale relationships between pathogen, host and vector.

Lastly, the impact that host and the pathogens, *B. burgdorferi* and *Anaplasma phagocytophilum*, have on the tick pathobiome was examined. *Anaplasma phagocytophilum* and *B. burgdorferi* significantly impacted the tick pathobiome by possibly removing or reducing some species and decreasing overall diversity. How those impacts were made, whether through direct competition or other methods, is not completely understood and should be investigated further. The relationship between these two pathogens should also be investigated further as this study presented evidence that there is significant interaction between the species. Additionally, rodent host was shown to impact tick pathobiome ecology. Specifically, *T. striatus* was again shown to be important to TBD, as this species not only harbored a larger amount of TBD infected nymphs but the ticks collected from *T. striatus* also had higher loads of bacteria than ticks collected from other rodent reservoirs.

The information from this dissertation adds to the complexity of the eco-epidemiology of TBD. As with much research there were more questions raised from these findings than any answers given. Future research can build off of these finding by continuing to study the relationships between microorganisms that exists on the pathobiome. There are also more unanswered questions about the changing ecology of ticks and hosts in the Twin cities metro and how those changes could impact the pathobiome and transmission risk for TBD in the future.

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